POSSIBLE ROLE OF LYSOSOMES IN CELLS OF "THE EPITHELIOID CELL SYSTEM"

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Granulomatous lesions which were induced with living BCG, heat-killed BCG and mycobacterial fraction (glycolipid) in rabbit lungs were studied by electron microscopy. The transformation of macrophages into epithelioid cells and giant cells observed in granulomatous lesion was named "the epithelioid cell system". According to lysosomal function, this system can be divided into three stages: (1) heterophagic stage, (2) intermediate stage and (3) autophagic stage. At every stage dense cuplike structures in the plasma membrane and the Golgi-associated tubular system in cytoplasm were observed. By biochemical assay of granulomatous lesion lysosomal cathepsin B revealed a correlative increment of specific activity on time course after pulmonary inoculation. Cathepsin B and lysozyme showed a high level of specific activity. However, specific activity of lysozyme in granulomatous lesion induced with living BCG was higher than that in granulomatous lesion induced with mycobacterial glycolipid.

Since Metchnikoff described the presence of epithelioid cells and giant cells in tuberculous lesions (17), many investigators have attempted to observe these cells. These cells are found in granulomatous lesions as well as in lymph nodes of patients suffering from not only tuberculosis, but sarcoidosis, silicosis and berylliosis. There is general agreement that macrophages transform to epithelioid cells and multinucleated giant cells (5, 28, 29).

The author has reported that alveolar macrophage specifically transformed to epithelioid cell and multinucleated giant cell after ingestion of *Mycobacterium* and glycolipid from *Mycobacteria*, and has proposed the term "epithelioid cell system (ECS)" for this series of gradation (20, 26).

Ultrastructure of cells in epithelioid cell system revealed two significant characterizations, one an abundance of lysosomes in cytoplasm and the other the architecture of the processes which are projected from the cell surface of the epithelioid cell (19).

The present study documents ultrastructural and cytochemical studies on granulomatous lesions induced with living and heat-killed BCG (*Mycobacterium bovis* strain BCG) and glycolipid from *Mycobacterium smegmatis*, and biochemical studies of lysosomal enzymes in granulomatous lesion. Attention is focused on lysosomes in the epithelioid cell system and the possible role of lysosomes in this system is discussed.

MATERIALS AND METHODS

Granulomatous lesions which were induced with living BCG, heat-killed BCG

and glycolipid (cord factor) were prepared for electron microscope observation (24). Granulomatous lesions were produced in rabbit lung after pulmonary injection through the chest wall of materials (living or heat-killed BCG and glycolipid) with Bayol F.

Primary fixation was carried out with 2% glutaraldehyde in 0.13 M phosphate buffer, pH 7.4 for 1 hour, and tissues were post-fixed in 2% osmium tetroxide in the same buffer. After dehydration, tissues were embedded in Epon. Thin sections were stained with uranyl acetate and lead hydroxide, and examined with a Hitachi HU-11Ds electron microscope operated at 100 KV or 75 KV, or otherwise indicated.

Some sections were electron stained with phosphotungstic acid (PTA) after being bleached with periodic acid (1), and subjected to Silver impregnation technique (14).

For cytochemical observation, tissues were incubated in Gomori's lead medium for acid phosphatase activity (7), in Nagata's incubation medium A containing Tween 80 for lipase activity (18) and in Hanker's incubation medium for non-specific esterase activity (8). For nonspecific esterase activity catalytic osmiophilic polymer generation from 3,3'-diaminobenzidine (DAB), following incubation of granulomatous lesions in 2-thiolacetoxybenzanilide (TAB)-copper-ferricyanide medium was carried out.

For biochemical studies of lysosomal enzymes, treated rabbit lung with living BCG and glycolipid was homogenized in 0.3 M sucrose containing 1 mM EDTA and fractionated by Hoffman's preparation method (11). Lysosomal acid hydrolases (acid phosphatase, β -glucuronidase, cathepsin B and lysozyme) were investigated in whole lesion preparation and fractionated lesion homogenate. α -N-Benzoyl-L-arginine ethyl ester hydrochloride (BAEE) as substrate for cathepsin B activity was used.

