AN ULTRASTRUCTURAL LOCALIZATION OF LYSOSOMAL ACID PHOSPHATASE ACTIVITY IN AGING MOUSE SPLEEN: A QUANTITATIVE X-RAY MICROANALYTICAL STUDY

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The fine ultrastructural localization of acid phosphatase (AcPase) activity in mouse spleen ages; 1 day, 1 and 2 weeks, and 1, 2 and 10 months was carried out by cytochemistry, using cerium as the trapping agent. The AcPase enzyme activity was observed in the red pulp. The enzyme reaction product precipitates appeared dense and homogeneous, and were localized in the lysosomes of macrophages, phagocytic reticular cells and littoral cells. The presence of cerium in the reaction product precipitate was confirmed by X-ray microanalysis. The spectral line was present at $La=4.84$ KeV and did not interfere with osmium emission line at $Ma=1.914$ KeV. The results demonstrate variation in the degree of AcPase activity in different ages of the animal studied. The enzyme activity increased progressively from day one, recorded a peak on the first week and thereon, decreased gradually until the 10th month.

Endocytosis and lysosomal degradation play an important role in turnover of red blood cells. Erythrocytes are produced in the bone marrow, brought in circulation for a minimum number of days (about 120 days) and eventually sequestered. At physiological condition the spleen is the organ primarily responsible for the removal of "aged" and/or effete red blood cells from the circulation (44) and in lesser degree, relegated to other organs of the reticuloendothelial system (46). The spleen is a highly vascularized organ (47) and it acts as a filter for the blood. The passage of the blood through the sinusoidal system enables the formed element and the plasma to come in intimate contact with the splenic macrophages (11, 29, 36, 45, 49) thereby constantly checking on any damaged erythrocytes and/or foreign substances that might have gained access into the blood.

Acid phosphatase (AcPase) is one of the hydrolases contained in the lysosomes and has long been recognized as the marker enzyme for this organelle (8, 14, 27, 43). Moreover, the use of acid phosphatase activity as a marker for cell death and/or cell undergoing lysis have been established (5-7, 26).

In recent times, improved cytochemical methods for the demonstration of AcPase activity, using cerium as a capture agent have been reported (4, 23, 38). In

these methods, the enzyme reaction product is precipitated as cerium phosphate.

In the present undertaking, an attempt was made to determine the specific site of histochemical and ultrastructural localization of AcPase activity in mouse spleen. The degradation of normal "aged" red blood cells and acid phosphatase activity are investigated. Furthermore, it describes the changes in AcPase activity in different ages of the animal by X-ray microanalysis, using cerium as the trapping agent. The use of this metal is of advantage over the conventional lead in that its spectral line does not interfere with osmium.

MATERIALS AND METHODS

Experimental Animals: ddY strain mice (three animals per age group) ages; 1 day, 1 and 2 weeks, and 1, 2 and 10 months were kept on normal laboratory diet (Clea CE2, Tokyo, Japan) and free access to water *ad libitum*.

Fixation: The animals were killed by cervical dislocation. Thereafter, freshly removed spleens were cut into smaller pieces, fixed in 2% monomeric glutaraldehyde in 0.05 M cacodylate buffer (pH 7.2) containing 7.5% sucrose for 1 hr at 4°C. After fixation, the specimens were rinsed three times (30 min

each) at 4°C in fresh, sucrose-containing cacodylate buffer solution (pH 7.2). The tissue specimens were processed without freezing.

AcPase Cytochemistry: The Lanthanide-based method for the ultrastructural localization of acid phosphatase was employed (19). The specimens were pretreated with sodium borohydride (0.5 mg/ml) in 0.05 M Tris-maleate buffer (pH 7.2) for 60 min at room temperature, this being followed by the addition of 0.1% saponin under the same condition. The sections were then preincubated in a medium consisting of 0.05 M Tris-maleate (pH 5.2), 3 mM CeCl_3 and 10 mM 3-amino-1,2,4-triazolium for 1 hr at 37°C in a water bath, and then incubated in medium of the same composition containing 15 mM β -glycerophosphate for 2 hr at 37°C in a water bath. The specimens were washed overnight at 4°C in 0.05 M cacodylate buffer containing 7.5% sucrose (pH 6.0). As controls, tissue sections were incubated with a medium containing no substrate and incubated for 60 min at room temperature, in 0.05 M cacodylate buffer (pH 7.2) containing 50 mM NaF.

