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Demonstration of a phagocytic cell system belonging to the hemopoietic lineage and originating from the yolk sac in the early avian embryo

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Summary

It is well established that hemopoietic cells arising from the yolk sac invade the avian embryo. To study the fate and role of these cells during the first 2.5-4.5 days of incubation, we constructed yolk sac chimeras (a chick embryo grafted on a quail yolk sac and vice versa) and immunostained them with antibodies specific to cells of quail hemangioblastic lineage (MB1 and QH1). This approach revealed that endothelial cells of the embryonic vessels are of intraembryonic origin. In contrast, numerous hemopoietic cells of yolk sac origin were seen in embryos ranging from 2.5 to 4.5 days of incubation. These cells were already present within the vessels and in the mesenchyme at the earliest developmental stages analyzed. Two hemopoietic cell types of yolk sac origin were distinguishable, undifferentiated cells and macrophage-like cells. The number of the latter cells increased progressively as development proceeded, and they showed marked acid phosphatase activity and phagocytic capacity, as revealed by the presence of numerous phagocytic inclusions in their cytoplasm. The macrophage-like cells were mostly distributed in the mesenchyme and also appeared within some organ primordia such as the neural tube, the liver anlage and the nephric rudiment. Comparison of the results in the two types of chimeras and the findings obtained with acid phosphatase/MB1 double labelling showed that some hemopoietic macrophage-like cells of intraembryonic origin were also present at the stages considered. These results support the existence in the early avian embryo of a phagocytic cell system of blood cell lineage, derived chiefly from the yolk sac. Cells belonging to this system perform phagocytosis in cell death and may also be involved in other morphogenetic processes.

Key words: avian embryo, yolk sac chimeras, hemopoietic cells, macrophages

Introduction

During vertebrate ontogenesis, stem cells colonize the hemopoietic organs where the different classes of blood cells, both myeloid and lymphoid, differentiate. Moore and Metcalf (1970) reported the presence of such stem cells in the mouse yolk sac mesoderm during early development, and proposed that all blood cell precursors emerge from yolk-sac-derived stem cells. However, a different situation was demonstrated in birds (Dieterlen-Lièvre, 1975, 1984): hemopoietic cells do arise from the yolk sac mesoderm during the earliest developmental stages, but the definitive blood cells are derived from intraembryonic precursors. A similar pattern has been reported in some amphibian species, e.g. *Xenopus laevis*, in which erythrocyte precursors are supplied first by the ventral blood island mesoderm during early larval stages, and later by the dorsolateral plate mesoderm, which is responsible for adult stem cells seeding (Flajnik et al., 1984; Maéno et al., 1985).

Precursors of both white and red blood cells have been identified in the yolk sac mesoderm of chick embryos (Edmonds, 1966). Studies of quail embryos grafted on a chick yolk sac have revealed the fate of such hemopoietic cells in the embryo. Undifferentiated hemopoietic cells of yolk sac origin are found in the intraembryonic mesenchyme and in circulating blood. Until day 5 of incubation, yolk-sac-derived hemopoietic cells are more numerous in the blood than intraembryonic ones, which will later replace them (Dieterlen-Lièvre and Martin, 1981). The contribution of yolk-sac-derived erythrocytes to blood and their progressive replacement from day 5 onwards by other cells originating within the embryo proper, have also been documented (Lassila et al., 1982; Dieterlen-Lièvre, 1984). Finally, although hemopoietic cells of the yolk sac mesoderm are able to colonize the thymic rudiment under experimental conditions and give rise to lymphocytes (Le Douarin and Jotereau, 1975), they do not contribute to the lymphoid population during normal

development (Lassila et al., 1978; Martin et al., 1979; Dieterlen-Lièvre, 1984).

However, it has not been clearly elucidated whether leukocytes derived from yolk sac precursors play a role in embryo development. Previous studies using chick embryo→quail yolk sac chimeras have shown that a macrophage-like cell population of yolk sac origin invades several organs in avian embryos during morphogenetic stages (Cuadros et al., 1991a, b). In the present study, we analyze the topographical pattern of appearance of yolk-sac-derived cells in normal quail and chick embryos and in yolk sac chimeras constructed according the two inverse patterns at 2.5-4.5 days of incubation. Two types of hemopoietic cell of yolk sac origin were recognized at these developmental stages: undifferentiated hemopoietic cells and macrophage-like cells which perform active phagocytosis, mainly in regions of cell death. The latter cells, together with other hemopoietic cells of intraembryonic origin, may constitute a phagocytic cell system in early embryos.

Materials and methods

White Leghorn and JA 57 strain chick embryos, and quail embryos (*Coturnix coturnix japonica*) were employed in this study. The chick embryos were staged using the Hamburger and Hamilton (HH) (1951) tables, for the quail embryos, the Zacchei (Z) (1961) stages were used.

Acid phosphatase (AcPase) histochemistry

Chick embryos in HH stages 12-25, and quail embryos in Z stages 14-16, were fixed in 4% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4) and embedded in Technovit 7100 (Kulzer, FRG) or JB-4 (Polysciences, USA). Sections measuring 5 μm in thickness were incubated overnight at 37°C in the medium described by Namba et al. (1983), rinsed in distilled water, counterstained with hematoxylin, air dried and mounted in Eukitt (Kindler, FRG). As controls for the specificity of the histochemical reaction, some sections were incubated in medium without substrate (naphthol AS BI phosphate) or in complete medium containing 0.01 M sodium fluoride, a specific inhibitor of AcPase activity. No enzyme activity was found in these sections.

Electron microscopy

Regions containing large numbers of AcPase-positive cells in the head of chick embryos from HH stages 19 to 24 were dissected and fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 5 h. The specimens were rinsed in buffer alone, postfixed in 1% osmium tetroxide, dehydrated in a graded series of acetones and propylene oxide, and embedded in Epon. Semithin sections were cut and stained with alkaline 1% toluidine blue. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a Zeiss 10C electron microscope.

Microsurgery

Yolk sac chimeras were obtained as described by Martin (1990). Briefly, the central area of the blastoderm of a 7-13 somite chick or quail embryo was replaced with the same region of an embryo of similar developmental stage from the other species (Fig. 1). Altogether, more than 70 yolk sac chimeras were made. About 25% of the chick embryos grafted on a quail yolk sac (chick embryo→quail yolk sac chimeras) developed an apparently normal extraembryonic circulation; from these, 5 were fixed at HH stages 13-15, after 24 h of reincubation, and 9 at HH stages 18-24 (48-56 h after microsurgery). Five quail embryos→chick yolk sac chimeras (a quail embryo grafted on a chick yolk sac), representing about 40% of the operations performed, were removed from the egg two days after transplantation, at Z stages 14-17, equivalent to HH stages 18-23.

