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Distribution of macrophages during fish development: an immunohistochemical study in carp (*Cyprinus carpio*, L.)

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Abstract A monoclonal antibody against carp macrophages (WCL15) has been utilised in flow cytometry, immuno-histochemistry and immuno-electron microscopy to assess the distribution of monocytes/macrophages in developing carp lymphoid tissues. In suspensions of living cells WCL15 reacted strongly with cytoplasm and plasmic membrane of macrophages. It also cross-reacted with a subpopulation of thrombocytes, but this reaction could be neglected by double immunostaining in combination with a thrombocyte-specific marker. In Bouin-fixed tissues the antibody distinctly recognised macrophages. Macrophages were found from day 2 post-fertilisation in head kidney and in the dorsal portion of the yolk sac epithelium. From 1 week onwards macrophages were found scattered in thymus and gut and during the second week in spleen. Macrophages increased in number in all lymphoid tissues until the 6–8th week post-fertilisation, but they decreased except in thymus, where they became localised mainly in the cortical-medullary boundary, and in white pulp areas of head kidney. The role of macrophages in allowing an early non-specific defence in young fish and in co-operating during the differentiation processes of T-cells and B-cells is discussed.

Key words Monoclonal antibody · Monocytes/macrophages · Fish · Ontogeny

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Introduction

The study of the immune system of fish is having a great impetus, both for a better knowledge of the evolution of the vertebrate immune system and to improve the control of fish health in aquaculture. Teleost fish show analogy with other vertebrates concerning humoral, cellular and non-specific responses (reviewed by Manning 1994). Phagocytosis appears to play an important role in the non-specific defence against microbial and microsporidian infections, as well as in the elimination of damaged tissues (Secombes and Fletcher 1992). Fish phagocytic cells include granulocytes (especially neutrophils), monocytes and tissue macrophages. A large body of evidence suggests that fish macrophages also play an important role in induction of humoral immune responses by presenting antigens and secreting cytokines (Secombes 1991; Verburg-van Kemenade et al. 1995).

The characterisation and localisation of fish monocytes/macrophages was mainly based on functional (Faisal and Ahne 1990; Verburg-van Kemenade et al. 1994) and histological observations (Manning 1994; Koumans-van Diepen 1994). Specific monoclonal antibodies (MAbs) for leukocyte populations are available for T- and B- lymphocytes of different fish species (Koumans-van Diepen et al. 1995; Scapigliati et al. 1995, 1996; Passer et al. 1996; Rombout et al. 1997; Joosten et al. 1997), non-specific cytotoxic cells (Evans and Jaso-Friedmann 1992) and carp thrombocytes (Rombout et al. 1996); however, MAbs for monocyte/macrophages were still lacking.

In this study a MAb (WCL15) reactive with monocytes/macrophages has been characterised and used to describe the distribution of such cells in lymphoid organs of carp (thymus, head kidney, spleen and gut) from 2 days post fertilisation (p.f.) until 60 weeks p.f. The positive cells were detected by avidin-biotinylated peroxidase immunostaining with nickel enhancement on paraffin sections, by indirect immunofluorescence and by immuno-gold electron microscopy.

Materials and methods

Animals

Wild-type carp, *Cyprinus carpio* (Linnaeus, 1758), from 2 to 420 days p.f. were bred and kept in the laboratories of the Wageningen School of Animal Sciences of the Agricultural University of Wageningen, The Netherlands. They were reared in recirculating, filtered, UV-sterilised water at 23°C and fed with *Artemia salina* nauplii for a period of 2 weeks followed by Trouvit K 30 pellets (Trouw, Putten, The Netherlands) at a daily age-dependent ratio of 10% (2-week-old) decreasing to 2% at 30 weeks.

Before the dissection of head kidney, thymus, gut and spleen, fish were killed with an overdose of buffered 0.03% tricaine methane sulfonate (TMS; Crescent Research Chemicals, Phoenix, USA) in water (w/v).

Production and selection of monoclonal antibodies against carp macrophages

The MAb WCL15 was raised in order to have a marker against carp macrophage-like cells (Weyts et al. 1997). The antibody was produced and tested in a permanent leukocyte line originated from carp blood leukocytes (CLC) selected by Faisal and Ahne (1990). The cells of the CLC line shared functional properties with monocytes and macrophages (e.g. adherence to plastic and phagocytosis of iron particles) and were WCL15-positive. The production of antibody is described by Weyts et al (1997). Briefly, a macrophage-enriched fraction (density range 1.02–1.07) from 1-year-old carp head kidney was utilised to immunise intraperitoneally Balb/c mice. Mouse spleen cells were isolated 3 days after the last treatment, fused with SP2/0-Ag-14 myeloma cells (Schullman et al. 1978) and cultured according to the procedure described by Schots et al. (1992). First selection was carried out by immuno-cytochemical labelling of cytocentrifuge slides with fluorescein isothiocyanate-conjugated rabbit anti-mouse Ig serum (RAM-FITC, Dakopatts, Glostrup, Denmark) of Ig^+ and Ig^- cell fractions of peripheral blood leukocytes (PBL) obtained after a magnetic sorting procedure previously described (Rombout et al. 1996). Both Ig^+ and Ig^- cell fractions were controlled with a fluorescence-activated cell sorter (FACStar, equiped with a 5 W argon laser tuned at 488 nm, Becton Dickinson Immunocytometry Systems, Mountain View, Calif., USA) and subsequently cytocentrifuge slides were made for each fraction. Hybridoma clones reactive with a subpopulation of Ig^- cells were selected and further analysed by flow cytometry testing the reactivity of their supernatants on cell suspensions from different lymphoid tissues (head kidney, PBL, spleen and thymus). Among these supernatants, WCL15 was selected as the MAb reactive with monocyte-like cells.

Cell preparations

Cell suspensions from the lymphoid tissues of 60-week-old carp were prepared in c-RPMI (270 mOsm/Kg) containing 0.1 M sodium azide by teasing out each tissue through nylon gauze filter (50 µm mesh size). Blood was sampled from the caudal vein using a syringe containing 0.5 ml heparin solution (10 IU/ml) in c-RPMI. The buffy coat was collected and cell suspensions were layered over a discontinuous gradient of Percoll (Pharmacia AB, Uppsala, Sweden) diluted in c-RPMI to yield densities of 1.02 and 1.07 g/ml. After centrifugation (840 *g*, 30 min, 4°C), cells between 1.02 and 1.07 density layer were collected, washed twice (680 *g*, 10 min, 4°C) and resuspended in c-RPMI containing 1% BSA and 0.1 M sodium azide.

