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Histochemical in situ identification of bovine embryonic blood cells reveals differences to the adult haematopoietic system and suggests a close relationship between haematopoietic stem cells and primordial germ cells

Accepted: 28 January 2004 / Published online: 24 February 2004
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Abstract Cryostat sections of bovine embryos of exactly known age (obtained from artificial insemination), ranging from 32 to 60 days post-insemination, were treated with a wide range of antibodies directed against cell surface antigens or lineage-specific factors in order to demonstrate different types of fetal blood cells and their precursors. An antibody specific to bovine c-kit (bk-1) stained not only presumptive haematopoietic stem cells in the dorsal aorta and the embryonic liver, but also a subpopulation of putative primordial germ cells in the gonadal anlage, the latter being further characterised by a positive labelling with the lectins STA, WFA and WGA and a histochemical reaction for alkaline phosphatase. The antibody against CD 45, commonly regarded as a pan-leukocyte marker, reacted in the bovine embryo with different types of blood cells, as well as with presumptive vasculogenetic cells and a subpopulation of putative primordial germ cells. CD 61 immunoreaction proved to be a useful tool for demonstrating megakaryocytopoiesis in the embryonic liver, in addition to the lumen of blood vessels and the mesonephros. Staining with BM-2 was restricted to a single population of medium-sized, round to oval cells, forming small groups within the parenchymal strands of the liver. Characterised furthermore by a U-shaped nucleus, this BM-2-positive cell type apparently represents a developmental stage in the granulopoietic lineage. B-lymphocytopoiesis in the bovine liver was detected with antibodies directed against WC-4 and IgM, but not until day 58 post-insemination. Using antibodies to CD 14, no positive results could be obtained in embryonic tissues, although anti-CD 14-positive macrophages were easily recognised in lymph nodes of adult bovines. The antibody against CD 68, however, identified

two populations of primitive macrophages in our samples. One population was located in parenchymal strands of the embryonic liver, probably acting as nursing cells for haematopoietic foci, and the other was observed intravasally in the sinusoids of the liver, most probably representing primitive Kupffer cells.

Keywords Haematopoietic stem cells · c-kit · Primordial germ cells · Embryonic haematopoiesis · Bovine

Introduction

In recent years, the development of a wide range of monoclonal antibodies directed against bovine cell surface molecules (see, for example, Naessens and Howard 1993; Rathkolb et al. 1997) and the crossreactivity of a number of antibodies specific for human leucocyte surface antigens (Jones et al. 1993; Ackermann et al. 1994; Brodersen et al. 1998) led to a number of meticulous studies on the adult bovine immune and haematopoietic systems (see, for example, Murakami et al. 1999; Van Kampen et al. 1999; Rebelatto et al. 2000). However, very little is known about the expression of blood cell surface antigens in bovine embryos and the immunohistochemical application of the corresponding antibodies in developmental studies. The aim of this investigation was, therefore, to test a number of antibodies for their suitability to discriminate between myeloid cells, macrophages, megakaryocytes and their precursor and stem cells in bovine embryos. The first haematopoietic stem cells with the ability of adult reconstitution are located in the aorta-gonad-mesonephros region (AGM) in vertebrate embryos and display a c-kit⁺/CD 34⁺/lineage (lin)⁻ phenotype in mouse and man (Medvinsky and Dzierzak 1996; Sanchez et al. 1996; Labastie et al. 1998; Tavian et al. 1999). C-kit is a proto-oncogene which encodes a transmembrane tyrosine kinase receptor (Yarden et al. 1987). C-kit mutation of the W locus in the mouse affects gametogenesis, pigmentation, haema-

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topoiesis and mast cell development during murine development (reviewed in Broudy 1997; Lyman and Jacobsen 1998). Consistent with these results, the expression of c-kit mRNA (Yarden et al. 1987; Geissler et al. 1988; Qiu et al. 1988; Nocka et al. 1989; Tan et al. 1990) and c-kit protein (Horie et al. 1991, 1992; Yoshinaga et al. 1991; Maeda et al. 1992) was detected in the ovary, testis, fetal liver, bone marrow, mast cells and skin. Additionally, the embryonic expression of c-kit includes a variety of tissues in which no obvious phenotypic changes have been detected in W mutant mice, for example, gut and central nervous system (Keshet et al. 1991; Motro et al. 1991).

Recently, the cDNA for bovine c-kit (bokit) has been cloned (Kubota et al. 1994) and six bovine-specific monoclonal antibodies (bk1–6) have been raised (Hikono et al. 1999). These antibodies have been used in flow cytometry for characterisation of bokit-positive fractions of bone marrow and peripheral blood (Hikono et al. 2001a, b). In the present study, we applied bk1–6 and a commercially available c-kit antibody with broad species crossreactivity, including ovine embryonic tissue (Tisdall et al. 1999). Haematopoietic stem cells and primordial germ cells (PGCs) are both expected to display c-kit immunoreactivity. Therefore, WFA, WGA, STA or alkaline phosphatase (AP) histochemistry was used to label putative PGCs (Wrobel and Süß 1998), and a bovine-specific antibody directed against the pan-leucocyte marker CD 45 was used to mark haematopoietic cells.

Morphological and functional differences between the embryonic and adult haematopoietic system are evident; most obvious is the formation of nucleated erythrocytes in the yolk sac or the development of fetal macrophages not via monocytes, but by intercalation of a specific precursor cell type designated as primitive macrophage (Faust et al. 1997; Naito et al. 1997). The availability of molecular tools such as specific antibodies allowed an accurate description of separate haematopoietic lineages and their precursors during different stages of human embryonic and fetal development (see, for example, Timens et al. 1990). On the other hand, little is known about species-specific differences in embryonic haematopoiesis. One should expect characteristic adaptations of the embryonic haematopoiesis depending on growth rate of the yolk sac, mode of placentation, morphological structure of the

AGM region or the timeline for acquisition of immunological competence. For an analysis of ongoing lineage-specific blood cell proliferation and differentiation in the bovine embryo, we tested a wide range of antibodies (BM1, BM2, CD 11b, CD 14, CD 34, CD 45, CD 61, CD 68, c-kit, IgM, myeloperoxidase, WC-4) in order to discriminate between myelopoiesis, megakaryocytopoiesis, B-lymphopoiesis and macrophages.

Materials and methods

Embryo collection and tissue preparation

For the present study, we used 12 bovine embryos of known age. Heifers of the Fleckvieh breed were artificially inseminated at the end of oestrus. Embryos were collected at slaughter in a local abattoir. The age of the embryos was determined in days post-insemination (dpi) and ranged from 32 to 60 dpi (Table 1).

Immediately after collection, the fetal membranes and the abdomen were opened prior to fixation. The fixation was carried out in two steps. Fixative I (0.5 h) contained 4% paraformaldehyde, 15% v/v saturated picric acid and 0.1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. Fixative II (1.5 h) had the same composition, but without glutaraldehyde. Following fixation, the complete embryos were washed (3×10 min) in 0.1 M phosphate buffer and transferred into a graded series of saccharose (10%, 20%, 30%, 40%). Embryos were oriented in the desired position on brass holders, enclosed in Tissue TEK OCT compound (Miles, Elkhart, IN, USA) and frozen in liquid nitrogen. Cryostat sections (12 µm thick) were mounted on gelatine/chrome-alum-coated slides and air dried for 2–3 min before further processing.

Histochemistry

Alkaline phosphatase was demonstrated using the methods of Gomori (1952) and McGadey (1970).

Lectin histochemistry was performed with biotinylated STA, biotinylated WFA and biotinylated WGA. (STA: *Solanum tuberosum* lectin; binding characteristics: β-D-GlcNac. WFA: *Wisteria floribunda* lectin; binding characteristics GalNac 1,6 Gal >αGalNac. WGA: *Triticum vulgare* lectin; binding characteristics: [β-D-GlcNac]_n linked 1→4 Neu Nac). Source of lectins: Sigma, Deisenhofen, Germany.) All steps of lectin histochemistry were carried out in a moist chamber on cryostat sections surrounded by water-repellent PAP-PEN (Science Services, Munich, Germany) as follows: (1) blocking of endogenous peroxidase with 10% methanol and 3% H₂O₂; (2) rinsing (3×10 min) in PBS; (3) preincubation (45 min) with blocking buffer containing 10% fetal calf serum (Seromed, Munich, Germany) and 0.005% cold water fish gelatine (Biotrend, Köln, Germany) in PBS; (4) incubation with the optimal

Table 1 List of investigated bovine embryos. All embryos resulted from controlled inseminations

Number of embryo	Crown–rump length (cm)	Age (days post-insemination)
229	0.9	32
159	1.3	34
227	1.3	34
228	1.7	34
212	1.9	36
128	1.6	37
204	2.0	43
211	3.4	45
186	2.5	45
126	3.5	50
105	5.4	58
104	6.6	60

Table 2 List of antibodies tested

	Specificity	Clone (source)	Species (cross)reactivity
Haematopoietic stem cells	C-kit	Polyclonal (Santa Cruz) bk-1 to bk-6 (H. Hikono)	Mouse, rat, human, ovine Bovine
	CD 34	Polyclonal (Santa Cruz)	Mouse, rat, human
Leukocytes	CD 45	CC1 (Biosource)	Bovine
Megakaryocytes	CD 61	Y2/51 (Dako)	Human
Myelopoiesis	CD 11b	M1/70.15.5.2 (Developmental Studies Hybridoma Bank, Iowa)	Mouse, human
	BM-1	NCL-BM1(Novocastra)	Human
	BM-2	NCL-BM2 (Novocastra)	Human
	Myeloperoxidase	Polyclonal (Novocastra)	Human
			Polyclonal (Dako)
B-lymphopoiesis	IgM	Polyclonal (acris)	Bovine
	WC-4	CC55 (acris)	Bovine, ovine
Macrophages	CD 14	VPM 65 (Biosource)	Sheep, bovine
	CD 68	EBM 11 (Dako)	Human, bovine

lectin concentrations (STA: 2.0 $\mu\text{g/ml}$; WFA: 0.5 $\mu\text{g/ml}$; WGA: 0.062 $\mu\text{g/ml}$) in blocking buffer overnight at 4°C; (5) rinsing as in step 2; (6) incubation in streptavidin-peroxidase (1:200; Jackson via Dianova, Hamburg, Germany) for 60 min at room temperature; (7) rinsing as in step 2; (8) developing with a DAB (diaminobenzidine tetrahydrochloride) kit (Immunotech, Hamburg, Germany) supplemented by 0.002% $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ and 0.04% $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ (Kujat et al. 1993); (9) rinsing twice in PBS; (10) dehydration in graded alcohol, clearing in xylol and mounting in DPX (Serva, Heidelberg, Germany). Control sections were incubated: (1) without lectin and (2) with lectins that had been diluted with PBS containing 0.2 M of the adequate inhibitory sugar (Sigma). These control sections remained unstained.

