Afferent Migration of the Kurloff Cells via Lymphatics into the Thymus of Estradiol-Treated Guinea Pigs

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Summary. The spatial distribution and migration of Kurloff cells containing PAS-positive large inclusion bodies in the thymus of estradiol-treated guinea pigs were histochemically studied by a combination of light and electron microscopy. Male guinea pigs were examined at various intervals from 7 days to 3 months after a single subcutaneous injection of estradiol. Differentiation of lymphatics from blood capillaries was performed by a 5'-nucleotidase (5'-Nase) staining method and the occurrence of Kurloff cells within 5'-Nase-positive lymphatics was confirmed by ultrastructural histochemistry. Several Kurloff cells first appeared at 7 days within lymphatics in the thymic capsule or interlobular connective tissues. At 12-15 days after estradiol administration, a lymphatic accumulation, a so-called "lymphatic center", was seen in the thymic septa or interlobular connective tissues. At 12-15 days after estradiol administration, a lymphatic accumulation, a so-called "lymphatic center", was seen in the thymic septa or interlobular connective tissues. At 12-15 days after estradiol administration, a lymphatic accumulation, a so-called "lymphatic center", was seen in the thymic septa or interlobular connective tissues. At 21 days after estradiol, Kurloff cells were preferentially accumulated along the corticomedullary junction extravascularly. Later the distribution was more diffuse. The conspicuous accumulation of Kurloff cells in the corticomedullary region could reflect an inability of Kurloff cells to use blood vessels as a route for migration. These findings strongly suggest the afferent migration of Kurloff cells into the thymus via lymphatics.

Mononuclear cells, characterized by large intracytoplasmic PAS-positive inclusions, appear in the blood, thymus, spleen and bone marrow in estrogenized or pregnant guinea pigs (Izard et al., 1976; Revell, 1977) and are often referred to as Kurloff cells (Kurloff, 1889; Foa and Carbone, 1889). Previous reports have shown that Kurloff cells develop a cytotoxic natural killer activity (Eremin et al., 1980; Debout et al., 1984) and have a pan-T lymphocyte immunophenotype (Debout et al., 1991). Their inclusion bodies exhibit both intense acid phosphatase (Landemore et al., 1987) and alpha naphthyl acetate esterase activity (Buat et al., 1988). Although Kurloff cells are either absent or minimal in lymph nodes and the thoracic duct lymph (Ernstrom and Sandberg, 1971; Revell et al., 1971), Simmons (1965) suggested the export of Kurloff cell from the thymus via the lymphatics. Recently, sandberg and Hageman (1990) demonstrated light microscopically that peculiar cell accumulations, the so-called "lymphatic centers" appear between ordinary thymic lobules in estradiol-treated guinea pigs. These lymphatic centers were surrounded by a marginal sinus, which communicated with interlobular or extrathymic lymphatics. From the orientation of lymphatic valves and the concentration of Kurloff cells within the lymphatic centers, the authors suggested the existence of an afferent migratory pathway for Kurloff cells. The present study therefore, describes the histochemical analysis and the ultrastructural organization of the lymphatic centers and Kurloff cells in the thymus of estrogenized guinea pigs. Special attention is paid to the possibility of the afferent migration of Kurloff cells into the thymus via lymphatics.

MATERIALS AND METHODS

Animals

Forty male Hartley guinea pigs weighing 250-300 g were injected subcutaneously with 1 mg estradiol (Sigma) suspended in 0.5 ml olive oil. All experiments were performed in accordance with the Guidelines.
The animals were deeply anesthetized with ether, and the thymus and other organs, including the spleen, bone marrow and cervical lymph nodes, were excised 7, 10, 12, 15, 21, 30 days and 3 months (3 or more animals per interval) after the injection of estradiol. Untreated normal animals and animals 15 days after the subcutaneous injection of olive oil without estradiol served as controls (total 6 animals).

General histology

For light microscopy, several organs were fixed with 10% formalin either by immersion or by perfusion via the aorta (Kato and Schoefl, 1987), and embedded in paraffin according to standard histological procedures. The sections were stained by the PAS method for Kurloff inclusion bodies, and counterstained with hematoxylin or methyl green.

