

Characterisation of Kupffer cells in some Amphibia

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ABSTRACT

A study on the Kupffer cells (KCs) of Amphibia was undertaken in order to compare these cells with those of endothermic animals. Liver tissue and isolated and cultured KCs were studied by light microscopy and by transmission and scanning electron microscopy. We have shown that amphibian KCs can be divided into 2 principal types: 'small' and 'large'. Both cell types possess the distinctive KC morphology. They show nonspecific esterase activity, weak endogenous peroxidase activity in the nuclear envelope and in the rough endoplasmic reticulum, and the ability to engulf naturally present cell debris or experimentally administered zymosan or latex particles. The principal difference between the small and the large cells consists in the substantial quantity of inclusion bodies that exist only in the latter. We conclude that amphibian KCs, apart from their ability to build melanosomes and synthesise melanins, are very similar to mammalian KCs.

Key words: Macrophages; mononuclear phagocyte system; reticuloendothelial system.

INTRODUCTION

Kupffer cells (KCs) are macrophages anchored to sinusoid endothelium in the liver, and are particularly numerous in the portal area. The principal characteristics that distinguish them from other sinusoid cells are their distinctive morphology; the presence of certain enzyme activities, such as nonspecific esterase and endogenous peroxidase, phagocytic and pinocytotic abilities (for the use of the term pinocytosis, see Discussion); the presence of membrane receptors for the Fc region of IgG and for complement, and their stainability with specific monoclonal antibodies. But because of their recognised heterogeneity (Ginsel, 1993), all these requirements are rarely expressed together; for this reason, the presence of at least 3 of the above-mentioned features is considered sufficient to characterise a cell as a macrophage (Leenen & Campbell, 1993). Currently KCs are included in the 'mononuclear phagocyte system' (MPS, Van Furth et al. 1972).

Mammalian KCs, particularly those in man, are of interest because of the numerous important functions that they subserve (for reviews, see Leenen &

Campbell, 1993; McCuskey, 1993; Turpin & Lopez-Berestein, 1993).

The ultrastructure of mammalian KCs has been described and reviewed in several publications (Widmann et al. 1972; Wisse, 1972, 1974, 1980; Motta & Porter, 1974; Motta, 1975; Widmann & Fahimi, 1975; McCuskey & McCuskey, 1990; Naito et al. 1997). Conversely, our knowledge of the structure and ultrastructure of amphibian KCs is very limited. Moreover, the excellent reviews by Manning & Horton (1982) and Turner (1988) show that the literature relevant to Amphibia is largely devoid of structural and ultrastructural studies with respect to the immunological functions of macrophages.

Generally, the authors who have studied amphibian macrophage structure and ultrastructure, have focused principally on their phagocytic activity (Fey, 1967; Turner, 1969; Campbell, 1970; Curtis et al. 1979; Zapata et al. 1982; Sichel et al. 1997; Guida et al. 1998). As has been known since the last century, pigment cell clusters are present in the liver of ectotherms, localised principally in portal areas. After numerous studies, we believe that these cells, in Reptilia and Amphibia, derive from KCs that pro-

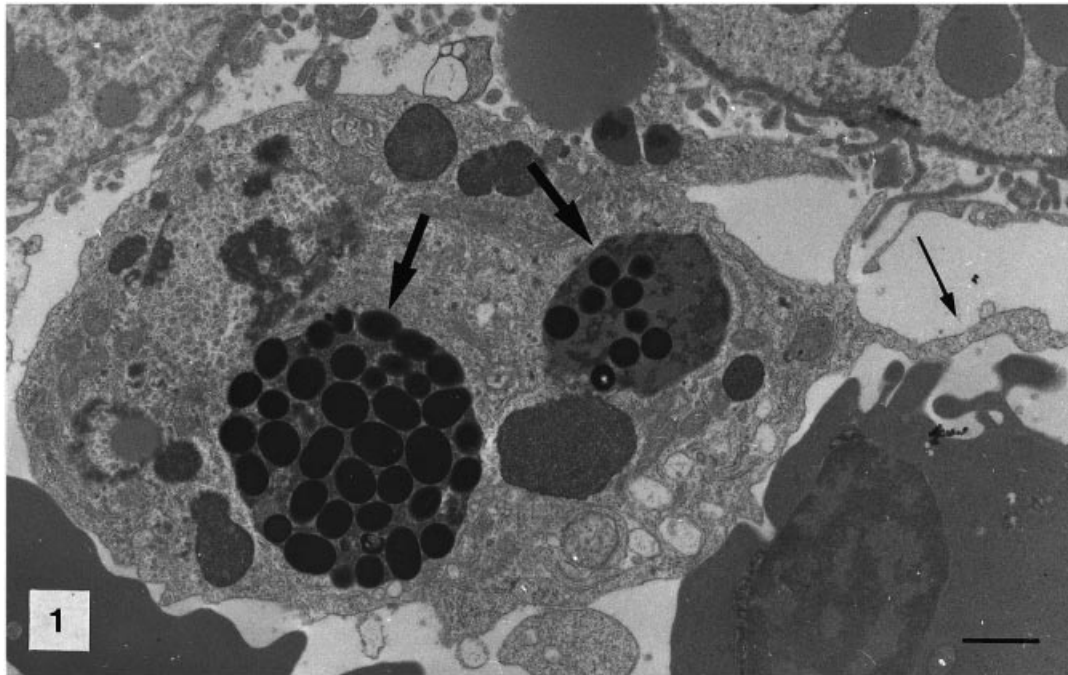


Fig. 1. Pigmented *Xenopus* liver. Kupffer cell in endothelial position, showing a pseudopodium in contact with an erythrocyte (thin arrow) and melanosomes in lysosomes (thick arrows). Bar, 1 μ m.