Double immunofluorescence staining and flow cytometry

For double labelling, cells were incubated in 250 µl suspensions in different tubes with the MAb WCL15 (diluted 1:50, 45 min at 0°C). After rinsing in TBS^+, cells were incubated for 30 min with RAM-FITC (Dakopatts, Glostrup, Denmark) diluted 1:100. After washing, cells were then incubated for 30 min at 0°C with normal mouse serum diluted 1:25. Cells were again washed and incubated (45 min at 0°C) with the biotinylated MAbs WCI12 (diluted 1:100) or WCL6 (diluted 1:100). WCI12 reacts specifically with carp Ig heavy chains and Ig-containing cells (Koumans-van Diepen et al. 1994) and WCL6 is specific for carp thrombocytes (Rombout et al. 1996). After washing, cells were finally incubated (45 min at 0°C) with phycoerythrin-conjugated streptavidin (Southern Biotechnology Associates, Birmingham, Ala., USA) diluted 1:5, and were analysed with a FACStar flow cytomer using the Data-MATE software analysis package (Applied Cytometry Systems, Sheffield, UK). All necessary controls for non-specific reactions were carried out. For each analysis 10^4 cells were measured.

Immuno-gold electron microscopy

Fractionated cells were obtained from adult lymphoid tissues and labelled with WCL15 as described above. Labelled cells were washed in c-RPMI and incubated (30 min at 4°C) with 25 nm gold-conjugated goat anti-mouse Ig (Aurion, Wageningen, The Netherlands) diluted 1:5. The cells were washed, resuspended in 1 ml of c-RPMI and centrifuged (1000 *g*) to yield a compact pellet. The pellet was fixed for 1 h as previously described (van Diepen et al. 1991). After fixation, the pellets were dehydrated through a series of graded alcohol and embedded in EPON 812 (Fluka Chemie, Buchs, Switzerland). Ultrathin sections (70 nm) were examined with a Philips EM 208 electron microscope.

Immunohistochemistry

Immunohistochemistry was performed as previously described (Scapigliati et al. 1995). Briefly, freshly dissected tissues were immersion-fixed in Bouin's liquid, dehydrated, cleared in toluene and embedded in paraffin. Serial transverse sections (7 µm) were rehydrated, washed in 0.1 M, pH 7.3 phosphate-buffered saline (PBS), followed by 20 min incubation with PBS containing 0.5% hydrogen peroxide to quench endogenous peroxidase activity. Adjacent sections were incubated (18 h at room temperature) with WCL6 (diluted 1:10 in c-RPMI), WCL15 (diluted 1:50 in cRPMI) or c-RPMI (control). Slides were then rinsed three times in PBS and incubated for 1 h with biotinylated horse anti-mouse Ig serum (Vector, Burlingame, Calif., USA) diluted 1:1000 in PBS containing 0.1% sodium azide and 1% BSA. Slides were then incubated for 1 h with avidin-biotinylated peroxidase complex (ABC, Vector, Burlingame, Calif., USA), with the avidin and biotinylated horseradish peroxidase solutions diluted 1:2000 in 50 mM, pH 7.6 TRIS-buffered saline (TBS). Sections were incubated in 50 mM TRIS buffer containing 0.4% nickel ammonium sulphate, 0.02% diaminobenzidine and 0.015% hydrogen peroxide, then dehydrated and mounted. From each animal, 5 sets of 2 consecutive sections (10 sections per tissue) of each lymphoid organ were differentially immunostained with WCL6 and WCL15, respectively. An observer who was unaware of the treatments made the counts of immunoreactive cells (nucleated only). Estimates of the number of macrophages present in the various tissues were then calculated by averaging ± standard deviation (SD) the cell numbers from 3–5 animals at each developmental age. WCL6 immunoreactive cells were examined in adjacent sections to estimate the cross-reaction of WCL15 with thrombocytes.

Analysis

Numerical results were analysed by two-tail Student's *t*-test for unpaired data. Cell measurements were obtained with a computer-assisted image analysis system [Leitz Aristoplan microscope, TK-1070E colour video camera (JVC, Japan) interfaced through TARGA 16 plus (AT&T) with a 486 PC, and Image ProPlus software package (Media Cybernetics, Silver Spring, Md., USA)].

