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Hemocyte production in trematode-infected *Lymnaea truncatula*

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Abstract. In trematode-infected *Lymnaea truncatula,* as in other lymnaeids, hemocytes are formed in the connective tissue. Mitoses are found singly in blood vessels or connective tissue or occur in hemocyte nodules, developing along the mantle epithelium or associated with blood sinuses. The so-called hemocyte-producing organ in *L. truncatula* is not equivalent to that in *Biomphalaria glabrata,* but rather involves the proximal part of the kidney sac. It has a dual structure: the main part, containing podocytes and broad hemal spaces, is adapted for hemolymph filtration; the apical portion, adhering to the mantle and pericardial epithelia, has a thicker connective-tissue frame in which hemocyte nodules may develop. The role of this region of the kidney in hemocyte formation is discussed.

Molluscs infected with trematodes or other organisms show an increased number of circulating hemocytes. The production of hemocytes in lymnaeids may be diffuse, occurring throughout the entire connective tissue (Sminia 1974; McReath et al. 1982; Sminia et al. 1983), along blood sinuses (Pan 1958) or in the connective tissue of the lung and the mantle (Müller 1956; Sminia 1974, 1981; Satdykova et al. 1978).

In other snails, hemocyte production is localized in a lymphoid organ, the hemocyte- or amoebocyte-producing organ (HPO or APO), the localization and structure of which vary. In *Biomphalaria glabrata,* the HPO, whose size varies, occurs in the wall between the pallial and the pericardial cavities (Pan 1958, 1965; Lie et al. 1975, 1976, 1980; Jeong et al. 1983; Joky et al. 1985). In snails exposed to trematode miracidia, resistant individuals show an active HPO in which dividing and differentiating hemocytes form thick nodules and maturing cells are released into a sinus (Jeong et al. 1983; Joky et al. 1985).

In various species of *Bulinus,* the lymphoid organ is located beneath the mantle, between the pericardial cavity and the saccular kidney; it consists of a few nodules of hemocytes in a connective-tissue frame including blood sinuses (Kinoti 1971). A lymphoid organ of similar localization has been described in various species of *Lymnaea* (Rondelaud and Barthe 1981, 1982; Sindou et al. 1986; Ruellan et al. 1987); it has a central lumen that is continuous with the kidney lumen (Rondelaud and Barthe 1981, 1982; Ruellan et al. 1987).

In this report we describe the ultrastructure of the so called HPO of *L. truncatula* and show that this organ is the proximal part of the kidney sac, a site of urinary ultrafiltration similar to that observed in *B. glabrata* (Matricon-Gondran 1990a). To determine whether hemocyte production in this species is diffuse or localized, we also studied the distribution of mitoses and hemocyte nodules in both healthy and trematode-infected snails.

Materials and methods

We used adult *Lymnaea truncatula* O.F. Müller that had been collected in Ambazac (Haute-Vienne, France). Most of the snails were naturally infected with rediae of *Fasciola hepatica* L., rediae of unidentified notocotylids or secondary sporocysts of *Haplometra cylindracea* Zeder. Other snails collected in Lussac-les-Châteaux (Vienne) had few parasites (notocotylids and viruses).

Light microscopy studies

Snails were fixed by immersion in Carnoy's fluid for 3 h and embedded in paraffin. Serial sections (7.5 µm thick) were stained with toluidine blue in acetate buffer (pH 4.6) for visualization of young basophilic hemocytes. Hemocyte divisions were studied in snails from Lussac-les-Châteaux that had been incubated for 16–20 h in 0.05%, 0.1% or 0.5% colchicine in water before fixation. Mitotic cells were visualised using the Feulgen nuclear reaction.

Electron microscope studies

The reno-pericardial region was isolated and immersed for 1 h in 1% glutaraldehyde in 0.05 M cacodylate buffer (pH 7.3). Samples were then rinsed in buffer, postfixed for 2 h in 2% osmium tetroxide in the same buffer, and embedded in Epon. Ultrathin sections contrasted using uranyl acetate and lead citrate were observed with a Philips EM 300 microscope.