Postincubation Processing: After washing, the tissue sections were postfixed in 1% OsO_4 plus 1.5% $\text{K}_4\text{Fe}(\text{CN})_6$ in 0.01 M cacodylate buffer (pH 7.2) for 1 hr at room temperature. The postfixative solution was prepared by adding one volume 2% OsO_4 in distilled water to one volume 3% $\text{K}_4\text{Fe}(\text{CN})_6$ in 0.2 M cacodylate buffer shortly before use (24). The tissue sections were rinsed several times in cacodylate buffer, dehydrated in a graded series of alcohol and acetone, and embedded in Epon 812. Thin sections (0.2 μm) were examined without poststaining in Hitachi H-700 electron microscope at 200 kV.

X-Ray Microanalysis:

A. Qualitative Analysis: Point analyses were done using JEM 4000EX electron microscope, with attached Tracor Northern TN-5400 X-ray microanalyzer, loaded with routine software. At TEM, the reaction products (precipitate) in 100 lysosomes per animal were analyzed for 100 s live time at 300 kV. The use of high accelerating voltage in X-ray microanalysis recorded a significant increase of peak-to-background (P/B) ratio (31). The size of the beam was maintained at about 0.5 microns. The spectral peaks were identified and labelled automatically by the computer.

B. Quantitative Analysis: The X-ray count of cerium (main spectral line) was determined at the range between 4.680 and 4.980 KeV, while the background was determined at the range between 9.400 and 9.800 KeV. The peak (cerium) and the background ratio was computed automatically by the

computer.

Statistical Analysis: Significance was evaluated by 2-tailed student's t-test for the difference between means.

RESULTS

Cytochemical AcPase Reaction

After the incubation of the tissue sections in cytochemical AcPase medium, ultrastructural localization of acid phosphatase activity was observed in the reticular cells of mouse spleen. The morphological structure of these cells are varied, however, they all showed positive reaction with AcPase enzyme (Figs. 1-3). As shown in Fig. 3, the characteristic micropinocytic vesicles were distinct and distributed along the periphery of the cytoplasm. Likewise, the reticular fiber along the cytoplasmic process was prominent and could be observed in some cells. The mitochondria were present in moderate number and were spherical in shape. The vacuoles were conspicuous in some cells. In other reticular cell types, remnants of large erythrocytes could still be identified (Fig. 1). The enzyme reaction product in different reticular cells and in different age group of the animals exhibited an apparent homogeneous staining pattern. However, greater activity was demonstrated in 1-week-old mice, as manifested by the higher intensity of staining reaction (Fig. 2).

Likewise, a dense precipitate indicative of acid phosphatase activity was demonstrated in the lysosomes of a macrophage-like cell. In Fig. 4, the macrophage appeared somewhat polymorphic and with the characteristic ruffling cell membrane. The cytoplasm contains lysosomes, vesicles and vacuoles. The mitochondria, with well-developed cristae were numerous. The lysosomes exhibited variation in size, some attaining larger dimensions, stained heavily and appeared homogeneous.

Acid phosphatase activity was also observed in the littoral cells. As shown in Fig. 5, heavy deposits of enzyme reaction product were localized in the lysosomes. The cell exhibited a meshwork-like appearance located near, and lining the lumen of the sinus.

No localization of acid phosphatase activity was observed in tissue specimens treated with NaF.

Variation in the size, number and shape of the lysosome positive with AcPase was noted in different cells and in different age group of the animals studied.