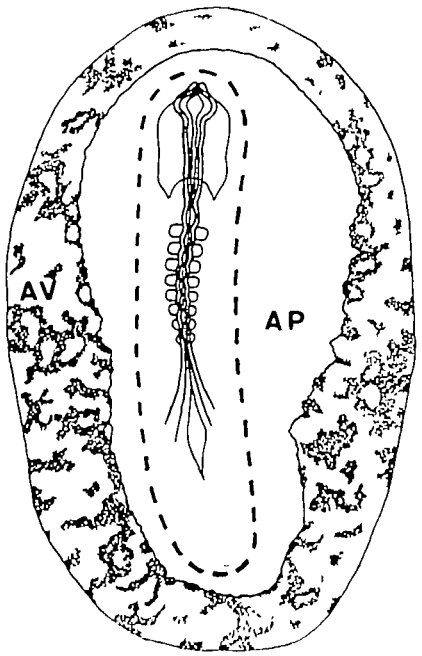


Fig. 1. Schematic drawing showing the boundaries between the grafted embryonic region (inside the dotted line) and the host yolk sac in yolk sac chimeras. AV, area vasculosa; AP, area pellucida.

To elucidate if the surgical procedures affected the results obtained in yolk sac chimeras, two chick embryo→chick yolk sac and two quail embryo→quail yolk sac chimeras were fixed 48 h after microsurgery and processed, respectively, for AcPase histochemistry and immunostaining.

Immunocytochemistry

We used two antibodies, MB1 (Péault et al , 1983) and QH1 (Pardanaud et al., 1987), which are specific for quail cells of blood and endothelial lineage. These antibodies do not react with chick cells.

Quail embryos ranging from Z stages 10 to 18, and chick embryo→quail yolk sac and quail embryo→chick yolk sac chimeras, were fixed in Bouin's fluid, embedded in paraffin and sectioned at 5 μm. Some quail embryos were embedded in Araldite and sectioned at 1.5-2 μm. The sections were treated with 0.3% hydrogen peroxide in phosphate-buffered saline (PBS) to eliminate the endogenous peroxidase activity, washed in PBS and incubated overnight in MB1 ascites fluid diluted 1 1000. After extensive washing in PBS, the sections were successively incubated in anti-mouse IgM biotinylated antibody (Amersham), and streptavidin-peroxidase complex (Vectastain, Vector Laboratories). To reveal peroxidase activity, a solution containing 0 05% diaminobenzidine and 0.02% hydrogen peroxide in PBS was applied Finally, the sections were counterstained with hematoxylin, air dried and mounted in Entellan (Merck). For QH1 immunostaining, after rehydration, the sections were incubated 60-90 min in

purified QH1 monoclonal antibody conjugated to fluorescein isothiocyanate (dilution 1:100), washed in PBS and mounted in 1:1 glycerol-PBS.

Double labelling AcPase/MB1

To determine whether the AcPase-positive cells were also recognized by MB1 antibody, some sections were processed for both AcPase histochemistry and MB1 immunostaining. Staining of sections from plastic-embedded embryos was unsuccessful with the MB1 antibody, thus double labelling was performed on paraffin sections. In paraffin-embedded material incubated in the same medium as plastic sections, all cells showed AcPase activity; the addition of sodium tartrate (an inhibitor of some types of AcPase activity) abolished or weakened the AcPase reaction in nearly all cells, with the exception of some that showed a distribution pattern and morphological features identical to those of the AcPase-positive cells seen in plastic sections. Therefore, sodium tartrate was added to the medium to obtain specific labelling of these cells.

Five micrometer thick paraffin sections from yolk sac chimeras and normal quail embryos fixed in 4% formaldehyde in PBS were incubated for 16-18 h in the medium described by Namba et al. (1983) containing 0.01 M sodium tartrate. The sections were washed and incubated for 30-40 min in MB1 ascites fluid (dilution 1:1000), and then incubated in anti-mouse IgM coupled with fluorescein isothiocyanate (Amersham). The sections were finally mounted in glycerol containing 0.1% paraphenylenediamine. The AcPase reaction was observed with transmitted light microscopy, and MB1 labelling was observed in the same section using epifluorescence. The proportion of AcPase-positive cells also recognized by MB1 antibody was determined in 16 sections from different parts of the embryo. Two quail embryo→chick yolk sac chimeras, two chick embryo→quail yolk sac chimeras and two normal quail embryo were analyzed.

Results

Normal chick embryos

In embryos embedded in plastic, the histochemical technique for detecting AcPase activity revealed the presence of cells showing intense enzyme activity in embryos from 2 to 2.5 days of incubation onwards (Fig. 2A). Numerous AcPase-positive cells were located in the mesenchyme, where they were first noted; positive cells were also found in some regions of ectoderm and endoderm, the neural tube and some organ primordia, such as the liver and the nephric rudiment. Although AcPase-positive cells might display different morphological features, frequently they were large cells containing numerous cytoplasmic inclusions (Fig. 2B). Electron microscopic observations revealed that phagocytic, macrophage-like cells were frequent in regions where AcPase-positive cells concentrate (Fig. 3). Since numerous AcPase-positive cells lay in regions showing degenerating cells, and some phagocytic inclusions were recognizable as dead cell fragments (Fig. 3), AcPase-positive cells apparently take part in the removal of dead cell debris.

No immunoreactivity to MB1 or QH1 antibodies was found in chick embryo tissues, as already reported (Péault et al., 1983; Pardanaud et al., 1987).

Normal quail embryos

A pattern of AcPase-positive cell distribution similar to that described in chick embryos was found in quail embryos.

MB1 and QH1 antibodies showed similar reactivity on quail embryo tissues (Pardanaud et al., 1987; Péault et al., 1988). Both antibodies reacted with endothelial cells of the vessels, and with blood cells (except mature erythrocytes). Outside the vessels, some isolated cells were also labelled in the mesenchyme. Serial sections showed that these single cells were not linked to endothelia, and therefore they were designated "free cells".

The immunolabelled cells showed variable morphological features. Some of them were round, with a large nucleus, and were seen both inside the vessels, often lying in blood cell islands, and in the mesenchyme (Fig.

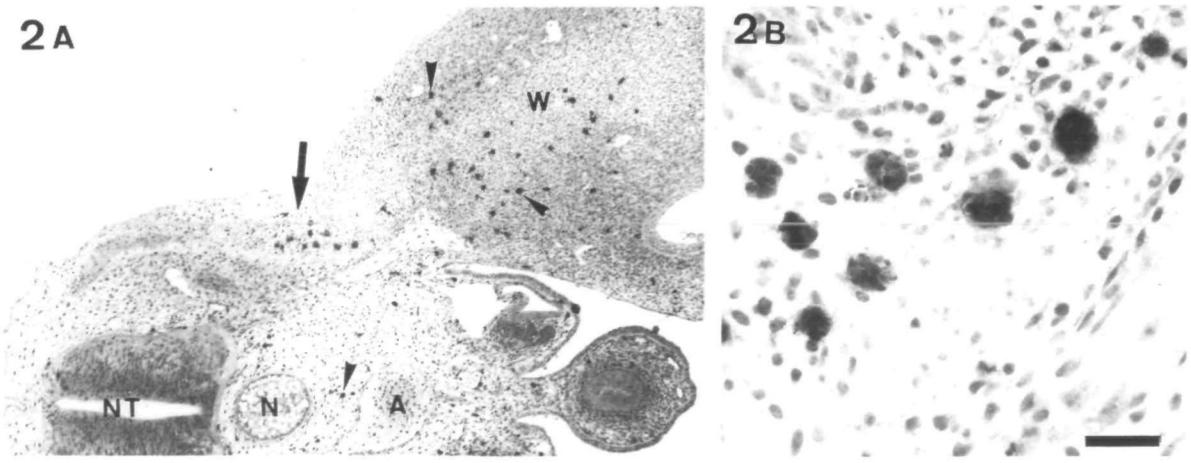


Fig. 2. AcPase staining of an HH stage 23 embryo (trunk level) embedded in plastic. (A) Numerous AcPase-positive cells (arrowheads) appear in the mesenchyme, and concentrate in some regions such as the wing bud (W) and around the ventrolateral boundary of the dermomyotome (arrow). NT, neural tube; N, notochord; A, dorsal aorta. Scale bar in B, 160 μm. (B) High magnification of the region marked by an arrow in A, showing large AcPase-positive cells which contain cytoplasmic inclusions. Scale bar, 25 μm