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Results

Organization of the proximal part of the kidney in Lymnaea truncatula

The kidney sac of *L. truncatula* lies transversally (left to right) in the dorsal wall of the mantle. Its proximal part, which covers the pericardial cavity (Fig. 1), is followed by a main portion that is characterized by nephrocytes; the kidney ends in a ureter.

The proximal part has a lacunar aspect due to large hemal spaces; its irregular central lumen is continuous with the main kidney lumen (Figs. 1, 2). At the ultrastructural level, the lumen is seen to be lined by podocytes (Fig. 3), forming a deeply infolded epithelium that is continuous with the nephrocyte epithelium of the main part of the kidney. Two ill-defined regions can be recognized: the larger one lies near the lumen and shows large hemal spaces supported by a light connective-tissue frame, whereas contiguous to the mantle and pericardial epithelia, the most apical region exhibits smaller hemal spaces and a denser array of cells among which groups of podocytes at the end of epithelial infoldings, connective-tissue cells and hemocyte nodules can be distinguished (Fig. 2). A conspicuous reno-pericardial duct connects the pericardial cavity to the main part of the kidney sac (Fig. 1). The blood supply of this region would appear to be venous rather than arterial; no renal artery has been detected in any species of *Lymnaea* studied.

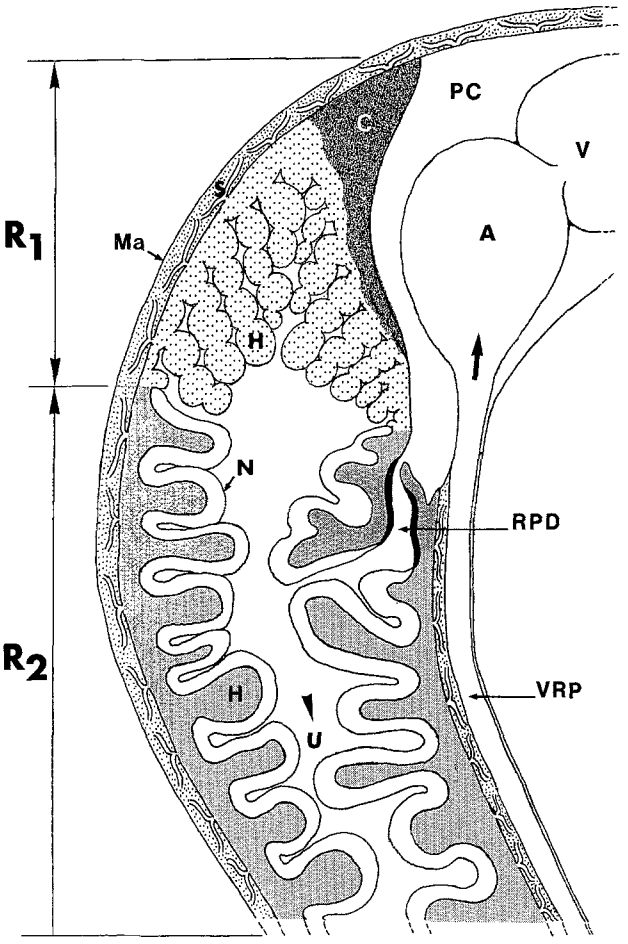


Fig. 1. The reno-pericardial region in *Lymnaea truncatula* (parasagittal section). The proximal region of the kidney, or filtration zone (*R1*), is located between the mantle (*Ma*) and the pericardial cavity (*PC*). It comprises large hemal spaces (*H*) projecting into the central urinary lumen (*U*); its apical portion (*C*) has a denser cellular structure. The main portion of the kidney, or elaboration zone (*R2*), consists of epithelial folds of nephrocytes (*N*). *A*, Auricle; *RPD*, reno-pericardial duct; S, afferent sinuses; *V*, ventricle; *VRP*, vena reno-pulmonalis

Components of the proximal kidney. Podocytes are rather flat epithelial cells, the perinuclear regions of which protrude into the urinary lumen (Fig. 3). Wide baso-lateral spaces extend between the typical pedicels and the apical regions connected by septate junctions. The irregularly spaced pedicels, measuring 0.15–0.25 μm in thickness, contain microtubules and attach to the basal lamina by microfilaments and hemidesmosomes; they are bound by thin diaphragms (Fig. 4).