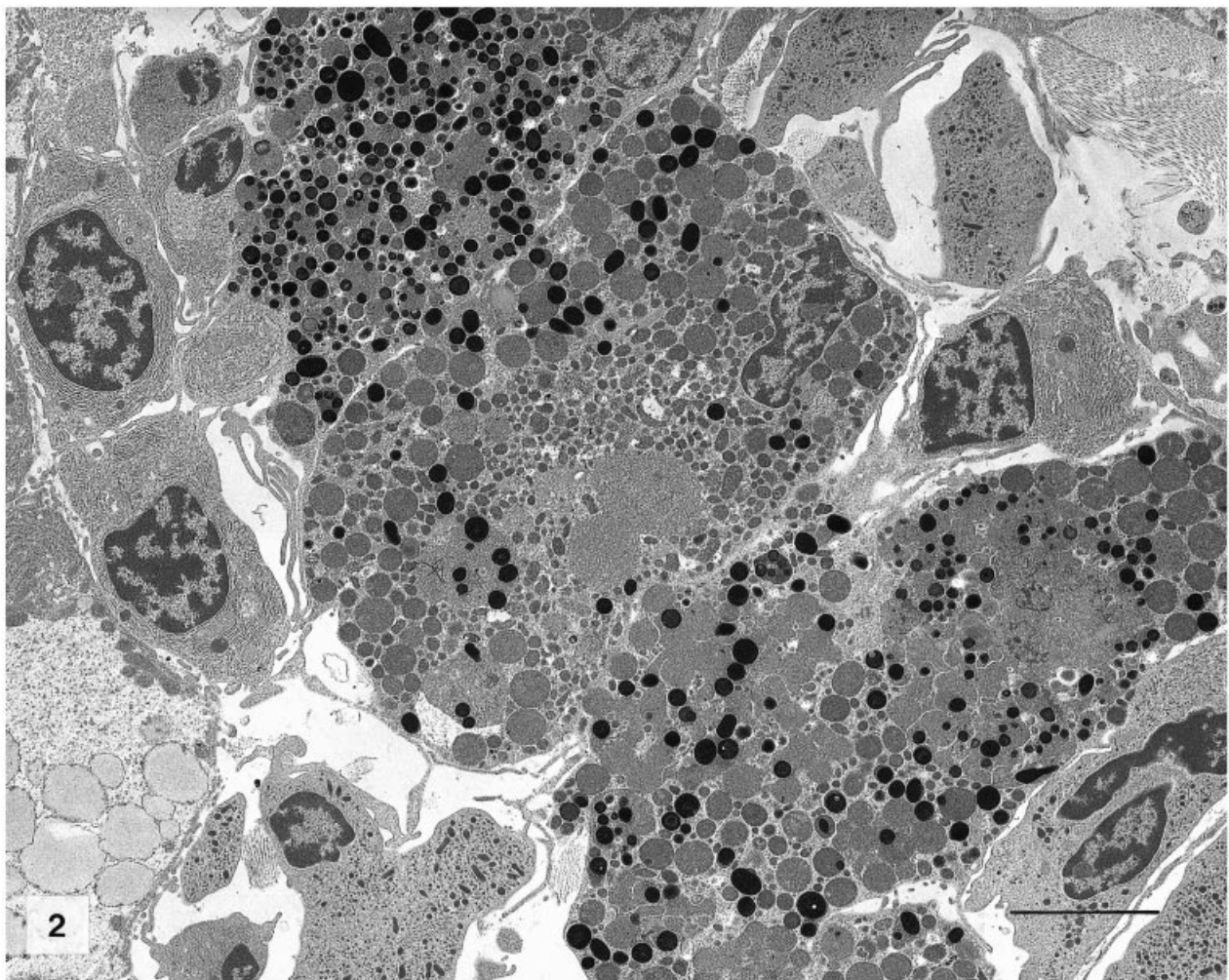


Fig. 2. Pigmented *Xenopus* liver. Small cluster of pigmented Kupffer cells in portal area. Bar, 2 μ m.

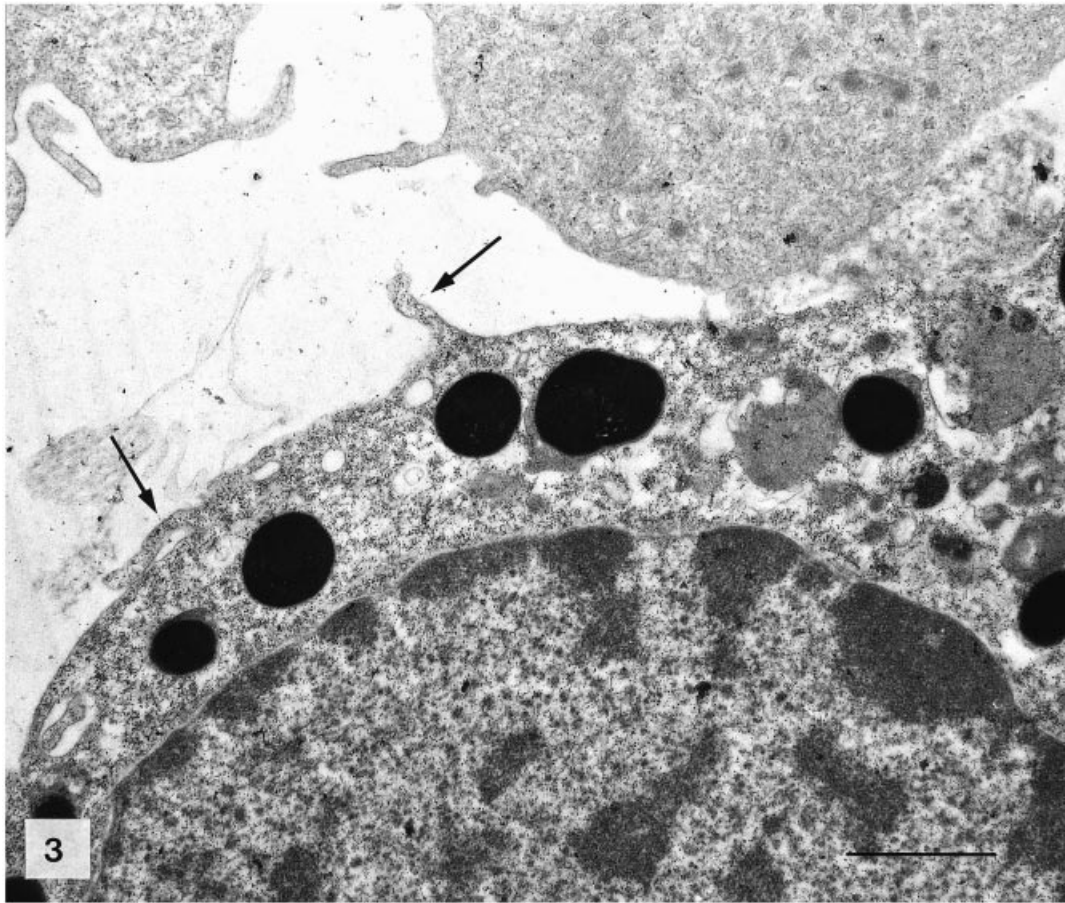


Fig. 3. Pigmented *Axolotl* liver. Kupffer cell containing few melanosomes and few inclusion bodies. Small microvillous-like structures are visible (arrows). Bar, 1 μ m.

gressively become pigmented (for a review, see Sichel, 1988), and we have been able to demonstrate, particularly in Amphibia, that the pigmentation of KCs depends chiefly on their ability to produce melanosomes and synthesise melanin, and only in part on melanosome phagocytosis (Sichel et al. 1997).

The particular behaviour of these cells (which are professional phagocytes, *sensu* Smythe, 1996, like those of mammals and birds) and which are also able to synthesise melanin, together with the general lack of knowledge about them, led us to carry out this study. Our aim is to contribute to the knowledge of the distinctive morphology of amphibian KCs, and their pinocytotic and phagocytic abilities, to verify the presence of nonspecific esterase and endogenous peroxidase activity in order to compare KCs of ectothermic and endothermic animals.