Immunohistochemistry

Immunohistochemistry was carried out in a moist chamber on cryostat sections surrounded by water repellent PAP-PEN as follows: (1) blocking of endogenous peroxidase; (2) rinsing; (3) preincubation (see blocking, preincubation and rinsing for lectin histochemistry); (4) overnight incubation at 4°C with the primary antibody (0.01 $\mu\text{g/ml}$) in blocking buffer (see Table 2); (5) rinsing as in step 2; (6) incubation (60 min) in the secondary antibody, i.e. 1:100 goat anti-mouse/biotinylated IgG (Jackson) or 1:200 goat anti-rabbit/biotinylated IgG (Jackson); (7) rinsing as in step 2; (8) incubation in avidin-biotin-peroxidase complex, ABC (Vector via Linearis, Wertheim, Germany) after Hsu et al. (1981) for 60 min; (9) rinsing as in step 2; (10) developing as in step 8 of lectin histochemistry; (11) rinsing twice in PBS; (12) dehydration in ethanol and mounting in DPX. Control sections were incubated without primary antibody and remained unstained.

Double-labelling of putative PGCs with anti-CD 45 and WFA

Double-labelling of CD 45 and WFA was performed on selected sections in one 34-dpi embryo in order to provide direct evidence for the identification of CD 45-positive cells as putative PGCs. All steps were carried out in a moist chamber on cryostat sections surrounded by water-repellent PAP-PEN. First, anti-CD 45 immunohistochemistry was carried out according to immunohistochemistry steps 1–5. Then, sections were placed in peroxidase-coupled goat anti-mouse IgG (Jackson) diluted 1:100 in blocking buffer for 1 h at room temperature. After rinsing with PBS, CD 45 immunoreactivity was visualised with DAB/Ni/Co/ H_2O_2 (for details see step 8 lectin histochemistry). After rinsing with PBS, sections were covered with a coverslip. Staining was immediately documented on a Zeiss Axiolab microscope equipped with a digital camera (Axio Cam MRc; Zeiss) using Zeiss MR grab software version 1.0. Next, the coverslip was removed and sections were incubated for 1 h with blocking buffer, followed by overnight incubation with biotinylated

WFA (0.5 $\mu\text{g/ml}$ in blocking buffer) at 4°C. After rinsing with PBS, sections were incubated with alkaline phosphatase-coupled ABC kit (ABC-AP; Vector) for 1 h. Lectin binding was visualised using a Vector Red alkaline phosphatase substrate kit I (Vector) as chromogen supplemented with levamisole for quenching endogenous alkaline phosphatase. Finally, sections were rinsed in A. dest. and mounted with Aquatex (Merck, Darmstadt, Germany). Staining was digitally documented. In control sections, both anti-CD 45 IgG and biotinylated WFA were omitted. These sections remained unstained.

Results

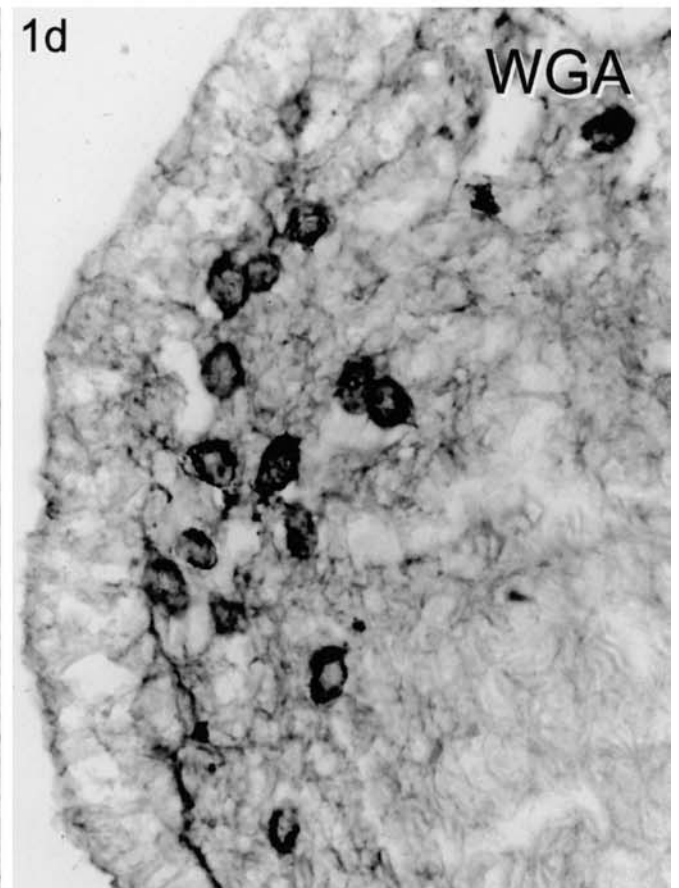
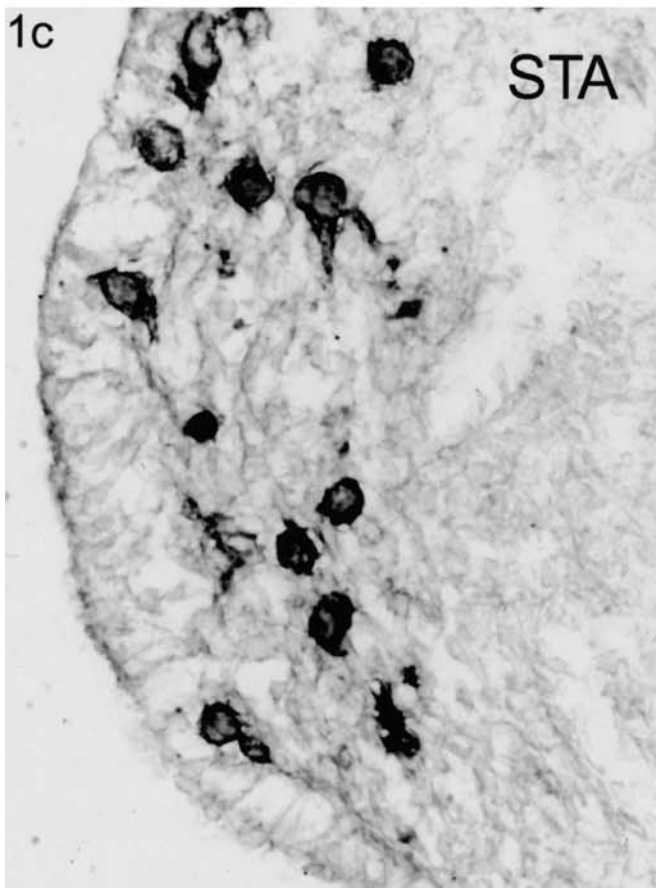
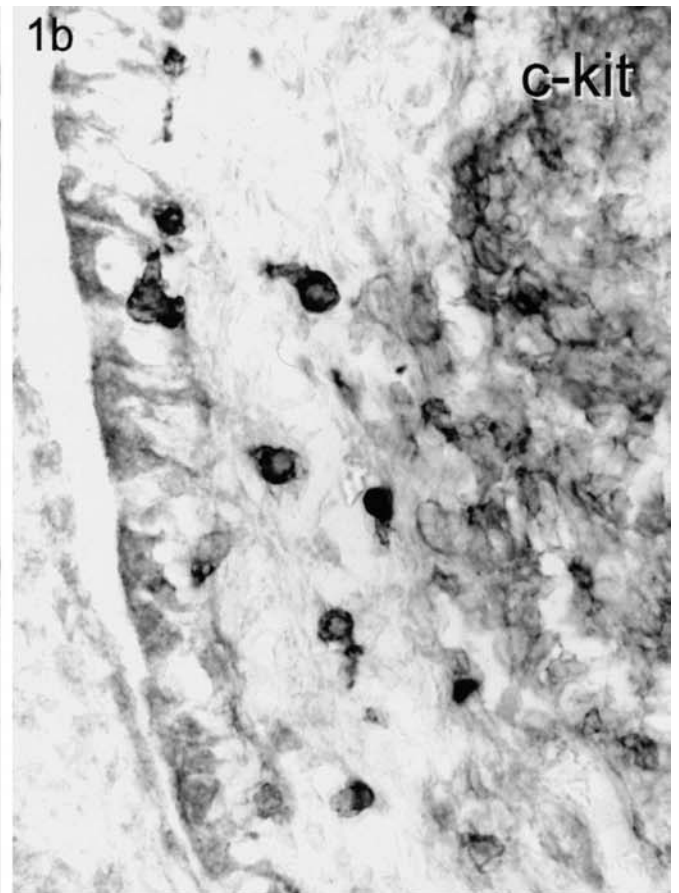
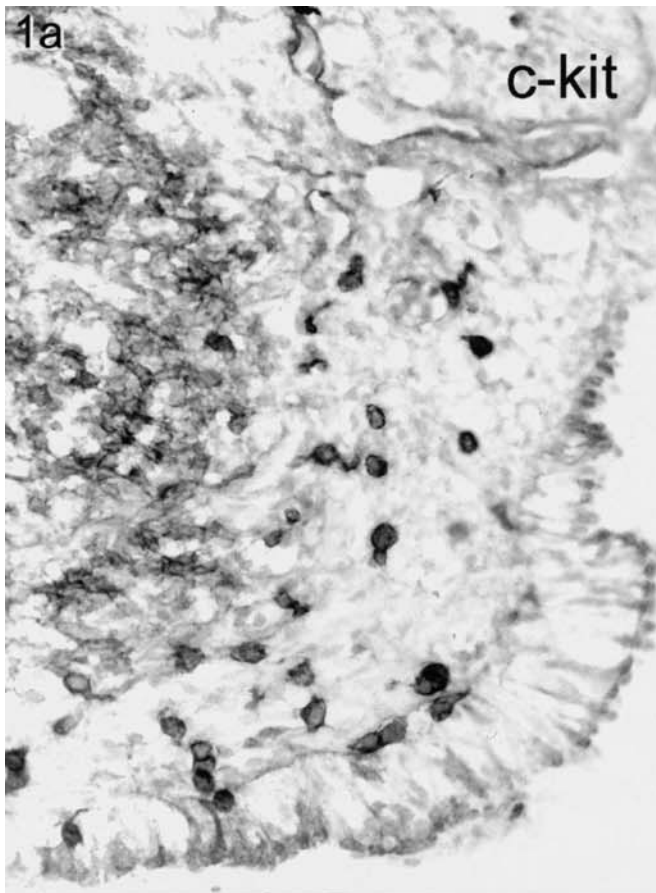
Identification of haematopoietic stem cells