Hemal spaces are filled with hemolymph characterized by hemocyanin macromolecules and contain variable numbers of hemocytes at various stages of development. The podocyte basal lamina acts as a barrier to hemolymph components (Fig. 4): it is very thick (up to 1.5 μm) and has a lamellar or fibrillar structure (1–6 lamellae). Hemal spaces are supported by a connective-tissue frame consisting of fibroblasts, muscle cells, fixed phagocytes and a few calcium spherule cells.

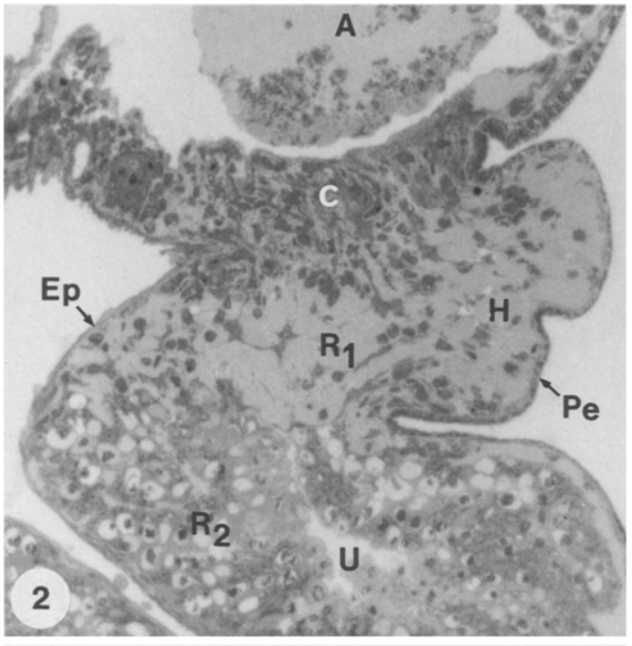
Fixed phagocytes are an important component of this region, although they also exist in other parts of the body. These large cells display long, thick extensions that are attached to the podocyte basal lamina or to collagen bundles. The clear cytoplasm contains a granular endoplasmic reticulum, dictyosomes and mitochondria. The major organelles are large secondary lysosomes, in which whole cells are degraded, and numerous residual bodies (Fig. 5).

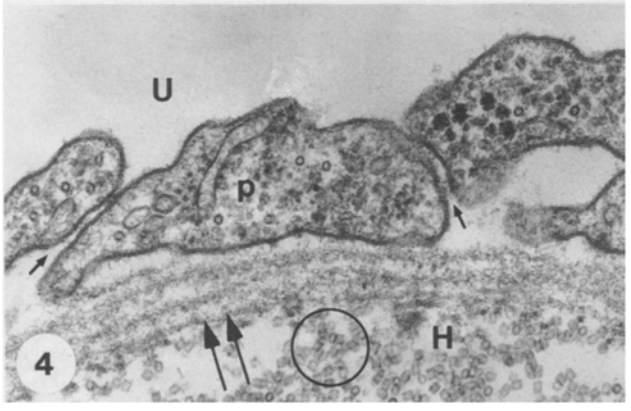
The hemocytes randomly scattered in the hemolymph have the same aspect as those observed elsewhere in the snail's body. They can be dividing cells, juvenile cells containing few secretory vesicles, or differentiating cells exhibiting glycogen deposits and secretory vesicles increasing in size and number (Fig. 6). In the proximal kidney, hemocytes exhibit long, extended filopods.

In the dense apical region, hemocytes are grouped into small nodules: some consist of juvenile cells and dividing cells associated with fixed phagocytes (Fig. 9); others contain more advanced cells (Figs. 7, 8). In the nodules, hemocyte filopods appear to be retracted or folded and the hemocytes are closely apposed to each other over large surfaces of plasma membrane (Figs. 7, 8).