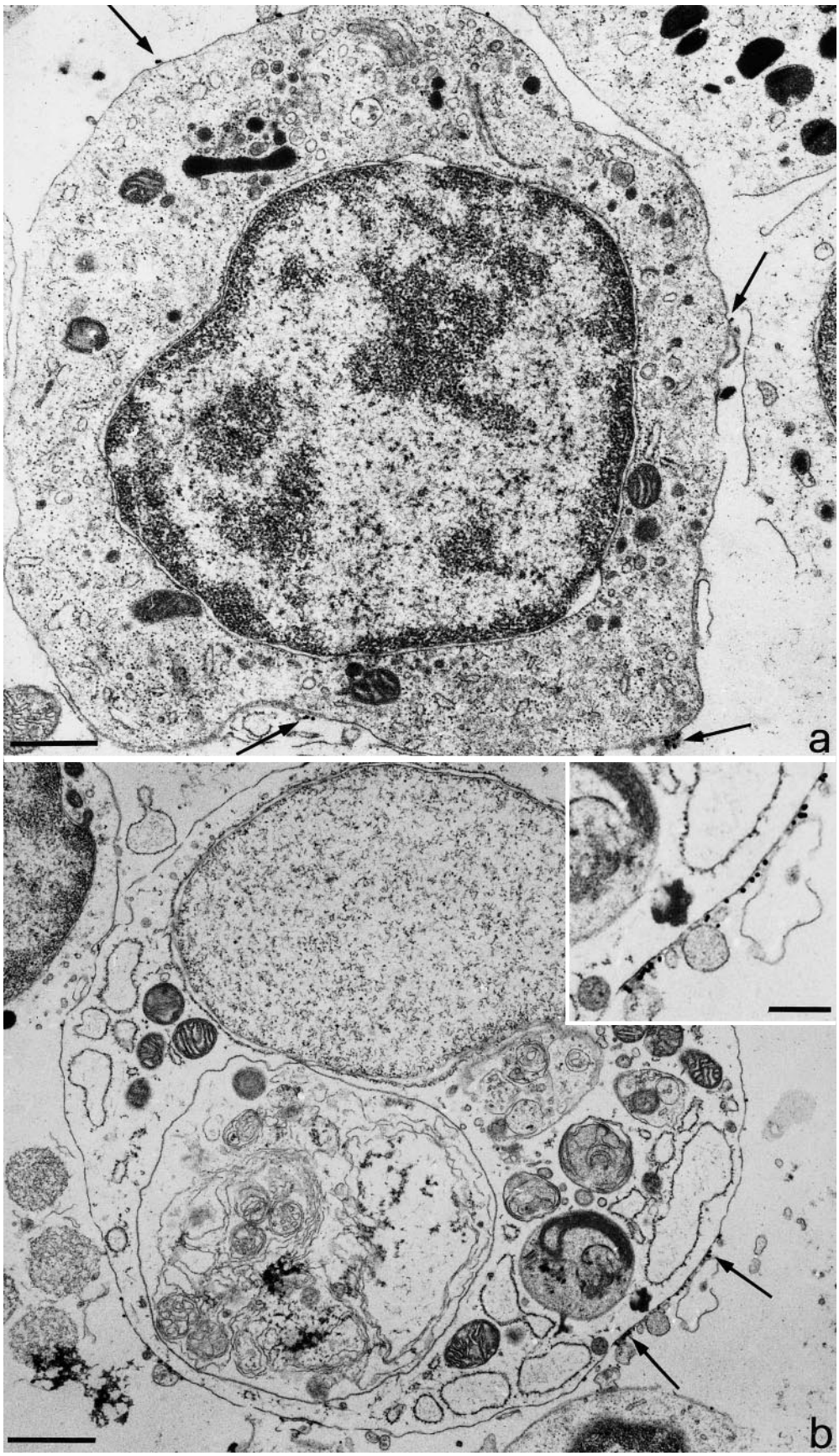


Fig. 1 Pre-embedding immuno-electron microscopy of cell suspensions from head kidney (**a**), gut (**b**). **a** A monocyte shows numerous gold particles on the plasmic membrane (*arrows*). *Bar* 750 nm. **b** An intestinal macrophage with numerous phagosomes shows gold particles (*arrows*) distributed around the plasmic membrane (*bar* 1 µm), which are magnified in the *inset* (*bar* 150 nm)

Results

Characterisation of WCL15

Immuno-electronmicroscopy of Percoll-enriched cell fractions from lymphoid tissues of juvenile carp confirmed that WCL15$^+$ cells had the morphological pattern of monocytes or macrophages. WCL15$^+$ monocyte-like cells of thymus (not shown) and head kidney (Fig. 1a) contained a considerable number of lysosome-like vesicles, whereas macrophages showed phagosomes with membranous bodies, especially in the intestine (Fig. 1b). Some thrombocytes in spleen and blood were also positive (not shown).

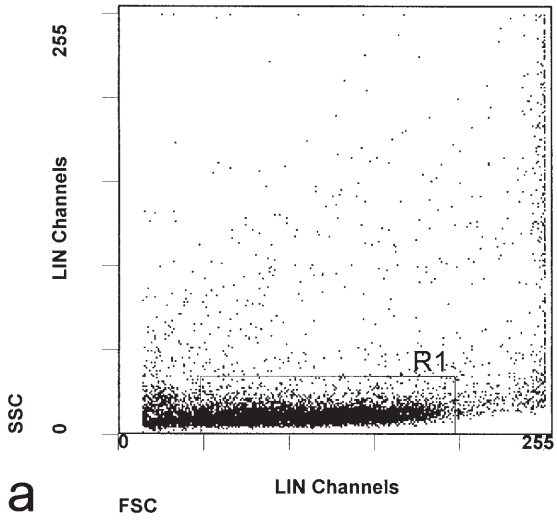
Flow cytometric double staining confirmed that WCL15 was not reactive with WCI12$^+$ cells (Fig. 2a, b) but cross-reacted with the majority of WCL6$^+$ thrombocytes (Fig. 2a, c). In contrast to thrombocytes, macrophages displayed a strong cytoplasmic immunoreaction (Fig. 3). In PBL and splenic suspensions WCL6$^+$ cells had to be subtracted, but in suspensions of other organs the number of WCL6$^+$ cells was negligible.

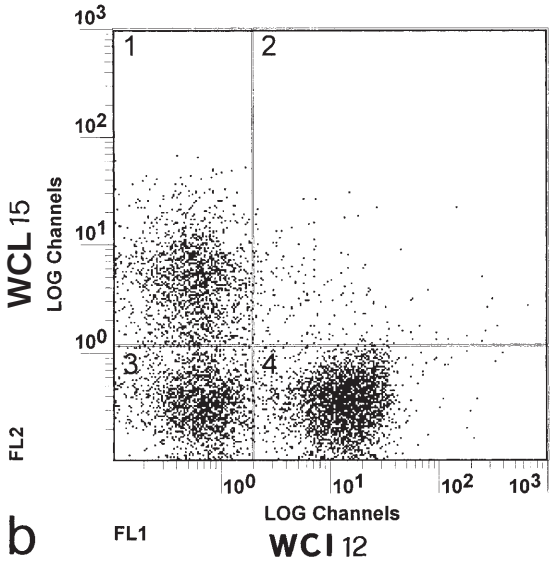
Immunohistochemical studies in developing carp

Numerical results after ABC-peroxidase staining were expressed as mean ±SD in Table 1. It was not necessary to subtract WCL6$^+$ cells in tissues, because the immunoreaction on thrombocytes was weak and hardly influenced the count of macrophages. The presence of WCL15$^+$ cells outside the lymphoid tissues was scarce in the nervous system, gills, liver and pancreas.

Head kidney

At day 2 p.f. the paired head kidney anlage consisted of two pronephric tubules. The inter-tubular tissue had no lymphoid appearance until day 3 p.f.. Immunohistochemistry revealed macrophages in carp near hatching time (2 days p.f.) localised in tubular epithelium of head kidney anlage (Fig. 4a) and in the dorsal portion of the yolk sac (Fig. 4b). At day 4 p.f. haematopoietic components appeared in the head kidney without any evident regionalisation. Immunohistochemistry revealed at this age some macrophages (Table 1) scattered in the parenchyma. At day 7 p.f. a higher number of macrophages





