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An Electron Microscopic Study on the Thymus of Larval and Metamorphosed Toads, *Xenopus laevis* Daudin

By

Saburo Nagata

Zoological Institute, Hokkaido University

(With 2 Text-figures and 7 Plates)

Recent extensive studies on the ontogenic and phylogenic aspects of immunity have established that the anuran amphibians are the lowest vertebrates whose immune system is comparable to that of higher vertebrates (for a review, see Du Pasquier, 1973). In *Xenopus*, the unusual feasibility of early larval thymectomy allows the definition of the critical larval stages for the establishment of immune responsiveness (Horton and Manning, 1972; Turner and Manning, 1974; Tochinai and Katagiri, 1975), together with the occurrence of the thymus-dependent and independent immune systems in this order of animals (Collie *et al.*, 1975; Tochinai, 1976).

Histological observations have been made on the thymus of *Xenopus* by Sterba (1950) and Manning and Horton (1969). However, no information has been presented for this organ on the ultrastructural level, in contrast with the case of other amphibian species (for a review, see Cooper, 1973), where fine structural aspects of the thymus are present. It seems therefore to be a necessary preliminary in *Xenopus* to establish the fine structural basis of the lymphocytes and the thymic structure, both for the precise integration of previous experimental results and for the future development of studies on the ontogeny of immunity in this animal.

The present study was intended to establish the fine structural basis of the thymus in the immunologically mature *Xenopus*, with particular attention to the lymphocytes and secretory epithelial cells.

Materials and Methods

The materials used were larvae and adults of the South African clawed toad, *Xenopus laevis* Daudin. The method for obtaining fertilized eggs and raising larvae to adults has been described previously (Nagata, 1976). Developmental stages were determined according to the Normal Table of Nieuwkoop and Faber (1956).

Thymuses were dissected carefully from stage 56 (36-day-old) larvae and metamorphosed (70-day-old) toadlets, with fine scissors and forceps. They were placed in ice-chilled 5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) in a paraffin-based Petri dish. The epidermis and connective tissue surrounding the thymus were removed, and the isolated thymuses were transferred to a large amount of the fixative. After fixation for 2 hr, the specimens were rinsed thoroughly with the buffer solution and postfixed in 1% osmium tetroxide for 2 hr. The osmificated specimens were dehydrated by graded acetone series and embedded in Epon 812 (Luft, 1961). Thin sections were cut with glass knives, stained with 1% uranyl acetate and lead citrate (Reynolds, 1963), and observed with a Hitachi HS-7 electron microscope. Some thick sections were mounted on glass slides, and stained with toluidine blue for light microscopic study.

For the ultrastructural localization of carbohydrates, the periodic acid-chromic acid-silver methenamine (PA-CrA-Ag) method was used on the non-osmificated sections, according to the procedure by Rambourg and Leblond (1967): Sections mounted on uncoated stainless steel grids werd oxidized successively with periodic acid and chromic acid, and incubated in a freshly prepared silver methenamine solution for 30 min at 60°C. The control run consisted of staining with silver methenamine solution without preoxidation. In order to digest glycogen, some glutaraldehyde-fixed specimens were incubated *in toto* in 0.5% α-amylase (adjusted at pH 7.0 with 0.1 M sodium cacodylate buffer) for 1 hr at 37°C. They were processed for staining with the PA-CrA-Ag method in exactly the same way as described above.

Results

Thymus from stage 56 larvae

As described by Manning and Horton (1969) on the light microscopic lelvel, the thymus is fully differentiated in stage 56 larvae, consisting of an essentially lymphoid cortex (Fig. 1A) and epithelial medulla (Fig. 1B). The major cell types which constitute the larval thymus are free cells of lymphocytic series, and the epithelial cells forming a reticular meshwork and the thymic cysts. Besides these cells, there are myoid cells in the medulla and blood capillaries and melanocytes throughout the thymus.

Of the lymphocytic cells distributed among the epithelial reticular meshwork, the small lymphocytes are dominant in both the cortex and the medulla. In the outer part of the cortex (Figs. 1A and 3), the large lymphocytes tend to accumulate and undergo mitosis. Electron microscopically, the cytoplasm of lymphocytes is characterized by the abundant free ribosomes and the pausity of scattered mitochondria. Small lymphocytes (5–6 μm in size; Fig. 5) have a large nucleo-cytoplasmic ratio and hyperchromatic nucleus. Large lymphocytes (8–10 μm in size; Fig. 6), in addition to their smaller nucleo-cytoplasmic ratio, are easily distinguishable from small lymphocytes by a large prominent nucleolus.