C-kit immunoreactivity

From the one polyclonal (sc-168) and six monoclonal (bk1–6) antibodies directed against c-kit which were applied immunohistochemically only one, namely bk-1, produced a reliable staining of distinct cell types in a variety of bovine embryonic tissues. In the gonadal fold and early gonad, large round cells which often displayed characteristic finger-like processes were moderately to intensely stained with bk-1 (Fig. 1a, b). Similar cells in consecutive serial sections were positive with the lectins STA and WGA (Fig. 1c, d), thus representing PGCs. Blastema cells in the centre of the gonad showed weak to moderate immunostaining (Fig. 1a, b). However, bk-1 failed to demonstrate extragonadal PGCs in the paraortic mesenchyme and especially in the coelomic bay, where numerous putative PGCs are expected to be present close to the gonadal fold.

Presumptive haematopoietic stem cells clustered together and attached to the wall of the dorsal aorta were intensely labelled with bk-1 (Fig. 2a, b). In the embryonic liver, bk-1-positive cells of various sizes occurred in the parenchymal strands (Fig. 3), most probably representing here also haematopoietic stem and progenitor cells.

Moderate to intense immunostaining with bk-1 occurred in the nervous system, including the neural tube, the developing spinal ganglia and the peripheral spinal nerve (Fig. 2a). Podocytes of the bovine mesonephros



displayed bk-1 immunoreactivity from 45 dpi onwards (Fig. 4), but were completely unstained in the developing metanephros. The endodermic epithelium of the gut, the developing lung buds (Fig. 5a, b) and the gall bladder also displayed intense bk-1 immunostaining. A network of capillaries with moderately to intensely stained endothelial cells as well as single cells with a stellate appearance due to multiple processes encircled the cranial embryonic gut, especially the lung buds (Fig. 5a, b).

The mesenchyme underlying the future cutis and subcutis, where melanoblasts are expected to be present, was completely devoid of bk-1-positive cells.

CD 34 immunoreactivity

The polyclonal antibody directed against CD 34 is known to display a broad species reactivity (mouse, rat, human; manufacturer's specification). In our material, it labelled almost all intravascular blood cells. Therefore, it is not suited as a marker for bovine haematopoietic stem cells or blood cell precursors.

Identification of CD 45-positive cells

Antibodies directed against CD 45 are commonly used as pan-leucocyte markers (Figs. 6, 7, 8, 9, 10, 11). In the bovine embryo, CD 45-positive cells were frequently observed in peripheral blood (Fig. 6) and liver (Fig. 7), the latter being the principal site of haematopoiesis during the first gestational trimester. Labelled cells displayed various sizes and shapes, thus indicating different types of blood cells such as haematopoietic stem cells, megakaryocytes, macrophages and granulocytes. Surprisingly, anti-CD 45 also labelled non-blood cells in bovine embryos. In the gonadal anlage, CD 45-positive cells were identified as putative PGCs by their large round somata and typical finger-like processes (Fig. 9a). In consecutive sections, the same cell type was labelled with the alkaline phosphatase reaction product (Fig. 9b) and the lectin STA (Fig. 9c), respectively. Double-labelling of selected sections first with anti-CD 45 (Fig. 10a, d) and second with the lectin WFA (Fig. 10b, e, f) revealed that a subpopulation of intragonadal CD 45-positive cells was also stained with WFA (Fig. 10d, e), thus providing direct evidence for putative PGCs expressing CD 45. Besides CD 45⁺/WFA⁺ cells, also CD 45⁺/WFA⁻ (Fig. 10d, e) putative PGCs, which displayed the same morphology as double-labelled cells and CD 45⁻/WFA⁺ (Fig. 10f) cells were present.

Fig. 1a–d Female embryo, 45 days post-insemination (dpi). **a, b** C-kit. **c** STA. **d** WGA. Low power photomicrograph (**a**) and detail (**b**) of c-kit-labelled gonad. Putative primordial germ cells (PGCs) display finger-like processes and are strongly positive. The same cells are also stained with the lectins STA (**c**) and WGA (**d**). Furthermore, somatic cells in the interior of the gonad show a moderate c-kit immunoreactivity. **a** ×360; **b–d** ×580

