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Distribution of Lysosomes in Cultured Cells and Ascites Tumor Cells[1),2),3)]

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(With 1 Plate)

Working with cell fractionation of non-specific acid phosphatase in the rat liver, de Duve and his colleagues have concluded that the liver contains a special group of lytic particles, which are recovered mainly with small mitochondria, but also to some extent with microsomes. The particles are designated as 'lysosomes' in view of that they are morphologically distinct entities. Essner and Novikoff (1961) studied electron-histochemically acid phosphatase rich particles occurring in peribiliary positions of hepatic cells, and stated that "lysosomes" reported by de Duve and 'dense bodies' demonstrated by means of electron-microscopy were the same entities. Ogawa *et al.* (1960) reported in cultured neural cells that cytoplasmic granules stained supravitally with neutral red gave positive reaction for acid phosphatase, suggesting that they are lysosomes. The staining method applied in tissue sections for acid phosphatase activity (Gomori, 1952) produced reliable information in cold formol-calcium fixed and frozen-sectioned tissues (Baker, 1946).

Recently, our histochemical data on the enzyme changes showed the following facts in the liver of rats fed 3'-methyl-4-dimethylaminoazobenzene: Use being made of Gomori's acid phosphatase technique, the activity of acid phosphatase was demonstrated in the peribiliary position in parenchymal cells of the normal rat liver. In regenerating areas of experimental livers, lysosomes appeared in the peribiliary position in some hepatic cells, while in others they showed irregular distribution. In the regions of hypertrophy and of hepatoma, a dispersion of lysosomes was detected in the cytoplasm. The lysosome distribution as mentioned above seems to be associated with a morphological expression of anaplastic changes taking place in the course of azo-dye carcinogenesis.

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2) This paper is dedicated to Professor Sajiro Makino, Zoological Institute, Faculty of Science, Hokkaido University, Sapporo, in honor of his sixtieth birthday, June 21, 1966.

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The present study was undertaken to identify hepatic cells in culture, and further to examine histochemically cellular changes induced by *in vitro* cultivation, following Gomori's acid phosphatase technique. In parallel, the acid phosphatase activity was investigated in ascites tumor cells as well as in cell strains in culture.

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Materials and methods

Methods of culture: Liver cells in primary culture from white rats, and two tissue culture strains cells from HeLa and H–99, were furnished materials for this investigation. H–99 is a tissue culture strain of a rat hepatoma origin established recently by Drs. S.H. Hori and M. Sasaki in Makino laboratory. The cells were grown at 37°C in a medium containing 15 per cent of bovine serum and 85 per cent of a nutrient medium which consists of 0.5 per cent lactalbumin hydrolysate, 0.1 per cent yeast extract in Earle's saline solution, 200 mg/l of glutamine, and 100 units/ml of penicillin. Cultured cells were treated with 0.2 per cent trypsin in Rinaldini's saline solution for disjoining cells from the glass wall. After washing with Rinaldini's saline solution the cells were planted on 12 × 32mm coverslips inserted in modified Leighton tubes. To each tube was added one milliliter of the medium. The two strains cells were subcultured by means of the stationary tube culture method. Liver cells were subjected to a roller tube technique, no subcultures having been used.

On the 3rd or 4th day in subculture, the coverslips with adherent cells were removed from the tubes, and rinsed in isotonic saline.

The following 5 kinds of rat ascites tumors, Yoshida sarcoma, Takeda sarcoma, GTD–4, H–96 and MTK–III, provided also materials for the present study. GTD–4 is an ascites tumor strain established from a hepatoma in the Institute of Cancer Immunopathology. H–96 is an ascites tumor strain established recently by Dr. S. H. Hori in Makino laboratory from a rat hepatoma. MTK-III is an ascites tumor strain of a rat hepatoma origin and has serially been transferred for several years in the laboratory. On the 4th day of intraperitoneal transfer of tumor cells, ascites tumor cells were smeared on coverslips and dried at room temperature.