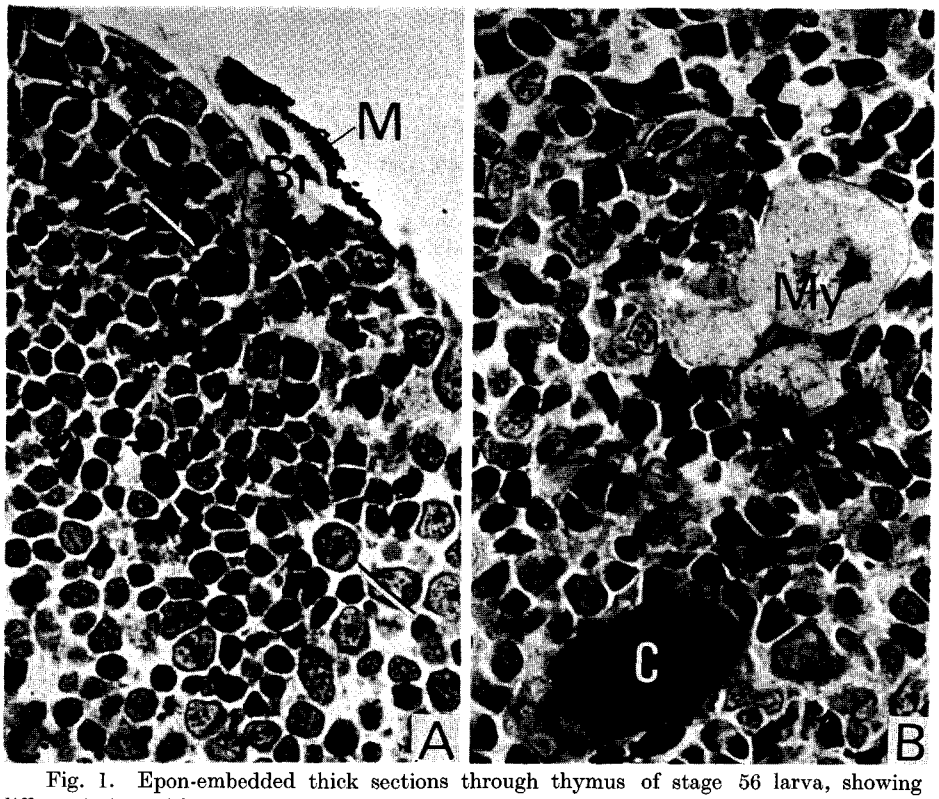

Fig. 1. Epon-embedded thick sections through thymus of stage 56 larva, showing differentiation of lymphoid cortex (Fig. A), and epithelial medulla (Fig. B) with thymic cyst (C) and large myoid cell (My). Note preferential localization of large lymphocytes (arrows in Fig. A) in outer part of cortex. Bl, blood capillary; M, melanocyte. Stained with toluidine blue. × 800.

There are intermediate or medium-sized lymphocytes showing the transitional features of the above-mentioned cell types. Regardless of their size, the state of the cytoplasmic organelles in lymphocytic cells does not vary significantly, although the occurrence of polyribosomes tends to be more frequent in large than in small lymphocytes. There are few, if any, strands of endoplasmic reticulum (ER), either with or without attached ribosomes.

Epithelial cells are identified by their pale cytoplasm and the desmosomal junctions which connect their irregularly branched cytoplasmic processes to each other. The cells contain various cytoplasmic organelles including filaments and glycogen particles as well as various sizes of vacuoles and granules. The entire surface of the organ facing the mesenchymal capsule is coated by the filamentous basal lamina which is deposited by the highly extended cytoplasmic processes of the

epithelial cells (Fig. 4). Some cells in the cortex contain a very large membrane-bounded body (Fig. 3), a possible phagosome, which contains membranous, granular or amorphous materials. The cells in the medulla display highly variable features (Fig. 7). Some of them contain an intracellular lumen (Figs. 7 and 8) delimited by an irregularly indented membrane (a possible intracellular cyst, Curtis *et al.*, 1972). Around the cystic lumen, there are several dense granules, bundles of filaments and in some cases clusters of mitochondria. Granulated ER and Golgi complexes are well developed in these cells.

The most conspicuous structure in the medulla is the intercellular cyst (Fig. 9a), which is lined by a number of microvillous projections from several epithelial cells closely joined to each other by desmosomes. The ciliation may also occur on the surface of the lumen (Fig. 9b). Immediately beneath the apical cell surface, there are membrane-limited, dense granules of various sizes. Treatment with the PA-CrA-Ag method proved that the specific staining occurs in the amorphous materials dispersed in both the inter- (Fig. 10) and the intracellular (Fig. 11) lumens, the cytoplasmic granules, as well as the folded membrane of the cystic lumen. The stainability in these structures was not affected by the saliva treatment.

Large myoid cells (Figs. 1B and 12a) are distributed in the medulla, either singly or in an aggregate of several cells with various degrees of striation. The size of the cells is highly variable, ranging from 10–25 μm, and the cell surface is joined with the neighbouring epithelial cells or myoid cells by desmosomes. Bundles of myofilaments closely resemble those in muscle cells in that they are composed of thick and thin filaments (Fig. 12b).

The endothelial cells forming the blood capillary walls are mutually joined by tight junctions. The basal lamina at the outer surface of these cells stains well with the PA-CrA-Ag method. It is noticed that the small lymphocytes found in the capillary lumen possess unusually long pseudopodial extensions (Fig. 13). Small lymphocytes were also seen frequently in the capillary wall in the medulla, crossing the narrow space between the cytoplasmic processes or being enclosed by the outstretched processes of endothelial cells (Fig. 14).

Melanocytes are distributed along the wall of the blood capillaries and among the mesenchymal capsule surrounding the thymus (Figs. 1A and 3).

