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A Metabolic Difference Between Two Lines of Lymphoma 6C3HED Cells in Relation to Asparagine.* (30169)

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Lymphoma 6C3HED cells of Gardner's original line (here designated Lymphoma 6C3HED-OG)(1) have long been carried in this laboratory through repeated transfers—either subcutaneously or intraperitoneally—in C3H mice. These cells are regularly susceptible to the effects of guinea pig serum *in vivo*(2), recent findings having indicated that the asparaginase activity of guinea pig serum is responsible for its antilymphoma effects(3) and that the susceptible cells may depend upon asparagine for growth *in vitro* (4). A subline of 6C3HED cells (here designated Lymphoma 6C3HED-RG1) was obtained several years ago from a mouse given "sub-curative" amounts of guinea pig serum (5). The cells of the RG1 subline are morphologically identical with those of the OG line and they resemble the latter closely in biological behavior, yet they are completely refractory to the effects of guinea pig serum *in vivo*(5), and evidence has been presented that they do not depend upon asparagine for growth *in vitro*(3).

In the present work, we have compared cells of the two lines for ability to incorporate L-valine-C^{14} *in vitro,* in the absence of added asparagine and in the presence of asparagine in various concentrations.

Materials and methods. Lymphoma cells of each type were removed with aseptic technique from the peritoneal cavities of C3H mice of the Heston line procured from the Jackson Laboratory, Bar Harbor, 5 days following implantation of one million cells. The harvested cells were washed twice in Eagle's basal medium(6) in which phosphates were increased by 10 times and $CaCl_2$ had been omitted and which contained 5% horse serum. The concentration of cells was then adjusted to 2 million per ml with the aid of a Coulter electronic cell counter. One ml of the cell suspension was next added to 1 ml Eagle's basal medium to which certain additions had been made so that the final incubating medium contained: 5% horse serum, uniformly labeled L-valine-C^{14} (Nuclear-Chicago Corp.) 0.1 μc, 24 μg per ml, and, in certain tubes, L-asparagine-H_2O (Mann Research Lab.) in various concentrations.

Incubation was carried out during 4 hours at 37°C, following which the cells were removed from the suspension by centrifugation and washed once with cold Eagle's medium without serum. Cold 10% trichloroacetic acid (TCA) was next added; the cellular precipitate was washed again with 10% TCA, once with 100% ethanol, and finally dissolved in 2 ml hydroxide of Hyamine.† Counts were then made in a PPO-POPOP‡-toluene mixture with a Packard Tri Carb liquid scintillation spectrometer, each determination being made on triplicate or quadruplicate specimens. Counting efficiency was about 55%.

Results. Table I shows the results of two representative experiments in which RG1 and OG cells incorporated L-valine-C^{14} in the

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† Hyamine = [p-(diisobutylcresoxethoxyethyl)-dimethylbenzylammonium chloride], Rohm and Haas.

‡ PPO = 2, 5-diphenyloxazole; POPOP = 1, 4-bis-2-(5-Phenyloxazolyl)-Benzene, (Nuclear-Chicago Corp.)

TABLE I. Incorporation of L-Valine-C^{14} *in vitro* by Lymphoma 6C3HED-OG and -RG1 Cells in Absence or Presence of Added Asparagine.

Exp	Cells	Added asparagine, mg/liter	Incorporation of L-valine-C^{14}, cpm per 2×10^6 cells (*average*)	(range)
1	RG1	0	*3618*	(3408-3950)
		.02	*3921*	(3121-4321)
		.22	*3760*	(3080-4340)
		2.2	*3567*	(3061-4060)
		22.0	*3752*	(3643-3960)
2	RG1	0	*4455*	(4250-4840)
		.02	*4234*	(3853-4660)
		.22	*3980*	(3750-4210)
		2.2	*4185*	(4035-4335)
		22.0	*4035*	(4020-4050)
1	OG	0	*738*	(645- 848)
		.02	*891*	(812- 984)
		.22	*1317*	(979-1640)
		2.2	*3404*	(2800-3920)
		22.0	*2870*	(2508-3033)
2	OG	0	*954*	(950- 958)
		.02	*1339*	(1311-1367)
		.22	*1431*	(1341-1511)
		2.2	*3104*	(2992-3296)
		22.0	*2481*	(2373-2590)

cpm = net counts per min.

presence and absence of added L-asparagine.

It can be seen from the Table that regardless of the presence or absence of added asparagine the RG1 cells incorporated an abundant and relatively constant amount of valine. On the other hand, in the absence of added asparagine, the OG cells incorporated a relatively small amount of valine. As the concentration of asparagine was increased the incorporation of valine by OG cells also increased and maximum incorporation occurred at 2.2 mg asparagine/liter. At one tenth this concentration of asparagine, valine incorporation was less than half of maximum.