X-Ray Microanalysis of Lysosome

A. Qualitative X-Ray Microanalysis

In the spleen tissue sections treated with

cytochemical AcPase medium, using cerium chloride (CeCl_3) as the trapping agent, a dense enzyme reaction precipitate, as a result of acid phosphatase activity with cerium was demonstrated in the lysosomes of macrophages, phagocytic reticular cells and littoral

cells. In these precipitates, the presence of cerium was confirmed by X-ray microanalysis (Fig. 6). As shown in the emission line spectrum, calcium and iron were also identified and labelled, suggestive of its presence in the reaction product.

FIGS. 1-5. Fine ultrastructural localization of AcPase activity in mouse spleen.

FIG. 1. A reticular cell showing positive reaction with AcPase enzyme in 1-day-old mouse. Remnant of large erythrocytes (E) is present within the cell. Unstained section. Bar = $1\ \mu\text{m}$

FIG. 2. Electron micrograph of a reticular cell in 1-week-old mouse. Vacuole (V). Unstained section. Bar = $1\ \mu\text{m}$

FIG. 3. Electron micrograph of a reticular cell in 2-week-old mouse. Vesicles (arrowheads) and reticular fiber (arrow) are distinct. Unstained section. Bar = $1\ \mu\text{m}$

Postfixation with OsO_4 plus $\text{K}_4\text{Fe}(\text{CN})_6$ for 1 hr improved the membrane contrast and did not interfere with cerium precipitation.

B. Quantitative X-Ray Microanalysis

The lysosomes positive with AcPase enzyme in

macrophages, phagocytic reticular cells and littoral cells were analyzed by point analysis. In the recorded spectra, peak label L_a and L_b from element cerium were present at 4.84 and 5.26 KeV, respectively. However, only the principal spectral line of cerium at

FIG. 4. A macrophage demonstrating extensive AcPase activity in 1-week-old mouse. Mitochondria, with distinct cristae are numerous. Unstained section. Bar= $1\ \mu\text{m}$

FIG. 5. Localization of AcPase activity in a littoral cell in 2-month-old mouse. The lysosomes are stained heavily and appeared homogeneous. Unstained section. Bar= $1\ \mu\text{m}$

$L_a = 4.84$ KeV was used in the computation of the peak-to-background (P/B) ratio, done automatically by the computer. Earlier, de Bruijn *et al.* (10) reported the spectral line of cerium at $L_a = 4.893$ KeV. The spectral line of cerium did not interfere with the emission line of osmium at $M_a = 1.914$ KeV (17).

The average X-ray count of cerium (main spectral line) and the background were computed separately,

and plotted against the age of the animal. As shown in Fig. 7, cerium and background peaks were prominent in 1-week-old mice.

The computed average peak-to-background ratio in 1 day, 1 and 2 weeks, and 1, 2 and 10 months old mice were compared. In Fig. 10, an increase in AcPase enzyme activity was noted from first day after birth, reaching a peak on the first week and from thereon, it progressively declined until the 10th

FIG. 6. A representative EPMA spectrum from lysosomal precipitate in 1-week-old mouse. Cerium (Ce) main spectral line at $L_a = 4.84$ KeV. Background X-ray count was done at point ($\rightarrow$). Other elemental peaks: Os (osmium), Ca (calcium), Fe (iron). Accelerating voltage = 300 kV.

FIG. 7. Average X-ray count of cerium (Ce) and background (B) plotted against the age of the animal.

FIG. 8. Quantitative distribution of Ce measured in terms of P/B ratio in different age group of mice studied.

month. At all ages examined, the changes in enzyme activity in one day, one week and second week old mice were found significant at $p < 0.05$.

DISCUSSION

To date, no system of nomenclature for the lymphoid cells is entirely satisfactory. Furthermore, reports on morphological studies done on spleen particularly of the red pulp are diversified.

The localization of AcPase activity in the spleen has been confirmed by light microscopic studies. Most investigators agree that in the lymphoid tissues the highest activity for the lysosomal enzymes, i.e. acid phosphatase occur in the red pulp of the spleen. However, views on the specific cellular localization of this enzyme are varied and obscure. In the rat, it has been localized in macrophages (33) and in both macrophages and reticular cells (21). In addition, Barka *et al.* (3) showed the localization of this enzyme in sinus endothelial cells. Similarly, this was demonstrated in human, guinea pig and monkey (16, 15). In mouse spleen, AcPase was reported to have been localized in reticulum cells (28).