Thymus from metamorphosed toadlets

The gross structure of the thymus in metamorphosed (70-day-old) toadlets is essentially the same as in the stage 56 larvae, although the former contain more voluminous medulla and a relatively thinner cortex. As compared with the larval thymus, there is no significant difference in the distribution and the ratio of small-, medium-sized- and large lymphocytes. The myoid cells now measure about 30 μm in diameter, containing highly developed sarcomeres. The lumens of the thymic cysts have been much increased in size. A notable feature in the adult thymus is the occurrence of several types of free or epithelial cells which are not

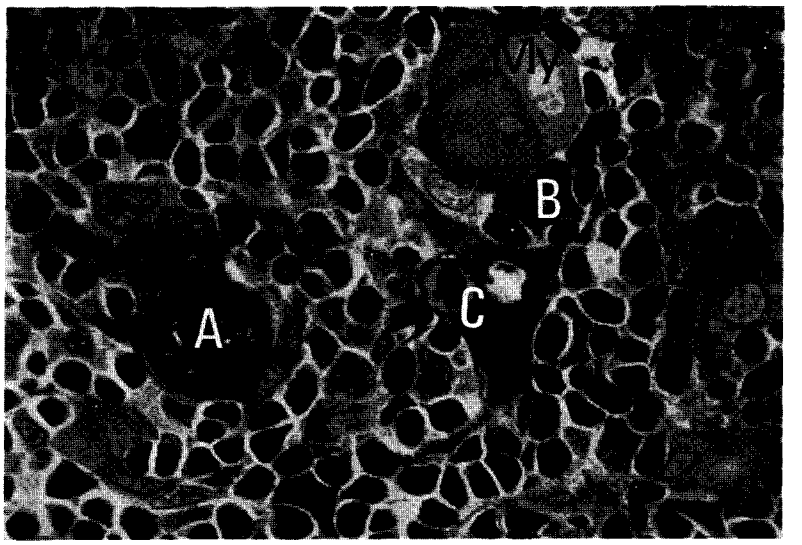

Fig. 2. Epon-embedded thick section through medulla of thymus of metamorphosed toadlet. Among lymphocytes, there are an enlarged myoid cell (My), a possible intracellular cyst (C), and cells containing rod-shaped granules (A) and metachromatic granules (B). × 800.

present in the larval thymus (Fig. 2).

The free cells (Fig. 15) possessing extremely long microvillous projections show an ultrastructure similar to the basophilic granulocyte in *Rana pipiens* (Campbell, 1970). They are distributed mainly in the medulla, but may also be found in the cortex. Their cytoplasm is filled with dense granules which are stained metachromatically with toluidine blue (Fig. 2).

There are epithelial cells containing a number of characteristic rod-shaped granules (Fig. 16) in the medulla. These cells (about 30 μm in size) are present either singly, in an aggregate of similar cells, or in contact with an elongated epithelial cell sheet. The prominent Golgi complexes and the well developed granulated ER suggest that they belong to the secretory cells. Less frequently, there are other epithelial cells as shown in Figs. 17 and 18. The cell represented in Fig. 17 contains zymogen-like granules (0.2 μm in diameter), and the cell shown in Fig. 18 possesses large dense granules and the unusually dilated cisternae of granulated ER containing electron-opaque substances.

Discussion

The electron microscopic features of the thymic lymphocytes shown in the present study are quite similar to those observed in higher vertebrates (Weiss, 1972) and other amphibian species (Klug, 1967; Kapa *et al.*, 1968; Curtis *et al.*,

1972), in that they have a large nucleo-cytoplasmic ratio and an abundance of free ribosomes with a dearth of other cytoplasmic organelles. The exact relationship between the large-, medium-sized-, and small lymphocytes cannot be stated from the present study, although the higher mitotic activity of the large lymphocytes in the outer cortex suggests a gradual interior deposition of small lymphocytes concomitant with their maturation, as discussed in mammalians and birds (*cf*, Goldschneider, 1974). Besides this lymphocytogenesis, a transfer of small lymphocytes either into or out of the thymus through the blood circulatory system will take place, as indicated by the occurrence of lymphocytes crossing the blood capillary walls in the medulla. One of the reliable markers for the differentiated lymphocytes will be the cell surface immunoglobulins. According to Du Pasquier *et al.* (1972), the *Xenopus* thymic lymphocytes are unusual in that over 80% of them at stage 56 larvae possess surface immunoglobulins. These membrane-bounded immunoglobulins are first detectable at stage 46–47, when the thymectomy does not completely abrogate the animal's ability to reject allografts (Horton and Manning, 1972). Electron microscopically, however, the major cell type found in the stage 46–47 thymuses is a large lymphocyte (unpublished observation). To fill the gap between these immuno-histochemical and ultrastructural observations, it seems extremely important to determine the ultrastructural localization of immunoglobulins on the thymic lymphocytes observed here.

Both intra- and intercellular cysts of *Xenopus* thymus show similar ultrastructural features to those observed in *Rana pipiens* (Curtis *et al.*, 1972). The cytochemical technique employed in the present study provided evidence that the medullary epithelial cells constituting the thymic cysts show the activity of producing and secreting carbohydrates into the cystic lumens. This is a confirmation of the previous light microscopic observation (Kapa, 1963; Curtis *et al.*, 1972) in that the secretory substances in the anurans are PAS-positive. In view of the observation that these substances are either acidophilic or basophilic in nature, they may consist of heterogeneous materials. As discussed by Curtis *et al.* (1972) for *Rana pipiens*, the substances in the thymic cysts could be regarded as a functional candidate for the humoral activity of the thymus. However, no direct evidence has hitherto been available for the presence of thymic humoral factors in cold-blooded vertebrates (*cf*, Dardenne *et al.*, 1973; Trainin, 1974). An experimental system to answer this important question is now available in *Xenopus*, since certain types of their immune responsiveness are completely abrogated by the early larval thymectomy (Turner and Manning, 1974; Tochinai and Katagiri, 1975), and its restoration is made possible by the implantation of the thymus from histocompatibly syngeneic animals (Tochinai *et al.*, 1976).

