Lysosomal Stimulation and Inhibition of the Growth of Cells in Tissue Culture.* (32379)

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A number of agents such as vitamin A(1), anoxia(2), and ultraviolet irradiation(3,4) that disrupt the lysosome *in vitro* and in the living cell are assumed to damage the cells as a result of the release of the hydrolytic enzymes of the lysosome. To obviate some of these assumptions concerning the lysosomes, Allison and Paton(5) added photosensitive dyes to cells in tissue culture. Uptake of the dye by the lysosomes of the dye-treated cells, followed by photoexposure, resulted in an increased number of chromosomal breaks. More direct evidence of the damaging effects due to the lysosome was obtained by Desai and Tappel(6) who observed hemolysis of rat erythrocytes by liver lysosomes. Many of the suggestions and hypotheses regarding the function of the lysosome assume that the hydrolytic enzymes of the lysosome damage the cell. Therefore, it appeared desirable to obtain more direct evidence of the activity of lysosomes by studying the effects of isolated lysosomes on the growth of cells in tissue culture.

Methods and materials. Stock strain L cultures (NCTC clone 929, clone of Strain L) and Detroit 6 CC13 were maintained in a modified Waymouth's medium MB752/1(7). The medium was modified by replacing the salt solution with the tris-citrate salt solution described by Paul(8,9). To 100 ml of the modified medium were added 5 mg of Neomycin sulfate, 0.2 mg of amphotericin B and fetal calf serum, 10% c/v. The pH of the medium was adjusted to pH 7.6 at the beginning of all experiments.

Stock cultures were grown in the above medium for 5 days in Blake bottles. The medium was removed and the cells detached by rubber policemen and suspended by agitation. The cell suspension, after counting in a Coulter counter, was adjusted to 1×10^5 cells/ml. After washing once by centrifugation, the cells were suspended in the modified Waymouth's medium containing fetal calf serum. Two milliliters of the medium containing 1×10^5 cells/ml were pipetted into Leighton tubes. After incubation for 48 hours and 96 hours, the cells were detached from the tubes with 0.25% trypsin (1:300, Baltimore Biological Laboratories) in a citrate buffer and counted in the Coulter counter.

Rat (Sprague-Dawley, male, 250-300 g) liver lysosomes were prepared by the method of Sawant, Shibko, Kumta, and Tappel(10) except that a Potter-Elvehjem homogenizer was used instead of a Waring Blendor. Each preparation of liver lysosomes was analyzed for acid phosphatase(11), total cathepsin (12), aryl sulfatase(13) and protein(14). Before analysis and use in the tissue culture studies, the membrane of the lysosomes was disrupted by freezing and thawing 10 times. The specific activity of the acid phosphatase of the lysosomal preparations used in this study was from 13,000 to 14,000 mμmoles nitrophenylphosphate hydrolyzed/min/milligram of protein.

Results. The addition of rat liver lysosomes to Strain L and Detroit 6 cells (Table I) results in a marked inhibition of the cells at 50 μg lysosomal protein/ml. More surpris-

TABLE I. Rat Liver Lysosomes on Growth of Strain L and Detroit 6 Cells.

	Strain L		Detroit 6	
Lysosome conc. (μg protein/ml)	2 days	4 days	2 days	4 days
	(cells/ml × 10^3)			
50.0	51.*	39.*	81.*	69.*
25.0	94.*	191.*	96.	142.*
5.0	141.	446.	110.	174.
2.5	143.	483.	107.	226.
1.0	126.	532.*	106.	285.*
.0	122.	456.	112.	213.

Each value is the average of 8 determinations.

* Values which are significantly different from the control $P < 0.05$ by Mann Whitney U Test(15).

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ing is the significant stimulation of the cells at lower concentrations of lysosomes (1 μg/ml). The inhibition of growth at the higher concentrations of lysosomes is accompanied by swelling of the cells and the appearance of dark bipolar areas in the cell that give a strongly positive stain for acid phosphatase.

To determine if the inhibitory factor(s) is unique to the lysosomes, liver was homogenized and fractionated by centrifugation as described by Sawant, Shibko, Kumta, and Tappel(10). Each of the fractions was resuspended and recentrifuged in the culture medium to remove the sucrose before use. All fractions were then added to Strain L cells at 50 μg protein/ml in a total volume of 0.1 ml (Table II). Although some inhibition occurs in the total homogenate as well as in the lysosome fraction, this is to be expected because of the lysosomal content of the total homogenate. Desai and Tappel observed most of the hemolytic activity of the lysosome is associated with the membrane of the lysosome(6). Lysosomal membranes were prepared as described by these authors and unlike the hemolytic activity, the growth-inhibiting activity of the lysosome is apparently not associated with the membrane of the lysosome (Table II).

To determine if the activity was associated with macromolecules, liver lysosomes were dialyzed against distilled water and the dialyzable and non-dialyzable material added to cultures of Strain L cells. The non-dialyzable material, from 100 μg of lysosomes, produced a marked inhibition (Table III). The dialyzable material, from 100 μg of the lysosomes, resulted in considerably less inhibition. At the lower concentration of 10 μg/ml the dialyzable and non-dialyzable material appear to cause stimulation of growth as was previously observed (Table III).

The heat stability of the growth inhibiting factors was compared by heating the lysosomes at 25°, 50°, 85°, 100°, for 30 minutes and incubating the heated lysosomes with Strain L Cells (Table IV). The result of this experiment suggests that the inhibitory material is heat labile.