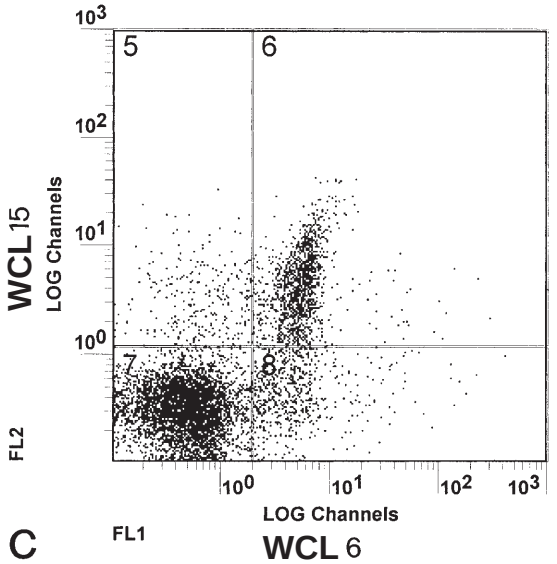


Fig. 2a–c Flow cytometric analysis of juvenile carp PBL. The 90° forward/side scatter (**a**) shows the gate used to calculate the percentages of immunoreactive cells. **b** Double labelling with WCL15, WCI 12 (anti-carp Ig) shows that these antibodies recognise two different leucocyte subpopulations: WCL15$^+$ monocytes/macrophages in quadrant 1 (24.8%), WCI 12$^+$ in quadrant 4 (44.6%). The proportion of double-positive cells in quadrant 2 (2.7%) is negligible. **c** Double labelling with WCL15, WCL6 (anti-carp thrombocyte) shows that the majority of thrombocytes (17.7%, quadrant 6) are double-positive. Only 8.3% of cells is WCL6$^+$15$^-$ (quadrant 8), 3.8% of cells WCL6$^-$15$^+$(quadrant 5)

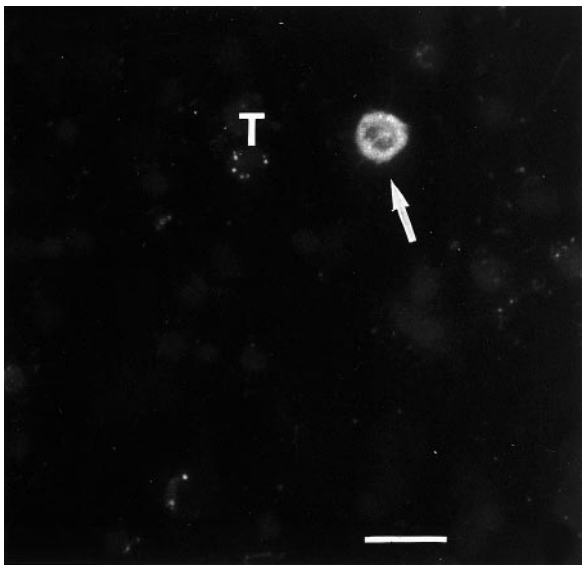


Fig. 3 Immunofluorescence staining with WCL15 in PBL suspension shows a marked cytoplasmic reaction in a monocyte (*arrow*), faint membrane reaction on a thrombocyte (*T*). *Bar* 20 µm

Table 1 Relative density number (N/100000 µm^2) of WCL15-immunoreactive cells in different organs (*HK* head kidney, *SP* spleen, *GUT* gut, *TH* thymus) at various days p.f. Each group is the mean±SD of 3 specimens

p.f. day	HK	SP	GUT	TH
2	332±17	–	0	–
3	75±21[a]	–	0	–
4	330±49[b]	184±16	14±6	0
7	120±73[c]	73±12	80±13[c]	41±16
14	30±1[d]	96±8[d]	10±4[d]	66±9
21	29±13	15±1[e]	24±3[e]	81±28
28	53±8	57±21	8±1[f]	49±40
35	36±14	68±7	7±2	40±1
42	53±11	39±9[g]	38±11[g]	15±9
56	61±4	72±18[h]	70±10[h]	64±1[h]
132	68±8	42±7[i]	56±5[i]	73±8
adult	70±22	51±2	46±5	107±35

[a] Significantly different (P<0.05) from day 2
[b] From day 3
[c] From day 4
[d] From day 7
[e] From day 14
[f] From day 21
[g] From day 35
[h] From day 42
[i] From day 132

was found in the head kidney, whereas in thymus and gut the number of macrophages was low. From 3 weeks until 5–6 weeks p.f., the head kidney (Fig. 4b) showed a large haematopoietic tissue and numerous macrophages were observed (cell diameter 9±1.6 µm, n=60). After 4 weeks p.f., the number of WCL15$^+$ cells per section in head kidney stabilised and reached the adult values (Table 1). Regional differences were observed in the intertubular tissue of juveniles from 11–22 weeks onwards, when lymphoid areas became organised around blood vessels of the head kidney and they were identifiable from the myeloid tissue. WCL15$^+$ macrophages (cell diameter 12±2.4 µm, n=80) were mainly localised in the lymphoid areas.

Thymus

At day 4 p.f. paired thymic anlagen first appeared in the dorsal part of gill chambers, but the first lymphoid appearance was established at the end of day 4 p.f. At day 7 p.f. the first macrophages were found between thymocytes (Fig. 5a). From week 2 p.f. onwards the thymus bulbed out into the gill chambers and contained numerous lymphoid cells and isolated, scattered macrophages. At week 3 p.f. numerous macrophages (diameter 7.5±1.6 µm, n=20) were in the parenchyma, either isolated or in small groups of 2–3 cells (Fig. 5b). Between 4 and 6 weeks p.f. a medullary and cortical region became visible. In this period, numerous macrophages with a considerable diameter (7–8 µm, Fig. 5d) were mainly distributed in the cortical-medullary boundary (Fig. 5c). Their presence peaked in 8–16 weeks p.f., then at 20 weeks p.f. macrophages decreased in number in the cortical-medullary boundary and became scattered again in the parenchyma (Table 1). In juveniles, the localisation of macrophages (size: 12–15 µm diameter) was similar to those in 20–22 weeks p.f. carp, moreover, a few WCL15$^+$ cells apparently were closed to non-pigmented and unreactive melano-macrophage centres (not shown).

Intestinal tract

At day 2 p.f. the intestinal tube started to differentiate as a columnar epithelium ventrally to the first pronephric tubules and dorsally to the yolk sac (Fig. 4a). Immunostaining occasionally revealed macrophages in the dorsal portion of the yolk sac, whereas the intestinal tube did not show any WCL15$^+$ cells (Fig. 5a). From day 4 p.f. some macrophages were recognisable in the growing intestinal epithelium surrounding the yolk sac (Fig. 6a). From day 7 p.f. onwards, the gut developed folds in which some positive intraepithelial macrophages were observed (Fig. 6b). From 3–6 weeks p.f. onwards, the cellular diameter of macrophages in the lamina propria of intestine became larger (15.6±5.8 µm, n=60) than in earlier stages and compared with other lymphoid tissues (Fig. 6e). The number of macrophages reached a peak at 6–8 weeks p.f., then slightly decreased and stabilised from 18 weeks onwards. In juveniles, large macrophages (10–20 µm diameter) were localised mainly throughout the intestinal mucosa, while smaller WCL15$^+$ macrophages were shown in the submucosa (7–10 µm diameter).

