tumors and red vascular lesions were observed in many of the tumor bearing animals. The macrophage cultures lacked detectable fibroblastic elements. Free virus was absent in the supernatant fluid of most of the virus treated macrophage cultures. More than one-half of the cultures whose cells induced tumors did not contain free virus at any time when their supernatant fluids were tested. Whenever detected, the titre of free virus was less than 10^2 v.f.u./ml of supernatant fluid. This value was about 1/1000 that of the free virus in infected fibroblast cultures which had a titre of 10^4 to 10^5 v.f.u. per ml of supernatant fluid. Sarcoma virus antigens were not observed in the virus treated macrophage cultures when tested by the fluorescent antibody staining technique including cell samples from macrophage cultures positive for tumor induction.

1. Rangan, S. R. S., Exp. Cell Research, in press.
2. Carrel, A., Ebeling, A. H., J. Exp. Med., 1924, v43, 461.
3. Fischer, A., Compt. rend. soc. de biol., 1925, v92, 109.
4. Ludford, R. J., Am. J. Cancer, 1937, v31, 414.
5. Sanford, K. K., Likely, G. D., Bryan, W. R., Earle, W. R., J. Nat. Cancer Inst., 1952, v12, 1317.
6. Jacoby, F., Cells and Tissues in Culture Methods Biology and Physiology, Willmer, E. N., ed., Academic Press, New York, 1965, v2, 1.
7. Bang, F. B., Warwick, A., J. Path. & Bact., 1957, vLXXIII, 321.
8. Franklin, R. M., Virology, 1958, v6, 81.
9. Bang, F. B., Levy, E., Gey, G. O., J. Immunol., 1951, v66, 329.
10. Bang, F. B., Foard, M., J. Nat. Cancer Inst., 1963, v30, 457.
11. Hanafusa, H., Hanafusa, T., Rubin, H., Proc. Nat. Acad. Sci. U. S., 1962, v49, 572.
12. Borsos, T., Bang, F. B., Virology, 1957, v4, 385.
13. Temin, H., Rubin, H., ibid., 1958, v6, 669.
14. Coons, A. H., General Cytochemical Methods, ed., Danielli, J. B., 1958, vI, 400.
15. Dougherty, R. M., Virology, 1961, v14, 371.
16. Rubin, H., ibid., 1955, v1, 445.
17. Rous, P., Am. Philosophical Soc. Proc., 1912, vLI, 205.
18. Harris, R. J. C., Adv. Cancer Res., 1953, v1, 242.

Received February 27, 1967. P.S.E.B.M., 1967, v125.

Toxicity of L-Asparaginase to Resistant and Susceptible Lymphoma Cells *in vitro*.* (32156)

WILLIAM C. DOLOWY, LLOYD M. ELROD, ROBERT N. AMMERAAL, AND ROBERT SCHREK
Medical Research Laboratory, University of Illinois, Chicago, and Tumor Research Laboratory, Veterans Administration Hospital, Hines, Ill.

It has previously been shown that cells of 6C3HED lymphoma are susceptible to guinea pig serum L-asparaginase *in vitro*, correlating with their susceptibility *in vivo*(1).

It has previously been shown that the blood lymphocytes of patients with chronic lymphocytic leukemia are more susceptible to death *in vitro* than are lymphocytes from normal volunteers when incubated with L-asparaginase from *E. coli*(2).

The present report describes the relative susceptibility to a wide range of concentrations of *E. coli* L-asparaginase *in vitro* of normal murine spleen and thymus lymphocytes and tumor cells from a strain of L-asparaginase-susceptible lymphoma (6C3HED) and from a strain of a derived *E. coli* L-asparaginase-resistant tumor (6C3HED-ECLAR1).