Localization of hemocyte production in L. truncatula

Although snails with low levels of trematode infection had been treated with colchicine, mitotic activity was observed. Mitoses were also visible in untreated parasitized snails. In both cases, mitoses were either dispersed





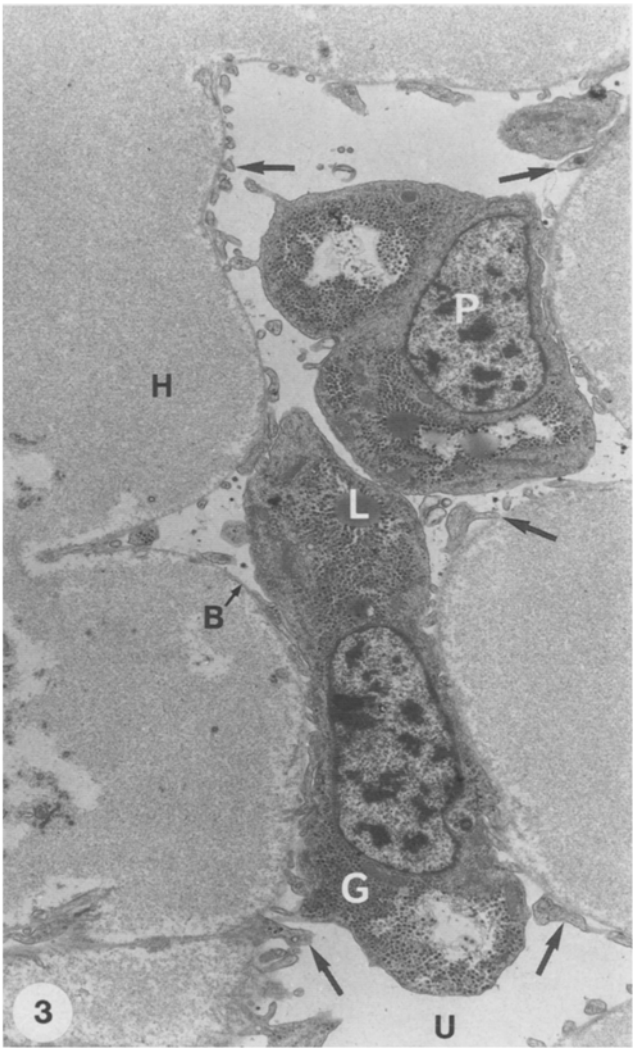


Fig. 2. Semi-thin section of the reno-pericardial region. The proximal kidney (*R1*) shows a dense apical region (*c*) associated with the mantle (*Ep*) and the pericardial epithelia (*Pe*). *A*, Auricle; *H*, hemal spaces; *R2*, nephrocyte region; *U*, urinary lumen. ×250. **Fig. 3.** The proximal kidney: large hemal spaces (*H*) are covered by podocytes (*P*), which form pedicels (*arrows*) interdigitating on the surface of the basal lamina (*B*). *G*, Glycogen; *L*, lipid globules; *U*, urinary lumen. ×5500. **Fig. 4.** Podocyte pedicels (*p*), irregularly spaced, are bound by thin diaphragms (*arrows*). The basal lamina varies in thickness and is often plurilamellar (*double arrow*). In the hemal spaces (*H*), hemolymph proteins, notably hemocyanin molecules (*circle*), are retained behind the basal lamina. *U*, Urinary lumen. ×40000

in connective tissue or localized in various nodular regions, affecting both juvenile and maturing cells.

Single hemocytes were found dividing in blood vessels or sinuses or associated with the connective tissue of open visceral spaces (Fig. 9). Hemocyte nodules varied in composition. Dividing mature hemocytes were found in cellular aggregates in the digestive gland in association with the lysis of secondary sporocysts of *Haplometra cylindracea* (Fig. 10).