For transmission electron microscopy (TEM), after immersion or vascular perfusion with a mixture of 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.4), the specimens were excised, cut into small pieces and immersed in the same fixative. They were postfixed for 2 h with 1% OsO4 in the cacodylate buffer, dehydrated through a graded series of ethanol and embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate, and examined in a JEM 1200 EX electron microscope (JEOL).

For scanning electron microscopy (SEM), after being cut into paraffin sections, the residual tissue blocks were deparaffined, fixed with 2.5% glutaraldehyde in the phosphate buffer, and postfixed in 1% OsO4. They were dehydrated through a graded series of ethanol and dried by the t-butyl alcohol freeze-drying method (Inoue and Osatake, 1988). The specimens were coated with gold using an ion-coater, and examined in a Hitachi S-800 SEM.

Enzyme histochemistry

Some of the unfixed tissue samples were directly frozen in OCT compound (Miles, USA) and cut with a cryostat (Bright 5030 microtome, Bright Inst. Co. Ltd, Huntingdon, England), while others were fixed with cold acetone and embedded in soft paraffin (low melting point: 42-44°C, Merck) as previously reported (Kato et al., 1993). In order to distinguish lymphatics from blood vessels, frozen or soft paraffin sections were fixed with a formaldehyde-CaCl2 fixative (2% paraformaldehyde-1% CaCl2) containing 7% sucrose and subjected to 5'-nucleotidase (5'-Nase) staining for light microscopy (Kato and Miyachi, 1989; Kato, 1990a). Briefly, they were incubated for 50 min at 37°C in the standard medium (Wachstein and Meisel, 1957) for 5'-Nase activity with lead nitrate as the capture agent and 5'-adenosine monophosphate (AMP, sodium salt, Sigma Chemical Co., St. Louis, USA) as the substrate. The standard medium was supplemented with 2 mM L-tetramisole, an inhibitor of nonspecific alkaline phosphatase, in order to discriminate the specific reaction for 5'-Nase activity from others. For TEM, 70 μm-thick sections were prepared in a microslicer (Dosaka EM Co. Ltd., Tokyo, Japan) and incubated for 40 min at 37°C in the reaction medium for 5'-Nase activity according to the cerium-based method by Robinson and Karnovsky (1983) (Kato, 1990b; Kato et al., 1991). They were postfixed in 2% OsO4 for 1 h at 4°C and processed for TEM observation as mentioned above. The following was done to provide controls: a) the substrate (AMP) was
omitted. b) The specific inhibitor (50 mM NiCl₂) of the 5'-Nase activity was added to the standard medium.

RESULTS

Lymphatics and the lymphatic center

The lymphatics stained with 5'-Nase staining could be identified as irregularly shaped spaces with 5'-Nase-positive vessel walls in the tissue sections of the guinea pig. 5'-Nase reaction product appeared as a black precipitate of lead sulfide in the wall of the lymphatics in the thymic connective tissue septa, although the smooth muscle in the wall of blood vessels also revealed 5'-Nase activity. The 5'-Nase-positive lymphatics were commonly situated in close proximity to blood vessels with weak 5'-Nase activ-

Fig. 2. TEM views of the lymphatic (L) and the lymphatic space (LS) containing Kurloff cells in a so-called lymphatic center as a local cell accumulation in the thymic interlobular septa reacted to 5'-Nase. a. Ten days after estradiol. ×2,700. b. Further magnification of the area (asterisk) in Figure 2a. Note the fine, continuous reaction product (arrows) associated with the lymphatic endothelium (E). ×16,000. c. The 5'-Nase-positive lymphatic contains Kurloff cells with a large spherical inclusion body 15 days after estradiol. The reaction product is located not only on the luminal surface (arrows) of the lymphatic wall, but also on the basal aspect (arrowheads) of the endothelium (E). ×5,000

