Immunohistochemical Demonstration of Lysozyme in Tissues and Blood Cells of Domestic Fowl

I. A Preliminary Report

By

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Summary. The distribution of hen egg white lysozyme (LZM) in domestic fowl tissues and blood cells was determined by use of the direct antibody techniques both immuno-fluorescent and -enzyme (horseradish peroxidase) labeled methods. Immunospecific LZM reaction was found in the serous tubular gland cells of oviductus proprius (magnum), the proximal tubules of the kidney, intestinal epithelium, Lieberkuhn's gland, and macrophages in the lung, spleen and lamina propria of the digestive tract. In addition, LZM was also found in the myeloperoxidase-less pseudoeosinophilic leukocytes (heterophils), monocytes and myelocytes of blood peripheral and marrow smears. This distribution of LZM is discussed in relation to its possible physiological role in avian tissues and cells and particularly to its known antibacterial functions. Results of experiments with the blood cell LZM support the hypothesis that avian LZM is capable of being used as "marker" enzyme in the differentiation and maturation of granulocytes and monocytes.

Avian egg white proteins are composed of several noticeable proteins, such as ovalbumin, ovomucoid, conalbumin, lysozyme, avidin, etc, which provide the developing embryo with both nutrition and protection. Many previous histochemical studies have been reported on the correlation between egg components and secretion of oviduct segments in domestic fowl. In immunohistochemistry, on the other hand, fluorescence antibody studies have localized ovalbumin, ovomucoid, conalbumin, lysozyme or avidin in the tubular gland or epithelial cells in steroid hormone stimulated immature female chickens. Normally, these egg white proteins would not be synthesized until the onset of sexual maturity, which occurs in chicks 3 to 4 month-old, when the gonads commence the production and secretion of steroid hormones. Suzuki et al. reported localization of ovalbumin in spawning hen oviducts.

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initially observed on the serous gland in oviductus proprius (magnum). Following studies of ovomucoid and lysozyme immunohistochemistry appeared in laying hen tissues and cells. In general, lysozyme’s antibacterial action is well documented, and its wide but selective distribution in animal tissues and human tissues and cells has been determined by a variety of biochemical and histochemical studies. In addition, chicken polymorphonuclear leukocytes (PMNs), in contrast to mammalian heterophil PMNs, are lacking myeloperoxidase (MPO) and associated with lysozyme and at least three other cationic proteins. Detailed immuno-morphological analysis of hen egg white lysozyme may, therefore, be helpful in examining and understanding the general problem of antibacterial function and blood cell differentiation as the marker protein in domestic fowl.

This study further investigates localization of lysozyme in various tissues and peripheral and marrow blood cells of laying hens by use of direct immunohistochemical methods.

Materials and Methods

Antisera.

The antigen used in this study was crystalline lysozyme (LZM, grade 1, Sigma Co. Ltd.) prepared from hen egg white. One unit will produce an O.D. of 0.001/min at pH 6.24 at 25°C in a 2.6 ml suspension of Micrococcus lysodeikticus and usually 17,500 to 30,000 units/mg. Five milligrams of LZM dissolved in 2 ml of 0.15 M saline and equal volume of Freund’s complete adjuvant were injected into New Zealand male rabbits. Three weeks after the first immunization, injections of antigen mixed with Freund’s incomplete adjuvant were repeated at 2-week intervals in order to obtain as high a titer of antiserum as possible. Each rabbit was then bled from the carotic artery 8 days after the last intravenous booster injection of 3 mg of LZM, with a preliminary administration of 10 mg of diphenhydramine-HCl (“Restamine”, Kowa Pharmaceutical Co. Ltd.). The crude gamma globulin fraction of rabbit anti-LZM serum was prepared by precipitation using ammonium sulfate, and conjugated with fluorescein isothiocyanate (FITC, “isomer I”, Sigma Co. Ltd.) or horseradish peroxidase (HRP, “Type VI”, Sigma Co. Ltd.) according to the published procedures. Purification of the conjugated antibodies was performed on Sephadex G-25 and DEAE-cellulose ion exchange columns according to the methods of McDevitt et al. in the case of FITC, and Yamashita et al. in the case of HRP. The molar ratio of the conjugated solutions was 0.75 (F/P) and 2.3 (E/P), respectively. The antiserum obtained was titrated by the passive hamagglutination test of Stavitsky. As a result, the serum showed a titer of 1:327,680, using 8 mg of antigen. Gel diffusion tests carried out on the Ouchterlony agar plate, using 1% Noble agar (Difco Co. Ltd.), indicated that the contaminator of antigenic LZM was common to ovalbumin, ovomucoid and conalbumin. The anti-LZM serum was absorbed by above mentioned proteins (Fig. 1) prior to preparations of fluorescein or enzyme-labeled antibody solutions. The immuno-specificity of the antiserum was also tested by immuno-electrophoresis in 1% Noble agar in a Veronal-HCl buffer (0.05 M, pH 8.6, ionic strength=0.05). Antigenic LZM showed a single cationic precipitation arc by the anti-LZM serum treated with absorpton.