In contrast to the report by Kapa *et al.* (1968) on the adult *Rana esculenta*, plasma cells were not found in the present study. A possible reason for this failure might be due to the difference in age of the animals examined. The relative increase of epithelial medulla during stage 56 and metamorphosis as observed here is interpreted as indicating the functional degradation of this organ

accompanying the aging process. On the other hand, recent experiments have demonstrated that the immune system of this animal is established at a far earlier stage than stage 56 (Tochinai and Katagiri, 1975), and that this immune system functions through the metamorphosis (Nagata, 1976). Hence, several types of epithelial cells containing variable granules and the myoid cells which show an emergence or remarkable development during metamorphosis, seem to be less important with respect to their role in the immunological function of the thymus.

Summary

An electron microscopic observation was made on the thymuses of the larval and metamorphosed toad, *Xenopus laevis*.

In the thymus of stage 56 larva, lymphocytic cells with abundant free ribosomes and a small number of mitochondria constitute the major cell types in the cortex. Small lymphocytes, most dominant in the organ, measure 5–6 μm in size, and are characterized by their hyperchromatic nucleus. Large lymphocytes (8–10 μm in size) possess a characteristic large nucleolus, and are distributed and undergo mitosis in the outer part of the cortex. The epithelial cells, tightly joined to each other by desmosomes, form a reticular meshwork among lymphocytes. In the medulla, some epithelial cells form conspicuous intra- and intercellular cysts, which consist of carbohydrate-containing materials in both the cystic lumens and the secretory granules in the cytoplasm. Large myoid cells are also present in the medulla. In addition, small lymphocytes are seen in the blood capillaries and in the endothelial capillary walls.

The thymus in the metamorphosed toadlet shows a marked increase of the medulla including the occurrence of several types of cells which are not present in the larval thymus. In view of the recent evidence that this toad is immunologically mature in earlier larval stage than studied here, the cells appearing during metamorphosis seem to be less important in immunological aspects.

I express my sincere appreciation to Asst. Professor Ch. Katagiri for his appropriate advice throughout the course of this study and for critical reading of this paper. Thanks are also due to Mr. Y. Takakuwa for his technical advice.