Since some asparagine was doubtless present in the horse serum of the medium, further experiments of this type were done in which horse serum was omitted from the medium. The same pattern of incorporation of valine-C^{14} was found, though in the absence of horse serum lower values for valine incorporation were obtained.

Comments. The experiments clearly show: 1) that RG1 cells incorporate valine-C^{14} abundantly and without regard to the presence or absence of added asparagine, 2) that OG cells incorporate valine-C^{14} relatively poorly in the absence of added asparagine, and 3) that an optimal concentration of asparagine exists for OG cells below which valine incorporation is sharply limited.

The fact that OG cells incorporate valine relatively poorly in the absence of added asparagine indicates that under such circumstances protein synthesis in these cells may be inhibited by an inadequate supply of asparagine. It may be noted in this relation that the levels of asparagine at which valine incorporation was poor were levels at which OG cells cultured *in vitro* showed little or no growth(3). The ability of RG1 cells to incorporate valine in the absence of added asparagine suggests that these cells may be able to produce needed asparagine from other sources by a mechanism not operative in the OG cell.

It can be seen that the findings of this paper provide a means whereby asparagine-dependent cells can be readily distinguished from asparagine-independent cells *in vitro*. In this relation it should be noted that Lymphoma 6C3HED-OG cells are not unique in their susceptibility to the effects of guinea pig serum *in vivo*(2,7,8,9) and thus presumably are not unique in their dependence upon asparagine. Indeed, the observations of Neuman and McCoy provide direct evidence that cells of Walker carcinosarcoma 256 have a dual requirement for asparagine and glutamine *in vitro*(10). Further, the Jensen rat sarcoma and a rat hepatoma have also been found to require L-asparagine for growth *in vitro*(11,12).

Summary. A metabolic difference has been found *in vitro* between two lines of Lymphoma 6C3HED cells, by measuring the incorporation of L-valine-C^{14} in presence and absence of added L-asparagine. Cells of the RG1 subline, insensitive to the effects of guinea pig serum *in vivo* and independent of need for extrinsic asparagine for growth *in vitro*, incorporated a large and relatively constant amount of valine regardless of presence or absence of added asparagine. Cells of the original OG line, by contrast, being susceptible to the effects of guinea pig serum *in vivo*

and requiring added asparagine for growth *in vitro,* incorporated relatively little valine unless sufficient amounts of asparagine were added to the medium.

The significance of the findings is briefly discussed.

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Trichloracetic Acid Soluble Polysaccharide-Protein Complexes.* (30170)

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It was recently reported (Woodside and Kocholaty(4)) that glycogen was firmly bound to both human and bovine protein fractions of thrombocytes and to gelatin. These biopolymeric complexes were distinguished from unbound glycogen by their solubility in 0.83% trichloroacetic acid (TCA)—83% ethanol solutions. The purpose of this study was to extend these observations to other proteins and polysaccharides.

Materials and methods. The glycogen extraction and precipitation techniques, carbohydrate standards, and quantitative carbohydrate determinations were described earlier (Woodside and Kocholaty(4)). Protein-bound polysaccharide values represent the difference between the 30% KOH extractable polysaccharide (total polysaccharide value) and the 0.83% TCA—83% ethanol insoluble polysaccharide (unbound polysaccharide). Protein was estimated by the Lowry protein determination (Lowry *et al*(3)) with bovine plasma albumin as the standard. For protein determination of gelatin solutions, gelatin, U.S.P. (Difco Laboratory, Detroit, Mich.) was used as the standard.

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The following commercially available proteins were used: gelatin, U.S.P., purified pigskin and calfskin gelatin, collagen, casein, edestin, β-lactoglobulin, bovine hemoglobin, guinea pig complement, human poliomyelitis immune globulin, immune serum globulin, poly-L-proline and poly-L-hydroxyproline. Cohn fraction VI, E. R. Squibb and Sons, New York, was also made available to us by Dr. J. H. Pert, American National Red Cross, Washington, D. C. Commercially available polysaccharides were: glycogens, rabbit liver and shellfish glycogens, dextran, (M.W. 80,000) dextrin, amylodextrin, and inulin. Glycogen, C. P., Pfanstiehl Chemical Corp., has been used as the standard glycogen suspension over the past 5 years.

Ten human serum protein fractions separated on a DEAE cellulose anion exchange column according to Fahey *et al*(5) were supplied by Mr. B. D. Ashley of this laboratory.

All protein and polysaccharide solutions were suspended in distilled deionized water unless otherwise specified. Reaction mixtures for polysaccharide-protein binding studies contained 20 mg protein and 0.5 mg polysaccharide in a total volume of 1.0 ml. In