RESULTS

Granulomatous response to Mycobacterium and mycobacterial fraction

The injection of heat-killed BCG with Bayol F into the rabbit lung produced a granulomatous response at the site of injection. One week after pulmonary injection, light microscopic observation showed exudative pneumonia including mainly alveolar macrophage accumulation with infiltrations of small amounts of lymphocytes and neutrophils (PMN). This exudative lesion changed to granulomatous lesion faintly pink or grey and to firm lesion at 3 to 4 weeks after pulmonary injection, and some of them revealed necrotic foci. Granulomatous lesions mainly consisted of epithelioid cells, small amounts of fibroblasts and lymphocytes. Injection of living BCG induced a more extensive granulomatous response in the lung than injection of heat-killed BCG. A similar granulomatous response was produced with glycolipid from *Mycobacterium smegmatis* (cord factor, trehalose-6,6'-dimycolate).

Ultrastructure of granulomatous lesion

Three to 7 days after pulmonary inoculation with living BCG and heat-killed BCG, the lesion showed a granulomatous unit of a group of alveolar macrophages surrounded with lymphocytes.

Cytoplasm of alveolar macrophage contained a great number of lysosomes and phagosomes which contained tubercle bacilli and adjuvant droplets (Figs. 1, 2, 3). One characteristic of phagosomes was that they never contained both tubercle bacilli and adjuvant droplet, and each of them was phagocytized separately (Fig. 5). Acid phosphatase reaction product could be seen around a body of tubercle bacilli in a phagosome (Fig. 4) and on a degenerated intracellular organism in the digestive vacuole (Fig. 7). Lysosomes fused to part of the outer limiting membrane of the phagosome which contained adjuvant droplet (Fig. 1). Short, slender microvilli extended from the free surface and they were covered with a multi-ribboned layer, in which electron dense granules were aggregated. Golgi apparatus was well developed, however mitochondria and rough surfaced endoplasmic reticulum were few. The nucleus was round in shape. Two weeks after injection, telolysosomes increased in number, containing membrane fragment, myelin figures, amorphous aggregates and degenerated intracellular organism (Fig. 6).

Around 3 weeks after pulmonary inoculation, alveolar macrophage developed to epithelioid cell, which revealed two significant characterizations, one an abundance of lysosomes in the cytoplasm, and the other the architecture of the processes from the cell surface.

According to structure and distribution of lysosomes, epithelioid cell could be classified into two types, type A and type B (Figs. 8, 9). The cytoplasm of type A was rich in lysosomes, especially autolysosome which included cytolysosome and autophagic sequestration (Fig. 9). Several of them contained mitochondria, fragments of the endoplasmic reticulum, free ribosomes and ground cytoplasm segregated from the surrounding cytoplasm by a single membrane or sometimes by a multilayered membrane coat. Some membranes forming the coat still appeared to be continuous with the endoplasmic reticulum (Figs. 10, 11, 12) (4). Lysosomes filled with electron dense contents had an irregular shape, and were wrapped in a bit of cytoplasmic ground substance (16, 27).

Interdigitating cell processes projected from the cell surface and attached to adjacent cells. Some invaginations were formed by folding between the processes of the same cell projecting from opposite sides of the same cell surface (Fig. 13). Moreover, appearances similar to autophagic sequestration were formed by a part of the interdigitating cell processes projecting into the cytoplasm, and the interdigitating cell processes showed a vortex figure which contained a bit of ground cytoplasm, membrane element and mitochondria (Fig. 14).

The cytoplasm of type B cell was rich in free ribosomes, rough surfaced endoplasmic reticulum and well developed mitochondria; however lysosome and phagosome were few in number (Fig. 8).