Materials and methods. Enzyme. E. coli L-asparaginase was obtained from Worthington Biochemical Corp. The preparations were purified by salt fractionation and chromatography and had a specific activity of 5.0 to 6.7 international units per mg protein. The lyophilized enzyme preparations were reconstituted in normal saline and sterilized by filtration through a 220 mμ Millipore® filter. Protein was determined by the method

* Aided by grants CA 07541 from Nat. Cancer Inst., USPHS and PHS-GRSG 172.

of Lowry *et al.* The enzyme was assayed as described previously(3). It was also assayed at pH 6.3 and pH 8.5 to ascertain whether any non-antitumor L-asparaginase was present(4). The results of the assays at both pH levels were almost identical, indicating that the L-asparaginase was wholly or very predominantly analogous to the peak 1(4), the enzyme with antitumor activity. The salt fractionation probably was responsible for the removal of the non-antitumor L-asparaginase.

Cells. Tumor cells. Both lines of tumor cells were carried by subcutaneous transplantation in C3H mice.

The 6C3HED lymphoma, which was shown to be susceptible to *E. coli* L-asparaginase *in vivo*(6,7), was used as the L-Ase-susceptible line.

The 6C3HED-ECLAR1 tumor, a descendant of the 6C3HED lymphoma, was deliberately produced in our laboratory as an L-asparaginase-resistant tumor *in vivo* by undertreatment of five C3H mice bearing the 6C3HED tumor subcutaneously with 83 international units/kg (IU/kg) of *E. coli* L-Ase. Two mice in which the tumor reappeared were treated with 250 IU/kg of *E. coli* L-Ase. The tumor diminished, but regrew. This line was designated as the *E. coli* L-asparaginase-resistant lymphoma (6C3-HED-ECLAR1) and was transplanted without any further treatment for 4 transplant generations in C3H mice.

Normal cells. The normal tissues studied were the thymus and spleen which were excised from Swiss albino mice immediately after euthanasia.

To prepare cell suspensions, either normal or malignant tissues were minced with a scalpel in media consisting of 20% heat inactivated (56°C 30 minutes) sera of rabbits and 80% Fischer's media no. 147G (Grand Island Biological Co., Grand Island, N. Y.). The cells were washed once and resuspended in fresh media. The suspensions were diluted to contain 1,000 cells/mm^3.

Cell survival tests. Suspensions of cells were incubated at 37.5°C, with and without asparaginase, for 48 hours. Each tube contained 0.9 cc of cell suspension and either 0.1 cc saline alone or 0.1 cc saline containing asparaginase.

Cells were counted in 0.2 cc of the suspension, placed in a slide chamber, through an inverted phase contrast microscope(2) The number of viable cells was counted in the treated and untreated suspensions before incubation and 24 hours after and 48 hours after incubation.

The percent cytocidal effect (% C.E.) indicating the number of cells which died in the presence of L-Ase compared with cells incubated in the absence of L-Ase for the same length of time, was calculated using the following formula.

$$\% \text{ C.E.} = \left(1 - \frac{\text{NT}}{\text{NC}}\right) \times 100$$

NC = number of cells per cu mm of control culture

NT = number of cells per cu mm of L-asparaginase culture

The criteria for cell viability used for microscopic examination include the following: 1) A cell was considered viable if it had an intact cytoplasmic and an intact nuclear membrane, an irregular outline of both membranes and clearly visible nuclear and cytoplasmic structure (Fig. 1). 2) A cell was not considered viable if both the nuclear and cytoplasmic membranes were circular in outline (Fig. 2), or if these membranes were not intact, or if the cell contents were visible only as a granular mass, or if the nucleus was absent or was pyknotic and amorphous.

Results. The relative toxicity of L-Ase to a number of cell lines is shown in Table I. The 6C3HED cells were very susceptible to L-Ase with over 80% cytocidal effect at L-Ase levels of 1.67 IU/liter and above. They are more susceptible to 0.167 IU/liter L-Ase and greater concentrations of L-Ase than any of the other cells tested. The susceptibility of these cells was detectable in L-Ase concentrations as low as 0.167 IU/liter at 2 days at which time a 59% C.E. was evident. In contrast, the C.E. was not detectable in the spleen or 6C3HED-ECLAR1 cells unless a concentration of L-Ase of 167 IU/liter or higher was used for 2 days.

The thymus cells showed an intermediate

susceptibility; a 38% C.E. was observed at 1 day.