Spleen

From day 4 p.f. the spleen anlage develops close to the dorsal wall of the anterior intestine (Fig 6a). The developing spleen was initially composed of large haemopoietic cells. Macrophages (size 7–10 µm) became visible at day 4 p.f. (Fig. 6a, Table 1). At day 6 p.f. the number of lymphoid cells increased and macrophages were apparently more numerous. From 2 weeks p.f. many macrophages were observed between lymphoid cells (Fig. 6b).

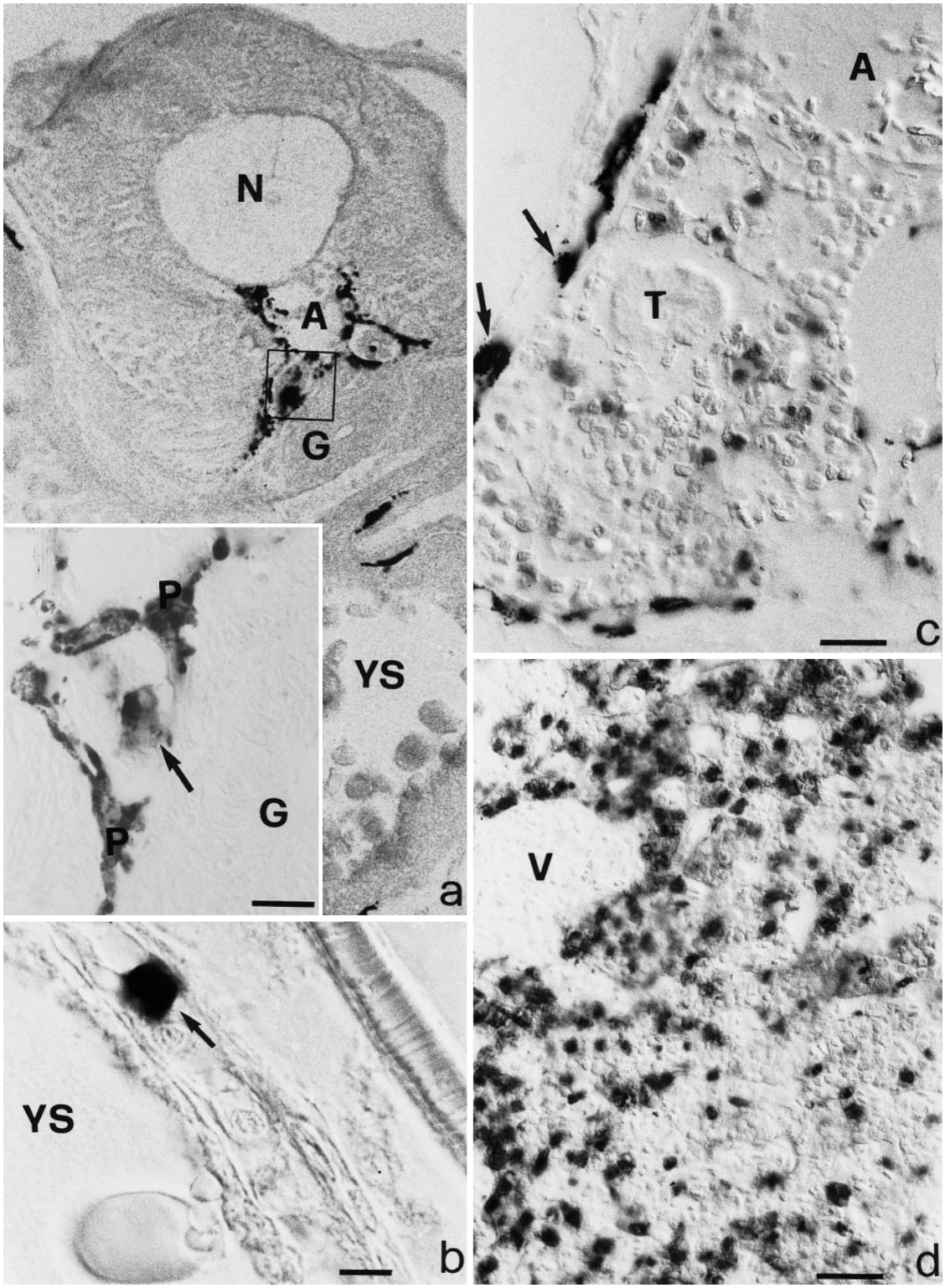


Fig. 4a–d Ontogeny of monocytes/macrophages in carp head kidney. **a** At day 2 p.f. the first two pronephric tubules are just beneath the dorsal aorta (*A*), above the gut (*G*; *YS* yolk sac, *N* notochord) ×600. In the epithelium of the kidney tubules a monocyte/macrophage is present (*arrow*), which is magnified in the *inset* (*bar* 10 µm). Positive cells were larger, devoid of dark granules compared with pigment cells (*P*) surrounding the head kidney. **b** At the same age (day 2 p.f.) a WCL15^{+} monocyte/macrophage (*arrow*) could be observed in the dorsal portion of the yolk sac (*YS*). Interferential contrast. *Bar* 8 µm. **c** At 3 weeks p.f., numerous monocytes/macrophages (*dark cells*) are localised in the intertubular tissue, whereas the kidney tubules (*T*) are devoid of WCL15-positive cells (*arrows* pigment cells, *A* dorsal aorta). Interferential contrast. *Bar* 20 µm. **d** At 5 weeks p.f., the intertubular tissue is rich in WCL15^{+} monocytes/macrophages (*dark cells*) intermingled with leucocytes (*V* blood vessel). Interferential contrast. *Bar* 20 µm