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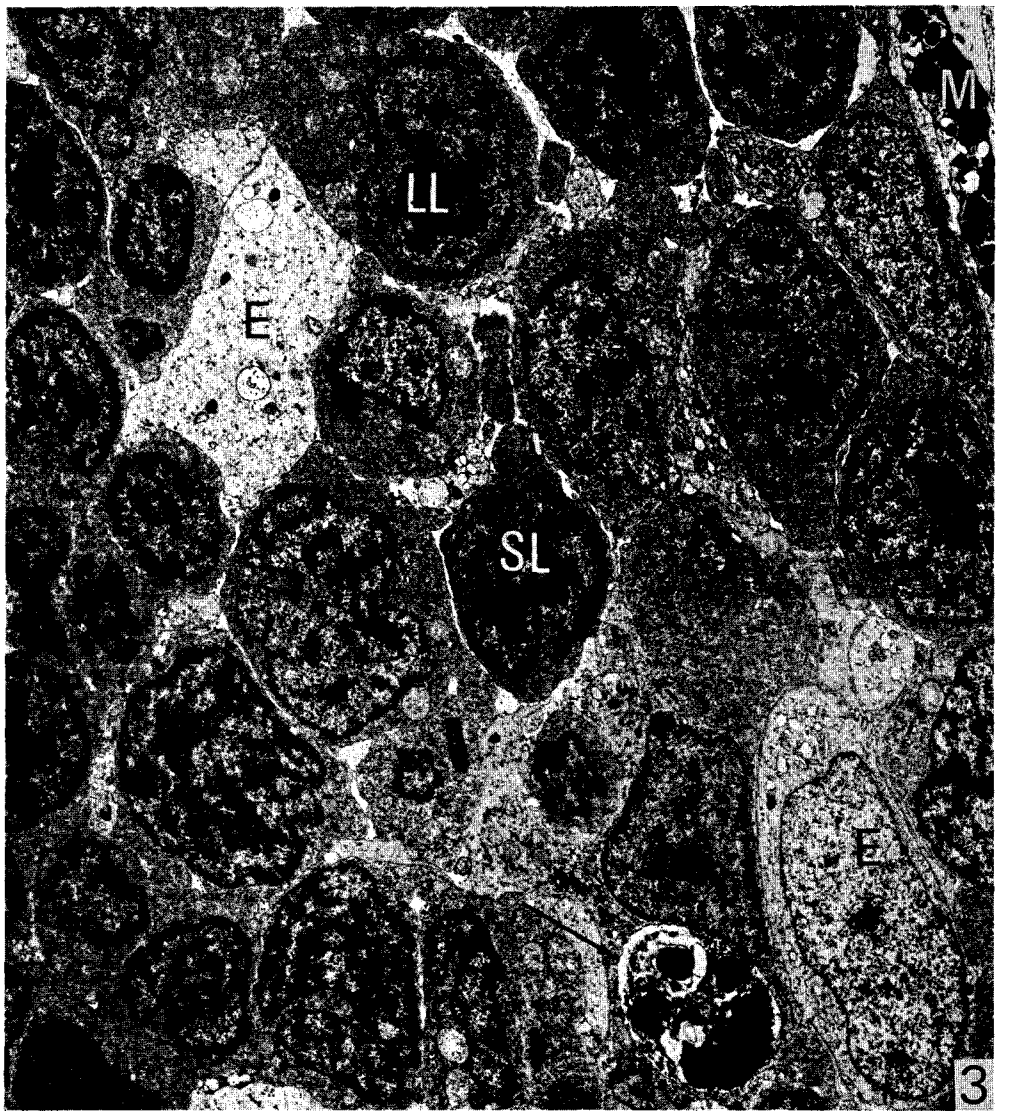
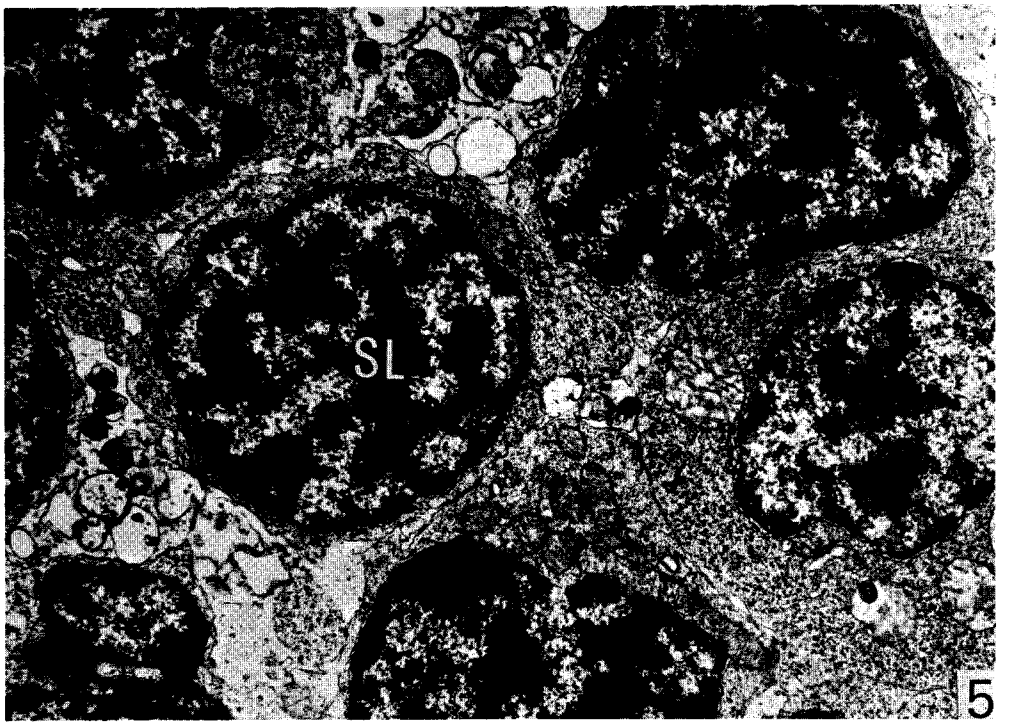
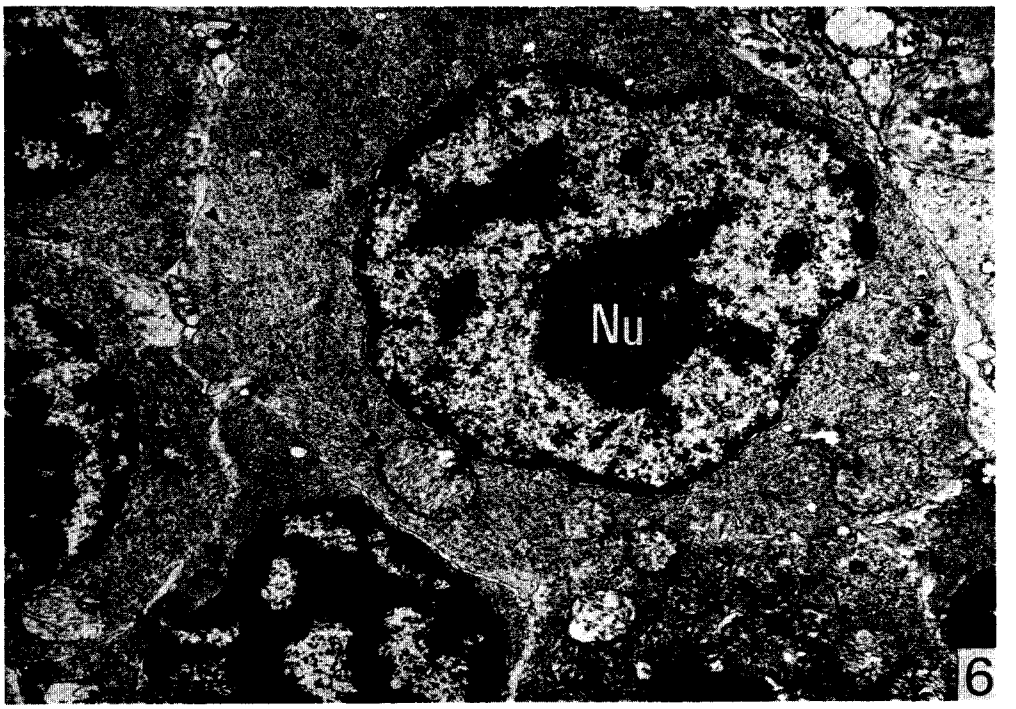