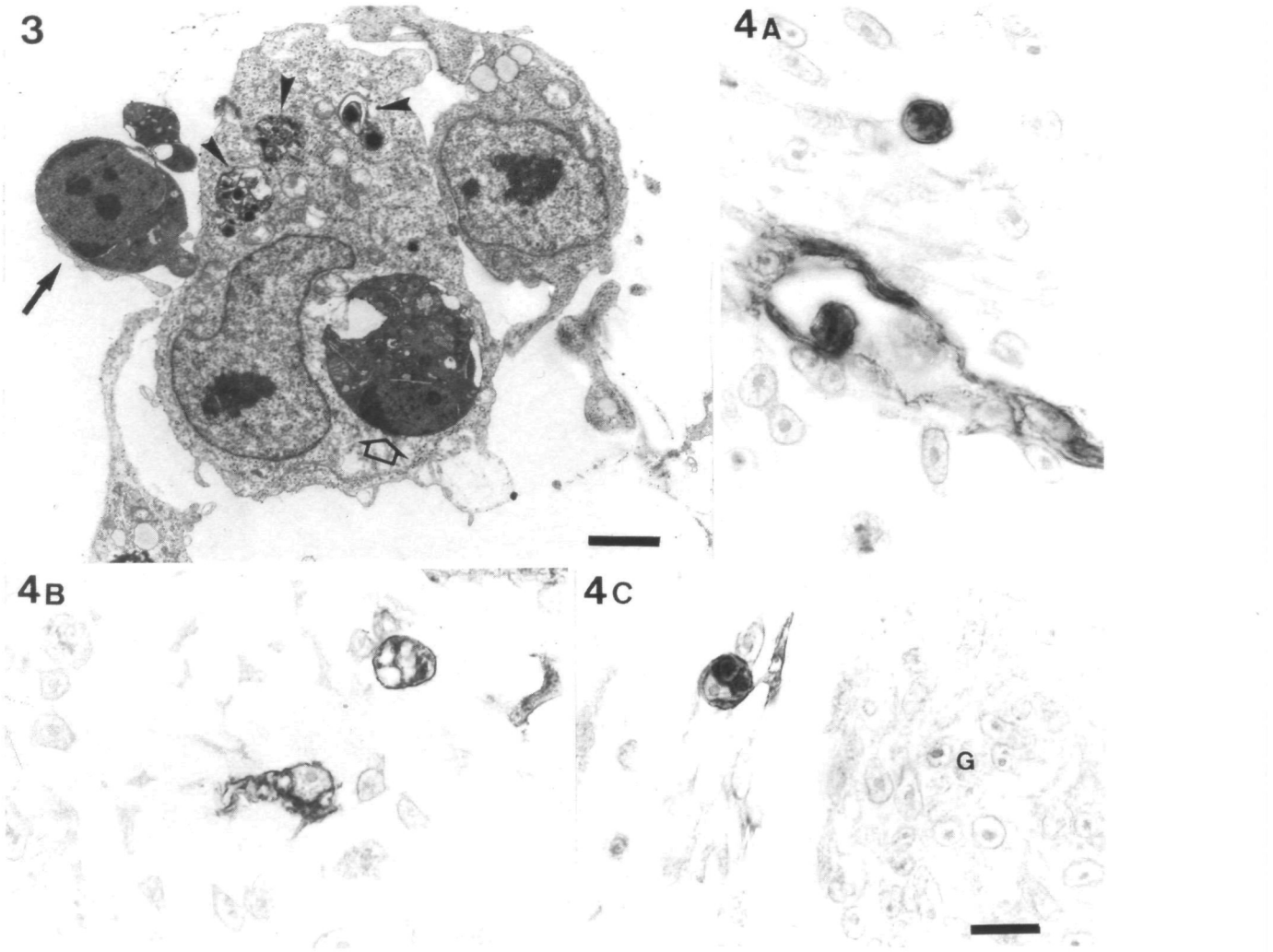


Fig. 3. Electron micrograph of the mesenchyme near the eye in an HH stage 21 embryo; in this region, numerous positive cells can be seen in AcPase-stained sections. The image shows a macrophage-like cell with vacuoles filled with different materials (arrowheads). The larger vacuole contains a dead cell fragment in which cytoplasmic organelles and a pycnotic nuclear fragment (open arrow) are recognizable. A cell process (arrow) has come into contact with another dead cell fragment Scale bar, 2 μm.

Fig. 4. MB1 immunostaining of sections from Z stage 14 normal quail embryos. (A) Two rounded cells, apparently undifferentiated hemopoietic cells, are seen in the cephalic region. One of them lies in the mesenchyme and the other is in a vessel. The endothelial wall of the vessel is also labelled. (B) Immunolabelled cells showing prominent vacuoles are seen in the mesenchyme. (C) A labelled cell with large inclusions in its cytoplasm appears near the trigeminal ganglion (G). Scale bar for A, B and C, 10 μm.

4A); these were probably pluripotent hemopoietic cells. In the mesenchyme, cells with different features were labelled: some were flattened, spindle-shaped or dendritic cells with one or two long cell processes (Fig. 4B). Other labelled cells in the mesenchyme were larger (up to 30 μm in diameter) and rounded in shape, and contained numerous vacuoles filled with various inclusions (Fig. 4C). These cells, which were similar to the AcPase-positive macrophage-like cells, apparently perform active phagocytosis. Free cells with intermediate morphological features between those of the cells just described were also found.

The distribution of the AcPase-positive cells matched that of the immunolabelled cells in quail embryos. To prove that the two techniques really labelled the same cells, we performed histochemical and immunohistochemical staining on the same section. Double labelling with AcPase/MB1 further distinguished the free cells in the mesenchyme from the endothelial cells: except in some vitelline vessels, the endothelial cells were devoid of AcPase reaction in sections incubated with the standard medium plus sodium tartrate. In contrast, both labelled cells within the vessels and free cells in the mesenchyme were AcPase and MB1 positive (Fig. 5A,B). In normal quail embryos, nearly all the AcPase-positive cells were also labelled with MB1 (all cells in one embryo and about 99% in another, Table 1) Some immunolabelled free cells were AcPase negative.