The apparent differences may be attributed to the structural changes this organ undergoes (25) and the marked difference in the morphological structures among animal species (49, 39). Likewise, it should be mentioned that the origin and the transformation of many of lymphoid tissue cells are not fully elucidated.

In the present study, the histochemical and ultrastructural localization of AcPase activity was observed in the red pulp. Deposits of reaction products were observed in the lysosomes of macrophages, phagocytic reticular cells and littoral cells. The results are similar to what have been reported earlier in LM.

In literature, types of reticular cells have been characterized based on various parameters (20, 22, 37, 42). Their morphological impressions partly affirmed the characteristics reticular cell type and other splenic cells observed in the present work.

Reticular cells share a functional and morphological characteristic of macrophages. Such that, identification of these cells appear difficult. Nevertheless, as observed in the present work, the visible presence of reticular fiber inherent to reticular cells may prove useful in distinguishing this type of cell from a macrophage.

Limited literature is available on the electron microscopic studies of AcPase activity in the spleen. Daems and Persijn (13) reported the demonstration of this enzyme in the splenic macrophages. Their obser-

vations were similar to what has been noted in this work. The heterogeneity of lysosomal AcPase staining reaction density has been reported (2, 39).

In LM, splenic littoral cells have been identified as a type of histiocytes and demonstrated strong acid phosphatase activity (9). Later observation, however, showed that littoral cells are distinct structures lining the sinusoidal lumen and not identical with histiocytes (16, 12).

The results of this investigation have confirmed that changes in acid phosphatase activity are age-related. The possibility that the increase in AcPase activity as a consequence of increase metabolic digestion of phagocytosed substance could not entirely be excluded (30). It must be noted that X-ray microanalysis determine quantitatively the extent of enzymatic activity within the lysosome based on the reaction product (1).

The decline in AcPase activity from 1-week-old mice and gradually until the 10th month as shown in the decline of the P/B ratio (Fig. 8), may be correlated partially with (a) the decrease in the number of splenic cells expressing positive enzyme activity (b) weakening of enzyme reaction activity in cells actually showing positive reaction with AcPase enzyme (30) and/or the role of the spleen in the degradation of normal "aged" and/or worn-out erythrocytes may have been relegated to other organs of the reticuloendothelial system at the later stage of life. The latter observation may have been the point of direction when Weiss (48) asserted that the adult spleen is not essential to life.

The observed calcium (Ca) peak in the X-ray emission line spectrum, may suggest that this element is present in the enzyme reaction precipitate. Calcium, being an innate substance of the red blood cells, further avouched its presence in the enzyme reaction deposit. However, further assessment at this point maybe difficult and futile. The methodology used may not be sufficient and/or appropriate to further elucidate its significance.

Henceforth, the presence of erythrocytic fragments in the lysosomes of the red pulp splenic macrophages and phagocytic reticular cells, would suggest that these organelles play an important role in the degradation of "aged" and/or effete erythrocytes and other blood cells (13, 32, 40). Erythrocytes digestion and acid phosphatase activity has been reported (18).

In rats, after gamma irradiation, damaged lymphocytes and erythrocytes were observed to have been phagocytosed by the splenic macrophages (35).

The iron (Fe) peak observed in the recorded emission line spectrum, suggest that this element is also present in the enzyme reaction precipitate. This could be

attributed to the degradation product of hemoglobin from the phagocytosed erythrocytes. It would be difficult, however, to affirm conclusively this contention. It should be mentioned that $K_4Fe(CN)_6$ was used in this work.

In view of the morphological similarities of the splenic cell types characterized and identified to have demonstrated acid phosphatase activity, immunocytochemical studies, i.e. the application of monoclonal technique should prove useful in further confirmation of the result of the present work.

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