Discussion. The susceptibility of 91.7% of 6C3HED tumor cells to death *in vitro* at concentrations of L-Ase at 1 day at 167 IU/liter correlates with the susceptibility of the same tumor *in vivo* in which it was shown that a dose of L-Ase (250 IU/kg), adequate to produce a plasma L-Ase level of at least 10 IU/liter for 24 hours, caused regression of the 6C3HED subcutaneous mouse lymphoma(8), and the same dose (250 IU/kg)

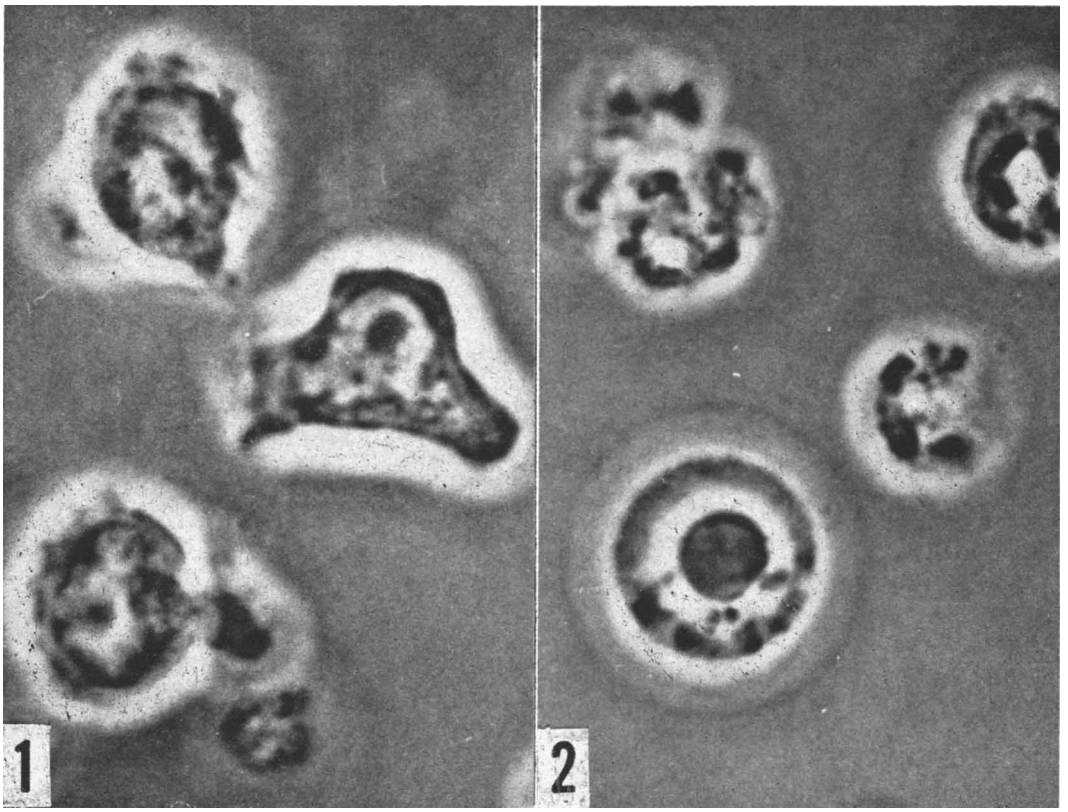

FIG. 1. Viable 6C3HED lymphoma cells with intact cytoplasmic and nuclear membranes, irregular outline of both membranes and discernible nucleus and cytoplasm. Live, unstained cells, phase contrast microscope. × 2,000.

FIG. 2. Nonviable 6C3HED lymphoma cell with nuclear and cytoplasmic membranes circular in shape. Unstained cell, phase contrast microscope. × 2,000.

TABLE I. Effect of L-Asparaginase on Lymphocytes of Normal Mouse Thymus and Spleen and on Tumor Cells of 6C3HED and 6C3HED-ECLAR1 Transplantable Lymphomas.