S. Nagata: Electron Microscopy of Toad Thymus





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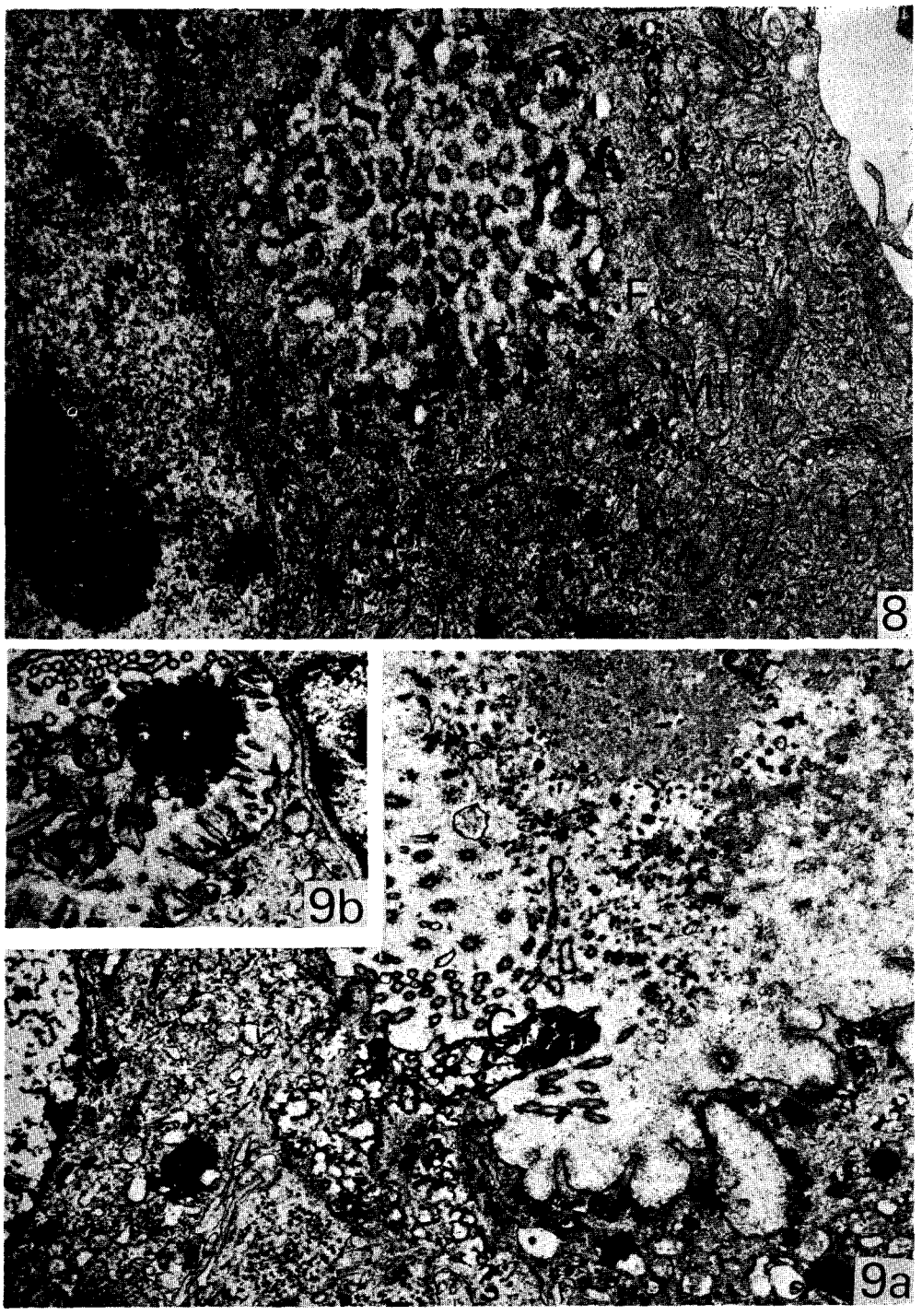

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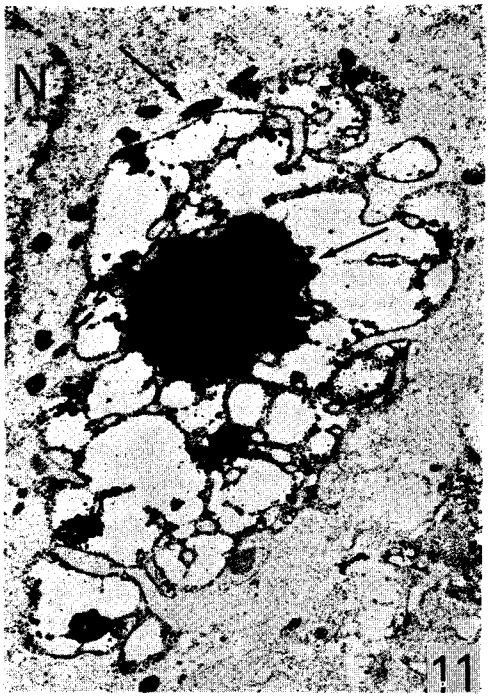