Occasionally, snails that did not appear to be parasitized by trematodes but were infected with viruses exhibited hemocyte nodules associated with the mantle, generally in the cephalic region (Fig. 11). Similar nodules occurred in the pulmo-renalis sinus, which runs along the kidney sac and the ureter. These contained basophilic hemocytes and showed randomly scattered mitoses (Fig. 12).

In the pericardial region, no special hemocyte concentration or nodule was found in the wall between the pericardial and the mantle cavities. As mentioned above, more or less juvenile hemocyte nodules were seen in the apical portion of the proximal kidney, associated with the pericardial epithelium (Figs. 7, 8).

Discussion

The present study of hemocyte formation in *Lymnaea truncatula* invaded by trematodes, a long-term reaction against the presence of foreign organisms, produced the following information: (a) the nature and functions of the region considered to represent a lymphoid organ are complex, and (b) the formation of hemocytes appears to be diffuse and seems to be associated with the connective tissues in this species.

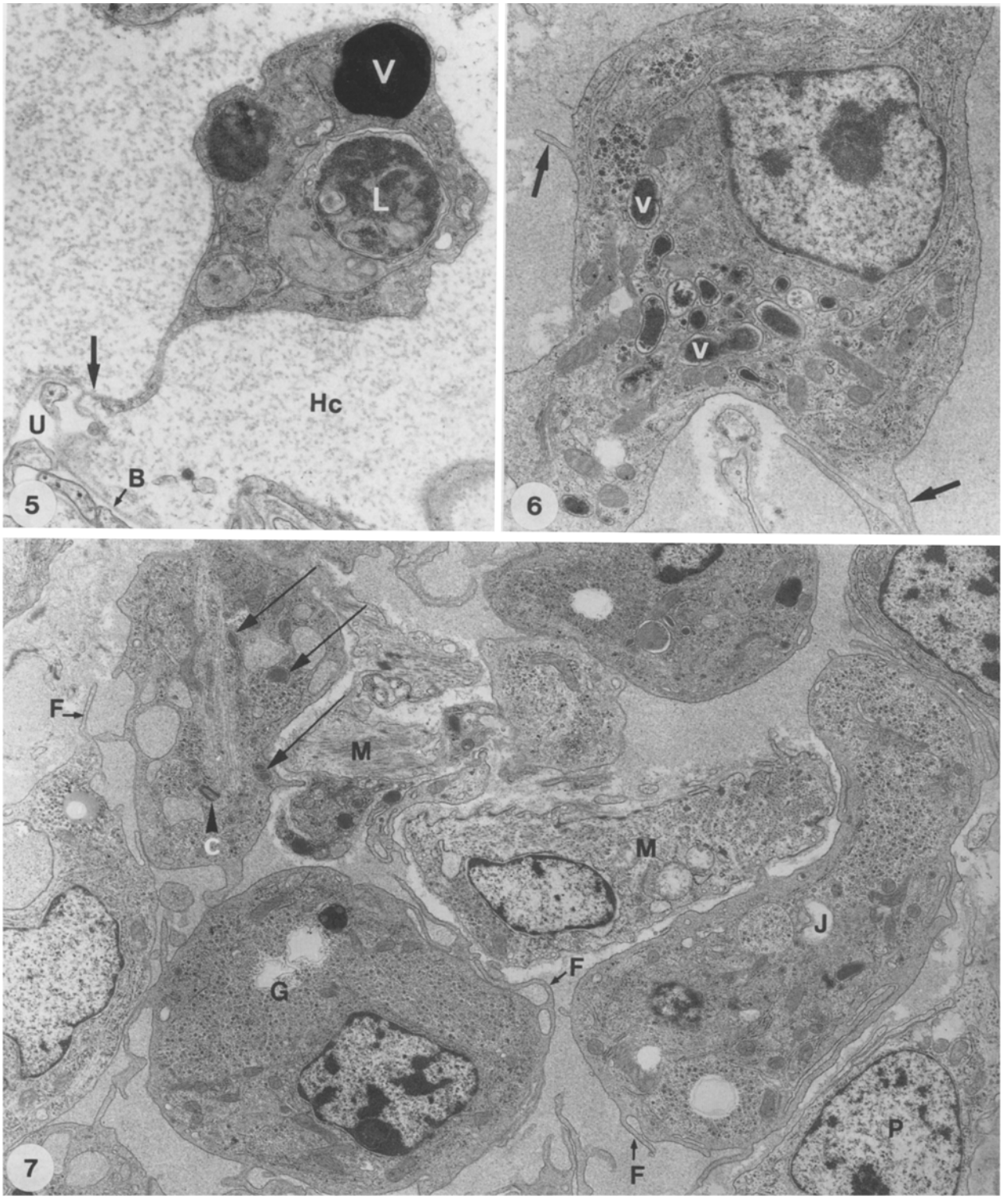


Fig. 5. Part of a fixed phagocyte: this irregularly shaped cell contains large lysosomes (*L*), residual bodies, and dense vacuoles (*V*); it is attached (*arrow*) to the podocyte basal lamina (*B*). *Hc*, Hemocyanin; *U*, urinary lumen. ×15000. **Fig. 6.** Hemocyte in the hemal space, showing extended filopods (*arrows*), a few glycogen particles and secretory vesicles (*V*). ×13000. **Fig. 7.** Part of a nodule containing a juvenile hemocyte (*J*) and more advanced cells, one showing glycogen deposits (*G*). A cell that contains secretory vesicles (*arrows*) is dividing. *C*, Centriole; *F*, filopods; *M*, muscle cell; *P*, podocyte. ×7000