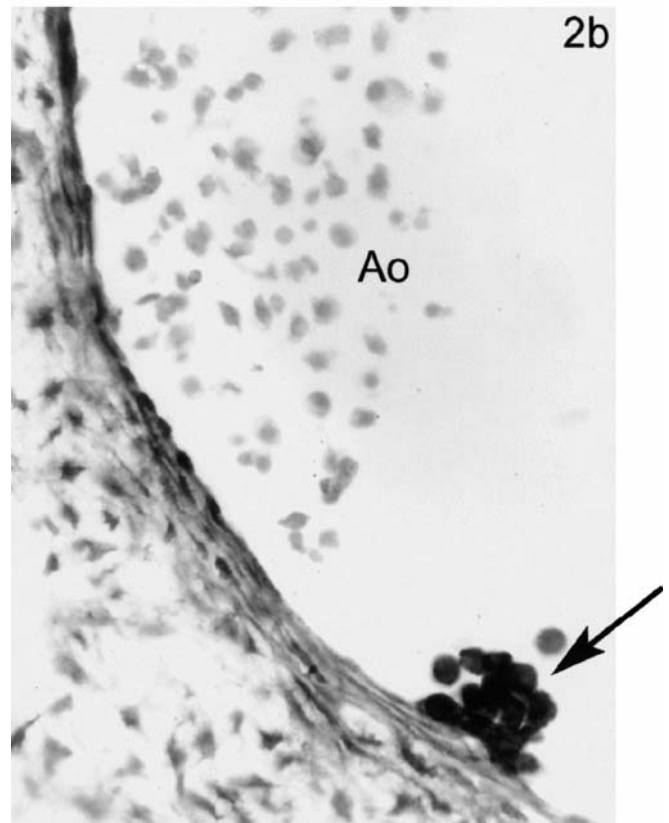
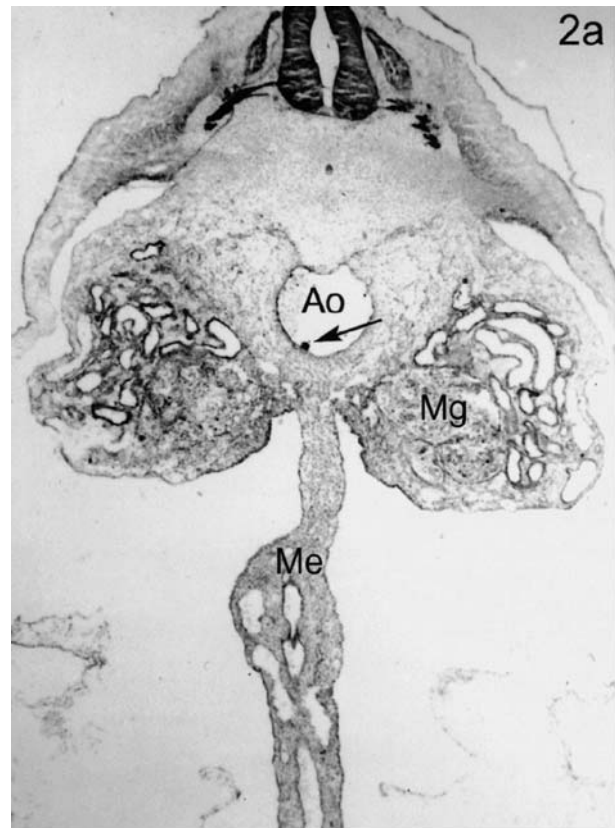
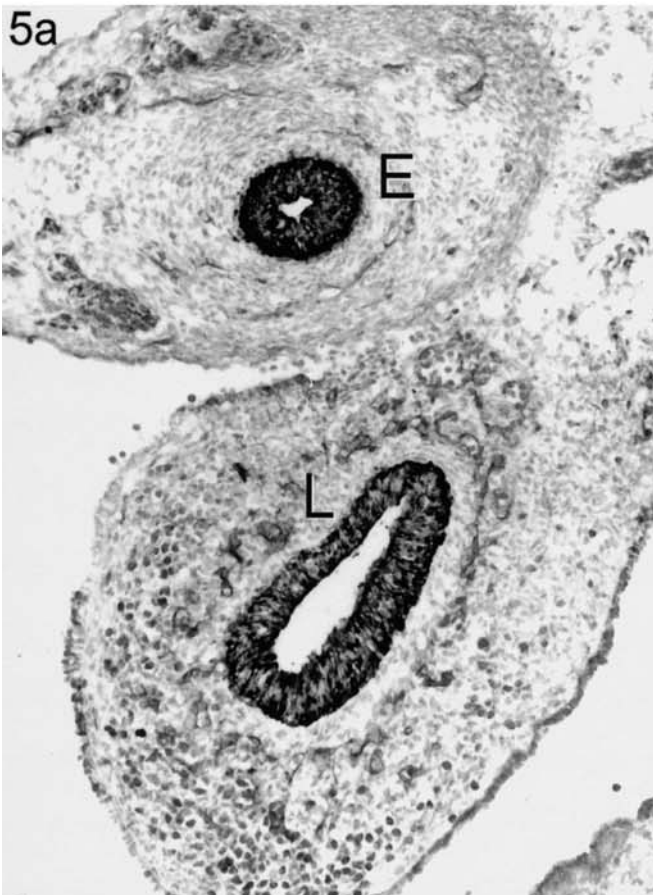
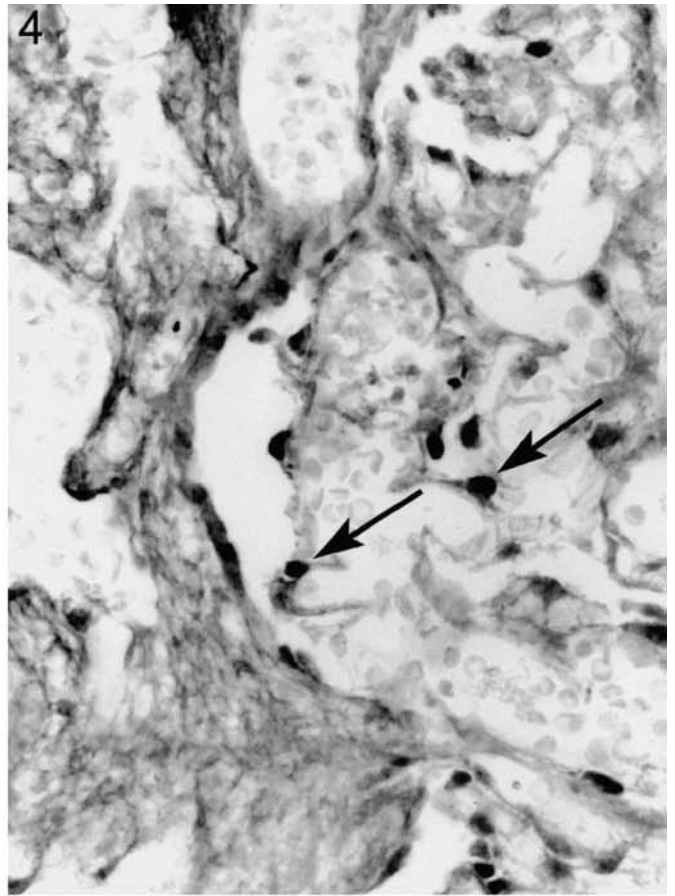
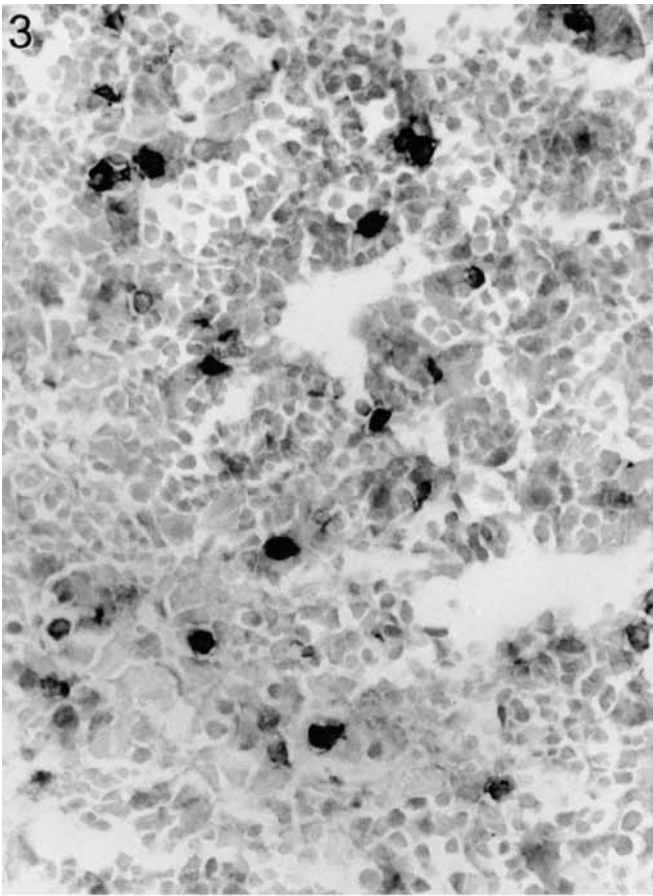


Fig. 2a, b Embryo 34 dpi, c-kit. An aortic cell cluster (*arrow*) comprised of presumptive haematopoietic stem cells is intensely c-kit positive. Note also the distinct labelling of neural tube, spinal ganglia and spinal nerve in **a**. *Ao* Aortic lumen, *Mg* mesonephros, *Me* mesentery. **a** ×36; **b** ×580



Putative PGC-like cells outside the gonads, especially in the coelomic bay and the paraaortic mesenchyme, were also CD 45-immunoreactive (Fig. 11a). These extragonadal CD 45-positive cells often also displayed the typical finger-like processes of PGCs. Cells of similar phenotype and position were labelled in consecutive sections with the lectin STA (Fig. 11b), corroborating their identification as putative PGCs. However, PGC-like cells represent only a subpopulation of CD 45-positive cells within the mesenchyme, mesenteria and mediastinum. Here, a population of smaller and irregularly shaped cells (for example, the CD 45-positive cells encircling the lung buds; see Fig. 8) may also comprise additional cell types including precursors of megakaryocytes, macrophages and presumptive vasculogenetic cells.

Identification of megakaryocytes and platelets

Anti-human CD 61 (clone Y2/51) is an antibody with established crossreactivity to bovine megakaryocytes in adult tissues. In our embryonic material, it also labelled very large and irregularly shaped megakaryocytes as well as platelets and platelet aggregates. Labelled megakaryocytes were most common in the trabeculae of the liver (Fig. 12a), but also frequently occurred elsewhere, mainly in an intravascular location (Fig. 12b).

Platelets and small platelet aggregates were most abundant in blood vessels, and larger platelet aggregations occurred in the coelomic cavity (not shown), possibly due to injuries during opening of the abdomen prior to fixation.

Myeloid cells/granulocytes

Human-specific antibodies to CD 11b, BM-1 and myeloperoxidase showed no crossreactivity with bovine embryonic tissue. However, BM-2, an antibody directed against human granulocytes, labelled a distinct cell type in bovine liver strands (Fig. 13a). These BM-2-positive cells showed a round to oval soma of medium size. Labelling with BM-2 usually obscured the nucleus of the cell, but sometimes a slightly paler U-shaped nucleus was identified (Fig. 13b). Two to five BM-2-positive cells were often loosely grouped together, most likely indicating areas of granulopoiesis.

Fig. 3 Embryo 45 dpi, c-kit. C-kit-labelled cells are located in the parenchymal strands of the liver and most probably represent haematopoietic stem and progenitor cells. $\times 360$

Fig. 4 Embryo 45 dpi, c-kit. The podocytes (*arrows*) in the mesonephros are intensely stained. $\times 460$

Fig. 5a, b Embryo 34 dpi, c-kit. The epithelia of the oesophagus (*E*) and of the developing lung buds (*L*) are distinctly labelled. Capillary endothelia (*arrow*) and presumptive vasculogenetic cells (*arrowhead*) in the periphery of the lung buds display a moderate c-kit immunoreactivity. **a** $\times 145$, **b** $\times 360$

B-lymphopoietic cells

Labelling with B-lymphoid-specific antibodies directed against bovine IgM and bovine WC-4 occurred in the liver of embryos older than 57 dpi. Anti-WC-4 demonstrated a few medium-sized or larger, irregularly shaped cells restricted to the liver strands, which, however, resembled more a subpopulation of megakaryocytes than B-cell progenitors (Fig. 14a). Cells labelled with anti-IgM outnumbered the WC-4-positive cells. The former were medium-sized and displayed a slightly irregular, ovoid shape (Fig. 14b).

Identification of macrophages

CD 14-positive cells

Bovine-specific CD 14 (VPM 65) antibody labelled the macrophages in control sections of adult bovine tissue (lymph nodes), but failed to stain cells in embryonic tissue.

CD 68-positive cells

Human-specific CD 68 (EBM-11) antiserum is known for its crossreactivity to adult bovine tissue. This antibody also produced a specific staining of macrophages in our embryonic specimens. CD 68-labelled cells were irregularly shaped and often displayed processes and protrusions. Such cells occurred in an intravascular or extravascular localisation throughout the mesenchyme (Fig. 15a), but were more numerous in liver strands (Fig. 15b).

Discussion

The present study reports for the first time the expression pattern of surface molecules of bovine embryonic haematopoietic cells and PGCs, thus establishing the methodological basis for further investigations of normal development and pathological conditions in these two important cell lines.

