Dr. Yamada: We could not confirm it by the observations of many sections (more than 1000).

Dr. Nakamura: What are the granules which appear at the peri-nucleolar area?

Dr. Yamamoto: It is not certain what may be the granules. The histochemical reactions did not give the exact performance probably by diffusion effects.

培養細胞のライゾソームについて

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Morphology and Cytochemistry of Lysosomes^{*}

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"Lysosomes" is a biochemically defined term meaning a separate class of cytoplasmic particles, entirely different from mitochondria and characterized essentially by their hydrolytic enzymes such as acid phosphatase, phosphoprotein phosphatase, acid ribonuclease, acid deoxyribonuclease, cathepsins A, B and C, non-microsomal β -glucuronidase, aryl-sulfatases A and B, and β -N-acetylglucosaminidase (1,2). Morphologically lysosomes have been tentatively identified with the peribiliary dense bodies (3,4), using mainly electron microscope, but their unequivocal morphological identification is still a controversial problem (5).

One of the main reasons why it is difficult to elucidate morphological entities of lysosomes is due to a rather small volume of cytoplasm in the cells in histological sections causing difficulty in correlative studies of various staining technics applied on sections. This difficulty can be to a considerable extent alleviated by using cultured specimens, for in tissue culture a cell of cuboidal form often settles down and becomes quite flat (6).

Materials and Methods

Fibroblasts or astrocytes grown, using a roller tube method (7), in cultures of cerebellar cortex obtained from new-born puppy, kitten and human embryo were used in the serial investigations performed in our laboratory. Various staining technics as listed in Table 1 were applied on these cultured cells. Details of staining technics have been described in previous publications (8-13).

Results

Results are summarized in Table 1. As can be deduced from the findings

obtained, cytoplasmic granules which take neutral red supravitaly (neutral red granules) are a phosphoprotein complex containing occasionally cholesterol and its esters (see Ogawa et al., 1960 (9) also), and further the neutral red granules

Table 1. Staining Properties of Lysosomes of Cultured Astrocytes.

	Ogawa et al. (1960)
A. Supravital stains.	
Janus green*	Mitochondria stained
Neutral red	Brick red
Methylene blue	Blue(orthochromatic)~purple(metachromatic)
Toluidine blue	Blue(orthochromatic)~purple(metachromatic)
Azure B	Blue(orthochromatic)~purple(metachromatic)
Sudan black in 50% ethanol	Black
B. Stains following fixation.	
Toluidine blue in 1% acetic acid	Unstained
Alcian blue	Unstained
Best's carmine	Unstained
Feulgen reaction	Negative
Plasmal reaction	Negative
Periodic acid-Schiff (PAS)	Positive
Chloroform-ethanol→PAS	Unstained
Sudan IV	Orange red (sudanophilic)
Sudan black (SB)	Black (sudanophilic)
Chloroform-ethanol→SB	Unstained
Acetone→SB	Unstained
Performic acid-Schiff (PFAS)	Positive
Osmium tetroxide or Kolatchev	Black (osmiophilic)
Modified luxol fast blue	Positive
Nile blue sulfate	Suggestive
Acid hematein (Baker)	Positive
Schultz	Positive
Naphthol yellow S	Cytoplasm diffusely stained
Millon	Cytoplasm diffusely stained
Phosphotungstic acid-hematoxylin (Mallory)	Mitochondria and occasionally granules stained
Acid fuchsin (Kull)	Mitochondria stained
Jacobson	Faintly reddish violet when retained
C. Enzymatic activity.	
Succinic dehydrogenase system	Intramitochondrial distribution
Lactic dehydrogenase system	Intramitochondrial distribution
Malic dehydrogenase system	Intramitochondrial distribution
Alkaline phosphatase	Positive
Acid phosphatase	Positive
Adenosine triphosphatase	Intramitochondrial distribution
Monoamine oxidase	Possibly intramitochondrial
Lipase (Tween 80)	Positive
Cholinesterase (true & pseudo)	Suggestive
Remarks	LYSOSOME: Phospholipoprotein complex containing cholesterol.

* Janus green, neutral red, methylene blue, toluidine blue and azure B are dissolved respectively in 0.25 M. sucrose.

are positive for acid phosphatase (Figs. 1-4), a part of alkaline phosphatase (Figs. 5-8), and lipase (Tween 80). In some cases alkaline phosphatase was also positive in the cytoplasmic area corresponding to the Golgi zone. Phosphatase positive granules (lysosomes) were observed even in mitotic cells (Figs. 4, 8). Lysosomes were observed in (Fig. 1) or around (Fig. 3) the Golgi zone, and in some cases scatteredly in the cytoplasm (Fig. 2).

The size of lysosomes in the acid phosphatase preparations varied from 0.1 to 1.3μ with an average of approximately 0.5μ .

Discussion

The findings obtained in the present investigation indicate that the neutral red granules are a phospholipoprotein complex and in reality correspond to lysosomes described biochemically by de Duve et al. in 1955 (1). This is in good agreement with the histochemical works performed by Holt (14) that the highest significant acid phosphatase activity in the rodent liver is associated with maximum retention of phospholipid. Furthermore, the fact that the neutral red granules are lysosomes is substantiated by the report that a majority of neutral red injected intravenously into the rat was recovered in the lysosomal fraction of liver homogenates (5).