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ity in the capsule or interlobular septa, but rarely seen within the thymic medulla. 5'-Nase activity in the lymphatic walls and PAS stainability of Kurloff inclusion bodies could not be detected simultaneously in the section stained with 5'-Nase and PAS double staining for light microscopy. For this reason, investigation of the relationship between the type of vessel and the distribution of Kurloff cells was carried out using 5'-Nase staining and PAS staining in serial tissue sections (Fig. 1a, b). These serial-section studies indicate that the vessels containing many PAS-positive Kurloff cells are lymphatics with 5'-Nase activity. Furthermore, TEM histochemistry for 5'-Nase activity was undertaken to identify the lymphatics precisely in addition to demonstrating the fine structure of Kurloff cells. At 10 and 15 days after estradiol administration, Kurloff cells with large granular or spherical inclusion bodies were seen in the capsule or interlobular septa, but rarely seen within the thymic medulla. 5'-Nase activity in the lymphatic walls and PAS stainability of Kurloff inclusion bodies could not be detected simultaneously in the section stained with 5'-Nase and PAS double staining for light microscopy. For this reason, investigation of the relationship between the type of vessel and the distribution of Kurloff cells was carried out using 5'-Nase staining and PAS staining in serial tissue sections (Fig. 1a, b). These serial-section studies indicate that the vessels containing many PAS-positive Kurloff cells are lymphatics with 5'-Nase activity. Furthermore, TEM histochemistry for 5'-Nase activity was undertaken to identify the lymphatics precisely in addition to demonstrating the fine structure of Kurloff cells. At 10 and 15 days after estradiol administration, Kurloff cells with large granular or spherical inclusion bodies were seen

Fig. 3. TEM views of the thymus in estrogenized guinea pigs. C cortex, A artery, V vein, Ca capillary. a. At 10 days, the lymphatic space (LS) contains many lymphocytes and Kurloff cells (arrows). A Kurloff cell (arrowshead) is seen among the vessels in the thymic interlobular connective tissue (ILC). ×2,000. b. At 15 days, Kurloff cells (KC) with large spherical inclusion bodies distend the lymphatic in the ILC. ×4,000
within 5'-Nase-positive lymphatics in the thymus (Fig. 2a, c). The cerium-based reaction product of the 5'-Nase activity was a dense granular precipitate localized on the luminal surface of the lymphatic wall and on the basal aspect of the endothelium (Fig. 2b, c). When the lymphatics in the thymic interlobular connective tissues contained numerous Kurloff cells and lymphocytes, they had an appearance similar to the tubular or saccular lymphatic spaces lined by attenuated endothelium (Fig. 3).

A peculiar accumulation of cells was seen in the thymic interlobular septae of estrogenized guinea pigs, but only rarely in normal controls. Local, slight cell accumulation first appeared in the thymic septa 10 days after estradiol administration (Fig. 3). Typical lymphatic accumulations, so-called “lymphatic centers” were apparent at 12-15 days (Fig. 4), but rarely seen at earlier or later periods following estradiol. To elucidate the structural organization of lymphatics and cell accumulation, the residual tissue blocks were cut into paraffin sections and examined by SEM (Fig. 5). The lymphatic accumulation often
Fig. 5. SEM view of the residual thymus block after cutting into the sections (Fig. 4). The areas a–d are further magnified respectively in Figure 6. An arrow indicates that the medullary tissue interrupts the cortical layer, extending to the interlobular septa. ×100
Fig. 6 a–d. Further magnifications of each area 1–4 shown in Figure 5 respectively. A artery, V vein.
a. Lymphatic space (LS) in the periphery of the lymphatic center, resembles a marginal sinus. ×330. b. The
tortuous canaliculi form labyrinthine connection. ×220. c. The LS communicates with the interlobular lymphatics (L). ×450. d. Note a lymphatic (L) with a valve (VL). ×480
extended centrally between ordinary thymic lobules, being surrounded by lymphatic spaces like the marginal sinus in a lymph node (Figs. 5, 6a). The spaces were conspicuous because of their irregular and branched appearance. A labyrinthine connection was seen between the extended structure and an adjacent lymphatic space (Fig. 6b, c). The irregular, tortuous canaliculi in the lymphatic accumulations communicated with interlobular lymphatics (Fig. 6c, d). Many Kurloff cells were found in the lymphatic accumulations and lymphatics when observed in the adjacent PAS staining tissue sections (Fig. 4) of the SEM tissue block (Fig. 5).