C-kit

It is well established that haematopoiesis in the yolk sac and fetal liver starts from cells that display a $c\text{-kit}^+/\text{lin}^-$ phenotype and give rise to $c\text{-kit}^-/\text{lin}^+$ mature cells (Ogawa et al. 1993). C-kit has been demonstrated in the haematopoietic tissues of the mouse (Orr-Urtreger et al. 1990; Motro et al. 1991; Ikuta and Weissman 1992; Palacios and Nishikawa 1992; Marcos et al. 1997) and human embryo (Teyssier-le Discorde et al. 1999). It has been shown that c-kit is essential for the proliferation of erythroid and myeloid progenitors in fetal mouse liver from day 12.5 of gestation on and for the generation of the

Fig. 6 Embryo 32 dpi, CD 45 immunoreactivity (IR). Intravascularly located CD 45-immunoreactive leucocytes (*arrow*) in the dorsal mesentery. The other blood cells in the vessel are negative. $\times 330$

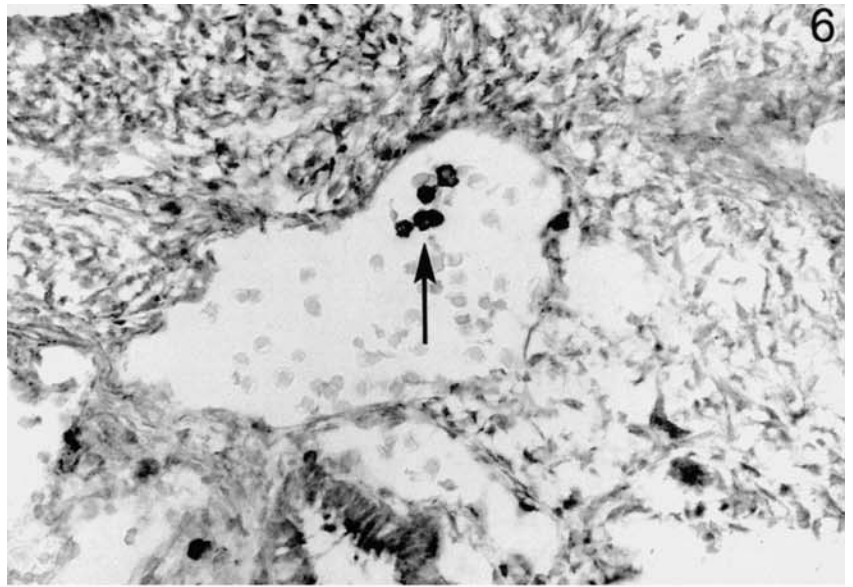


Fig. 7 Embryo 34 dpi, CD 45-IR. In the liver, numerous CD 45-labelled cells are present. These cells are located predominantly in the parenchymal strands where they display different sizes and shapes. The larger and more irregular cells (*arrows*) most probably represent cells of the megakaryocytic lineage, whereas medium-sized and more ovoid-shaped cells (*arrowheads*) may belong to the lineage of granulopoietic cells. $\times 425$

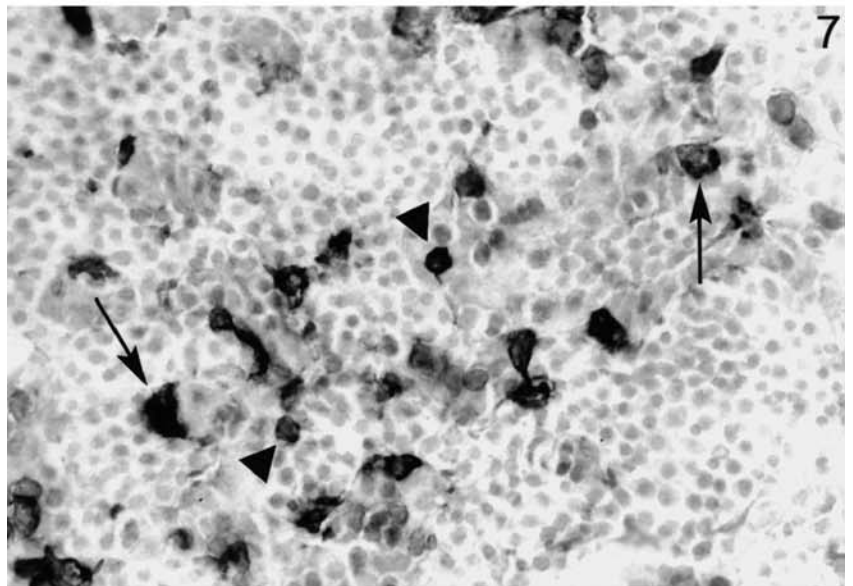
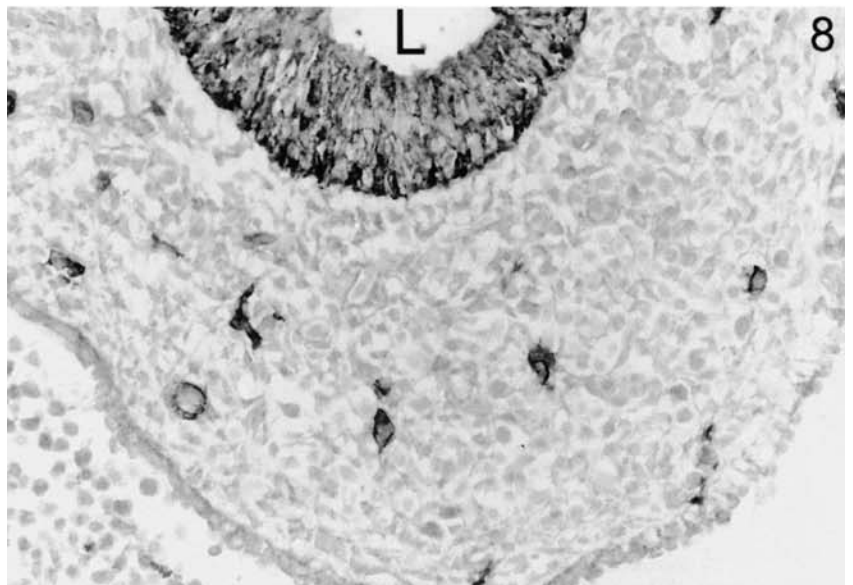


Fig. 8 Embryo 34 dpi, CD 45-IR. The epithelium of the lung buds (*L*) is distinctly CD 45 positive. Smaller CD 45-immunoreactive cells are scattered in the surrounding mesenchyme and may include vasculogenetic cells. $\times 340$



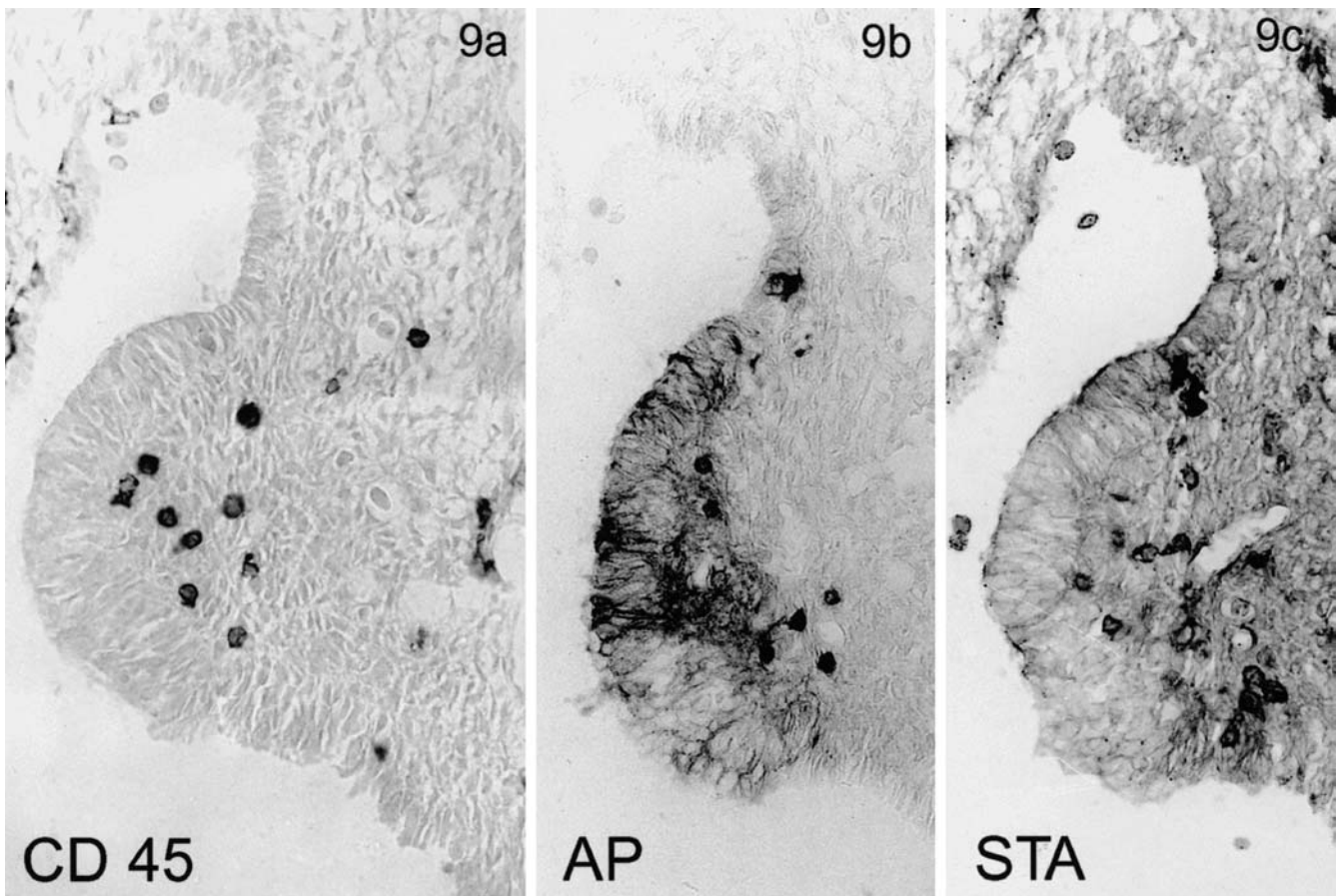


Fig. 9a–c Embryo 34 dpi. **a** CD 45-IR. **b** Alkaline phosphatase (AP). **c** STA. **a–c** Putative PGCs in the gonadal fold are positive for CD 45, AP and STA. $\times 360$

stem cell pool that initiates bone marrow haematopoiesis (Ogawa et al. 1993). Therefore, *c-kit* is a useful marker for identifying haematopoietic progenitors in early embryonic and fetal organs. The bovine liver is the main site of fetal haematopoiesis and accordingly, intensely *c-kit*-labelled cells could be demonstrated in liver parenchymal strands. In this species as well, they most likely represent haematopoietic stem cells and progenitor cells. The latter are known to display *c-kit* immunoreactivity and additionally the characteristic lineage markers (Delassus et al. 1999).