Much biochemical as well as cytochemical works have been done on mitochondria in the past few decades. Peculiarly enough, however, the chemical works on the neutral red granules, which are also an important cellular organelle, have been rather ignored. The present pioneering investigation seems to open the way for serial chemical works on the neutral red granules in future.

It should be mentioned here that a part of alkaline phosphatase and lipase (Tween 80) were demonstrated histochemically in lysosomes of cultured cells. Holt (15) also investigated that esterase showed a similar distribution pattern to that of acid phosphatase positive granules (lysosomes) in the rodent liver. According to tissue fractionation studies, however, both esterases (16) and alkaline phosphatase (17) have been found to be in the microsomal fraction. This discrepancy between biochemical and histochemical findings remains to be clarified.

As one of releasors of lysosomal enzymes in the cells, anoxia has been suggested by de Duve (5). This concept is supported by the finding obtained in our laboratory (18) that lysosomes are decreased in number in fibroblasts cultivated under anoxic condition with a concomitant appearance or increase in number of degenerative phospholipid granules devoid of phosphatase activity.

Although it has been suggested that the formation of lysosomes in the cells has some connection with pinocytosis (19, 20), a detailed mechanism of formation of lysosomes needs further investigation.

Summary

1. Lysosomes were studied from the morphological and cytochemical aspects, using cultured cells.
2. It was concluded that the neutral red granules are a phospholipoprotein complex and correspond in reality to lysosomes described by de Duve et al. in 1955.
3. Some properties of lysosomes were also briefly described.

Legends for figures

Figs. 1-4. Modified Gomori's method for the demonstration of acid phosphatase (β -glycerophosphate as substrate, pH 5.2).

Fig. 1. A fibroblast from a 10 day-old culture of puppy cerebellum. Acid phosphatase positive granules (lysosomes) are observed mainly in the Golgi zone. $\times 1500$

Fig. 2. A fibroblast from a 8 day-old culture of kitten cerebellum. Lysosomes are scattered in the cytoplasm. $\times 1500$

Fig. 3. A fibroblast from a 10 day-old culture of puppy cerebellum. Lysosomes are observed in the cytoplasmic area surrounding the Golgi zone. $\times 1500$

Fig. 4. A mitotic cell, possibly a fibroblast, from a 10 day-old culture of puppy cerebellum. $\times 1500$

Figs. 5-8. Modified Gomori's method for the demonstration of alkaline phosphatase (β -glycerophosphate as substrate, pH 9.2).

Fig. 5. A fibroblast from a 10 day-old culture of kitten cerebellum. Alkaline phosphatase is also positive in lysosomes. $\times 1500$

Fig. 6. A standard astrocyte from a 7 day-old culture of kitten cerebellum. $\times 1500$

Fig. 7. A fibroblast from a 10 day-old culture of puppy cerebellum. $\times 1500$

Fig. 8. A mitotic cell, probably a fibroblast, from a 10 day-old culture of kitten cerebellum. $\times 1500$

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Discussion

Dr. Tanaka: (1) How was the relationship between the cultural conditions and the granules? (2) The vital staining by acidic and basic dye stuffs revealed the intracellular granules, similar to the lysosome by Novikoff, and also myelin. The staining

may be due to the internment action of exogenous substance, which diffused into the cytoplasm, in granular structures having no relation to mitochondria and Golgi apparatus.

- Dr. Ogawa : These granules increased in number in parallel with the cultural time. (Ogawa et al : 1960). As to the decided mechanisms of lysosome formation, it seems to need further wide histochemical, cytochemical and electron microscopic studies. Your findings of myelin-like structures may coincide with our cytochemical results in terms of a phospholipoprotein nature but on the other hand, Pasteel and his co-workers have shown that the acid phosphatase positive β -granules were not myelin-like but granular.
- Dr. Hosoda : De Duve has shown other enzyme activities such as β -glucuronidase, RNA-ase and proteinase in lysosome fractions, and, on the other hand, our histochemical studies on spread preparations of subcutaneous tissues of rabbits and mice have revealed the disagreement of the β -glucuronidase positive granules with those of acid phosphatase. Have you any observation about the localization of these enzymes? How did you differentiate fibroblasts and macrophages?
- Dr. Ogawa : We have observed on alkaline phosphatase, acid phosphatase and lipase (by Tween 80). Further studies other enzymes will be performed later.
The differentiation of cultured macrophages and fibroblasts is very easy. (Ogawa ; 1960)

* Supported by a grant from the Rockefeller Foundation (CA MNS 60236)

Fast Green 染色による赤血球系細胞内の グロビンの定量について

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The Quantitative Estimation of Globin in Erythrocytic Cells by Fast Green Staining.

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There had been no suitable method for the quantitative estimation of cellular specific proteins, of which synthesis is an important problem in relation to the cell-differentiation and cell-maturation. The authors have succeeded in measuring the quantity of globin in each erythroblast by use of fast green FCF.

The present studies have reported the method and some results about the measurement of globin synthesis in erythroblast of rabbits.