MATERIALS AND METHODS

Animals

We used the following amphibian specimens: *Rana esculenta* L. caught near Catania (Italy); albino and pigmented neotenic specimens of *Xenopus laevis* Daudin; adult

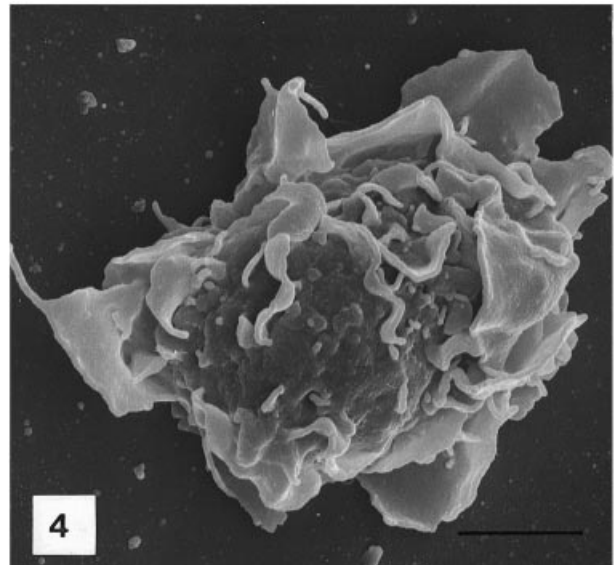


Fig. 4. SEM micrograph of a cultured Kupffer cell from *Rana* in which numerous lamellipodia are visible. Bar, 3 μ m.

specimens of *Triturus cristatus* Laurenti; albino and pigmented neotenic specimens of *Ambystoma mexicanum* Cope (axolotl).

All animals, except *Rana esculenta*, were purchased from commercial sources.

Chemicals

Carmine, glutaraldehyde, osmium tetroxide, caesium chloride, and hydrogen peroxide (perhydrol 30%) were purchased from Merck (Darmstadt, Germany); α -naphthyl acetate, pararosaniline, sodium fluoride, collagenase, 3,3'-diaminobenzidine tetrahydrochloride (DAB), latex beads (0.6 μm), zymosan A, sodium azide, β -glycerophosphate, trypan blue, and pronase E from Sigma Chemical Co. (St Louis, Mo); Durcupan ACM from Fluka (Chemika-Biochemika, Switzerland); HEPES, Medium E199, newborn calf serum, gentamycin, penicillin, streptomycin, and amphotericin B from Seromed. All other chemicals were of analytical grade.

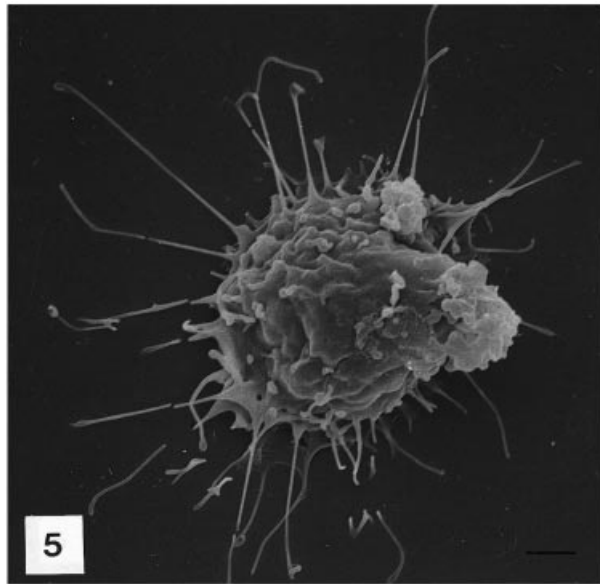


Fig. 5. SEM micrograph of a cultured Kupffer cell from *Rana* in which filopodia are shown. Bar, 2 μm .

Isolation and culture of Kupffer cells from *Rana* liver

All operations were carried out in conditions of absolute sterility. The experiments were performed according to the method of Guida et al. (1998) with minor modifications. The livers from *Rana*, after perfusion, were excised, minced and subjected to digestion (Pintucci et al. 1990), then filtered through a sterile nylon gauze. The filtrate supernatant, after being allowed to settle for 10 min, was centrifuged at 600 g for 5 min. The resultant pellet, after repeated washing with Medium E199 modified for amphibia, was recovered with 5 ml of the same medium and resuspended in 30% metrizamide in phosphate buffered saline (PBS) 0.8% in the ratio of 5:7 (vol:vol) and centrifuged at 1400 g for 15 min. We obtained a pellet and a top layer that were recovered separately and then washed; the cells were counted in a Bürker chamber and then seeded in culture medium at a density of 3×10^6 cells in 75 cm^2 T flasks for the pellet and 1.6×10^5 cells in 25 cm^2 T flasks for the top layer. The cells were incubated at 28 $^{\circ}\text{C}$ in the presence of 5% CO_2 . After 24 h incubation the culture medium was removed and replaced with fresh medium; this was repeated every 24 h for 3 d. About 20% of cells adhered to the flasks and these were used for our studies.

Routine transmission electron microscopy

The study was carried out both on slices of liver tissue from the studied amphibia and on KCs isolated from *Rana* liver. Liver tissue specimens were treated according to Sichel et al. (1997). For preparation of isolated and cultured KC specimens, the cells detached from the flasks after 24 h of culture by a cell scraper

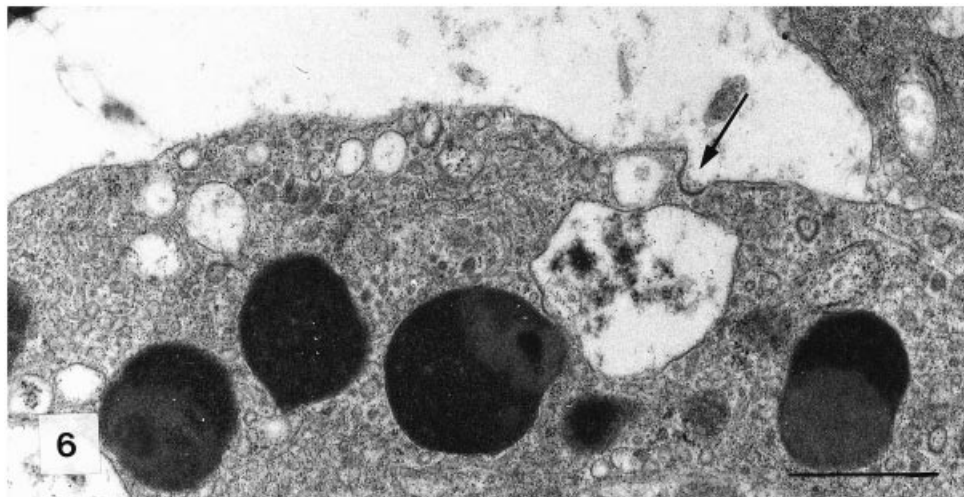


Fig. 6. Pigmented *Xenopus* liver. Surface of a large Kupffer cell in which the fuzzy coat is particularly evident in a pit (arrow). Bar, 1 μm .