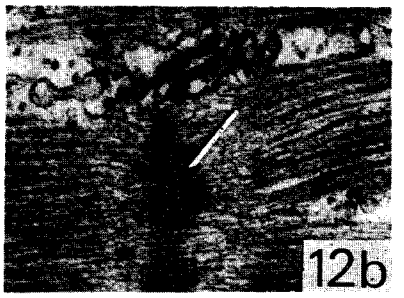

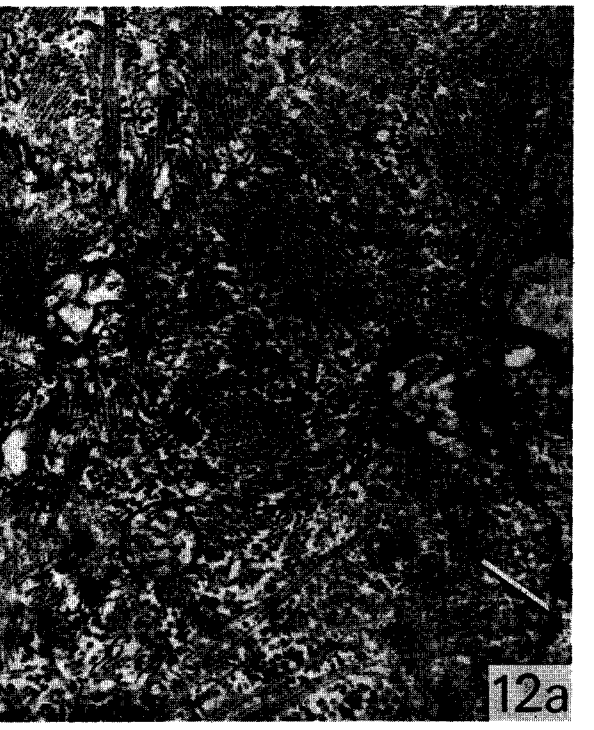

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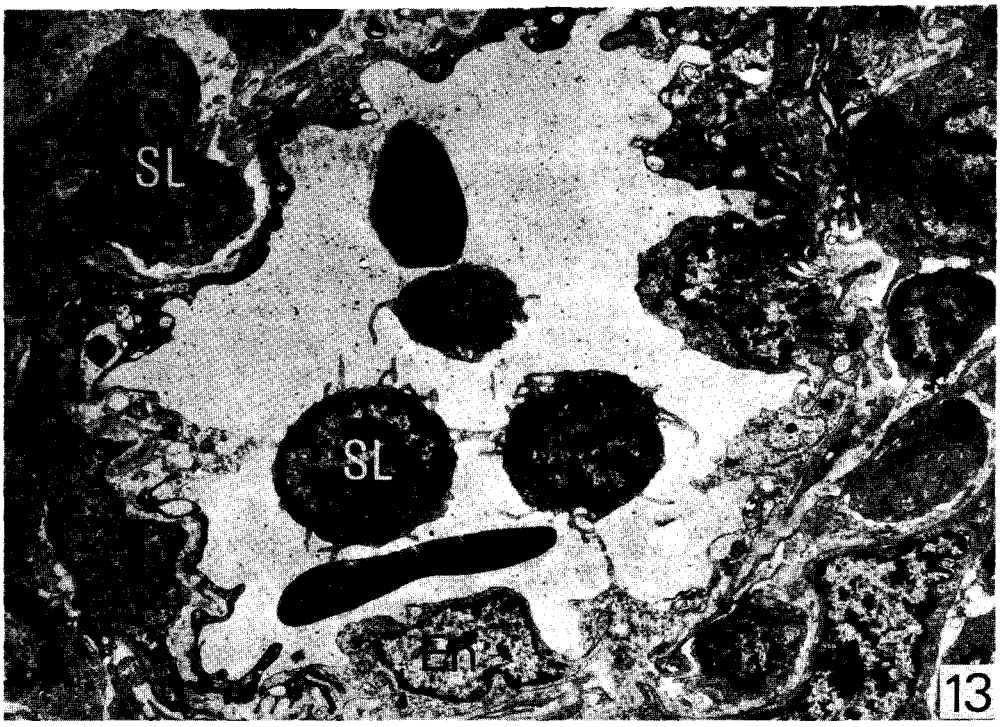

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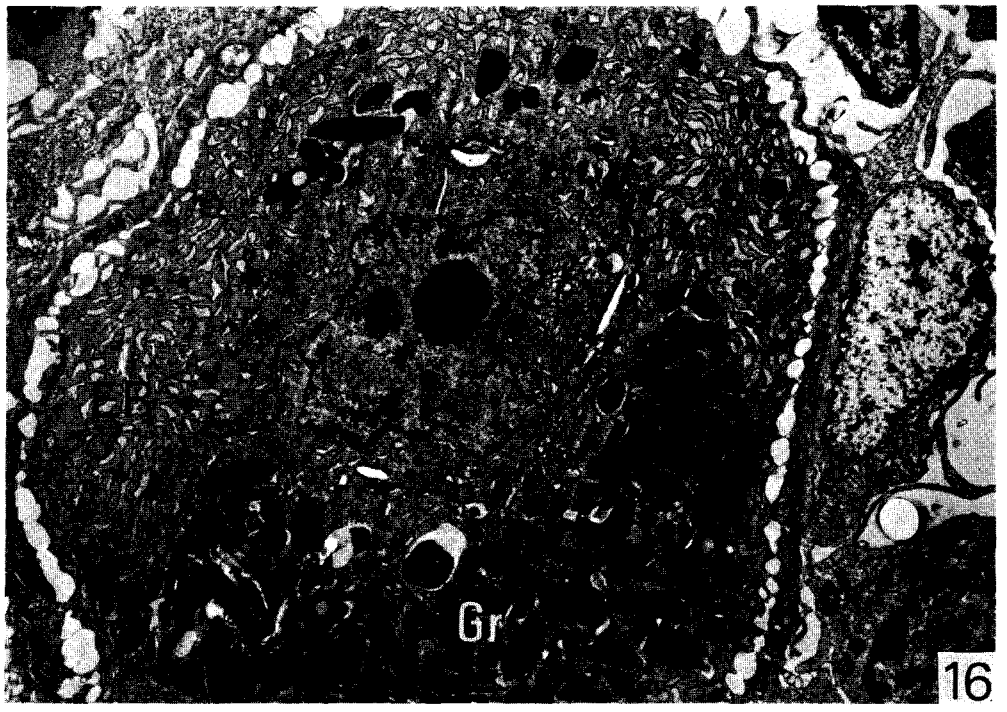