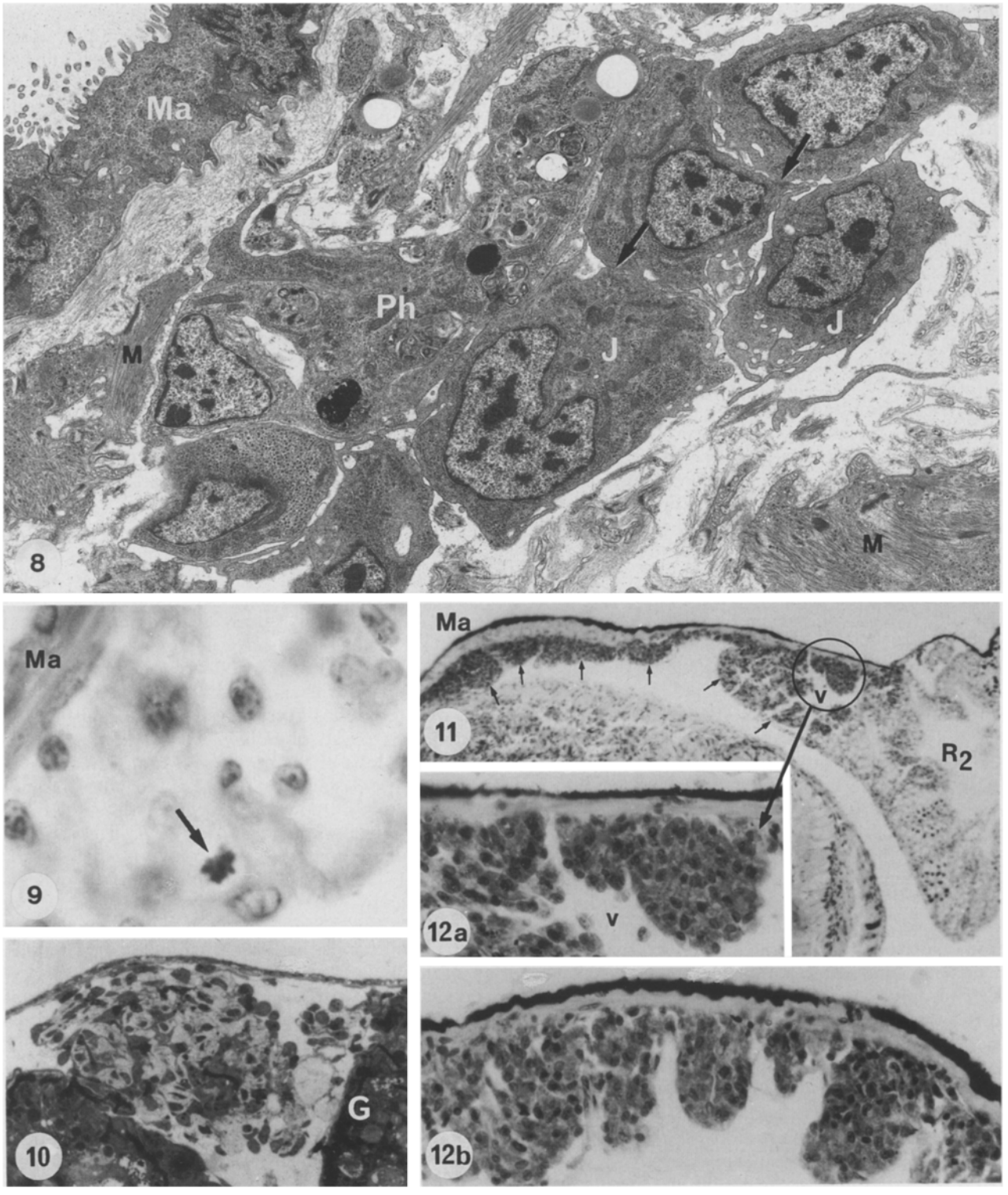


Fig. 8. Hemocyte nodule in the proximal kidney: juvenile hemocytes (*J*) are associated with a fixed phagocyte (*Ph*); they have retracted and folded filopods and show several mutual contacts (*arrows*). *M*, Muscle cell; *Ma,* mantle epithelium. ×6000. **Fig. 9.** Hemocyte mitosis (*arrow*) in a nodule associated with the mantle (*Ma*) of a colchicine-treated snail (Feulgen reaction). ×1600. **Fig. 10.** Cell aggregate in the digestive gland (*G*): a degenerating sporocyst of *Haplometra cylindracea* and its envelope are being invaded and disrupted by hemocytes. ×400. **Fig. 11.** Hemocyte nodules (*arrows*) in the cephalic region are associated with the mantle epithelium (*Ma*) or with the vena reno-pulmonalis (*v*). *R2*, Nephrocyte region of the kidney (toluidine blue staining). ×100. **Fig. 12a, b.** Details of nodules containing densely packed basophilic hemocytes. **a** Nodules protruding in the vena reno-pulmonalis (*v*). **b** Nodules associated with the mantle (toluidine blue staining). ×400

At the fine structural level, the "hemocyte-producing organ" (HPO) of this snail (Rondelaud and Barthe 1981, 1982) exhibits the typical structure of the filtration site of the kidney. A similar differentiation in a pulmonate kidney has recently been reported in *Biomphalaria glabrata* (Matricon-Gondran 1990a). Both of these organs consist of an epithelial layer of podocytes that separates the