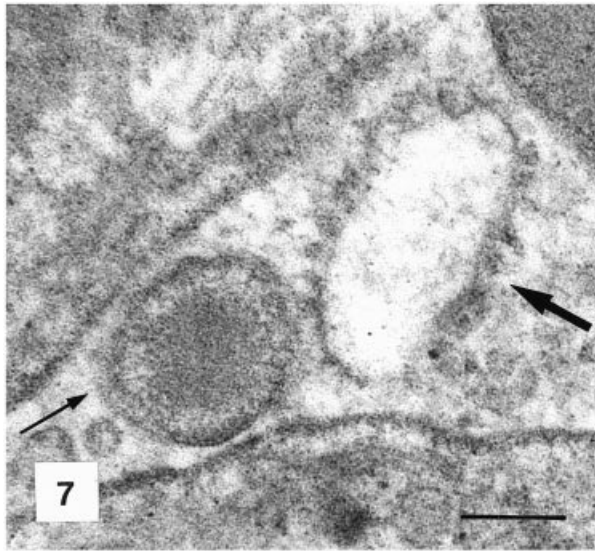


Fig. 7. *Rana* liver. Kupffer cell showing a coated vesicle (thin arrow) and a bristled vacuole (thick arrow). Bar, 100 nm.

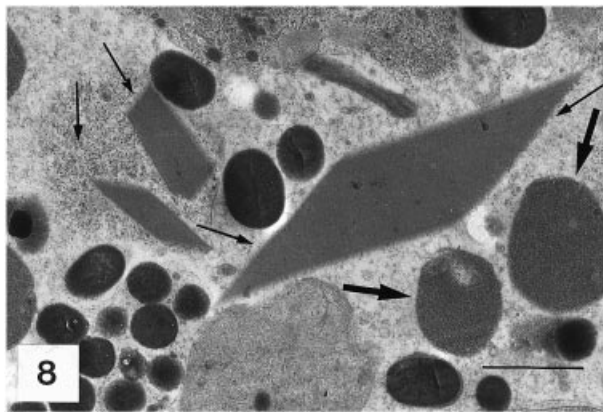


Fig. 8. Pigmented *Xenopus* liver. Particular of a Kupffer cell in which siderosomes (thick arrows) and crystal-like bodies deriving from haemoglobin (thin arrows) are visible. Bar, 1 µm.

were fixed at 4 °C with 1% glutaraldehyde in 0.12 M PBS (pH 7.2) for 30 min, washed in 0.8% PBS, postfixed in 1% osmium tetroxide for 1 h, dehydrated through a graded ethanol series and embedded in Durcupan ACM. Ultrathin sections were cut by a Reichert Ultracut E microtome and contrasted with uranyl acetate and lead citrate. All observations were carried out using a Hitachi H-7000 TEM.

Scanning electron microscopy (SEM)

For SEM, the cells, grown for 24 h on sterile circular cover glasses inserted into the culture flasks, were fixed for 30 min with 1% glutaraldehyde in 0.12 M phosphate buffer, pH 7.4, postfixed for 30 min in 1% osmium tetroxide, dehydrated in ethanol, critical

point dried and gold sputtered using an Emscope SM33. A Hitachi S-4000 field emission scanning electron microscope was utilised for the observations.

Cytochemistry

Nonspecific esterases (EC 3.1.1.1, 3.1.1.2, 3.1.1.6). The isolated and cultured cells from *Rana* liver were treated to determine the positivity for nonspecific esterases using the pararosaniline method after Gomori, cited by Oliver et al. (1991).

Acid phosphatase (AcPase) activity. This was detected according to the methods of Beaulaton (1967), Robinson & Karnovsky (1983), and Schraermeyer & Stieve (1991).

Peroxidase activity. Liver pieces from *Rana* were used to determine peroxidase activity using the method of Pino & Bankstone (1979), Wisse (1974) and Graham & Karnovsky (1966).

Pinocytosis and phagocytosis

Pinocytosis experiments were carried out on *Rana* using trypan blue, lithium carmine and ammoniacal carmine. The animals were divided into 3 groups of 5 individuals, each of about 50 g. Each frog was injected via their dorsal lymph sacs every other day for 1 wk with the following quantities of 'vital dyes': group 1 with 0.25 ml of 5% trypan blue in phosphate buffer; group 2 with 0.5 ml of 2.5% lithium carmine (Kawai et al. 1998); and group 3 with 0.5 ml of 0.5% ammoniacal carmine in PBS 0.8% (Langeron, 1934). All animals were killed 1 wk after the last injection and small pieces of their liver were processed routinely for optical microscopy (OM) and TEM study.

Phagocytosis experiments were carried out utilising both frogs and KCs isolated from *Rana* liver. In the first instance we proceeded according to our previous method (Sichel et al. 1997), in the second the cultured cells were treated with zymosan (20 µl from a stock suspension of 1 mg/ml of zymosan in PBS 0.8% for 1.6×10^6 cells) for 30 min at 28 °C, washed with PBS 0.8%, fixed for 30 min with 1% glutaraldehyde in PBS 0.12 M and postfixed in 1% osmium tetroxide. Subsequent processing followed routine procedures.

Experiments with opsonised zymosan were performed using the method of Gridley et al. (1991).

RESULTS

The amphibian KCs studied here are localised in the endothelial walls of the hepatic sinusoids, often interpolated between the endothelial cells, and especially numerous in the portal area (Figs 1, 2). Those

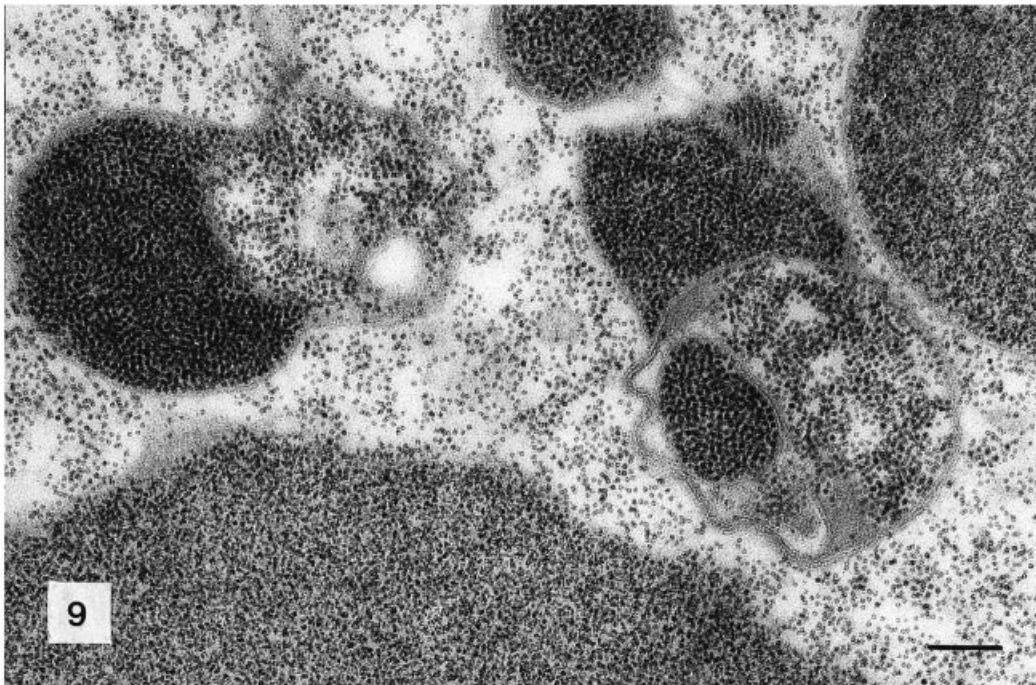


Fig. 9. Albino *Xenopus* liver. Composite siderosomes and numerous ferritin particles are visible. Bar, 100 nm.