In early quail embryos, the free cells cannot unequivocally be identified with MB1/QH1 immunolabel-

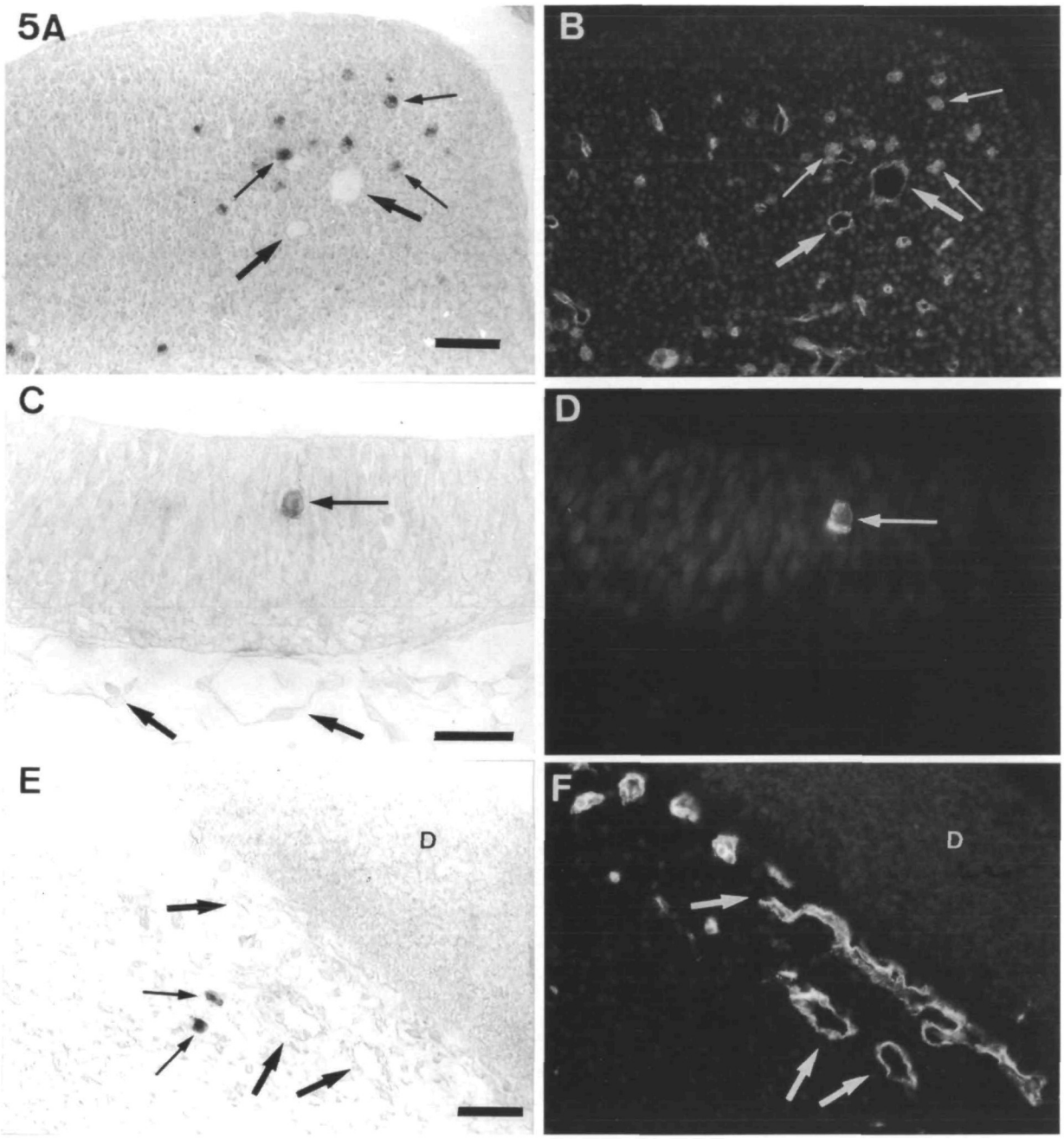


Fig. 5. Sections double stained for AcPase activity (A,C,E) and MB1 immunoreactivity (B,D,F). (A,B) In normal quail embryos, both AcPase-positive cells (thin arrows) and the vessels (thick arrows) are immunostained with MB1 antibody. Wing bud of a Z stage 16 quail embryo. Scale bar, 40 μm. (C,D) The AcPase-positive cell (thin arrow) in the rhombencephalic wall of an HH stage 19 chick embryo→quail yolk sac chimera is also labelled with MB1 antibody Note that no label is found in vessel endothelia (thick arrows). Scale bar, 25 μm. (E,F) In the head mesenchyme of a Z stage 15 quail embryo→chick yolk sac chimera, two AcPase-labelled cells (thin arrows) are devoid of MB1 immunolabelling; in contrast, the endothelial walls of the vessels (arrows) near the diencephalon (D) are labelled. Scale bar, 40 μm.

ling owing to the presence of single presumptive endothelial cells in the mesenchyme (Pardanaud et al., 1987; Coffin and Poole, 1988). Free cells became apparent as vascularization advanced, appearing in regions free of vessels at slightly later stages. No free cells were distinguished in 16-somite embryos (Z stage 10). Some of these cells were identified at the 20-somite stage (Z stage 11-12), but they did not show the morphology of macrophage-like cells. Free cells increased in number in subsequent stages and began to invade non-mesenchymal regions, appearing in the nervous tissue from Z stage 13 onwards, when vascularization of the brain either has not yet started or is restricted to regions different from those showing free cells. Numerous free cells in different areas of the embryo had macrophage-like morphological features at

Table 1. *Cells showing AcPase reaction and MB1 labelling were counted in two normal quail embryos, two chick embryo→quail yolk sac chimeras and two quail embryo→chick yolk sac chimeras*

	Number of cells		
	AcPase+/MB1−	AcPase+/MB1+	Total
Normal quail	3 (1 1%)	290 (98.9%)	293
Normal quail	0	381 (100 0%)	381
Chick→quail yolk sac			
X 41	23 (9 0%)	234 (91 0%)	257
X 42	10 (4 6%)	207 (95 4%)	217
Quail→chick yolk sac			
X 103	356 (73 2%)	130 (26 8%)	486
X 104	362 (74 8%)	122 (25 2%)	484

these developmental stages. As free cells were specifically labelled in chick embryo→quail yolk sac chimeras (see below) and their distribution in these chimeras correlated with that found in normal quail embryos, a more detailed account of their distribution and development will be given in the next section.

Chick embryo→quail yolk sac chimeras

In these chimeras, the endothelial cells in the embryo were largely devoid of label with MB1 and QH1 antibodies. However, 20-80 μm long patches of labelled endothelial cells were seen in the wall of some vessels in seven of twelve chimeras analyzed. The vessels with labelled endothelial cells were seen mainly in the cephalic region, such as those of the perineural plexus and the anterior cardinal veins. Chimeric vessels were sometimes found in the chick territory of the yolk sac, suggesting migration of endothelial cells along the vessel walls, as Christ et al. (1990) have recently described.

Free cells immunolabelled with MB1 and QH1 antibodies, derived from yolk sac precursors in these chimeras, were also found. To evaluate the proportion of AcPase-positive cells that were recognized by the antibodies in this type of chimeras, two chick embryo→quail yolk sac chimeras were fixed 48 h after microsurgery and processed for double labelling, to reveal both AcPase activity and MB1 immunoreactivity. More than 90% of the AcPase-positive cells were also MB1 positive (Fig. 5C,D; Table 1), thus showing that they originated in the quail yolk sac.