Tissues and blood smears.

Seven 15-month-old white Leghorn laying hens were used in this study. Each homogenate of the liver, pancreas,
small and large intestine, kidney, spleen and heart tissue, and blood plasma showed antigenic cross-reactions against the absorbed antiserum, revealed by double diffusion analysis. After killing, the tissue blocks of the hen oviducts were quickly frozen with dry ice-acetone. The sections, cut at 8 μ in thickness in a cryostat at −16°C, were fixed with various fixatives respectively for 30 min at room temperature to determine the most satisfactory fixative, according to the recommendation of Yasuda et al. Consequently, 0.5% glacial acetic acid-ethanol was found to be the most satisfactory fixative for preservation of the tissue antigen. Similarly, small tissue blocks of various organs for paraffin embedded sections and peripheral and marrow blood smears were fixed with the same fixative. All specimens were reacted with the labeled antibody solution in the direct method. The tissues were tested for fluorescence with an Olympus Fluorescence Microscope (Type BH-RFL) and for peroxidase reaction products using 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma Co. Ltd.) and hydrogen peroxide system in a 0.05 M Tris-HCI buffer at pH 7.6. The sections used as a control examination reacted in several ways according to the description of Yasuda et al. General histological and hematological appearances were also observed on slides stained with Mallory-azan or Giemsa staining, respectively.

Results

Similar results were obtained by both direct methods of immunohistochemistry. Specific sites of LZM localization both tissues and blood cells examined are illustrated in Figs. 2-24 and summarized as below.

In the infundibulum (tuba Fallopii), charaza forming portion, specific fluorescence was observed in the secretory portion of the serous tubular gland of lamina propria mucosae and the supranuclear region of epithelium (Fig. 2). Figures 3 to 7 illustrate the intense specific fluorescence and enzyme staining products associated with the serous gland cells which lie beneath the surface of the epithelial layer in the magnum, albumen-secreting portion, although both brilliant and pale fluorescent areas were distinctly found in the case of FITC-labeled antibody method (Fig. 3). Lysozyme was observed from the gland to the lumen, suggesting that the secretory products pass through the excreting duct and are excreted into the lumen of the oviduct. Also, immunoreactive LZM sites were occasionally seen coating the free surface of mucous epithelia but not epithelial cells in the upper magnum segment (Figs. 3, 4, 5 and 6). In the lower magnum segment, however, brilliant fluorescence was observed in the mucous epithelia and occasionally in the some cells of excreting duct (Fig. 7). In the isthmus (shell forming portion), LZM was found in some epithelia and on the surface of mucous membrane when the egg was located in the lower portion of the isthmus (Fig. 8). No immunoreaction was noted in the egg-shaped swelling part of the vagina.

In the kidney, LZM was identified in the proximal convoluted tubules of the cortex (Figs. 9, 10, 11 and 12). Occasionally, LZM was found in some epithelia of distal convoluted tubules (Fig. 11). The immuno-positive findings were small granular, with the granules located mainly near the apical region just below the brush border. No reaction was noted in glomeruli, Henle’s loop, and collecting tubules.

In the jejunum, moderate reactions to LZM were apparent in Lieberkühn’s gland (Fig. 13). Paneth cell was not found in the digestive tract of chicken at all. There were strong LZM immuno-positive findings in the apical cytoplasm, supra-
nuclear region, and striated border of epithelial cells in the both large and small intestine (Figs. 14, 15 and 16).

In the lung, the epithelia of intrapulmonary bronchi, the bronchial cartilage, the alveolar epithelium, and blood vessels were all devoid of the enzyme. Only weak immuno-HRP reaction products of LZM could be observed in the macrophages of the lung as the spleen and lamina propria of the digestive tract (Fig. 14).

In the peripheral blood smear, mature pseudoeosinophilic leukocytes and occasional monocytes consistently revealed a large amount of immunoreactive LZM (Figs. 17, 18, 19 and 20). There was no discernible enzyme in large and small lymphocytes (Fig. 20), basophilic leukocytes, platelets and nuclear erythrocytes. An occasional eosinophilic leukocytes showed a very weak immuno-positive reaction, but most of them showed no reaction. Bone marrow smears showed no immunoreactive findings of LZM in any erythrocyte precursors, basophilic cells, megakaryocytic elements, plasma cells, or reticuloendothelial cells (Fig. 21). All myelocytes and metamyelocytes showed LZM that usually was slightly more evident than that of the mature heterophils (Fig. 22). Horseradish peroxidase-labeled antibody method was applicable for detection of LZM in both smears even after inhibitory treatment for MPO in eosinophilic leukocytes with 0.3% H₂O₂-methanol (Figs. 23 and 24).