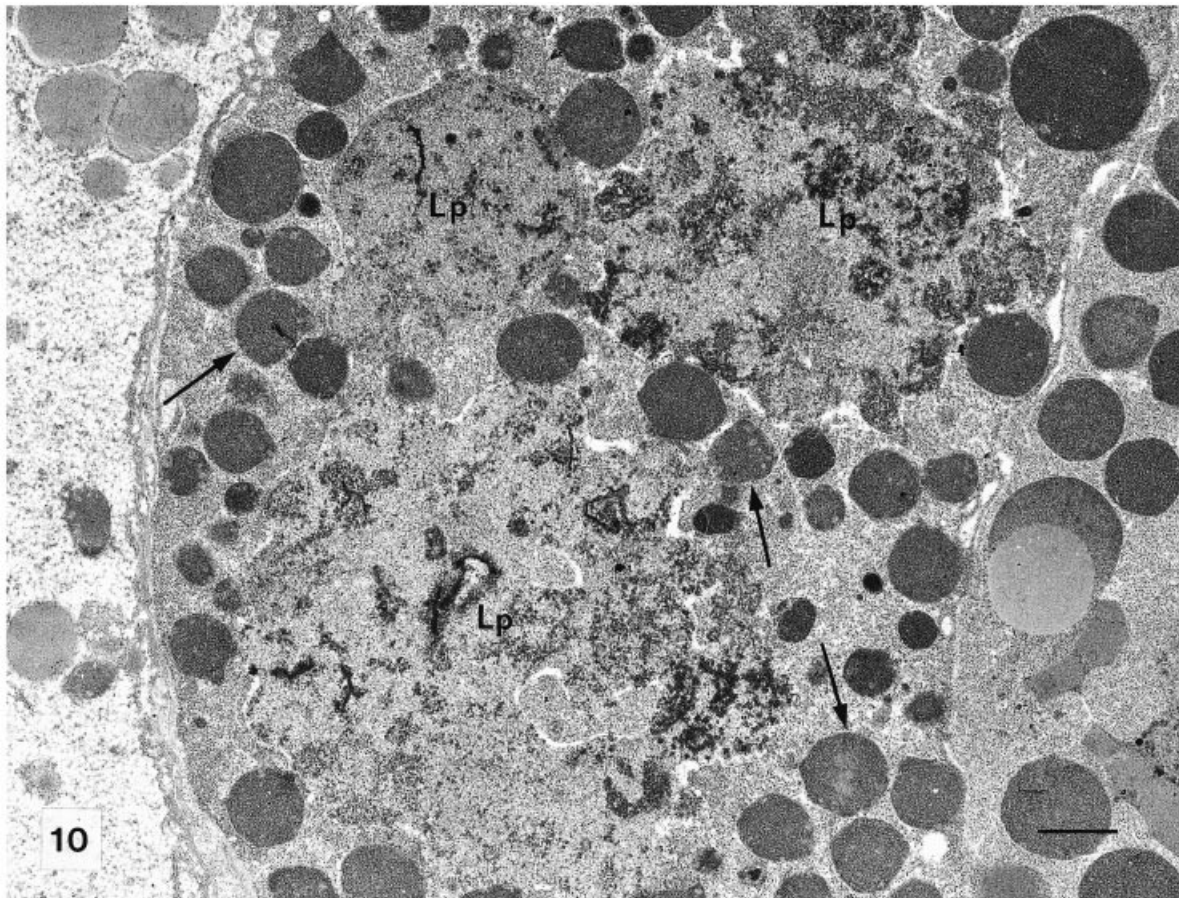


Fig. 10. Albino *Xenopus* Kupffer cells. Numerous lipofuscins (Lp) and lysosomes (arrows) are visible. Bar, 1 μ m.

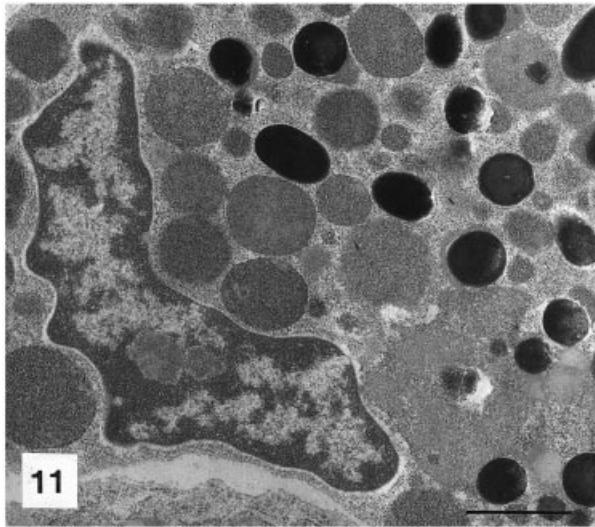


Fig. 11. Pigmented *Xenopus* liver. Kupffer cell with a nucleus in peripheral position, irregularly elliptic in shape, with numerous indentations. Bar, 1 μ m.

in an endothelial position, which are not particularly enlarged are $\sim 13.5 \mu\text{m} \times 4.5 \mu\text{m}$; we term these 'small Kupffer cells' (Fig. 1). Their exposed surface shows microvillous-like structures, sometimes in contact with the endothelial fenestrae, filopodia (particularly visible in cultured cells) and lamellipodia (Figs 3, 4, 5). We employ the term 'large Kupffer cells' for those that are present principally in the portal areas because they contain massive quantities of

melanosomes and numerous other inclusion bodies. Their dimensions are $\sim 18 \mu\text{m} \times 11 \mu\text{m}$. These KCs are normally aggregated in clusters but no organised junctions have been observed on their contact surfaces. Their surface is more regular than that of the small KCs, but numerous microvillous-like formations, filopodia and lamellipodia are also present.

A fuzzy coat, particularly evident on the surface of the pits (Fig. 6), covers the plasma membrane of both small and large cells. Coated, apparently empty, vesicles and bristled vacuoles are seen in the peripheral area of the cell (Fig. 7). Empty vacuoles and vacuoles containing few small dense granules are spread throughout the cytoplasm.

Despite our very numerous observations we have so far never seen those 'classic' structures referred to as 'worm-like structures' (Törö et al. 1962; Orci et al. 1967), or 'endocytosis vermiformis' (Matter et al. 1968), which are described in mammalian KCs.