Free cells of yolk sac origin were already present in chimeras fixed 24 h after microsurgery (HH stages 13-14). Their numbers varied from embryo to embryo, perhaps due to small differences in the size of the grafted area, but they were scarcer than in more fully developed chimeras. Most free cells were round and appeared inside the vessels; a few labelled cells showed macrophage-like morphology. At these developmental stages, immunolabelled cells were more frequent in the cephalic region. Many of these cells appeared near cerebral vesicles and in association with developing cranial ganglia and nerves (Fig. 6A). Rare free cells were observed in regions posterior to the otic vesicle and, when observed, they were always located at the lateral boundary of the somites and lateral plate mesoderm. Unlike in the cephalic region, no free cells appeared surrounding the neural tube at these posterior levels. This distribution was similar to that of the AcPase-positive cells in unoperated chick embryos. Circulating MB1/QH1-positive cells were also frequent in vitelline vessels. As Fig. 6B shows, labelled cells appeared in blood cell islands among unlabelled ones.

The MB1/QH1-labelled free cells increased greatly in number in all regions of chimeras fixed after 48-56 h of reincubation (HH stages 18-24). Although AcPase-positive/MB1-positive cells in mitosis were sometimes distinguishable, the increase in their numbers probably resulted from the arrival of additional cells from the yolk sac. Labelled cells were always found in vitelline and intraembryonic circulation, and they often ap-

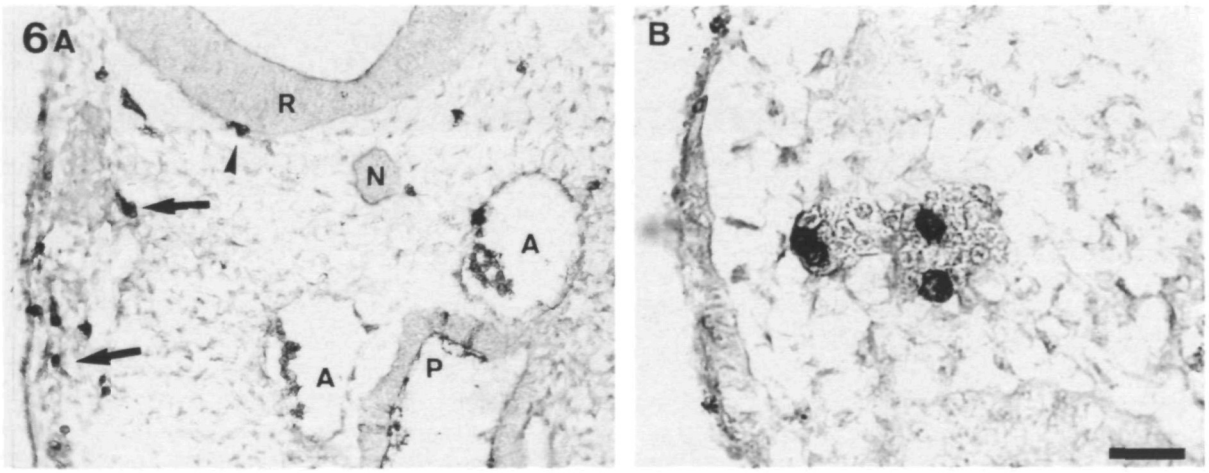


Fig. 6. MB1 immunostaining of sections from HH stage 14 chick embryo→quail yolk sac chimeras. (A) Labelled cells appear in the mesenchyme at the level of the rhombencephalon. One abuts the pial surface of the brain (arrowhead) and others (arrows) are in the region of the developing trigeminal ganglion and nerve. R, rhombencephalon; N, notochord, P, pharynx; A, dorsal aorta. Scale bar in B, 55 μm. (B) Some labelled cells can be observed among unlabelled ones in a blood cell island at the level of the neck. Scale bar, 20 μm.

peared on both sides of the endothelial wall, suggesting that they were able to traverse it.

In these chimeric embryos, MB1/QH1-positive cells showed a consistent distribution which was similar to that in normal quail embryos. At the level of the trunk, they appeared scattered in the mesenchyme around the neural tube and notochord, near the dorsal aorta and in the dorsal mesentery (Fig. 7A,B). However, neither the aortic clusters nor the cells budding from them into the aorta lumen (Dieterlen-Lièvre and Martin, 1981) showed immunoreactivity. In the head, in addition to labelled cells scattered throughout the mesenchyme (Fig. 7C,D), some cells were concentrated in regions of cell death, e.g., the central region and midline of the branchial arches (Glücksmann, 1951) and some developing cephalic ganglia, such as the trigeminal ganglion (Fig. 7C). In these cases, a large proportion of labelled cells showed macrophage-like morphological features, and apparently phagocytosed cell debris. Immunolabelled cells were always present in regions showing cell death, such as the limb bud (see below) and the necrotic zone described in the tail region by Schoenwolf (1981) (Fig. 7E).

In chimeras fixed at 48-56 h of reincubation, labelled free cells were seen within the central nervous system (Fig. 7B,F). The labelled cells were found consistently in the same locations: in the ventricles, in dorsal regions of the brain, especially the rhombencephalon (Fig. 7F) and trunk neural tube, and in regions with a well-formed marginal layer. Although labelled cells in the nervous system were sometimes found near vessels, they often also appeared in regions where vascularization had not started; thus free cells in these locations could not have reached the nervous tissue via the bloodstream. Some images suggested that labelled cells may enter the nervous system from the mesenchyme, passing through the basement membrane (Fig. 7F). Free cells were also observed associated with developing peripheral nerves, at both cephalic and trunk levels (Fig. 7C).

The antibodies also reacted with cells situated in some non-nervous organ primordia. Numerous immunolabelled cells were seen in the liver rudiment, where the cells lay on the cords of hepatoblasts (Fig. 8), in the pericardial cavity, and in the nephric rudiment (Fig. 7A,B).

Owing to the existence of well-identified cell death processes in the limb primordia (Hinchliffe, 1981), the distribution of labelled cells in leg and wing buds was studied in detail. In HH stages 18-20, when cell death processes have not begun in these structures, few labelled cells appeared in the bud; those that were found were mostly elongated (Fig. 9A). Increasing numbers of cells were seen in successive stages. In HH stage 23, when cell death processes are prominent in both the leg and the wing buds, the cells were more widely distributed (Fig. 9B), and frequently showed macrophage-like morphological features, containing large cytoplasmic inclusions (Fig 9C). It therefore seems that MB1/QH1-positive and AcPase-positive cells are the mesenchymal macrophages described in the limb bud necrotic zones (Dawd and Hinchliffe, 1971; Hurle and Hinchliffe, 1978).

Quail embryo→chick yolk sac chimeras

In these chimeras, a staining pattern complementary to that observed in the inverse chimeras was obtained with the MB1 and QH1 antibodies. The endothelia of the vessels in the embryo were labelled; no unlabelled endothelial cells were observed. In the dorsal aorta, the cells forming the aortic clusters and those that were entering the circulation from the clusters were recognized by the antibodies.