Discussion

In the findings presented above, we have successfully shown the immunohistochemical localization of the LZM in various tissues and blood cells of domestic fowl at the light microscopic level by use of both direct FITC-labeled and HRP-labeled antibody methods. Previously, successful localization of human and rat LZM was accomplished by use of immunoperoxidase, immuno-fluorescent, and substrate film methods revealed by light microscope and the unlabeled HRP-anti-HRP (PAP) method at the ultrastructural level.

A few immunohistological studies have been made of LZM purified from hen egg white in the tubular gland cells of female sex steroid-stimulated chicken oviducts. In our present study of laying hen tissues and cells, we have confirmed the presence of high concentrations of LZM in the albumen-secreting serous gland of the oviduct, proximal tubular cells of the kidney, tissue macrophages, and blood granulocytes of peripheral and marrow components.

Egg white is secreted mainly by the anterior portion of the oviduct. Fluorescent antibody studies have localized ovalbumin, LZM, ovomucoid, and conalbumin on magnum tissue of chicks given estrogen plus progesterone. This combination of hormones gives the maximal rate of induction of each of the protein. Much of the protein is formed in the magnum during the intervals between the passage of successive eggs down the oviduct, possible via an intermediate lipid phase. Because of the conspicuous granules they contain, these proteins are presumed to be synthesized primarily in the tubular serous gland cells after hormone stimulation. However, one of the minor proteins, avidin, is localized in the epithelial cells. In this study, brilliant specific fluorescence and HRP-reacted materials were observed in the gland on magnum and in the mucous epithelium on upper magnum segment and isthmus as the ova passed through the oviduct. In the latter immuno-positive area, it is assumed that the secretory products excreted into the lumen may be absorbed again in epithelial cells (Figs. 7 and 8), and their immuno-
positive LZM of bright green fluorescence and brownish small granular HRP-reacted materials were observed in the apical cytoplasm of these cells.

The major role of the kidney in the renal handling of LZM filtered through the glomeruli has been well documented in previous studies. Available evidence indicates that LZM is reabsorbed by the proximal and occasional distal tubules (Figs. 9, 10, 11 and 12) and excreted urine in the same manner as that of humans and rats, except for some cases of monocytic or myelocytic leukemia and nephrosis. The ultimate fate of the reabsorbed LZM is not clear, but it may be returned to circulation or be catabolized in situs.

The finding of LZM in Paneth cells of the small intestine in humans and other species not corresponds with finding in domestic fowl because of without Paneth cell in chicken. The specific function of LZM in Paneth cells is unknown, but an antibacterial activity seems possible. We have also observed weak but apparently immuno-specific LZM in Lieberkühn’s gland (Fig. 13) and the apical cytoplasm of intestinal epithelial cells (Figs. 14, 15 and 16). Its presence on the intestinal surface and cytoplasm may be reabsorbed with intraluminal LZM produced and secreted from upper parts of the digestive tract.

The detection of LZM in tissue macrophages and PMNs was conformable to the principal function of antibacterial and nonspecific reticuloendothelial system in the domestic fowl.

The aforementioned results of blood smears show evidence that mature pseudoeosinophilic granulocytes (heterophils) (Figs. 17, 18, 19 and 20) and monocytes contain LZM in all peripheral blood elements. In mammalian blood smears, MPO is usually restricted to granulocytes. Although avian heterophil PMNs appear to function in vivo in a manner generally comparable to that of mammalian PMNs, chicken heterophils lack MPO and alkaline phosphatase. Profiles of chicken granular leukocytes, however, showed strong MPO activity in eosinophils. Because of the difficulty in distinguishing between heterophils and eosinophils in smears of chicken blood, the HRP-labeled antibody technique was applied after inhibitory treatment with 0.3% H2O2-methanol to identify cell types in this blood smear study (Figs. 19, 20, 23 and 24). On the other hand, Brune et al. showed that large granules (band III), rod-shaped and MPO-negative granules from chicken peritoneal exudate cells clearly originate in the heterophil PMNs and contain large amounts of basic proteins and LZM. Our results agree reasonably well with previous observations of the antibacterial properties of avian PMNs granules isolated and characterized by analytical cell fraction methods. Immuno-positive LZM was not observed in eosinophils and basophils during light microscopy.