The most abundant inclusion bodies, except for the melanosomes, present in amphibian KCs consist of material derived from red blood cells (RBCs). Whole RBCs, or their irregular fragments can be seen internalised in the cytoplasm, together with a series of other bodies such as rectangular or trapezoidal crystals, probably derived from haemoglobin (Fig. 8). These features confirm previous reports that demonstrated, by histochemical methods, that amphibian KCs contain iron (Sichel, 1953).

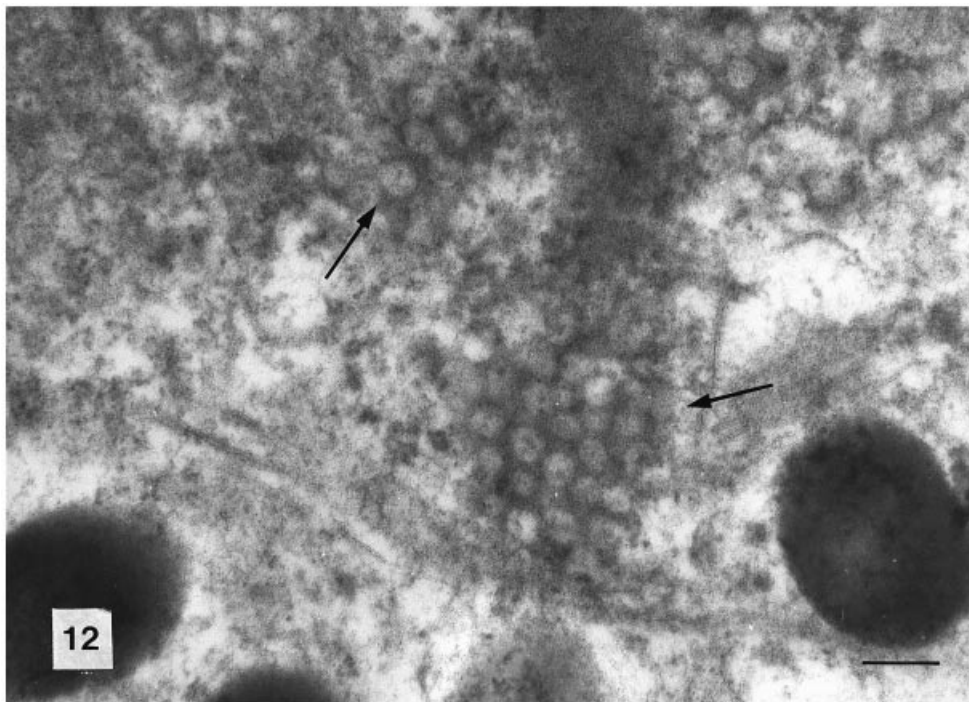


Fig. 12. *Rana* Kupffer cell. Tangential section of a nuclear envelope in which numerous pores are visible (arrows). Bar, 100 nm.

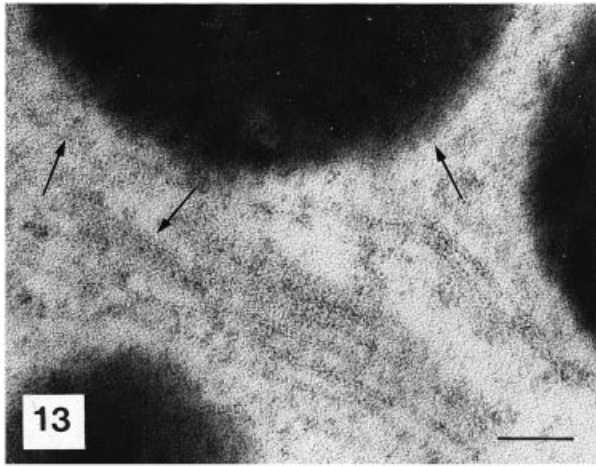


Fig. 13. *Rana* Kupffer cell. Lysosomes surrounded by membranes in which AcPase activity (arrows) is present (Beaulaton's method). Bar, 50 nm.

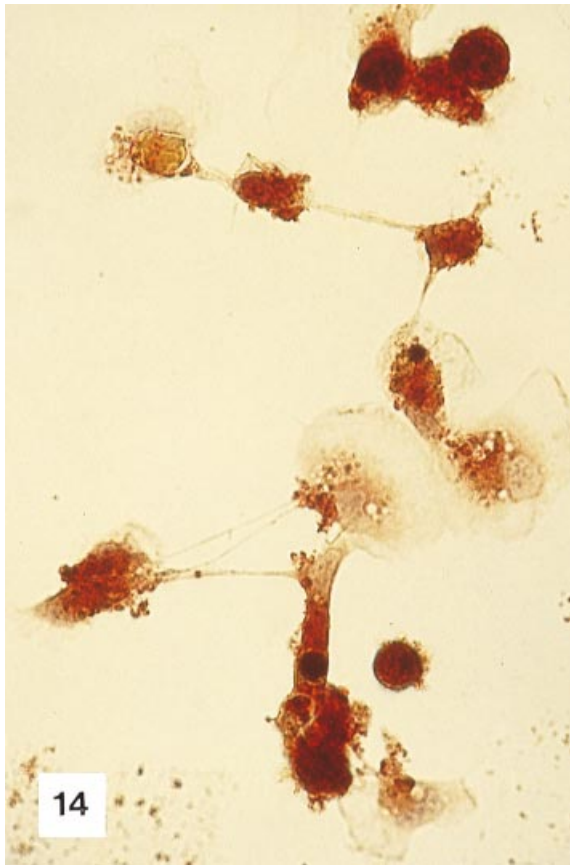


Fig. 14. Cultured Kupffer cells from *Rana*. Nonspecific esterase activity is present. Melanin granules in the cytoplasm of the cells are visible. $\times 200$.

According to David & Freytag (1963), who described ferritin-like bodies in the KCs of urodele amphibians, in our animals numerous electron dense ferritin-like particles were also seen both randomly spread throughout the cytoplasm and frequently grouped to form siderosomes in the same cell;

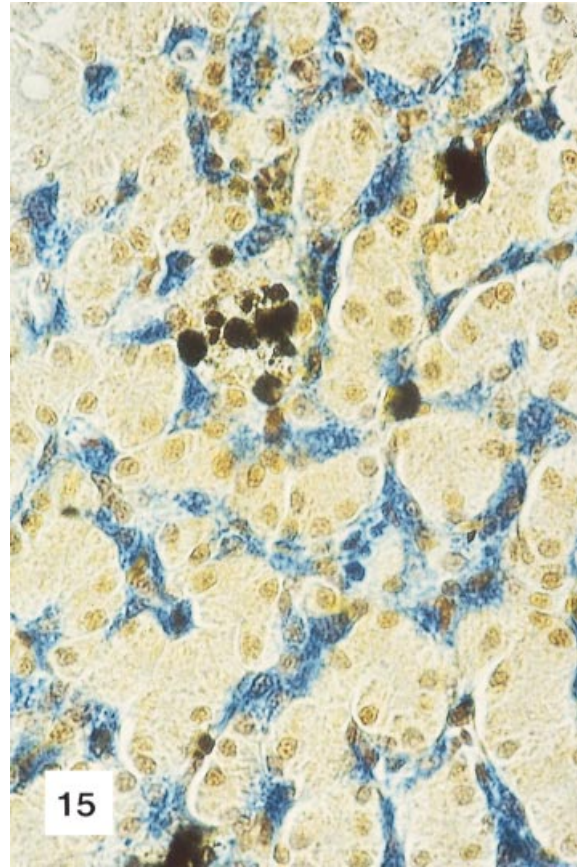


Fig. 15. *Rana* liver. Sinusoidal cells in which trypan blue is stored are shown. Many of these cells contain melanin granules. $\times 200$.