**Localization of Kurloff cells**

None of Kurloff cells could be found in the thymus of normal control male guinea pigs. A few days after the injection of estradiol in the male guinea pigs, Kurloff cells appeared in the thymus as well as in the

![Figure 7](image)
splenic red pulp and bone marrow but not in the lymph nodes (cortex). Their localization and number in the thymus conspicuously differed by time periods after the estradiol injection. At 7 days after the administration, very few Kurloff cells were recognized within the lymphatics, and none of them in the thymic parenchyma. At 10 days, many Kurloff cells were found within the lymphatics along the thymic surface and in the periphery of the cortex (Fig. 7a), but they were absent or still few in number within the thymic parenchyma (Figs. 7a, 8). At 12-15 days, a number of Kurloff cells were located preferentially not only in the periphery of the lymphatic center and in the tortuous lymphatic space (Fig. 4), but also in the narrow area of the medullary tissue, which interrupted the cortical layer (Fig. 5, arrow). Ultrastructurally, the Kurloff cells were often found in an immediate vicinity of the lymphatic endothelium or presumably in transit across the vessel wall (Fig. 10). Lymphatics in the thymic adipose tissue contained numerous Kurloff cells whereas arterial and venous vessels contained none or few. Some Kurloff cells were sporadically found in the perivascular space around the postcapillary venule (Fig. 7c). At 21 days, Kurloff cells were located preferentially along the corticomedullary junction in the thymus, apparently extravascularly (Fig. 9). At 30 days, a large number of Kurloff cells appeared throughout the involuted thymus whose corticomedullary junction had partly disappeared (Fig. 7d). An amount of their debris was seen in the Hassall’s corpuscles. At 3 months, a few Kurloff cells could still be confirmed in the thymus, the spleen and the bone marrow.

Fig. 8. Further magnification of the area shown by asterisk in Figure 7a. Note many PAS-positive Kurloff cells within the lymphatic (L) around the blood vessel (A) in the thymic capsule and in the periphery of the cortex, but no Kurloff cells in the thymic parenchyma at this time. ×800

Fig. 9. Guinea pig thymus sections 21 days after the injection of estradiol. C cortex, M medulla. a. 5'-Nase staining of a cryostat section. There is a 5'-Nase-positive lymphatic (arrow) in the medulla. The pale cell accumulation indicated by arrowheads would be analogous to the PAS-positive Kurloff cells shown in Figure 9b. b. PAS staining of a paraffin section. Note the conspicuous accumulation of PAS-positive Kurloff cells (magenta) in the corticomedullary region of the thymus from the same animal as shown in Figure 9a. ×130
DISCUSSION

The present study has demonstrated that a large number of Kurloff cells appeared fairly uniformly within the 5'-Nase-positive lymphatics after the injection of estradiol (7-10 days). We have used PAS-stained tissue sections for light microscopy, which are contiguous to the SEM tissue block as shown in Figure 5. This combined method of light and scanning electron microscopy allowed extensive observation of the lymphatics and the spatial distribution of the cells in question.

It is of interest that a lymphatic accumulation containing Kurloff cells developed in the thymic interlobular septa, and at that time, the cells were absent or still few in number in the thymic parenchyma (Figs. 7a, 8). The Kurloff cells-rich lymphatic accumulation with the tortuous lymphatic canaliculi apparently corresponds to the "lymphatic center" proposed by SANDBERG and HAGELIN (1990), since it was located between thymic lobules and possessed a distinct marginal sinus (Figs. 4, 6a). As noted by previous authors, the lymphatic center was continuous with ordinary thymic tissue but lacked a typical cortex and medulla. Accordingly, this is not considered to be a parathymic lymph node in mice and rats, regardless of the existence of an interior lymphatic sinus communicating with extrathymic lymphatics.