Coverslips carrying culture cells and ascites tumor cells were fixed 30 to 60 minutes at 0°C to 4°C in the following fixatives: (1) 4% formaldehyde, and 1% $CaCl_2$; (2) 4% formaldehyde, 7.5% sucrose, and 0.067M phosphate buffer, pH 7.4 (Holt and Hicks, 1961); (3) 4% formaldehyde in Rinaldini's saline solution; (4) 6.5% glutaraldehyde in 0.1M phosphate buffer, pH 7.4; (5) 3% formaldehyde, 1.5% glutaraldehyde, 7.5% sucrose, and 0.1M phosphate buffer, pH 7.4; (6) 2.5% formaldehyde, 2.5% glutaraldehyde, 7.5% sucrose, and 0.1M phosphate buffer, pH 7.4.

Histochemical method: The acid phosphatase activity was demonstrated by means of Gomori's technique (1952). Following fixation, cultures or ascites were rinsed briefly in distilled water, and incubated 60 minutes in a freshly prepared substrate medium. At the end of the incubation period, coverslips were rinsed in distilled water, immersed for a few minutes in a dilute ammonium sulfide solution, rinsed briefly in distilled water, dehydrated

in graded ethanol, cleared in tetrachloroethylene, and mounted in a synthetic resin.

As controls, the above subjects were incubated with the substrate medium containing 10 mM NaF or the medium lacking in sodium β-glycerophosphate.

Results

Culture cells fixed with the following solutions (1), (2) and (3), for 1/2 hours, showed a considerable shrinkage of the cytoplasm, while an intense acid phosphatase activity was demonstrated in the whole cytoplasm. On the other hand, specimens fixed in fixative (4) showed better preservation of cells, though this fixative was more inhibitory to acid phosphatase than formaldehyde containing fixatives.

Based on the above facts, it is expected that a mixture of formaldehyde and glutaraldehyde would be favorable for demonstrating acid phosphatase in cultured cells. The cultured cells fixed with fixative (6) showed less shrinkage of the cytoplasm than those with (5), along with a moderate acid phosphatase activity. For ascites tumor cells, the materials fixed with fixative (1), (2) and (3) exhibited the intense activity of acid phosphatase, while less activity occurred in those fixed with (4), (5) and (6).

Fixation was found to be prerequisite for acid phosphatase staining, because unfixed materials subjected to the same histochemical procedure were negative. Control coverslips always gave negative reaction for acid phosphatase activity.

Normal liver: On the 3rd to 4th day following a primary culture of rat liver, epithelium-like cells migrated from the explant margin forming a monolayer. Various patterns of the acid phosphatase activity were found in those cells. In general, the cells far distant from the explant showed weaker acid phosphatase activity. Some cells in the vicinity of the explant showed intensely stained fine granules (lysosomes) surrounding the nucleus, while in some others lysosomes distributed in the periphery of the cytoplasm in the same fashion as in those found in liver cells *in vivo*. At the periphery of the outgrowth, lysosomes located exclusively in the nuclear hof, or otherwise they scattered evenly throughout the cytoplasm as discrete fine particles. In still other cells, lysosomes occurred in certain places of the cytoplasm outside the nuclear hof. In mitotic cells, a few masses of lysosomes were observed in the periphery of the cytoplasm. Nuclei were always negative.

H–99: Lysosomes in interphasic cells were circumferentially disposed, not in the nuclear hof. In some cells, the lunar cluster of lysosomes surrounded the nuclear membrane. The vast majority of mitotic cells, 95.3 per cent in frequency, showed the acid phosphatase activity.

HeLa: Most satisfactory and consistent results were obtained in cells growing in monolayer. Cell growth within three to four days after subculture was most favorable for study. Staining pattern varied from cell to cell according to different mitotic stages. In most interphasic cells, the acid phosphatase activity was

shown by fine particles surrounding the nucleus, while a few cells showed discrete coarse and fine particles evenly distributing in the whole cytoplasm. In 27.1 per cent of mitotic cells, lysosomes were agglutinated into some masses and located in the periphery of the cytoplasm. Damaged cells were remarkable by a diffuse intense staining of the whole cytoplasm and nucleus.

Yoshida sarcoma, Takeda sarcoma, GTD-4, H-96 and MTK-III: Ascites tumor cells derived from Yoshida sarcoma, Takeda sarcoma and MTK-III showed a rather similar distribution pattern of lysosomes to that in HeLa cells: in most cells lysosomes occurred in the nuclear hof, while in some others they were scattered evenly within the cytoplasm. A series of intergardes was observed. H-96 cells were classified into two groups on the basis of the lysosome distribution. H-96 cells usually proliferate in the form of islands. Some islands consisted of cells rich in lysosome, whereas other islands contained cells strikingly poor in lysosomes. GTD-4 showed in most cells a circumferential distribution pattern of the lysosomes in the cytoplasm.