Fig. 16. *Rana* liver. Small Kupffer cell in which an internalized zymosan particle is visible. Bar, 2 μ m.

sometimes they also form structures in which the ferritin particles appear associated with a membranous substrate (Fig. 9). The ferritin particles (for a review, see Iancu, 1992) can, moreover, be ordered to produce crystal-like structures. These particles should be the ferric-hydroxy-phosphate core of ferritin; they show variable dimensions from 7 nm to 8 nm. The siderosomes or the composite siderosomes sometimes contain clusters of smaller, electron-dense particles;

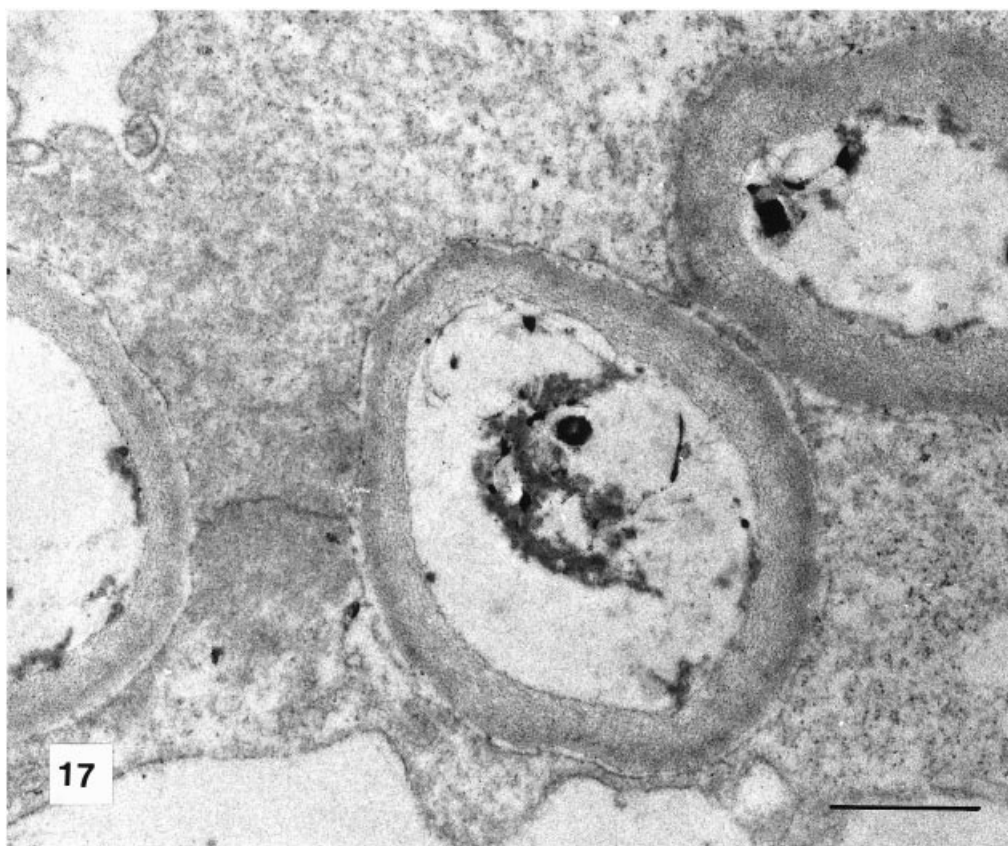


Fig. 17. Cultured Kupffer cell from *Rana*. Engulfed zymosan particles are visible. Bar, 1 μ m.

we consider these structures to be haemosiderin. In the composite siderosomes, the ferritin granules can be associated with cellular debris.

With regard to other inclusion bodies, lipofuscin is generally present in the large KCs intermingled among the siderosomes, melanosomes, lysosomes, etc. Lipofuscin is particularly abundant in the albino specimens (Fig. 10) whose liver, as shown by Corsaro et al. (1995), contains a quantity of unsaturated fatty acids greater than that of the pigmented liver. These lipopigments are almost absent in the small KCs.

Dense bodies (lysosomes) are visible throughout the cytoplasm (Fig. 10). Composite lysosomes, containing cellular debris with melanosomes, are also seen.

In this study we will not describe the 'melanosomogenesis centres', the premelanosomes and melanosomes, which are present in the large KCs, because they have already been considered by Sichel et al. (1997).

Mitochondria appear normally distributed in the small KCs, but in the large KCs they appear to be less numerous because of the large quantity of the internalised material, and are visible around the inclusion body collections. The mitochondria generally show an elliptic section and are $\sim 1 \mu$ m long

and $\sim 0.3 \mu$ m wide; their cristae are perpendicular to the major axis of the organelle and their content, which is not very electron-dense, is constituted by very thin granules immersed in a matrix.

Rough endoplasmic reticulum (RER) is plentiful in the small KCs and regularly distributed throughout the cytoplasm as flattened cisterns or small vesicles. Conversely, it appears scantily present in the large KCs, perhaps due to the cell volume. In this case the RER is represented by small vesicles or flattened vesicles principally localised near the nucleus. We have never encountered the 'annulate lamellae' observed by Wisse (1977) in mammalian KCs.

Golgi complexes are numerous in the small KCs, but appear to be very few in the large KCs, probably due to their volume. In these cells the trans Golgi network is also implicated in melanogenesis (Sichel et al. 1997).