hemal spaces from the urinary space. Podocytes in both snails show wide baso-lateral spaces, irregularly spaced pedicels connected by thin diaphragms, and thick, multilayered basal laminae that are assumed to form the ultrafiltration barrier.

As in *B. glabrata,* the hemal spaces in *L. truncatula* contain hemolymph and hemocytes at various stages of development and are supported by a light connective-tissue frame that is rich in fixed phagocytes. Although the ultrafiltration site in *B. glabrata* has an arterial blood supply (Matricon-Gondran 1990a), we did not find any renal artery in the various *Lymnaea* species studied. As described in *L. stagnalis* (Bekius 1972), hemolymph should arrive at the kidney through afferent pulmonary sinuses and then be drained from its proximal part through the vena renalis minor. Thus, the hydrostatic pressure necessary for hemolymph ultrafiltration may be obtained by a system of valves.

The proximal part of the kidney in *L. truncatula* exhibits the same location and general aspect as structures previously reported in *L. stagnalis*, such as the portions of renal infoldings with a squamous epithelium observed by Wendelaar Bonga and Boer (1969) and the "nephridial gland" mentioned by Bekius (1972); all of these structures may represent the same region of the kidney and have the same functions. The "lymphoid organ" observed by Kinoti (1971) in various *Bulinus* species may have a similar significance.