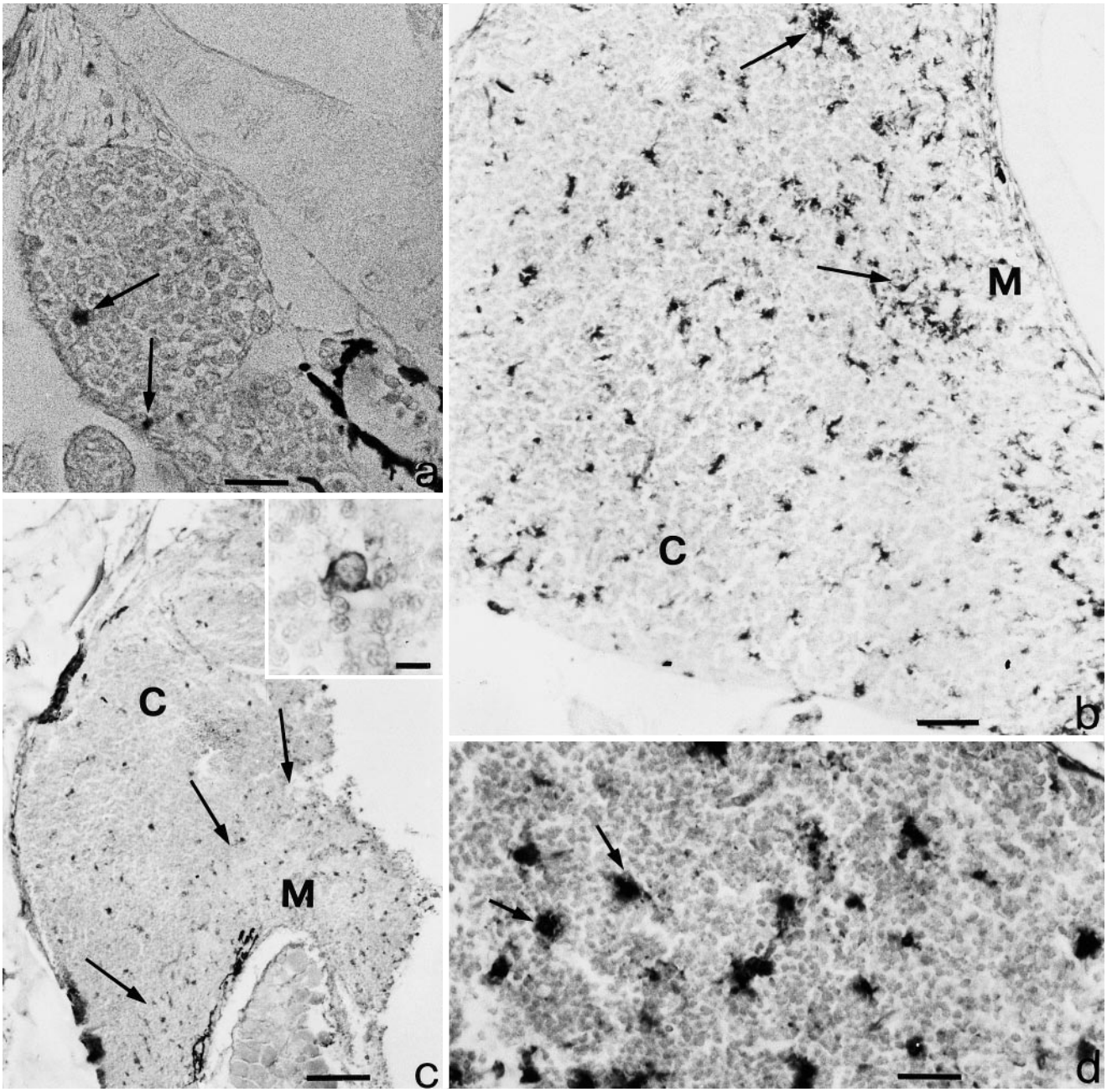
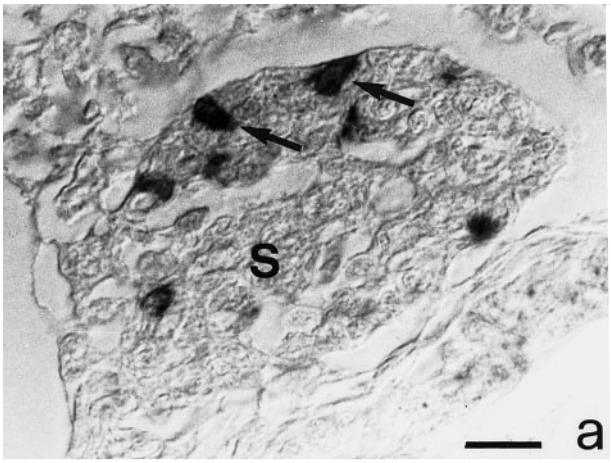


Fig. 5a–d Ontogeny of monocytes/macrophages in carp thymus. **a** At 4 days p.f. the first anlage of thymus starts to differentiate, first shows immunoreactive macrophages (*arrows*). *Bar* 20 µm. **b** Between weeks 3 and 4 p.f. the differentiation first occurs between cortex (*C*), medulla (*M*). Macrophages (*dark cells*) are scattered in the parenchyma, some of them concentrated in the cortical-medullary boundary (*arrows*). *Bar* 80 µm. **c** At week 6 p.f. the thymus has an elaborate morphology, shows numerous macrophages (*dark cells*) mainly located in the cortical-medullary boundary (*arrows*), in the medulla (*M*; *C* cortex). *Bar* 100 µm. A medullary macrophage (*dark cell*) is shown in the *inset* (*bar* 5 µm). **d** Macrophages (*arrows*) in the medullary region are isolated or in small groups of two to three cells. *Bar* 40 µm