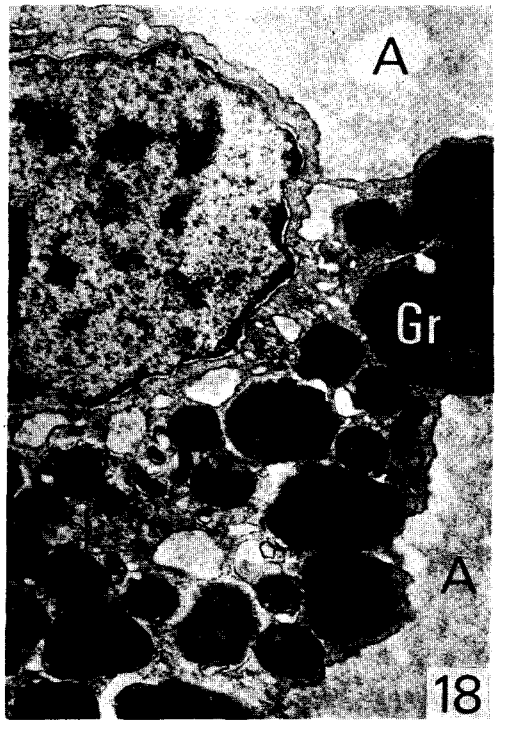

S. Nagata: Electron Microscopy of Toad Thymus

Explanation of Plates XIV-XX

Figs. 3–14 and 15–18 are sections from thymuses of stage 56 (36-day-old) larvae and metamorphosed (70-day-old) toadlets, respectively.

Abbreviations:

C, thymic cyst	Gr, granules	My, myoid cell
E, epithelial cell	g, glycogen particles	N, nucleus
En, endothelial cell	LL, large lymphocyte	Nu, nucleolus
ER, endoplasmic reticulum	M, melanocyte	SL, small lymphocyte
F, cytoplasmic filaments	ME, mesenchymal cell	
G, Golgi complex	Mt, mitochondria	

Fig. 3. Outer part of thymic cortex, showing small (SL) to large (LL) lymphocytes distributed among the epithelial reticular meshwork. Thymic surface to upper right. Arrow, epithelial cell containing a possible phagosome. × 4,800.

Fig. 4. Outermost part of thymus, showing basal lamina (arrows) on epithelial cell (E) surface facing mesenchymal sheath (ME). × 12,000.

Fig. 5. Small lymphocyte (SL), showing densely-packed chromatin and large nucleo-cytoplasmic ratio. × 12,000.

Fig. 6. Large lymphocyte, showing abundant cytoplasm and a large prominent nucleolus (Nu). Note heterochromatin is less distinct than in small lymphocyte (see Fig. 5). × 12,000.

Fig. 7. Thymic medulla, showing an epithelial cell containing a possible intracellular cyst (C) among small lymphocytes. Arrow, desmosome. × 6,000.

Fig. 8. Higher magnification of possible intracellular cyst (C) delimited by numerous microvillous projections. Secretory granules (Gr), mitochondria (Mt) and filaments (F) are rich in the surrounding cytoplasm. × 16,800.

Figs. 9a and 9b. Parts of intercellular cysts, showing amorphous materials accumulated in the lumen and microvillous projections coated with filamentous materials. Note numerous secretory granules (Gr) in cytoplasm immediately beneath plasma membrane (9a). Ciliation is also seen in Fig. 9b. × 12,000 and × 16,800, respectively.

Figs. 10 and 11. Occurrence of carbohydrates in thymic medulla, as demonstrated by the PA-CrA-Ag method. Besides the contents of inter- (Fig. 10) and intracellular cysts (Fig. 11), specific staining (arrows) is seen also on secretory granules and the plasma membrane of epithelial cells. × 4,800 and × 16,800, respectively.

Figs. 12a and 12b. Part of a large myoid cell in the medulla, containing glycogen particles (g) among myofilaments (12a), as well as a z-line substance (arrow in Fig. 12b) connecting thin filaments to each other. Arrow in Fig. 12a, desmosome. × 16,800 and × 45,000, respectively.

Fig. 13. Blood capillary lumen surrounded by endothelial cells (En), found in medulla. Note high degree of pseudopodial extensions in small lymphocytes (SL) in the lumen. × 4,800.

Fig. 14. A small lymphocyte traversing the capillary endothelium. Capillary lumen to upper left. × 16,800.

Fig. 15. A free cell containing metachromatic granules. Note long pseudopodial extensions of cytoplasm (arrow). × 8,000.

Fig. 16. A large epithelial cell containing rod-shaped granules (Gr) and distended granulated ER. × 6,000.

Fig. 17. Part of an epithelial cell containing zymogen-like granules (Gr). Granule formation in Golgi complexes (G) is apparent. × 16,800.

Fig. 18. Part of an epithelial cell containing dense granules (Gr) and dilated cisternae of granulated ER (A). × 16,800.