In the bovine embryo, intensely *c-kit*-immunoreactive cells also occur in large clusters attached to the wall of the dorsal aorta. *C-kit* immunoreactivity has also been reported for aortic clusters of the mouse (Yoshida et al. 1998; Yokomizo et al. 2001) and *c-kit* mRNA has been detected in CD 34⁺ aortic clusters of human embryos (Labastie et al. 1998). These intraaortic clusters of spherical cells which adhere to the aortic endothelium are characterised as embryonic haematopoietic stem cells by their expression of typical surface antigens, transcription factors and oncogenes (for review see Péault et al. 2002). Their emergence at day 28 to day 38 in humans and at day 9.5 to day 11.5 in mice coincides with the appearance of functionally defined long-term culture-

initiating cells (reviewed in Marshall and Thrasher 2001). Recently, the AGM region turned out to be a potent intraembryonic source of haematopoietic stem cells for fetal liver colonisation. However, not all the *c-kit*-positive cells in the AGM region are haematopoietic stem cells. Putative PGCs are also located in this region, especially in the gonadal folds. Cells with PGC-typical histochemical properties are observed in the periaortal mesenchyme and the coelomic bay and, to a lesser extent, in the dorsal mesentery (Wrobel and Süß 1998). While intragonadal bovine PGCs display a distinct *c-kit* immunoreactivity, the presumptive extragonadal PGCs are *c-kit* negative in this species. These findings contrast with those in the mouse, where *c-kit* mRNA has been detected in migrating PGCs of mice ranging from 10.5 to 12.5 dpi. Strong *c-kit* immunofluorescence has also been reported for migrating PGCs in the wall of the gut in 9.5 dpi mouse embryos (Bernex et al. 1996). The *c-kit*-negative putative extragonadal PGCs with unknown developmental potential in the AGM region of bovine embryos may probably never reach the gonads and undergo apoptosis due to the lack of trophic factors. *C-kit* is such a factor known to be important for survival and proliferation and perhaps also for the migration of PGCs (Pesce et al. 1993; Donovan 1994). As shown for the mouse, extragonadal

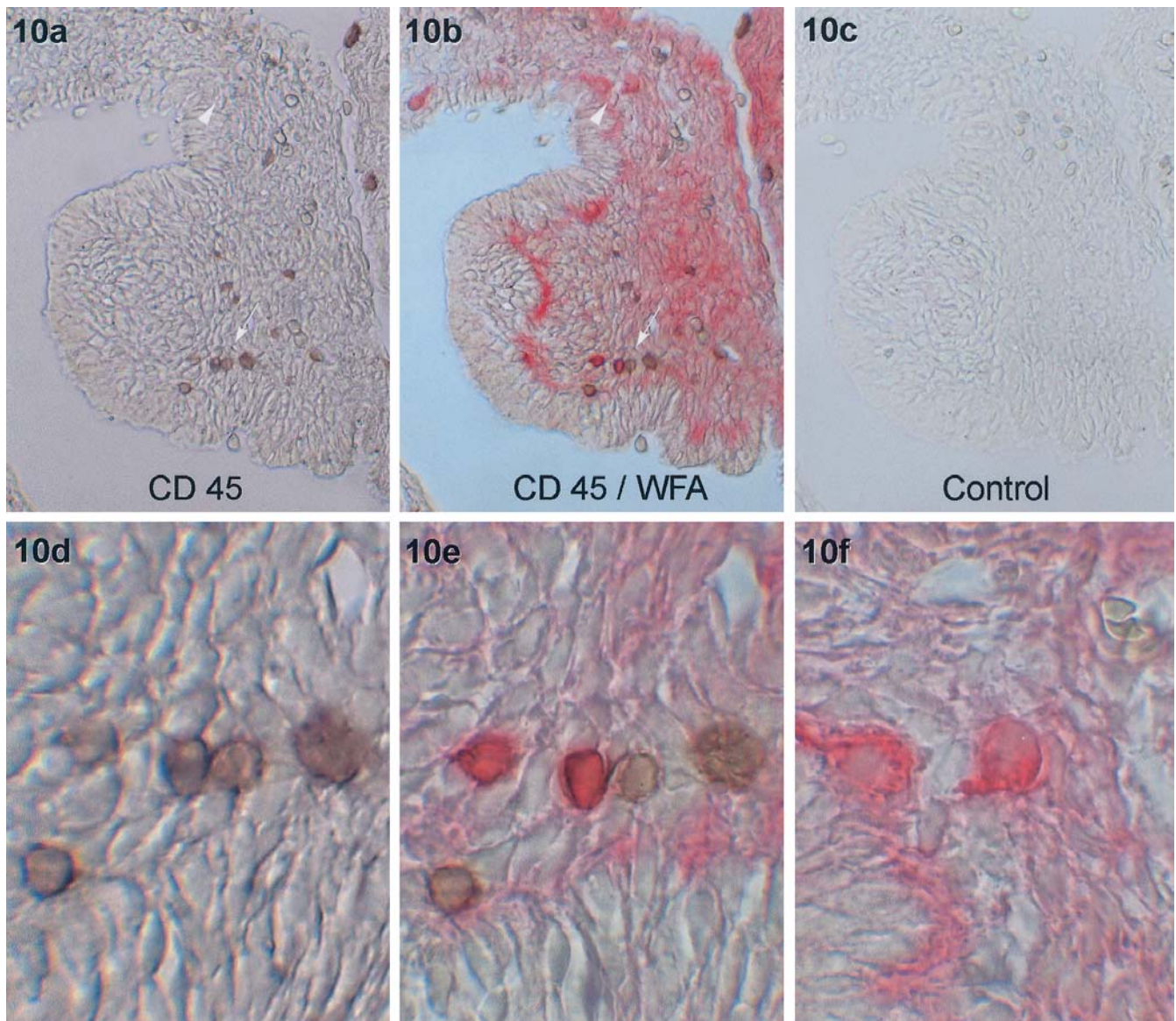


Fig. 10a–f Embryo 34 dpi. **a** CD 45-IR. **b** Double-labelling with anti-CD 45 and WFA (same section as **a**). **c** Control processed according to the double-labelling protocol with omission of primary antibody and biotinylated lectin. **d** CD 45-IR (high power micrograph of **a**). **e, f** Double-labelling with anti-CD 45 and WFA (high power micrograph of **b**). Labelling of the same section with first

anti-CD 45 (**a, d**) and then additionally with WFA (**b, e, f**) reveals three differently labelled types of putative PGCs: CD 45⁺/WFA⁻ cells, CD 45⁺/WFA⁺ cells and CD 45⁻/WFA⁺ cells. *Arrows* in **a, b** point to a group of three CD 45⁺/WFA⁻ and two CD 45⁺/WFA⁺ cells (enlarged in **d, e**). *Arrowheads* in **a, b** point to two CD 45⁺/WFA⁺ cells enlarged in **f**. **a–c** $\times 240$; **d–f** $\times 1,100$

PGCs located in the distal dorsal mesentery fail to reach the gonadal ridge (Molyneaux et al. 2001).

Paralleling findings in the mouse, c-kit immunoreactivity was additionally located in various bovine embryonic tissues, for example, the epithelia of gut, lung buds and gall bladder, and podocytes of the mesonephros, but the functional significance of c-kit in these sites has yet to be elucidated. The distinctly labelled capillary network encircling the developing lung buds is another interesting c-kit localisation because these vessels develop in mice by true vasculogenesis (Schachtner et al. 2000), i.e. via de novo differentiation of endothelial cells in the mesoderm. However, whether the c-kit-positive peribronchial capil-

laries in the bovine embryo also develop from individual angioblasts cannot be concluded from our material. This could be determined by careful examination of a close and uninterrupted series of bovine embryos at stages between 28 dpi (where no capillary network or individual angioblasts are present around the sprouting tracheal bud; unpublished observations) and 32 dpi (where the peribronchial capillary network is already established). The developing dorsal aortae in 22 dpi bovine embryos represent another site of vasculogenesis and are also c-kit positive (unpublished observation), pointing to c-kit as a general vasculogenetic marker. On the other hand, in the bovine embryo the c-kit antibody failed to label cells of