From 3 to 6 weeks p.f., a growing number of macrophages, isolated or in small groups, were observed close to splenic capillaries; however their relative number per area apparently decreased (Fig. 6c, Table 1). Considerable numbers of WCL6$^+$ thrombocytes were visible; however the cytoplasmic reaction of WCL15$^+$ macrophages was stronger compared with the reaction of the thrombocyte membranes (Fig. 6c, d) and hence discrimination of positive macrophages was possible. Regional differences in white and red pulp areas could not be clearly detected in the spleen before 8–10 weeks p.f., although lymphoid cells began to accumulate. From 6 weeks onwards, the number of macrophages (size 10–20 µm), slightly decreased and remained constant from 18 weeks onwards (Table 1).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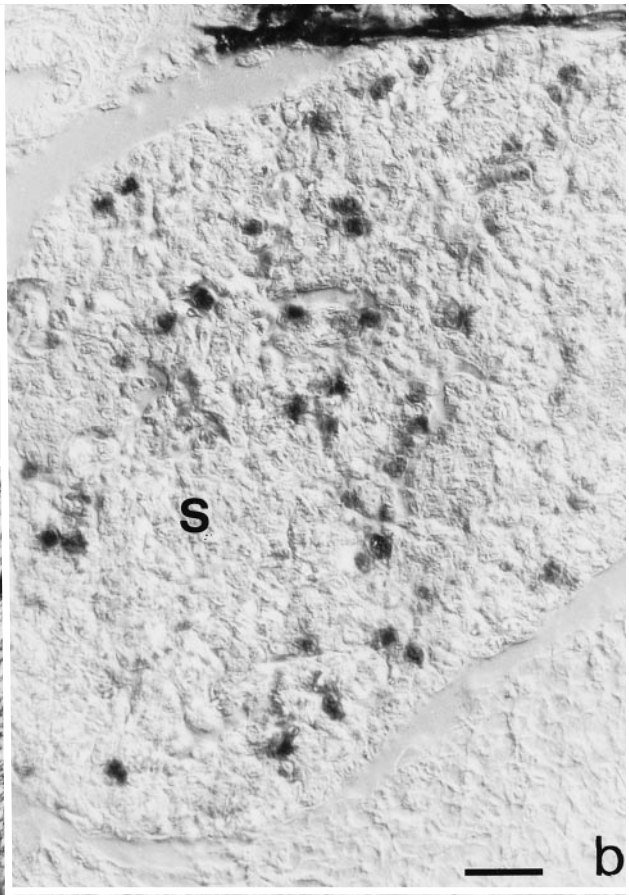
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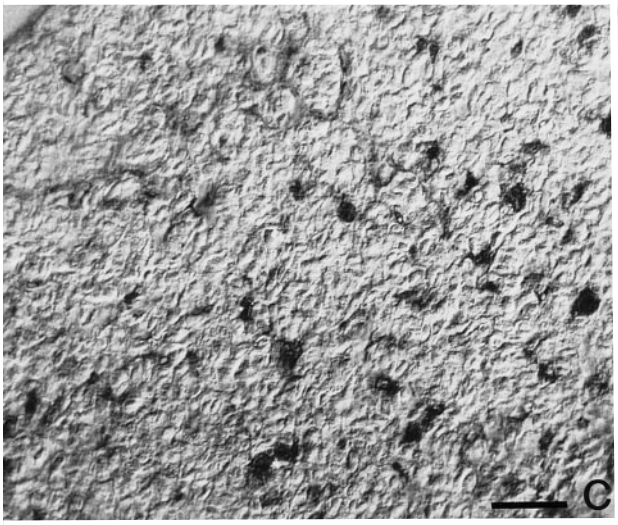
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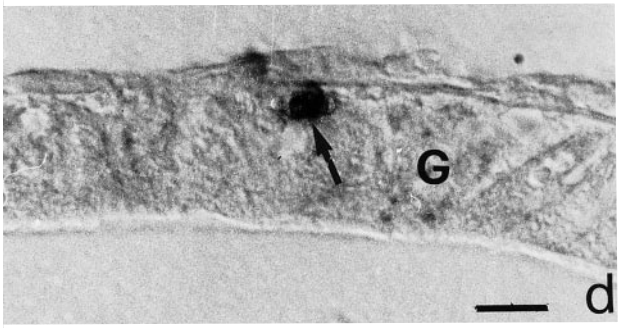
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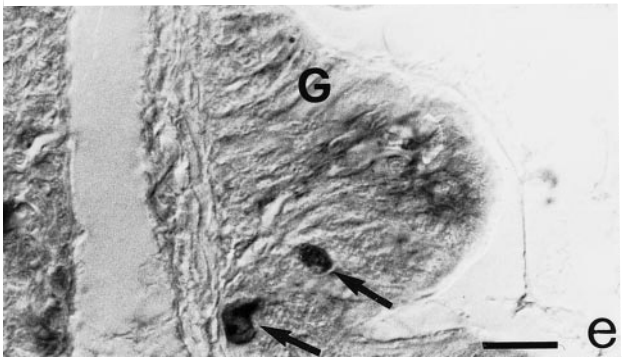
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Discussion

Macrophages and neutrophils in fish are the principal phagocytic cells, which phagocytose inert or antigenic materials, exert cytotoxic activity and stimulate lymphocyte proliferation by secreting interleukin-1-like factors (Secombes and Fletcher 1992; Verburg-van Kemenade et al. 1995). As in mammals, fish monocyte/macrophages could play a central role in cell-mediated immunity as they are involved in antigen presentation and regulatory functions (Secombes and Fletcher 1992; Roitt et al. 1993). Previous characterisation of monocytes and macrophages in adult or larval fish was made by functional (Faisal and Ahne 1990; Sveinbjornsson and Seljelid 1994; Verburg van-Kemenade et al. 1994) or (ultra)structural studies (Zapata and Cooper 1990; Koumans-van Diepen et al. 1994). Both cell types were evidenced and a lineage relationship was suggested, but never proved. A suitable marker for monocytes and macrophages, WCL15, was recently reported by Weyts et al. (1997), showing that a permanent cell line originating from carp peripheral blood leukocytes (Faisal and Ahne 1990) having morphological and functional characteristics of macrophages was reactive with this MAb. In the present study, we utilised WCL15 on cell suspensions of different tissues of juveniles or sections from developing organs of carp in order to study the timing and localisation of monocyte/macrophages during ontogeny. A more detailed characterisation confirmed that WCL15 reacted with cells having the morphology of carp monocytes and macrophages (Koumans-van Diepen et al. 1994) and with $WCL6^+$ thrombocytes. The cross-reaction of WCL15 with thrombocytes in non-fixed cell suspensions from PBL or spleen could be avoided in fixed paraffin-embedded material because of the strong cytoplasmic reaction of monocyte/macrophages and the negligible reaction with the membranes of thrombocytes. With immuno-electron microscopy, cross-reaction of WCL15 with lymphoid cells and neutrophils could be excluded. Monocyte/macrophages apparently were larger (around 10 µm diameter, or more) than activated non-specific cytotoxic cells (Meseguer et al. 1994; Mulero et al. 1994). Moreover, the double staining with WCL15 and WCI12 confirmed the negligible cross-reactivity of WCL15 with B-lymphocytes.