Quantitative measurement of autolysosome including autophagic vacuole, cytosegresome and wrapping mechanism by lysosome indicated two classifications of epithelioid cell according to distribution of lysosomes (Table 1). This showed that type B cell was situated at the intermediate stage between the stage of macrophage and the type A cell of epithelioid cell. Therefore, the epithelioid cell system can be divided into three stages according to a functional point of view for lysosomes; that is, heterophagic stage, intermediate stage and autophagic stage (20).

At every stage, dense cuplike structures (29) appeared in the plasma membrane (Fig. 15), and similar vesicles were located under the plasma membrane. A

complicated network of branching tubules which may correspond to the Golgi-associated tubular system (13) was found in the cytoplasm (Fig. 16), and some ends of tubules showed continuity with the plasma membrane.

The injection of glycolipid from *Mycobacteria* showed similar findings except phagosome in macrophage contained only adjuvant droplet (Fig. 21).

Studies on electron staining of the epithelioid cell

Floating thin sections were stained with PTA after bleaching with periodic acid (1). When a high pH value of PTA solution (higher than pH 1.9) was employed, their stainability decreased. When the staining was applied to a sample

FIG. 1. Heterophagosomes(P) in alveolar macrophage in granulomatous lesion induced with heat-killed BCG. There are a moderate number of lysosomes (L) and phagosomes (P) in the process of fusing. The photograph was taken by HS-6 electron microscope.

FIG. 2. Alveolar macrophage in granulomatous lesion induced with living BCG fills numerous phagosomes in cytoplasm.

FIG. 3. The intracellular organism in alveolar macrophage of granulomatous lesion induced with living BCG shows degenerative change in morphology. One week after injection.

FIG. 4. Granulomatous lesion by living BCG incubated in Gomori's medium for acid phosphatase. Reaction products are seen around bacteria in digestive vacuoles (arrows). One week after injection.

FIG. 5. Phagosomes containing tubercle bacilli (P₁) and adjuvant droplet (P₂) separately in epithelioid cell.

FIG. 6. Higher magnification of telolysosomes in cytoplasm of epithelioid cell.

FIG. 7. Reaction products for acid phosphatase activities revealed in degenerative intracellular organisms in digestive vacuoles.

FIG. 8. Cytoplasm of epithelioid cell, which belongs to type B, contains well-developed RER, aggregated ribosomes and mitochondria. Both heterophagosome (P₁) and autophagosome (P₂) can be seen.

FIG. 9. Typical cytoplasmic appearance of epithelioid cell (type A) shows numerous lysosomes and well developed Golgi apparatus.

FIG. 10. Higher magnification of type A cell shows Golgi apparatus and autophagic vacuoles containing primarily endoplasmic reticulum, free ribosomes and unstructured cytoplasm.

FIG. 11. Outer membrane of autophagic vacuole which contains free ribosomes and unstructured cytoplasm shows continuity with outer membrane of mitochondria (arrows).

FIG. 12. Autophagic vacuole contains smooth membrane components with cytoplasmic matrix formed by a multilayered membrane coat.

FIG. 13. Invagination of cell processes shows zigzag array. All processes are composed by projections from the same cell.

FIG. 14. Whirl appearance by twisting of cell processes including cytoplasmic matrix.

FIG. 15. Cuplike structure in the plasma membrane (arrow).

FIG. 16. Complicated network of branching tubules which may correspond to the Golgi-associated tubular system.

FIG. 17. PTA staining shows strong stainability to lysosome. Photograph was taken by HS-6 electron microscope.

FIG. 18. Intensive impregnation of lysosome content can be seen by silver impregnation procedure. A photograph was taken by HS-6 electron microscope.

FIG. 19. Catalytic osmiophilic polymer generation from DAB, following incubation of granulomatous lesion induced with living BCG in TAB-copper-ferricyanide medium.

FIG. 20. Reaction products for lipase shown in lysosome and inside a vacuole membrane.

FIG. 21. ECS (epithelioid cell system) cell induced with mycobacterial glycolipid. Two weeks after injection. Phagosome (P) containing adjuvant droplet.