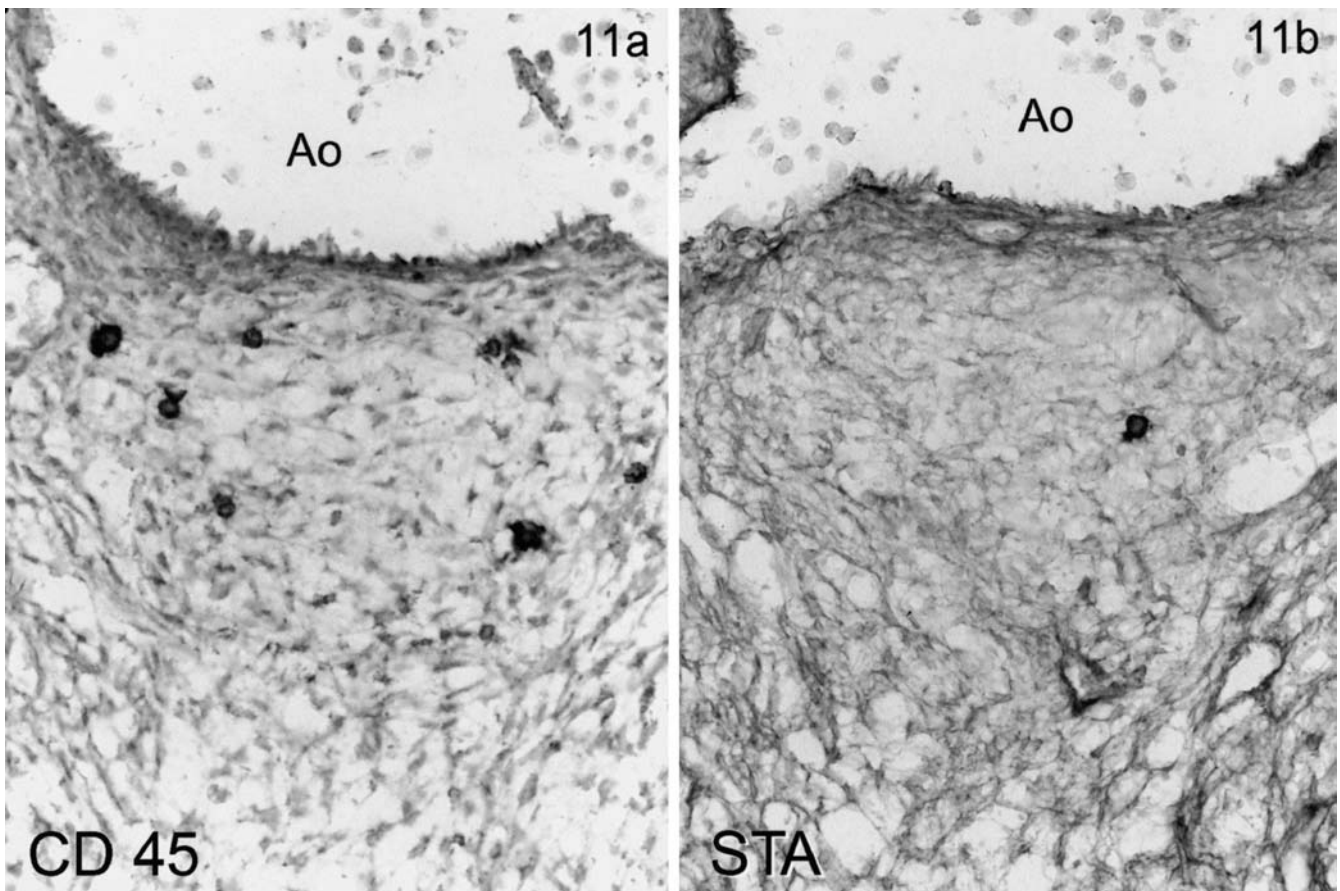


Fig. 11a, b Embryo 34 dpi. **a** CD 45-IR. **b** STA. Extragonadal putative PGCs in the periaortal mesenchyme can be recognised morphologically by their distinct finger-like processes and histo-

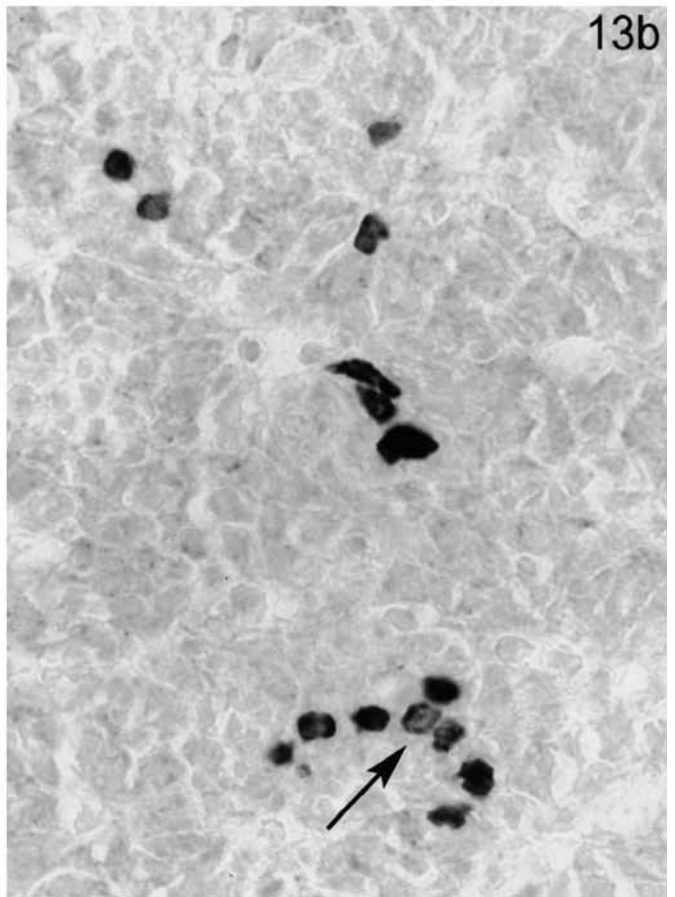
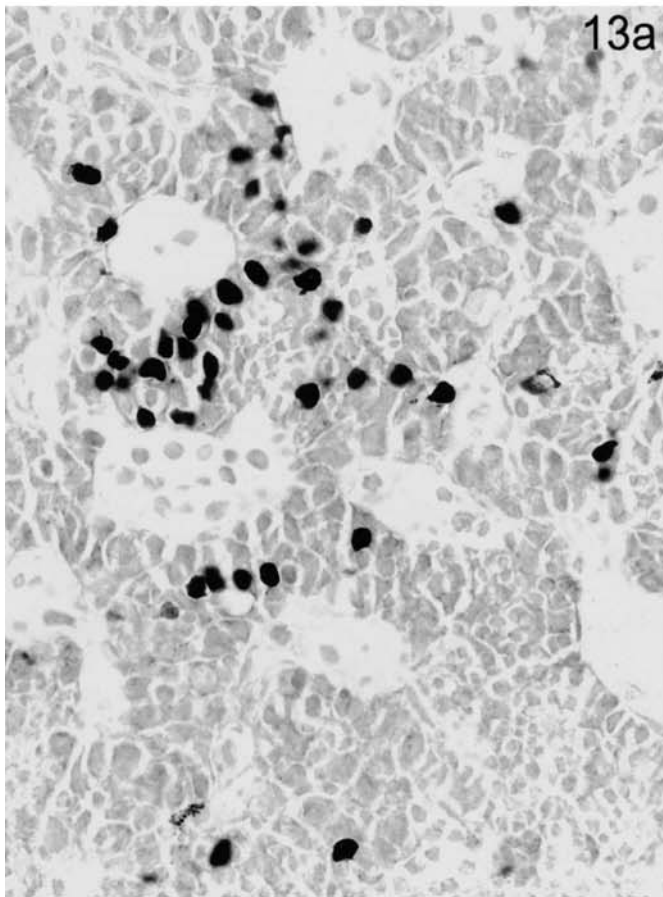
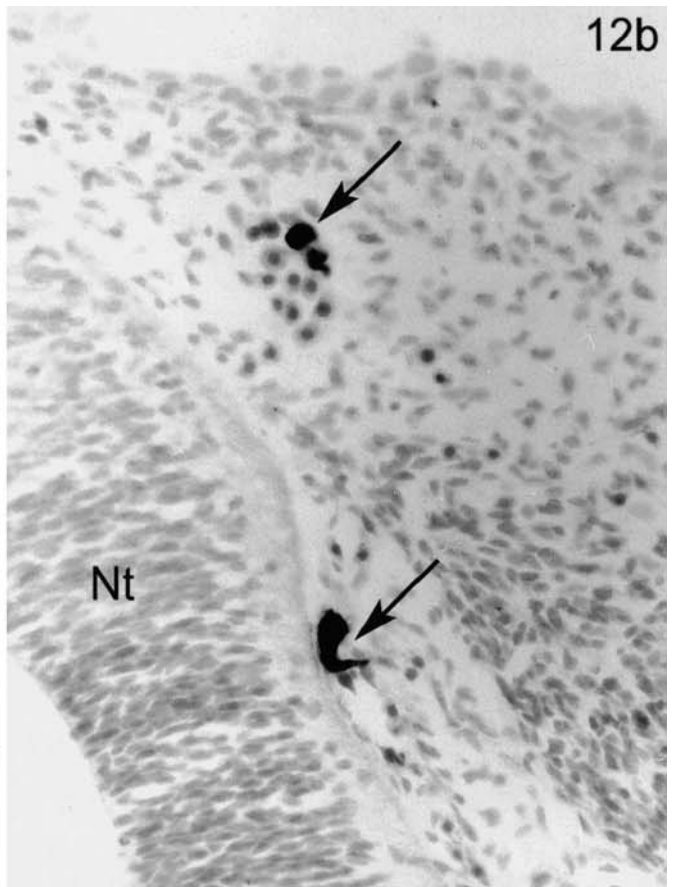
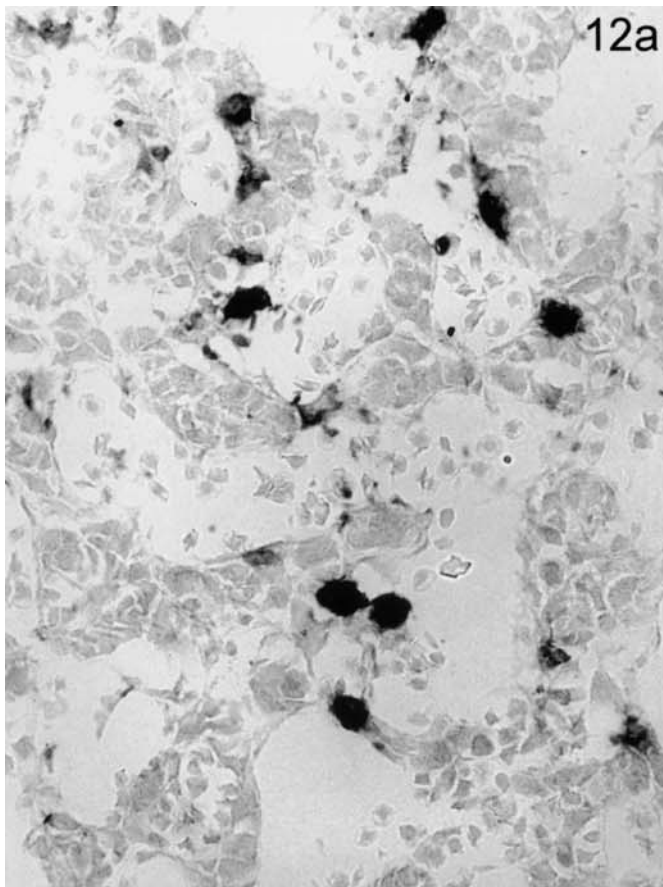
chemically by their reactions against CD 45 (**a**) and the lectin STA (**b**). *Ao* Aortic lumen. $\times 360$

the melanocytic lineage and interstitial cells of Cajal, both characterised by their distinct c-kit expression in the mouse (Manova and Bachvarova 1991; Opdecamp et al. 1997; Young 1999).