In normal bone marrow, Giemsa stain was used to identify immunofluorescence positive cells as pseudoeosinophilic myelocytes and metamyelocytes, and mature heterophils. The results of avian LZM tests confirmed the findings of human peripheral blood smears of acute pro-myelocytic leukemia and normal marrow smears, in which an immunofluorescence method, using human LZM purified from urine, milk or saliva was employed. There is some evidence that avian LZM also plays an important role in the intra-cellular destruction of certain phagocytized materials. As a result of our blood smear observations, we would like to suggest that chicken LZM is capable of being used as a “marker” enzyme in the maturation and differentiation of granulocytes.
Detailed characterizations of fine structured cell types using immuno-cytological methods of mature chicken granular leukocytes and monocytes will be discussed later in this journal.

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PLATES
Explanation of Figures

Plate I

Fig. 1. The agar gel-diffusion test on the Ouchterlony plate.
Antiserum absorbed with three contaminating proteins (AS) and lysozyme (L1, L2) system, indicating the formation of clear precipitine line between antibody and antigen.

Fig. 2. The infundibulum of the oviduct.
Specific immunofluorescence was found in the serous tubular gland (tg) and the apical region of epithelial cells (e). Non-specific autofluorescence of erythrocytes (black arrows) was also found. White arrow indicates the opening of the excretng duct to the lumen (L) of the oviduct. ×400

Figs. 3 and 4. The upper magnum segment of the oviduct.
Specific immunofluorescence was found in the serous gland cells although both brilliant (bf) and pale (pf) fluorescent areas were distinctly demonstrated. An immunoreacting material (black arrow) was seen in the lumen (L) and some cells of the excreting duct (ed) but not in epithelial cell layer. ×200

Figs. 5 and 6. The upper magnum segment of the oviduct.
Immuno-specific enzyme reaction-products were found in the serous gland cells but neither in the epithelium (e) nor the excreting duct (ed). Both sections of 5B and 6B were counterstained with the mixture of toluidine blue and methylene blue solution. ×200

Fig. 7. The lower magnum segment of the oviduct.
Specific immunofluorescence was found in the serous tubular gland, the apical region of the epithelium (e), and some cells (black arrows) of the excreting duct (ed). ×400

Fig. 8. The isthmus of the oviduct.
Specific immunofluorescence was found in some epithelial cells (e). Non-specific autofluorescence of erythrocyte (black arrow) was also found. m: Lamina propria mucosa. ×200 in 8A, ×400 in 8B.
Plate II

Figs. 9, 10, 11 and 12. The sections of the kidney.
Specific immunoreacting fluorescence and HRP-reaction products were identified in the proximal convoluted tubules (p) and some cells (black arrow) of the distal convoluted tubules (d). No reaction was noted in glomeruli (gl). The section of 10B was counterstained with the mixture of toludine blue and methylene blue solution. ×200 in Figs. 9 and 10, ×400 in Figs. 11 and 12.

Figs. 13 and 14. The sections of the small intestine.
Specific immunoreacting LZM was found in the Lieberkühn’s gland (Lg) and the supranuclear region and the striated border (s) of the epithelium (e). The entero-chromaffin cells (in Fig. 13, black arrows) were showed with autofluorescence. Tissue macrophages (in Fig. 14 black arrows) in the lamina propria mucosa (m) were immuno-positive findings. ×400 in Fig. 13, ×200 in Fig. 14.

Figs. 15 and 16. The sections of the large intestine.
Specific immunoreacting LZM was found in the supranuclear region of the epithelium but not in the lamina propria mucosa (m). The section of the 16B was counterstained with the mixture of toluidine blue and methylene blue solution. ×400 in Fig. 15, ×200 in Fig. 16.
Plate III

Figs. 17, 18, 19 and 20. The peripheral blood smears.
Specific immunoreacting fluorescence and HRP-reaction products were observed in the intracytoplasmic granules of the mature heterophils (h). The smear of Fig. 20 was prepared from buffy coat. In the case of immuno-enzyme methods, reaction products were revealed even after inhibitory treatment for MPO with 0.3% H₂O₂-methanol. No reaction was noted in nuclear erythrocytes, thrombocytes (t) and large lymphocyte (l). The blood smears of Figs. 19 and 20 were counterstained with Giemsa solution. ×200 in Figs. 17 and 18, ×400 in Fig. 19, ×1000 in Fig. 20.

Figs. 21, 22, 23 and 24. The marrow blood smears.
Specific immunoreacting fluorescence and HRP-reaction products were observed in the intracytoplasmic granules of the occasional promyelocyte (in Fig. 21, black arrow, p), myelocytes (m) and mature heterophils (h). In the case of immuno-enzyme methods, reaction products were revealed even after inhibitory treatment for MPO with 0.3% H₂O₂-methanol although a photograph of 24B was only stained with DAB-H₂O₂ substrate solution after inhibitory treatment mentioned above. The smears of Figs. 23 and 24 were counterstained with Giemsa solution. ×100 in Fig. 21, ×200 in Figs. 22 and 24, ×400 in Fig. 23.