Scale line indicates 1 μ m.

TABLE 1. *Quantitative measurement of autolysosomes in the epithelioid cell system*

Classification	Area of Autolysosome Area of Cytoplasm	Number of Autolysosome per 100 μ^2 of Cytoplasm
Transformed Type	$1.37 \pm 1.34 \%$	6.85 ± 3.36
Epithelioid Cell		
Type B	5.32 ± 4.96	6.08 ± 1.94
Type A	19.12 ± 8.25	39.96 ± 10.68

$p < 0.05$

which had been fixed only with glutaraldehyde, stainability of the outer limiting membrane of the lysosome was negative (Table 2). An elegant visualization of the elastic fiber and collagen fiber was obtained. Plasma membrane and other intracellular membrane systems such as membrane of lysosome, pinosome and multivesicular body showed stainability to PTA (Fig. 17) (25).

Silver impregnation procedure after oxidation with periodic acid was applied to thin sections of granulomatous lesion fixed in OsO_4 and embedded in methacrylate (14). A linear arrangement of silver granules showed on the plasma membrane, mitochondrial membrane and membrane system of endoplasmic reticulum. Intensive impregnation of lysosome content could be seen (Fig. 18).

Other cytochemical observations on granulomatous lesion

Electron-dense reaction products both for nonspecific esterase and for lipase were located on the lysosome, on the phagolysosome, on the plasma membrane and inside of the vacuole (Figs. 19, 20) in similar sequences.

Biochemical studies on lysosomal enzymes on granulomatous lesion

An investigation of acid hydrolases in granulomatous lesion induced with

TABLE 2. *Stainability of cytoplasmic organella in epithelioid cell (19).*

Fixative	Stain	Membrane system						Connective tissue	
		Plasma membrane	MVB & Pinosome	Lysosome	SER & RER	Mitochondria	Nucleus	Collagen fiber	Elastic fiber
OsO_4	—	+	+	+	+	+	+	—	—
	UA	++	++	++	++	++	++	—	++
	Pb(OH)_2	+++	+++	+++	+++	+++	+++	—	++
	PTA	++	++	++	+	+	+	—	+++
Glutaraldehyde	—	—	—	—	—	—	—	—	—
	UA	+	+	+	—	—	++	—	++
	Pb(OH)_2	+	+	+	—	—	++	—	+
Glutaraldehyde + OsO_4	PTA	++	++	+	—	—	+	+++	+++
	—	+	+	+	—	—	+	—	—
	UA	+	+	+	+	+	++	—	++
	Pb(OH)_2	+++	+++	+++	++	++	+++	—	+
	PTA	++	++	+	—	—	+	+++	+++

FIG. 22. Enzyme activities of granulomatous lesion induced with living BCG.

FIG. 23. Enzyme activities of granulomatous lesion induced with mycobacterial glycolipid (trehalose-6,6'-dimycolate from *Mycobacterium smegmatis*).

living BCG and glycolipid from mycobacteria was carried out. Acid phosphatase, β -glucuronidase, cathepsin B (BAEE esterase) and lysozyme were found in homogenized granulomatous lesion (Figs. 22, 23). In a crude lysosomal fraction from BCG stimulated lung, acid phosphatase, β -glucuronidase and cathepsin B (BAEE esterase) showed their highest specific activity compared with other fractions. In correlative observations between specific activities of acid hydrolases and time course after pulmonary inoculation, there was no relation to acid phosphatase activity and β -glucuronidase activity. However, cathepsin B and lysozyme activities increased correlatively. Lysozyme activity in BCG induced granulomatous lesion showed twice the increase of that in glycolipid induced granulomatous lesion.

DISCUSSION

The origin of macrophage has not been confirmed at the present time. Some researchers have reported that macrophage arises from connective tissue or endothelial cell precursors. Some reviews have indicated that macrophages are transformed from lymphocytes or monocytes (2). Recently, Sutton and Weiss (1966) (29) documented the transformation of monocytes in tissue culture into macrophages, epithelioid cells, and multinucleated giant cells. Van Farth et al. (1972) defined the term "mononuclear-phagocyte system (MPS)" (31). The term "epithelioid" cell is frequently used to describe a large mature macrophage (Carr, 1973) (2).