Because the above experiments suggest that the inhibitory factor(s) are thermolabile and non-dialyzable, it appeared probable that a protein(s) is responsible. To study this probability, 1.0 ml of a 0.25% trypsin (3500 N.F. units/mg) was added to 1.5 mg of lysosomal protein in a 0.02 M Tris buffer, pH 7.8 and incubated for 3 hours at 37°C. Control samples containing trypsin only and lysosomes only were also prepared and incubated. The 3 samples were dialyzed against 100 ml

TABLE II. Inhibitory Activity of Particulate Fractions of Rat Liver on Strain L Cells.

	Cells/ml × 10^3	
	2 days	4 days
Total homogenate	78.	213.
Microsomes	103.	183.
Lysosomes	73.	64.
" membrane	119.	294.
" soluble	101.	79.
No addition	124.	340.

Lysosomal membrane and soluble fraction prepared by freezing and thawing 10 times and centrifuging at 100,000 × *g* for 2 hr. Lysosomal membrane and soluble fractions added to cells at a concentration equivalent to 50 μg protein/ml of unfractionated lysosomes.

TABLE III. Dialysis of Lysosomes on the Inhibition of Strain L Cells.

	Cells/ml × 10^3	
Lysosomal protein/ml	2 days	4 days
100 μg dialyzable	130.	366.
non-dialyzable	120.	185.
10 μg dialyzable	145.	532.
non-dialyzable	164.	576.
No addition	138.	510.

Each value is the average of 4 separate determinations. Dialyzed 24 hr at 5°C against 100 volumes of distilled water in seamless cellulose. Samples lyophilized and reconstituted in culture medium to provide the equivalent concentrations.

TABLE IV. Heat Stability of the Growth Inhibiting Factor of Rat Liver Lysosomes.

	Cells/ml × 10^3	
Temperature	2 days	4 days
25°	90.	77.
50°	114.	104.
85°	148.	383.
100°	140.	390.
No addition	144.	441.

All experimental tubes contain lysosomal protein heated for 30 min to the indicated temperature and added to cells at a concentration of 50 μg lysosomal protein/ml. Each result in the Table is the average of 8 determinations.

TABLE V. Trypsin Effect on Inhibitory Factor(s) of Lysosomes.

	Cells/ml × 10^3	
	2 days	4 days
100 μg lysosome/ml	109.	116.
Idem + trypsin	134.	311.
Trypsin only	131.	305.

Each result is the average of 8 determinations.

of distilled water at 5° for 24 hours, and the non-dialyzable material lyophilized. Each sample was taken up in the Tris-Waymouth salt solution and the equivalent of 100 μg/ml of lysosomes added to Strain L Cells (Table V). It is apparent that the trypsin inactivated the inhibitory factor(s) present in the lysosomes.

Allison and Paton(5) suggested that the chromosomal breakage that they observed following dye uptake and photoexposure resulted from the release of DNAase. However, the addition of DNAase, 200 μg/ml, (Sigma, Bovine Pancreas 150,000 units/mg) to the tissue culture medium containing Strain L Cells caused no inhibition of the cells. This suggests that the inhibition is probably not due to DNAase.

Summary. These experiments provide evidence for the presence of a non-dialyzable, thermolabile, protein(s) in lysosomes which inhibits the growth of Strain L and Detroit 6 cells. This protein is not bound to the lysosomal membrane but is released upon rupture of the lysosome. The study tends to confirm the conclusions of other investigators, who have used a less direct approach to the problem, that lysosomes cause cell damage and inhibit cell growth. However, it has not been previously observed that the lysosomes may also stimulate cell growth when present at low concentrations. Since practically any type of cell damage may result in the rupture of the lysosomal membrane, it is of interest that the contents of the lysosome may either stimulate or inhibit the growth of the neighboring cells depending upon the number of injured cells or ruptured lysosomes.

1. Dingle, J. T., in Ciba Foundation Symposium on Lysosomes. A.V.S. deReuck & M. P. Cameron, ed., Boston, Little Brown, and Co., 1963, 384.

2. deDuve, C., in Subcellular Particles, T. Hayashi, ed., New York: Ronald Press, 1959, 128.

3. Weissmann, G., Fell, H. B., J. Exp. Med., 1962, v116, 365.

4. Weissmann, G., Dingle, J., Exp. Cell Res., 1961, v25, 207.

5. Allison, A. C., Paton, G. R., Nature, 1965, v207, 1170.

6. Desai, I. D., Tappel, A. L., Proc. Soc. Exp. Biol. & Med., 1965, v118, 496.

7. Waymouth, C., Nat. Canc. Inst., 1959, v22, 1003.

8. Paul, J., Cell and Tissue Culture, Williams & Wilkins, 3rd Ed., 1965, 86.

9. Gwatkin, R. B. L., Siminovitch, L., Proc. Soc. Exp. Biol. & Med., 1960, v103, 718.

10. Sawant, P. L., Shibko, S., Kumta, U. S., Tappel, A. L., Biochim. et Biophys. Acta, 1964, v85, 82.

11. Sigma Tech Bulletin, Sigma Chemical Co., St. Louis, Mo., 1963, 104.

12. Wasi, S., Murray, R. K., Macmorine, D. R. L., Movat, H. Z., Brit. J. Exp. Path., 1966, v147, 411.

13. Roy, A. B., Biochem, J., 1953, v53, 12.

14. Lowry, O. H., J. Biol. Chem., 1951, v193, 265

15. Siegel, S., Nonparametric Statistics, McGraw-Hill, New York, 1956.

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