	No. of days at 37°C	No. of exp	% of cells surviving in control suspension	% Cytocidal effect — International units/liter					
				1,670	167	16.7	1.67	0.167	0.0167
Spleen	1	8	47.3	40.8	28.5	16.5	14.9	17.5	6.0
	2	7	30.3	67.5	62.8	25.9	25.8	17.7	—13.2
Thymus	1	7	38.3	68.5	47.6	40.1	38.7	12.4	11.1
	2	2	10.6						
6C3HED	1	13	82.6	77.7	91.7	89.7	82.4	47.7	10.1
	2	8	53.1	85.1	98.4	98.3	95.8	59.0	1.5
6C3HED-ECLAR1	1	7	83.1	58.2	15.8	23.8	3.0	1.3	
	2	3	69.3	78.9	49.4	25.6	22.6	10.8	

caused regression of the majority of intracerebral lymphomas(5).

The resistance of the 6C3HED-ECLAR1 tumor *in vitro* to 167 IU/liter L-Ase at 24 hours correlated with the failure of the tumor *in vivo* to regress completely after inoculation of 250 IU of L-Ase per kg body weight.

Others(4) have shown that C3H mice can produce antibodies to *E. coli* L-Ase, and that subsequent treatment of these mice with *E. coli* L-Ase does not cause a newly implanted 6C3HED lymphoma to regress. They attribute this to the neutralizing effect (32 to 49%) of antibodies measured in the serum of their mice. In contrast, a group working with the EARAD1 tumor(9) state that in their experience, *E. coli* L-asparaginase was effective in retreatment of mice with recurrent leukemia or cured mice receiving a second transplant, and that they believe that sensitization to L-asparaginase does not occur under their conditions or that any antibodies which may have been formed do not interfere with enzyme activity.

Since our first line of resistant tumors was produced under *in vivo* conditions which could have included neutralization of subsequently administered L-Ase similar to the experience of others(4), we were interested in ascertaining whether resistant tumors would arise in the absence of prolonged exposure to possible antibodies within animals.

We subsequently developed another L-asparaginase-resistant line 6C3HED-ECLAR2 by treating 5 mice with 83 IU/kg of L-Ase; the tumors regressed, then regrew and were transplanted to new mice. When the tumors were 1 cm in diameter, the mice were treated with 167 IU/kg, and the tumors regressed, then regrew and were transplanted to new mice. The same sequence was followed in each subsequent transplant generation, using 250 IU/kg to treat new mice for 2 transplant generations.

In this series the tumor was developed to be resistant *in vivo* to a dose of L-Ase which always caused complete regression of the original tumor line.

Summary and conclusions. The 6C3HED lymphoma which is susceptible to *E. coli* L-Ase *in vivo,* is also susceptible to *E. coli* L-Ase *in vitro.* The 6C3HED-ECLAR1 lymphoma which is resistant to *E. coli* L-Ase *in vivo* is also resistant to *E. coli* L-Ase *in vitro.* At 2 days of incubation, these cells respond to L-Ase concentrations of 1,000 to 10,000 fold of that needed to exert a cytotoxic effect upon the susceptible cell line. Spleen cells are approximately as resistant as the 6C3HED-ECLAR1 lymphoma. Thymus cells are more susceptible than spleen cells, but more resistant than the susceptible tumor cell line.

1. Borella, L., Journey, L. J., Hiramoto, R., Cancer Res., 1966, v26, 2451.

2. Schrek, R., Dolowy, W. C., Ameraal, R. N., Science, 1967, v155, 329.

3. Dolowy, W. C., Henson, D., Cornet, J., Sellin, H., Cancer, 1966, v19, 1813.

4. Roberts, J., Prager, M. D., Bachynsky, N., Cancer Res., 1966, v26, 2213.

5. Schwartz, J. H., Reeves, J. Y., Broome, J. D., Proc. Nat. Acad. Sci., 1966, v56, 1516.

6. Dolowy, W. C., Cornet, J., Henson, D., Ammeraal, R., Proc. Soc. Exp. Biol. & Med., 1966, v123, 133.

7. Mashburn, L. T., Wriston, J. C., Arch. Biochem. Biophys., 1964, v105, 450.

8. Dolowy, W. C., Ammeraal, R. N., Cornet, J., Henson, D., to be published.

9. Boyse, E. A., Old, L. J., Campbell, H. A., Mashburn, L. T., J. Exp. Med., 1967, v125, 17.

Received March 2, 1967. P.S.E.B.M., 1967, v125.