The lymphatic accumulation found in this study was present commonly at 12-15 days after estradiol administration, but rarely at other times. There were few Kurloff cells in normal controls. This may be because such a structure is produced as the result of an adaptation for increased cell migration as suggested by SANDBERG and HAGELIN (1990). KOTANI et al. (1966) described, in the normal guinea pig thymus, a local accumulation of thymic lymphocytes in the interlobular connective septa or at the surface of the lobules. However, the structure reported by them was not so clearly isolated from the surrounding thymic tissue like the lymphatic center. As the lymphatic center communicates with the medullary tissue of adjacent lobules, this structure presumably was produced by the migration of thymic lymphocytes from the thymic medulla. BAACK and KATER (1975) reported the presence of the central thymus area (medullary parenchyma) in normal guinea pigs containing lymphatic sinuses and postcapillary venules, where lymphatics and venules originated. This thymic area may not be identical with the lymphatic accumulation or the lymphatic center since the central thymus area is an intralobular area, from which the other lobules branch.
Concerning the distribution of the intrathymic lymphatics, Harris and Templeton (1968) reported that there are two types of interlobular lymphatics in the normal guinea pig thymus: the lymphatics in the perivascular space and those independent of vascular bundles. Studies by Kotani et al. (1966) further suggested that the lymphatics in the normal guinea pig thymus begin in the cell accumulation, through which thymic lymphocytes appear to leave the thymus, although they did not refer to the occurrence of Kurloff cells. Congestion of lymph after ligation of the lymphatics draining the guinea pig thymus, often causes tiny lymphatics to become visible in the medullary tissue contiguous to the interlobular lymphatic aggregations (Kotani et al., 1985; Kotani, 1990). In the present study of the control and estradiol-treated animals, lymphatics were rarely recognizable within the thymic parenchyma in the corticomedullary region (Fig. 9a).

Previous publications have often focused on the structural organization of the intrathymic lymphatics and their role in relation to the efferent migratory route of thymic lymphocytes to general circulation (Kotani et al., 1967; Ushiki, 1986; Kato, 1988). It has been also reported that in calves (Chanana et al., 1971) and sheep (Miyasaka et al., 1990), thymic lymphocytes leave the thymus not only via blood vessels but also via lymphatics. However, our findings have shown that Kurloff cells mostly appeared progressively in an order from within the lymphatics, in the lymphatic center, at the cortical surface, and finally inside the lobule (Fig. 8). Considering the time of occurrence and the distribution pattern of Kurloff cells after estradiol administration, an afferent migratory pathway of those cells via the lymphatics into the thymus lobules may occur, although there is still no direct evidence for the existence of this tract.

Kurloff cells occur only several days after estradiol treatment, suggesting that the effect of estradiol may be indirect. Neither estradiol nor serum from estrogenized animals induced the formation of Kurloff cells in vitro (Sandberg and Hagelin, 1986). The real function of estrogen binding sites remains unknown, although low affinity estrogen binding sites could be detected in Kurloff cell cytosol (Landemore et al., 1988).

As for the organ principally responsible for the production of Kurloff cells, Simmons (1965) reported that they would be produced solely in the thymus as they were observed in large numbers in the thymic lymphatics. Sandberg and his coworkers suggested that Kurloff cells may proliferate within the spleen and migrate into the splenic efferent blood vessels (Sandberg, 1970; Ernstrom and Sandberg, 1971). In our previous study (Kato et al., unpublished data), however, we found that in splenectomized animals, estradiol increased the number of Kurloff cells in the thymus. Furthermore, there was evidence that the cells occur and increase in number in the spleen of thymectomized guinea pigs in response to estradiol stimulation. An increased number of Kurloff cells has been also reported in the blood of thymectomized guinea pigs (Ranlov et al., 1970), suggesting their extrathymic origin. Kurloff cells occur predominantly in the bone marrow and the splenic red pulp, and later in the thymus after estradiol administration. These findings may imply an active formation of Kurloff cells in the bone marrow.

In conclusion, we have investigated the possible migration of Kurloff cells into the thymus using the light and electron microscope. Our findings have substantiated previous conclusions that Kurloff cells can migrate into the thymus via lymphatics and lymphatic accumulation, the so-called "lymphatic center" (Sandberg and Hagelin, 1990). Further studies are needed since the precise pathway of Kurloff cells from lymphatics into the thymic parenchyma could not be monitored at present.

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REFERENCES


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