The small KCs contain a single nucleus in a nearly central position, $\sim 4.74 \mu$ m \times 3.1μ m; it is pushed nearer to the cell wall and it is 5.7μ m \times 2μ m in the large KCs (Fig. 11). In both types of cell it is almost elliptic in shape with indented boundaries and the chromatin, as thin granules, is scattered throughout the nucleoplasm; sometimes the granules are clumped

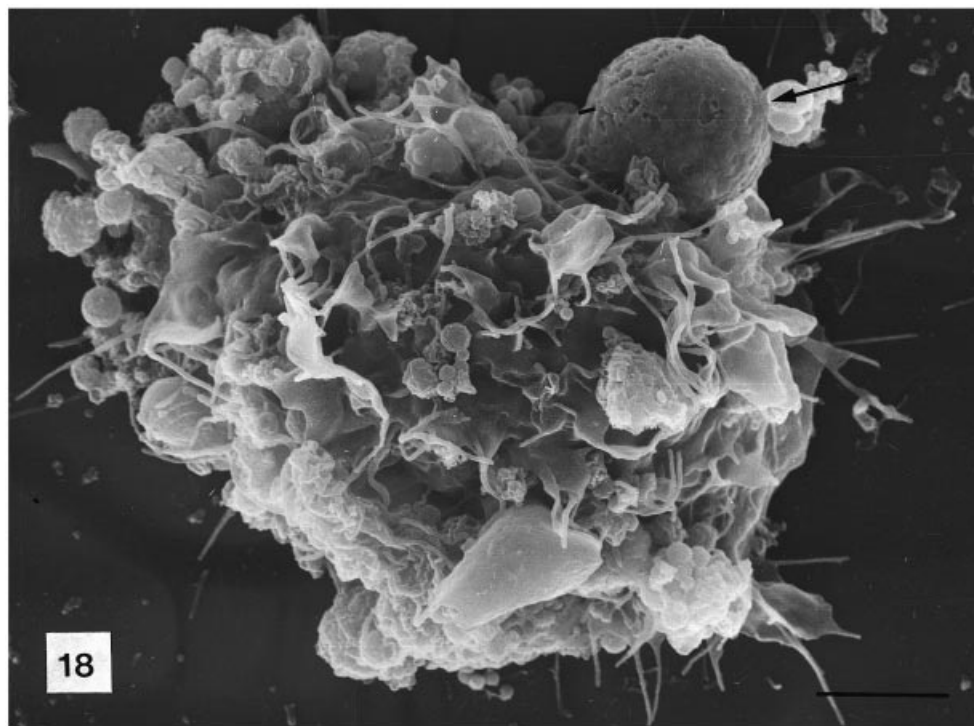


Fig. 18. Cultured Kupffer cell from *Rana*. SEM micrograph showing numerous filopodia and lamellipodia. A zymosan particle being engulfed is visible (arrow). Bar, 3 μ m.

and adhere to the nuclear envelope, which is well outlined, with a moderately sized perinuclear cistern. The nuclear pores are clearly visible, particularly in tangential section (Fig. 12).

From the histochemical standpoint, we have previously shown that acid phosphatase (AcPase) activity is present in the melanosomes which are, currently, considered to be lysosomes (Smit et al. 1993; for a review, see Orlow, 1995). AcPase activity is also present in the membranes that surround the lysosomes (Fig. 13).

Endogenous peroxidase activity is evident weakly in the nuclear envelope and RER in both small and large KCs of amphibians with all methods used; but is absent in the cultured KCs. Nonspecific esterases are also detectable (Fig. 14).

With regard to the internalisation experiments we chose to adopt the terms pinocytosis and phagocytosis as they have been classically used (see Discussion). Pinocytosis is demonstrated by the presence of coated vesicles and bristled vacuoles in the cytoplasm (Figs 6, 7); moreover, the injected 'vital dyes' are stored, as in mammals, in the cell lining the hepatic sinusoids and in those clustered in the portal areas (Fig. 15).

In a tissue location, phagocytosis is shown by the presence of both zymosan particles and latex beads in those cells that have the morphological characteristics of KCs (Fig. 16). Zymosan is also engulfed by cultured KCs (Figs 17, 18). We have not seen

differences in the phagocytosis of opsonised as compared with unopsonised zymosan. Results are summarised in Table 1.

DISCUSSION

The morphology of the cells studied here shows characteristics similar to those of the mammalian KCs: for instance, the presence of filopodia, microvillous structures, lamellipodia, a fuzzy coat, coated vesicles, bristled vacuoles and the habitual set of organelles. With regard to the inclusion bodies, in amphibian KCs it is also possible to observe the various bodies described in mammalian KCs; but, in amphibian KCs numerous lipofuscin bodies, red cells or red cell fragments, structures derived from haemoglobin and iron-protein complexes (i.e. ferritin or haemosiderin) are always present in quantities that in Mammalia are usually present only in pathological livers or in experimentally iron loaded livers (Iancu et al. 1978, 1985; Richter, 1978, 1984).

Apart from lipofuscin, red blood cells and their derivatives, iron-protein complexes, melanosomes and 'melanosomogenesis centres' (Sichel et al. 1997), we believe that the distinctive morphology of these cells permits us to consider them to be very similar to mammalian KCs. Moreover, they show nonspecific esterase activity and, even if weak, endogenous

Table. Some morphological and histochemical features of amphibian Kupffer cells in comparison with mammalian Kupffer cells

| | Amphibia | Mammalia |
|---|----------------|----------------------------------|
| Filopodia | Present | Present |
| Microvillous structures | Present | Present |
| Lamellipodia | Present | Present |
| Fuzzy coat | Present | Present |
| Coated vesicles | Present | Present |
| Bristled vacuoles | Present | Present |
| Lipofuscin bodies | Abundant | Only present in pathologic liver |
| Red cells and derivatives | Present | Absent in normal liver |
| Haemoglobin derived structures | Abundant | Usually absent |
| Iron-protein complexes | Abundant | Usually absent |
| Worm-like structures | Not detectable | Present |
| Melanosomogenesis centres and melanosomes | Present | Absent |
| Phagocytic and pinocytotic activity | Present | Present |
| Nonspecific esterase activity | Present | Present |
| Endogenous peroxidase activity | Very weak | Usually present |

peroxidase activity. At present, we cannot explain this weak peroxidase activity in amphibian KCs; but, although amphibian KCs are normally filled with red blood cells, their fragments, haemoglobin derivatives, etc., we think it noteworthy that in mammalian fetal macrophages, which have an avid haemophagocytic activity, peroxidase activity is also absent (Naito et al. 1997).

With regard to the term 'pinocytosis', as stated above, we have chosen to adopt the terminology defined by De Duve in a footnote (on p. 126) of a work by Cohn et al. (1963); for a discussion on this matter see Lloyd (1996).

Recently Kawai et al. (1998) have resumed the study on the classic vital staining method and, because lithium carmine is incorporated principally in the endothelial cells of the liver sinusoids and also in KCs, even if in a minor quantity, the authors reach the conclusion that a generalised cell system exists, based on its vital staining capacity. We are unable to share their opinion; vital staining is indicative of a widely diffused ability of a great number of heterogeneous cells and it is not a specific marker for macrophages.

Finally, it is known that mammalian macrophages are generally formed of heterogeneous populations, mainly distinguishable on the basis of morphological, histochemical and functional criteria (Witsell & Schook, 1991; Armbrust & Ramadori, 1996; for reviews, see Ginsel, 1993; Leenen & Campbell, 1993). Also, in our amphibian specimens, we believe we can affirm that 2 KC populations are present in the liver, one made up of small cells anchored to the sinusoid walls which contain a few or no melanosomes, the other comprised of large cells, apparently more differentiated, and filled with numerous inclusion

bodies, including a particularly large quantity of melanosomes. Because both cell types (small and large cells) show characteristics of Kupffer cells, we think it is very probable that they are different steps of a differentiation process, and the melanin production is the final activity of these cells.

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