Besides the labelled cells found within the vessels, scattered free cells could be also seen in the mesenchyme (Fig. 10). However, they were less numerous than in normal quail embryos and chick embryo→quail yolk sac chimeras of comparable development. Sections from two quail embryo→chick yolk sac chimeras were double labelled: about 75% of the AcPase positive cells showed no label with MB1 antibody, and were therefore thought to be derived from precursors in the chick yolk sac (Fig. 5E,F; Table 1). The AcPase-positive/MB1-positive cells, of intraembryonic origin, were intermingled with the AcPase-positive/MB1-negative ones, and showed similar morphological features. Therefore, no obvious differences of distribution were observed between cells arising from the yolk sac and those derived from intraembryonic precursors.

Although labelling of the endothelia made it difficult to identify immunolabelled free cells, which were less numerous, some of them were distinguishable in the same regions already noted in the previous section, i.e., the dorsal neural tube, mesenchymal regions showing cell death processes, tail, limb buds, etc.

Control chimeras

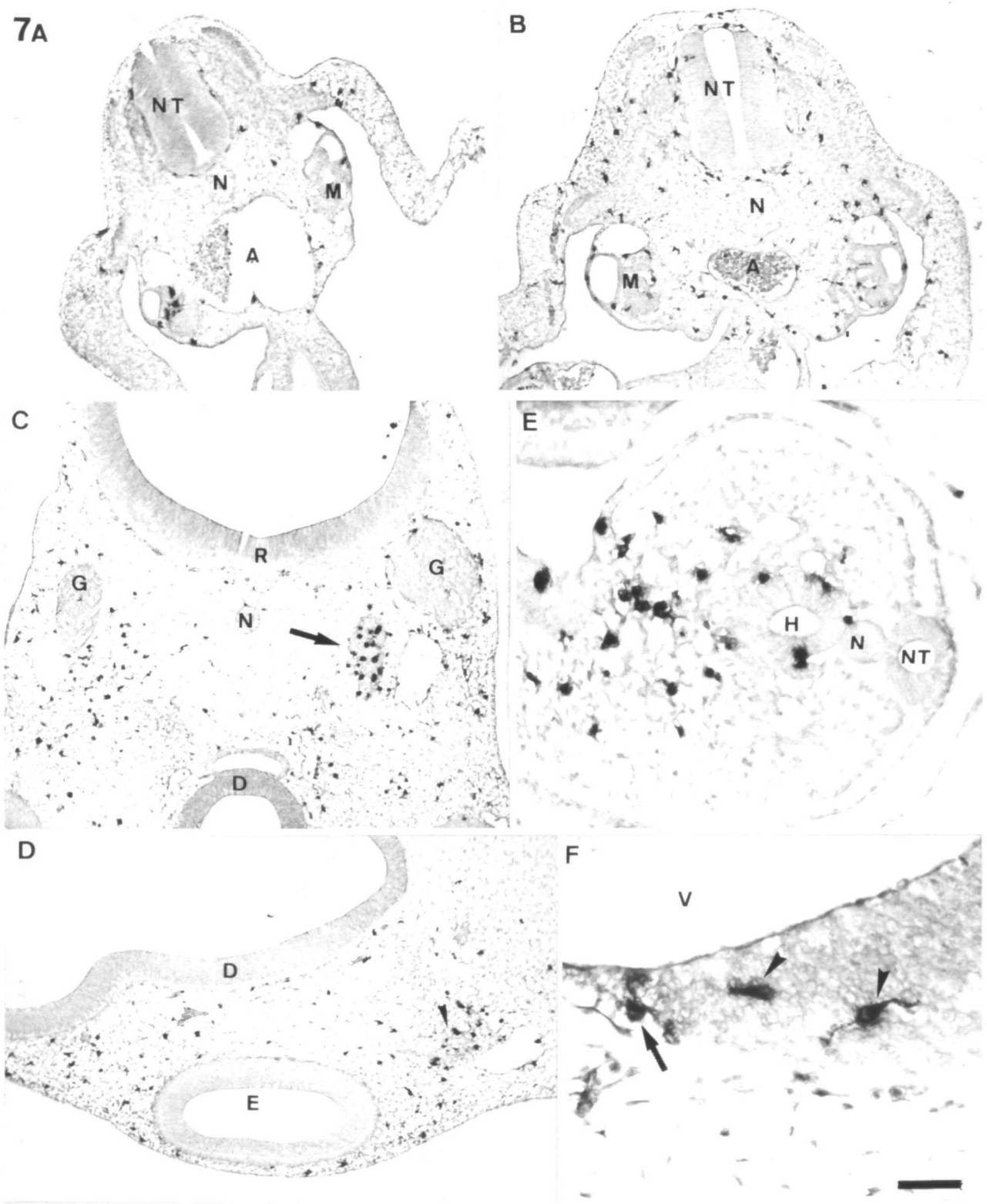
In the control chimeras (chick embryo→chick yolk sac and quail embryo→quail yolk sac) the distribution of the AcPase-positive, MB1/QH1-positive cells was similar to that described in normal embryos and heterospecific yolk sac chimeras.

Discussion

Yolk-sac-derived hemopoietic cells in the avian embryo

The results described above show that in normal quail embryos at 2.5-4.5 days of incubation, two populations were distinguishable among the hemangioblastic cells recognized by the MB1 and QH1 antibodies: (1) endothelial cells, which were largely devoid of AcPase activity when sodium tartrate was added to the incubation medium; and (2) cells circulating and cells lying free in the mesenchyme, which were both AcPase- and MB1/QH1-positive. Thus, the free cells contained tartrate-resistant AcPase, while the enzyme activity in other cell types was tartrate-sensitive. A few isolated MB1/QH1 labelled cells with no staining for AcPase were also observed.

The endothelial cells and free cells have different



origins, as revealed by results in the yolk sac chimeras. Most of the free cells were of yolk sac origin, since they were MB1/QH1 positive in chick embryo→quail yolk sac chimeras but lacked these antigens in inverse chimeras. By contrast, endothelial cells originated in the embryo, as shown by the presence of MB1/QH1 immunoreactivity in endothelial cells of quail embryo→chick yolk sac chimeras and the absence of such staining in the opposite chimeras. These results are consistent with recent reports on early vasculogenesis in avian embryos (Pardanaud et al., 1987; Coffin and Poole, 1988; Dieterlen-Lièvre et al., 1988), which noted that the early vascular network originated from angioblastic cells that differentiate "in situ". The significance

Fig. 7. Distribution of immunolabelled cells in chick embryo→quail yolk sac chimeras reincubated for 48-56 h. (A) Trunk of an HH stage 20 chimera, showing labelled cells in the mesenchyme. NT, neural tube, N, notochord; A, aorta; M, mesonephros Scale bar in F, 150 μm. (B) At a similar level, more labelled cells appear in an HH stage 22 chimera. Many cells are located around the neural tube (NT) and in the mesonephric region (M). For the remaining labels, see A. Scale bar in F, 150 μm. (C) HH stage 23 yolk sac chimera, at the level of the rhombencephalon. Numerous labelled cells are concentrated in one of the lobes (arrow) of the trigeminal ganglion (G). R, rhombencephalon, D, diencephalon; N, notochord. Scale bar in F, 150 μm (D) Labelled cells scattered throughout the cephalic mesoderm of an HH stage 21 chimera. Some immunolabelled cells are associated with the developing ophthalmic division of the trigeminal nerve (arrowhead). D, diencephalon; E, eye. Scale bar in F, 150 μm. (E) Macrophage-like labelled cells in the tail region of an HH stage 20 yolk sac chimera. NT, neural tube; N, notochord; H, hindgut. Scale bar in F, 40 μm. (F) Two labelled cells (arrowheads) in the neuroepithelial wall of the rhombencephalon in an HH stage 21 yolk sac chimera. A third cell (arrow) is seen on the pial surface. V, Fourth ventricle Scale bar, 25 μm.