Mycobacterium and mycobacterial fractions induced granulomatous lesion in which macrophage transformed into epithelioid cell and giant cell. *Nocardia* and its glycolipid fraction induced epithelioid cell granulomata. However, epithelioid cell proliferation does not occur by phospholipid fraction of *Nocardia asteroides* (21). Therefore, the author has proposed the term "epithelioid cell system (ECS)" (7), to describe cells induced with *Mycobacterium* and mycobacterial fraction, including monocyte, macrophage, epithelioid cell and giant cell.

From considerations of the general properties of lysosome (9), it is reasonable to search whether the epithelioid cell system possesses endocytosis, autophagy, crinophagy, residual bodies, exocytosis and extracellular digestion by lysosomal enzymes. From this point of view, epithelioid cell system can be divided into three stages, that is, heterophagic stage, intermediate stage and autophagic stage (20). In the heterophagic stage, it is supposed that several immunological phenomena may occur by BCG-phagocytized macrophage and BCG-stimulated macrophage. Macrophage may change to "activated macrophage" during this period, and immunological determinant also from digested mycobacteria and mycobacterial fraction may be released, revealing immunological information as well as information about T-lymphocyte.

It is suggested that the release of acid hydrolases from activated macrophage may induce tissue destruction in this stage. Davies et al. (3) mentioned that the interactions of inflammatory substance with macrophage or lymphocyte may lead to the release of neutral protease. The author reported here about cathepsin B enzyme activity in the epithelioid cell system. Tomino (1977) found new protease which resolved to substrate of elastin in experimental tuberculous cavity wall (30). In the granulation layer of experimental tuberculous cavity strong acid phosphatase reaction product for marker of lysosomal enzyme and nonspecific esterase reaction

products are found (22). Therefore, these results lead us to suggest that the release of proteolytic enzymes causes initiative reaction of cavity formation in the lung by immunological basis. On the other hand, the granulation layer surrounded by experimental tumor in mice which are injected with living BCG or BCG-Cell Wall Skelton (CWS) revealed acid phosphatase reaction product and nonspecific esterase reaction product (23). Keller suggests that activated macrophage produced a soluble factor which has a contact-mediated effect on target tumor cell (12). Hibbs also indicated that lysosomal constituents inject into target tumor cell (10). This information suggests some relationship between lysosomal enzyme from macrophage and tumor cell (15).

Sutton (29) suggested that the cupped structures represent lysosomes which discharge their content extracellularly. This secretory mechanism may coincide with eruptocrine secretion (13). Golgi-associated tubular system is a structure also associated with the secretory function. Thus it is necessary to consider that there is a multisecretory mechanism in the epithelioid cell system.

Autophagy may be the morphologic expression of one type of turnover. Ericsson mentioned several possibilities about autophagy (6). The autophagic stage of the epithelioid cell system may be the beginning of focal necrosis or cellular atrophy that leads to tissue atrophy, or it may be a representation of the degenerative process of the epithelioid cell system.

Membrane components in epithelioid cell showed different stainability to PTA. It is supposed that stainability to PTA depends upon the molecular structure of the membrane in each membranous organella, especially upon the various state, which may change according to the physiological conditions of the cells and the functions of the organella itself (25).

According to biochemical assay for acid hydrolases in granulomatous tissue, there is no relationship between biochemical enzyme specific activities and formation of reaction products on electron microscopic sections. Also there was no relationship on time courses of enzyme specific activities for acid phosphatase and β -glucuronidase. However, the activities of both cathepsin B and lysozyme revealed relative correlation on their time courses. Therefore, it is necessary to choose a suitable enzyme for the measurement of lysosomal function by lysosomal enzyme activity.

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