Comments

There is a variety of opinion on the optimal type of fixation for the demonstration of acid phosphatase. Recent data in this field have been reviewed by Gomori (1952), Novikoff (1955) and Pearse (1960). Eränkö (1951) reported that acetone fixed paraffin embedded tissues were unstatisfactory for use with the metal-salt method; he recommended that the technique was useful only for unfixed or formalin-fixed frozen sections.

Recently, lysosomes were demonstrated in cultured cells fixed with formaldehyde (Rose, 1961, Munro, 1964). Sabatini *et al.* (1963) noted that the fixation with glutaraldehyde gave a relatively undistorted result for cellular structure being superior to that obtained with formalin. Robbins and Gonatas (1964c) successfully used glutaraldehyde as fixative for acid phosphatase staining, but it inhibited the enzyme activity to considerable extent, and required long time of incubation. The present study resulted in that a mixture of formaldehyde and glutaraldehyde gave a consistently good preservation of morphology as well as of enzyme activity.

It is very difficult to identify hepatic cells by the exlcusive application of this technique. Referring to the present results in connection with those of other histochemical methods, such as for example PAS reaction, it seems apparent that a few cells possessing lysosomes in the periphery of their cytoplasm correspond to parenchymal cells of the liver. No such cells were observed in the periphery of the outgrowth in cultures. Evidence presented suggests that hepatic cells lost their characteristic nature during a short term cultivation, so far as the acid phosphatase activity is concerned.

Robbins and Gonatas (1964a, b) examined the ultrastructure in the course of mitotic division by means of electron microscopy, and reported that a complex that first made its appearance at prophase but became most prominent at metaphase

was a partially membrane bounded cluster of dense osmiophilic bodies. These clusters were shown to be derived from multivesicular bodies, being acid phosphatase positive. Novikoff (1962) described that, in the liver of new born rats, the non-dividing cells showed the typical pericanalicular distribution of lysosomes, while in dividing cells lysosomes lay adjacent to the nucleus.

The results of the present experiment, however, indicated that HeLa strain showed the acid phosphatase activity in 27.1 per cent of dividing cells, whereas H–99 strain cells showed the activity in 95.3 per cent of the mitotic cells. Cultured liver cells exhibited various types of lysosomal distribution in the interphasic cells.

Novikoff (1962) stated further that, in Novikoff hepatoma cells, lysosomes often appeared unrelated to the position of the Golgi apparatus. The latter was in the "nuclear hof" area, while the lysosomes were widespread in the cytoplasm. However, some ascites tumor cells contained lysosomes which showed an unusual concentration in the nuclear hof. He regarded these cells as the cells preparing for mitosis.

In striking contrast, data presented by the author's study indicated that ascites tumor cells so far examined, except GTD–4, showed lysosomes locating almost exclusively in the nuclear hof in the majority of cells. On the other hand, GTD–4 cells were characterized in most cases by a circumferential distribution pattern of the lysosomes.

Summary

The distribution pattern of lysosomes was investigated in cultured cells from HeLa, H–99 and normal liver, as well as in ascites tumor cells from Yoshida sarcoma, Takeda sarcoma. GTD–4, H–96 and MTK–III, use being made of Gomori's acid phosphatase technique. A mixture of formaldehyde and glutaraldehyde, and formol calcium were employed as fixatives.

No definite pattern of lysosome distribution was observed in cultured liver cells, H–99 and H–96 cells. Lysosomes were found preferentially in the nuclear hof in the majority of cells from Yoshida sarcoma, Takeda sarcoma, MTK–III and HeLa strain. GTD–4 cells were characterized in most cases by a circumferential distribution of lysosomes.

HeLa cells showed the acid phosphatase activity in 27.1 per cent of dividing cells, whereas in H–99 cells the activity was noted in 95.3 per cent of mitotic cells.

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Explanation of Plate XXII

Fig. 1. H–99.