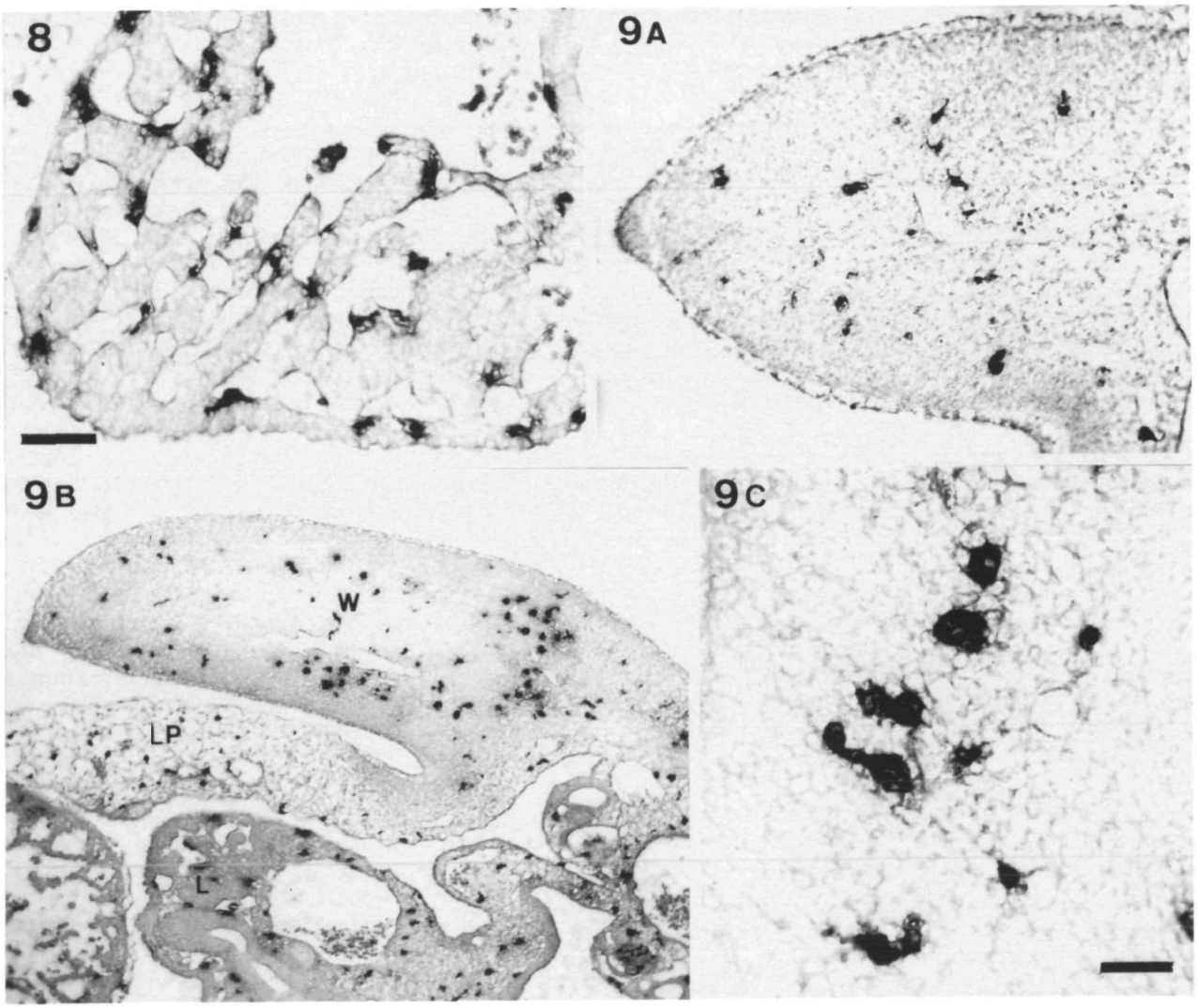


Fig. 8. MB1 labelled cells in the liver primordia of an HH stage 23 chick embryo→quail yolk sac chimera. The labelled cells resemble the Kupffer cells of the adult liver. Scale bar, 50 μm.

Fig. 9. MB1 labelled cells in wing buds of chick embryo→quail yolk sac chimeras. (A) In an HH stage 20 chimera, few cells are present in the wing bud. Scale bar in C, 60 μm. (B) Distribution of labelled cells in the wing bud of an HH stage 23 chimera showing that labelled cells concentrate in regions of cell death. Immunolabelled cells are also frequent in the lateral plate mesoderm (LP) and in the liver (L). Scale bar in C, 150 μm. (C) High magnification of immunolabelled cells in the wing bud mesenchyme in B. The cells are large and rounded, with dense inclusions. Scale bar, 20 μm

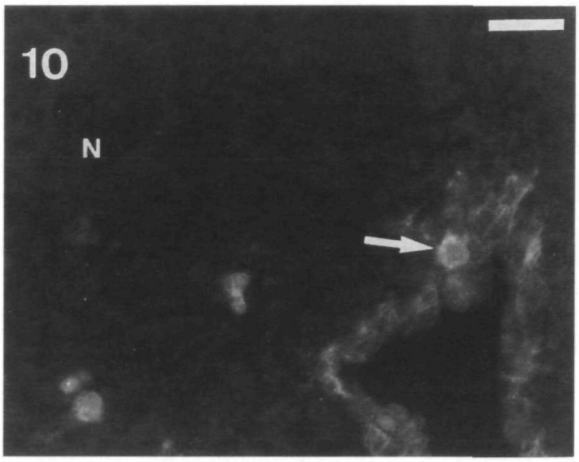


Fig. 10. QH1 immunostaining of a Z stage 16 quail embryo→chick yolk sac chimera. Two labelled cells are seen in the mesenchyme near the notochord (N) at the level of the trunk. Another positive free cell (arrow) is located in the endothelial wall of a branch of the aorta, which is also labelled. Scale bar, 40 μm.

of the occasional presence of yolk-sac-derived cells in the endothelia of vessels of some chick embryo→quail yolk sac chimeras is difficult to interpret. Because their occurrence was variable and we were unable to observe endothelial cells from the yolk sac in the quail embryo→chick yolk sac chimeras as noted earlier by Beaupain et al. (1979), it is probable that the yolk sac cell contribution to embryonic endothelia is an artifact due to the greater invasiveness of quail cells compared to chick cells.

Some of the labelled free cells and circulating cells showed the morphological features of undifferentiated hemopoietic cells as described by Dieterlen-Lièvre and Martin (1981); their state of commitment cannot be inferred from this type of detection. In embryos of 3-4 days of incubation, labelled cells with macrophage-like morphological features predominated; these cells had intense AcPase activity and often contained large vacuoles and phagosomes. Taken together, the morphological and histochemical observations suggest that they belong to the monocyte/macrophage lineage.