The proximal part of the kidney of *L. truncatula* differs from the HPO of *B. glabrata*. The hemocyte nodules in its apical region are dispersed in connective tissue between podocyte epithelial folds. There are no thick, proliferative zones in which hemocyte development proceeds from the pericardial epithelium towards a sinus into which maturing cells are released, as reported in *B. glabrata* (Jeong et al. 1983; Joky et al. 1985).

According to Rondelaud and Barthe (1980, 1981), the hemocytic response in *L. truncatula* occurs as late as 21 days after exposure to miracidia in snails infected with *Fasciola hepatica*. So there is no immediate HPO response (1–7 days after exposure) such as that observed in *B. glabrata* exposed to echinostomes to which they are resistant (Lie et al. 1976; Jeong et al. 1983; Joky et al. 1985). *L. truncatula* is susceptible to *F. hepatica* infection and the miracidial invasion probably does not trigger the hemocytic response. The late reaction observed by these authors may be triggered by excretory-secretory products of advanced stages of the parasite.

The increased abundance of hemocytes in the proximal kidney may reflect a general increase in hemocytes rather than a local production of the cells, but we cannot exclude the possibility of increased activity of the hemocyte nodules in the apical part of the kidney. The role of these nodules in hemocyte formation would be more accurately evaluated by studying healthy, laboratory-reared lymnaeids that have been experimentally exposed to trematodes to which they are resistant, beginning at the onset of infection.

Hemocytes observed in the proximal kidney were in various stages of development. Such a heterogeneity of the hemocyte population has also been observed in *L. stagnalis* (Dikkeboom et al. 1984). The hemal spaces of the proximal kidney of *L. truncatula* may also represent a site for either the maturation or the storage of hemocytes, as suggested for *B. glabrata* (Matricon-Gondran 1990b).

Thanks to the presence of numerous fixed phagocytes, the proximal kidney may participate in the internal defence system of the snail by sequestering and digesting foreign particles or organisms. Tripp (1961) and Brown and Brown (1965) have shown the manner in which fixed phagocytes accumulate foreign particles. The fixed phagocytes of *L. truncatula* share the characteristics of those of *L. stagnalis,* in which Sminia et al. (1979) demonstrated the importance of lysosomes and acid phosphatase activities.

Our observation of hemocyte divisions occurring singly in the connective tissue or hemal spaces of *L. truncatula* is in good agreement with Sminia's opinion (Sminia 1974; Sminia et al. 1983) that the production of hemocytes in *L. stagnalis* is diffuse. In addition, we found hemocyte nodules, which have not previously been recorded. Although all of the nodules contained dividing cells, their functional significance varied. Heterogeneous cell aggregates in the visceral spaces of snails harbouring secondary sporocysts of *Haplometra cylindracea* are involved in the lysis of the parasites (Monteil and Matricon-Gondran 1991) and can be assimilated into granulomas. The hemocyte nodules associated with blood sinuses in the cephalopedal region of *L. truncatula* recall the cell accumulations described in *Marisa cornuarietis* (Yousif et al. 1980). As we did not study them by electron microscopy, we cannot judge whether they are equivalent to those found in the proximal kidney. We also do not know whether these proximal kidney nodules play a role similar to that of the HPO in *B. glabrata.*

In conclusion, in *B. glabrata* most, but probably not all, hemocyte divisions following the exposure of resistant snails to trematodes occur in the HPO (Jeong et al. 1983; Sullivan et al. 1984; Joky et al. 1985). Hemocyte maturation may then take place in the hemal spaces of the proximal kidney (Matricon-Gondran 1990b). In lymnaeids, hemocyte production is diffuse, with cell divisions in connective tissue occurring singly or in nodules. The hemal spaces of the proximal kidney may also play a role in the differentiation or storage of these cells. General conclusions concerning the long-term cellular response to parasite infection must await further studies on other species of snails that serve as intermediate hosts to trematodes.

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