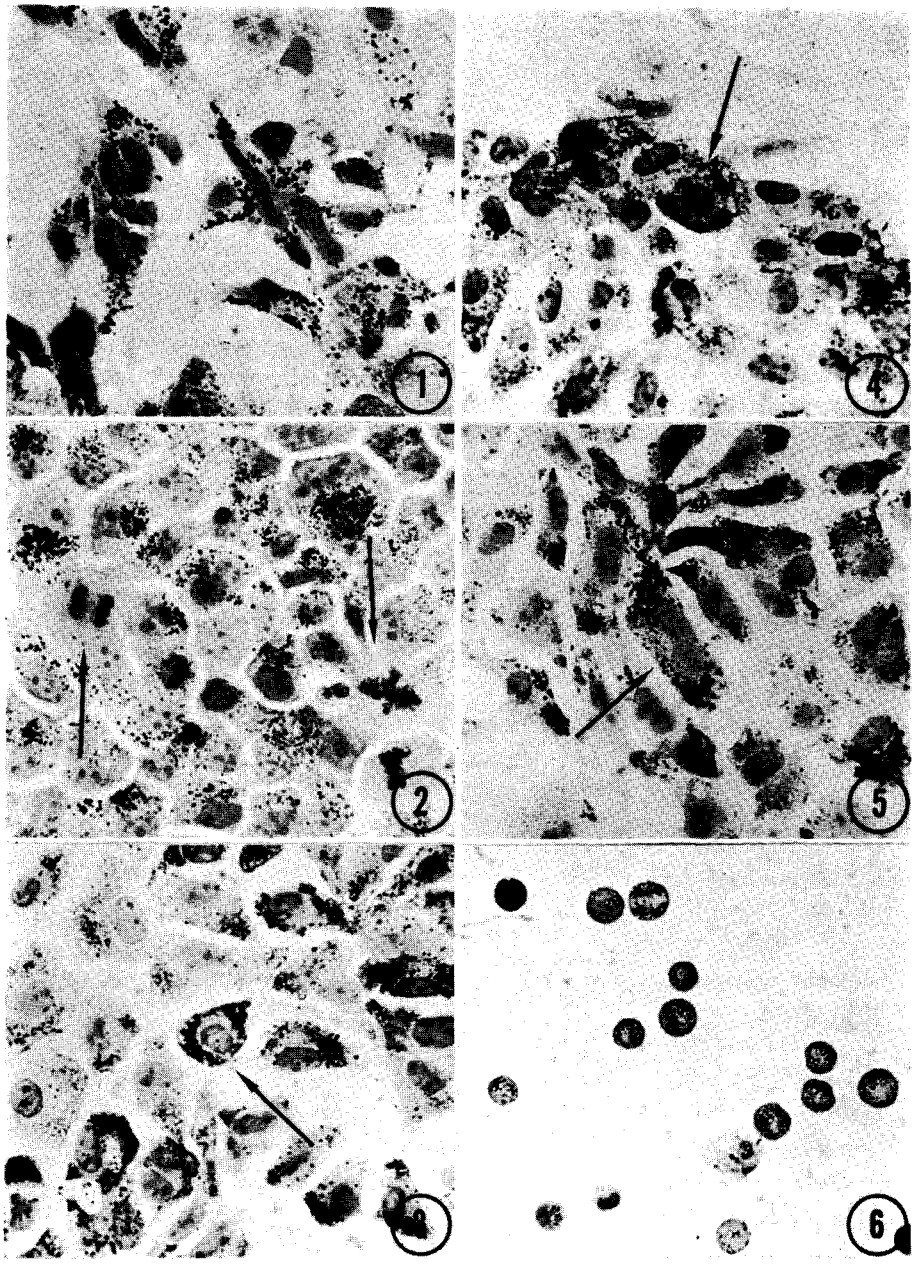
Fig. 2. HeLa cells. Note metaphasic cells, indicated by the arrows.

Fig. 3. Liver cells. 7 days after *in vitro* transfer. Note the parenchyma-like cell.

Fig. 4. Same material showing mitotic cells with the prominent lead sulphide particles.

Fig. 5. Same material showing the distribution of lead sulphide particles in the binucleated cell, indicated by an arrow.

Fig. 6. Yoshida sarcoma.



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