In the stages considered here, the free cells originated chiefly from yolk sac cells. Therefore, cells coming from the yolk sac invade the embryo from day two of incubation onwards. They may enter the embryo through the blood circulation and/or by migrating extravascularly through the mesoderm. The progressive increase in the number of free cells observed in most advanced embryos suggests that cells from the yolk sac continue to enter the embryo during all stages studied here.

However, as Table 1 shows, not all free cells were of yolk sac origin. About 5-10% of the AcPase-positive cells in the chick embryo→quail yolk sac chimeras, and about 25% in the inverse chimeras, were derived from precursors that had either entered the embryo before microsurgery, or most likely, differentiated from the intraembryonic mesoderm. The presence of cells committed to hemangioblastic lineage has been reported in the lateral plate mesoderm of 2-4 day quail embryos (Dieterlen-Lièvre, 1984; Dieterlen-Lièvre et al., 1988), which may be the earliest manifestation of intraembryonic hemopoiesis. Later, diffuse hemopoietic foci were observed within the mesenchyme, which will produce the precursors of definitive blood cells (Dieterlen-Lièvre and Martin, 1981). Therefore, the free cells of intraembryonic origin that we found in the chimeras may be early hemopoietic cells arising from intraembryonic mesoderm. These cells appeared in the locations already described (Dieterlen-Lièvre and Martin, 1981; Dieterlen-Lièvre, 1984), and their characteristics were similar to those of the cells of extraembryonic origin. The differences observed in the proportion of intraembryonic and extraembryonic free cells in the two types of chimeras may be explained by the fact that the quail cells proliferate and differentiate faster than the chick cells.

Hemopoietic phagocytes in the avian embryo

As noted above, many of the free cells appeared engaged in phagocytosis. Numerous authors have described specialized phagocytes that play a role in the removal of dead cell debris (Saunders, 1966; Dawd and Hinchliffe, 1971; Hurle and Hinchliffe, 1978; Schoenwolf, 1981; Hinchliffe, 1981; García-Porrero et al., 1984) and extracellular matrix remodeling (Bard et al., 1975) during early embryo development. These cells have frequently been described as macrophages on the basis of their morphological features. In their review of cell death phenomena, Beaulaton and Lockshin (1982) affirmed that two types of specialized phagocytes involved in the elimination of dead cells during development can be identified: true macrophages, derived from monocytes, and macrophages derived from the mesenchyme. Among the latter are the phagocytic cells that eliminate cell debris in areas of cell death that arise during limb morphogenesis in avian and mammalian embryos. Detailed descriptions of macrophage differentiation from mesenchymal cells in limb buds have been performed with ultrastructural and cytochemical methods (Ballard and Holt, 1968; Dawd and Hinchliffe, 1971; Hurle and Hinchliffe, 1978). In organ cultures of the posterior necrotic zone of the wing bud excised when the cells of this region are already committed to death, macrophagic cells appear and phagocytose cell debris (Fallon and Saunders, 1968); these macrophages apparently differentiate from mesenchymal cells concomitant with cell death processes, and later regress to their early fibroblast-like morphology (Brewton and McCabe, 1988).

Our study demonstrates that the specialized phagocytic cells reported to exist in regions of intense cell death are derived from hemopoietic cells, and can be described as "true macrophages". The studies cited above on the origin of macrophages in the limb buds do not contradict our results. Free cells of yolk sac origin were already present in the limb mesoderm at HH stage 18, and thus, they are included in the mesoderm regions excised for organotopic culture. Moreover, the mor-

phological transformations of cells phagocytosing dead-cell remains may be related to the different morphological types of immunolabelled cells observed: before the start of cell death, MB1/QH1-positive cells were similar to other cells in the mesenchyme; when abundant cell death occurred, numerous labelled cells with prominent inclusions in their cytoplasm were common. Therefore, the hemopoietic cells performing phagocytosis underwent the same morphological changes as have been described in mesenchymal macrophage differentiation.

Taken together, our results support the notion that, in the embryo, there is a widely distributed phagocytic cell system derived from the blood cell line. The macrophages must be conveyed by the blood circulation throughout the embryo, and probably exit the vessels and move through the mesenchyme in response to chemotactic signals (such as factors released during the cell death process) that determine their consistent distribution in the embryo. Our experimental results indicate that the blood cell line is the source of specialized phagocytes during development, although other embryonic cell types might act as non-professional phagocytes (García-Porrero and Ojeda, 1979; García-Porrero et al., 1984; Hurle et al., 1978; Beaulaton and Lockshin, 1982; Kalman, 1989). The hemopoietic phagocytes are mostly of yolk sac origin in the early embryo, as shown by the percentage of immunolabelled AcPase-positive cells in the two inverse chimeric patterns.

Jeurissen et al. (1988) have characterized an antibody that recognizes monocytes, interdigitating cells and macrophages (both macrophages in connective tissue and resident macrophages in organs) in the chicken. This finding led them to suggest that a mononuclear phagocytic system, comparable to the one described in mammals (Van Furth, 1980), may exist in birds. Although no definitive conclusions can be reached from our results, the macrophage cells reported here may belong to this system. This notion is supported by the observation that MB1/QH1-labelled cells located in some organ primordia occasionally showed similar morphological features to those of adult resident macrophages, which belong to the mononuclear phagocytic system (see Fig. 8).

Recently, the presence of macrophages has been reported in early developing vertebrate embryos. Ohinata et al. (1990) have described nonlymphoid leukocytes scattered throughout the mesenchyme prior to the establishment of blood circulation in *Xenopus*. These authors claimed that these cells were mostly macrophages, and argued that they might represent a transient population that performs phagocytic functions before the emergence of leukocytes from other sources. In mice, cells immunoreactive to the F4/80 antibody, which is specific for macrophages, are first seen on embryonic day 10 in the yolk sac, liver and mesenchyme, and thus are among the earliest hemopoietic cells to be produced (Morris et al., 1991).

In addition to their role in phagocytosis, macrophages may have other functions during development. Morris et al. (1991) propose that they take part in cellular interactions during hemopoiesis. At the developmental stages considered in this study, when morphogenetic processes are taking place, macrophages in avian embryos were clearly related with cell death. These cells are probably also involved in extracellular matrix remodeling associated with cell migration and cell rearrangement, as Bard et al. (1975) have proposed in the cornea. Finally, a similar role in hemopoiesis to those described by Morris et al. (1991) can be envisaged.

The phagocytic cell system that we detected in yolk sac chimeras was not observed in earlier experiments with this model (Dieterlen-Lièvre and Martin, 1981). In these experiments, a quail body was grafted on a chick yolk sac, and most chimeras were analyzed from day 5 of incubation onwards. Moreover the resolution of the quail nucleolar marker, which served as tracer of the origin of cells, is not as good for isolated cells as the MB1 and QH1 antibodies. These facts may explain why the yolk-sac-derived phagocytic cells were not detected. In the present work, no chimeras older than 4-5 days have been analyzed. It will be interesting to determine whether the yolk sac macrophages are progressively replaced by cells of intraembryonic origin, like other blood cells during development in birds (Dieterlen-Lièvre, 1984), or whether they become a long-lived resident population. We are now trying to elucidate the fate of the free cells of yolk sac origin and their relation with definitive cell populations in the adult.

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