Fig. 6a–f Ontogeny of monocytes/macrophages in carp spleen, intestine. **a** At 4 days p.f. the first anlage of the spleen (*S*) shows $WCL15^+$ macrophages (*arrows*). Interferential contrast. *Bar* 15 µm. **b** During the first week the spleen (*S*) rapidly grows, numerous $WCL15^+$ macrophages (*dark cells*) are in the parenchyma. Interferential contrast. *Bar* 30 µm. **c** At week 5 p.f. the splenic parenchyma display numerous monocyte/macrophages (*dark cells*). Interferential contrast. *Bar* 30 µm. **d** At 4 days p.f. the gut (*G*) consists of a columnar epithelium where WCL15-positive macrophages (*arrow*) can be seen. Interferential contrast. *Bar* 30 µm. e) In week 1 p.f. the gut epithelium (*G*) starts to form the first folds, some WCL15-positive macrophages (*arrows*) are observed. Interferential contrast. *Bar* 30 µm. **f** At week 6 p.f. the intestine displays numerous folds containing large macrophages (*arrows*). Interferential contrast. *Bar* 30 µm

The immunohistochemical results obtained in this study are in agreement with a previous ontogeny study on leucocytes isolated from different organs, where the proportion of $WCL15^+6^-$ cells (considered as monocytes/macrophages) was estimated from 1 to 30 carp weeks (Romano et al. 1997). Since a considerable number of macrophages was observed in cell suspensions from lymphoid tissues at 1 week p.f., we have now analysed the earlier distribution in lymphoid tissues of macrophages from hatching (2 days p.f) onwards. The first appearance of $WCL15^+$ cells in the head kidney and in the dorsal part of the yolk sac at 2 days p.f. confirms previous morphological observations (Botham and Manning 1981). Our study could not establish the localisation of macrophage precursors before 2 days p.f.. The yolk sac of fish stores maternal IgM and is apparently an early site of immune competence (reviewed Tatner 1996) where non-specific defence mechanisms arise, providing fries immune protection before maturation of specific immunity. In addition, the yolk sac is probably the earliest organ exhibiting haematopoietic capacities in fish embryos, as indicated for dogfish (reviewed Zapata et al. 1996) and carp (Botham and Manning 1981). Most authors agree that the kidney is the major haematopoietic organ and the main site of erythrocyte, granulocyte, B lymphocyte and monocyte differentiation (Ellis 1977; Zapata 1979; Bielek 1981; Botham and Manning 1981). On the other hand, the observation of erythrocytes and macrophages before development of the kidney suggested the existence of other haematopoietic foci in the embryo (Zapata et al. 1996).

Although it is generally assumed that circulating monocytes are precursors of vertebrate macrophages (Roitt et al 1993), this has been recently questioned for mammals and birds. In mammals, bipotential precursors for granulocytes and macrophages (Unanue 1993) are suggested. The MAb WCL15 does not react with carp granulocytes, which suggests that only already differentiated cells could be recognised or that such bipotential precursors are absent in fish.

During the first week p.f. macrophages can be observed in carp thymus. Their number grew reaching adult values from week 8 p.f., and their distribution changed from scattered (until 3–4 weeks p.f.) to small groups of cells mainly localised in the cortical-medullary boundary (from 5 to 20 weeks p.f.). From 20 weeks onwards, macrophages again dispersed in the parenchyma. Macrophages were localised in the “outer-inner” boundary and the outer zone of trout thymus by a strong positivity for acid phosphatase and esterase (Castillo et al. 1990). In addition, T-cell proliferation in the thymus of Atlantic salmon and catfish was dependent upon the presence of macrophages (Smith and Braun-Nesje 1982; Miller et al. 1986). Considering that the thymus seems to be the primary lymphoid organ for differentiation of T lymphocytes (Abelli et al. 1996; Romano et al. 1997), thymic macrophages could play a role in the maturation process, being also involved in the clearance of apoptotic thymocytes.

From day 4 p.f. a considerable number of macrophages appeared in the intestine. Their number increased during larval development, especially in the mucosal epithelium. Intraepithelial hindgut macrophages were larger (10–20 µm) than in the head kidney (7–10 µm), as previously observed in adult carp (Rombout et al. 1993; Koumans-van Diepen et al. 1994). Fish intestinal macrophages seem to express antigens on their membrane and their role in scavenging and immune-complex-binding was suggested (Rombout et al. 1993; Koumans-van Diepen et al. 1994), as demonstrated for mammalian intestinal macrophages (Lefrançois and Puddington 1995). The presence of numerous lymphocytes (mainly T-cells) in the gut-associated lymphoid tissue (Abelli et al. 1997; Joosten et al. 1997) suggests that cellular co-operation can occur with macrophages; however, this function can be neglected in earlier stages of carp because the first intestinal lymphocytes were observed after 1 week p.f. (Romano et al. 1997).

Scattered WCL15$^+$ cells were observed in spleen from 4 to 7 days p.f.. From week 2 p.f. onwards, numerous WCL15$^+$ cells, isolated or in small groups, were found close to splenic capillaries. The presence of macrophages peaked at week 8, just when they were also numerous in thymus, head kidney and gut. As in the head kidney, WCL15$^+$ macrophages were mainly localised in poorly developed lymphoid areas, where the majority of lymphoid cells are accumulated (Botham and Manning 1981; Abelli et al. 1996) together with WCL15$^-$ melanomacrophages. Although mammalian macrophages have a widespread distribution in non-lymphoid tissues, WCL15$^+$ cells are scarcely present in gills, liver, pancreas and nervous system. The localisation of macrophages in the fish central nervous system was previously established (Dowding and Scholes 1993). The possibility that some specialised tissue macrophages are not recognised by WCL15 is therefore suggested, or that there is a different distribution pattern of macrophages in fish.

The size of WCL15$^+$ cells varied among different tissues and increased throughout development; moreover, morphological differences among thymus, head kidney and hindgut macrophages were demonstrated in juvenile carp by immuno-electronmicroscopy. Therefore, heterogeneity of macrophages is suggested, but it is still unknown whether these differences are correlated with different functions. In mammals, macrophages are heterogeneous, vary in degree of differentiation and have distinctive proprieties in the different tissues (Unanue 1993). Possibly, similar proprieties are associated with fish macrophages, but functional proof is required.

The early and stable presence of macrophages inside lymphoid tissues (mainly thymus and head kidney) supports the idea that carp macrophages could play a role in the maturation of the fish immune system, being involved in antigen processing and negative selection. A role in the selection of T- and B-lymphocytes exerted by the resident population of monocyte/macrophages in thymus, spleen and bone marrow (mammals) and the bursa of Fabricius (birds) has been firmly established (Roitt et al. 1993). In younger fish, macrophages could play a role in secreting growth factors, in non-specific defence and in removing cell debris generated by physiological cell death during development.

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