CD 45

CD 45 is commonly accepted as a pan-leucocytic marker, and antibodies directed against this surface molecule are widely used for discrimination of haematopoietic and non-haematopoietic cell lineages (see, for example, Labastie et al. 1998; Jaffredo et al. 2000). As expected, also in the bovine embryo, CD 45 labels not only leucocytes of different sizes and shapes in intravascular localisations and in the developing liver, but also megakaryocytes, haematopoietic stem and progenitor cells. Rather surprisingly, cells of the non-haematopoietic lineage were also labelled, with the putative PGCs being the most conspicuous. Recently, it has been shown by Crosby et al. (2002) that progenitor cells of the biliary epithelial cell lineage in adult mice share the same markers (CD 34, c-kit and CD 45) as haematopoietic precursors from the bone marrow and embryonic liver,

indicating a more widespread distribution and a lower tissue specificity of CD 45 than previously thought. Identification of intragonadal CD 45⁺/WFA⁻ cells as well as CD 45⁻/WFA⁺ and CD 45⁺/WFA⁺ cells may reflect a stepwise differentiation of putative PGCs. The presence of the CD 45⁺/WFA⁺ subpopulation of PGCs in the embryonic bovine gonad corresponds well with the results of studies demonstrating the transdifferentiation capacity of alkaline phosphatase/SSEA-1-positive PGCs in different murine culture systems into erythroid, myelomonocytic, multipotential and primitive haematopoietic stem cells (Rich 1995; Ohtaka et al. 1999). Additionally, PGCs and intraaortic clusters of haematopoietic stem cells can both be labelled with the lectin STA (unpublished observation). Thus far there is no direct evidence of PGCs differentiating into haematopoietic cells *in vivo*. However, indirect evidence has accumulated over several decades (for a review see Woodruff et al. 1995; Motzer et al. 1998; Saito et al. 1998). Numerous case reports dealing with the clonal analysis of extragonadal germ cell malignancies in the mediastinum or in the nervous system associated with haematological malignancies indicate that both types of malignant cell populations arose from a common precursor. Therefore, it has been concluded that



extragonadal germ cell tumours represent aberrant germ cell migration to an “inappropriate” environment, which might result in a malignant transformation (see, for example, Chaganti et al. 1989; Heimdal et al. 1991; Scaravaglio et al. 1996). Additionally, under certain culture conditions, PGCs give rise to a population of cells which resemble pluripotent embryonic stem cells and are able to produce a wide variety of mature differentiated cell types including haematopoietic cells when injected into nude mice (Matsui et al. 1992). The apparent similarities between PGCs and haematopoietic stem cells have also led to the question of whether a common precursor may exist for these two cell types in normal development (De Felici and Pesce 1994). Arguments for such a common origin are derived from a clonal analysis of the extraembryonic mesoderm in the mouse embryo. While that part of the presumptive extraembryonic mesoderm which gives rise to blood islands is generally located more distally than the PGC precursors, there exists a small region of overlap in the extreme proximal part of the epiblast which gives rise to clones containing both types of cells (Lawson and Hage 1994). Nevertheless, direct evidence for a well-defined common haematopoietic stem cell/PGC precursor is still lacking. The markers shared by PGCs and putative haematopoietic stem cells could simply reflect the late final determination of the germ cell line in mammalian embryos, the so-called PGCs being a rather undifferentiated cell population with the capacity to still develop into tissues of all three germ layers (Durcova-Hills et al. 2001). Another possibility is that structural and functional similarities between the haematopoietic and PGC systems could have evolved in parallel because both cell types are subjected to similar problems, for example, homing, apoptosis, cluster formation during proliferation, etc.

Lineage markers

In this study, embryonic megakaryocytes and platelets have been successfully demonstrated with anti-CD 61 (clone Y2/51) which is an antibody with known species crossreactivity to adult bovine tissue (Brodersen et al. 1998). In the bovine embryo, megakaryocytes are most common in parenchymal liver strands, the main site of haematopoiesis during this gestational period. Surprisingly, developing megakaryocytes are also found in tissues

which are generally not considered to be haematopoietic sites, for example, in blood vessels of the body wall and in the mesonephros. The CD 61 antibody used in this study can be recommended as a valuable and reliable tool for the *in vivo* study of embryonic, fetal and adult megakaryocytopoiesis.

The only antibody for myelopoietic/granulopoietic cells which yielded convincing results in this study was BM-2, a monoclonal antibody primarily developed against human granulocytes. BM-2 also reacts with early precursor forms of human myeloid cells and is recommended for the detection of myeloid leukaemias and granulocytic sarcomas (manufacturer's information). In the bovine embryo, staining with BM-2 occurred solely in parenchymal liver strands and seemed to be restricted to a single population of medium-sized, round to oval cells. Due to the U-shaped nucleus of this cell type and the arrangement of the cells in small groups, the cells characterised by BM-2 may constitute granulopoietic nests. Occurrence of BM-2-positive cells in the liver coincides with the first appearance of myelocytes in bovine peripheral blood and liver (Winquist 1954). Since we were not able to detect BM-2-labelled myelocytes in peripheral blood and because of the uniform shape and size of BM-2-labelled cells in the liver, it can be concluded that BM-2 is expressed only in certain developmental stages of granulocytic precursors in the bovine embryo and not in mature granulocytes. Therefore, we consider BM-2 to be a useful marker for the detection and quantification of granulopoietic development.

B-lymphopoiesis in the bovine liver was detected with antibodies directed against WC-4 and IgM, but not until 58 dpi. Accordingly, Winquist (1954) described the first appearance of lymphocytes in peripheral blood in bovine embryos of 70 mm, corresponding to an age of approximately 60 dpi. WC-4 and IgM-labelled cells were scarce and displayed a large and irregularly shaped cell body. The same morphology has also been described in early mouse lymphopoiesis (Timens and Kamps 1997). Therefore, we consider WC-1 and especially IgM immunoreactivity as specific B-cell markers suited for the investigation of fetal B-lymphopoiesis.

The correct identification of different types of macrophages is of great importance in both veterinary diagnostics and experimental pathology. Therefore, markers specific for the monocytic lineage have been developed and well characterised for the bovine. Antibodies exist which direct against bovine CD 14 (Berthon and Hopkins 1996; Gupta et al. 1996; Sopp et al. 1996) and CD 68 (Ackermann et al. 1994; Zeng et al. 1996; Gutierrez et al. 1999). Using the antibody directed against CD 14, we were unable to detect macrophages in embryonic tissues, although these cells were easily recognised with anti-CD 14 in lymph nodes of adult bovines. Using anti-CD 68, numerous macrophages could be detected in the embryonic liver. The liver macrophages were identified as two populations, one being located in liver parenchymal strands where they may act as nursing cells in haematopoietic foci, and the other in the sinusoids, here

Fig. 12a, b Embryo 32 dpi, CD 61-IR. **a** Large and irregularly shaped megakaryopoietic cells are observed in liver strands or close to the margins of liver sinusoids. **b** Intravasal megakaryocytes (*arrows*) are located within the perineural mesenchyme. *Nt* Neural tube. $\times 360$

Fig. 13a, b Embryo 34 dpi, BM-2-IR. BM-2-labelled granulopoietic progenitor cells are present in the parenchymal strands of the liver and arranged in small groups. Most BM-2-positive cells are of ovoid shape and medium size. Sometimes, a paler U-shaped nucleus (*arrow*), similar to that of myeloid cells, can be recognised at higher magnification (**b**). $\times 360$; **b** $\times 580$

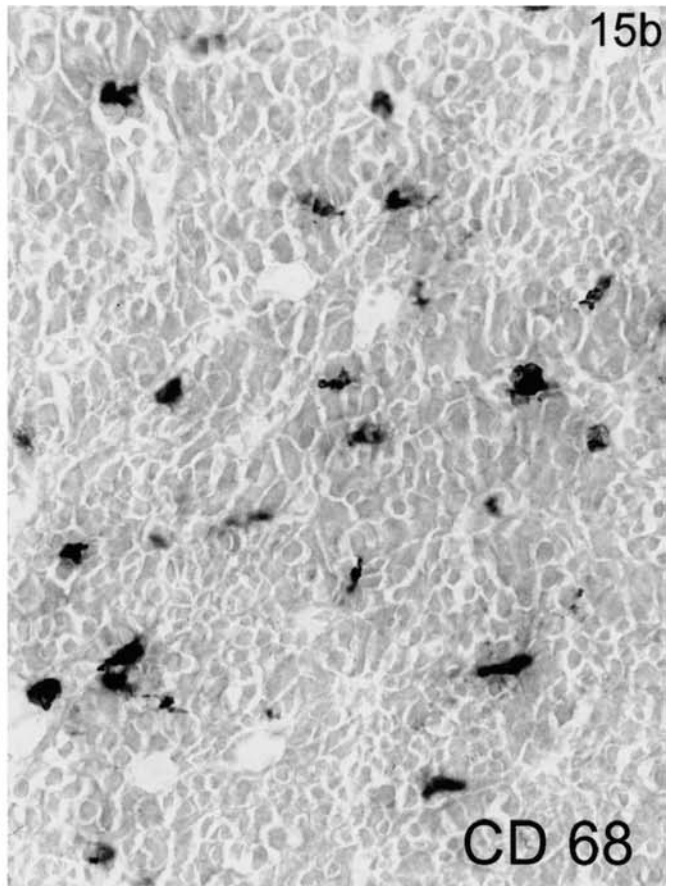
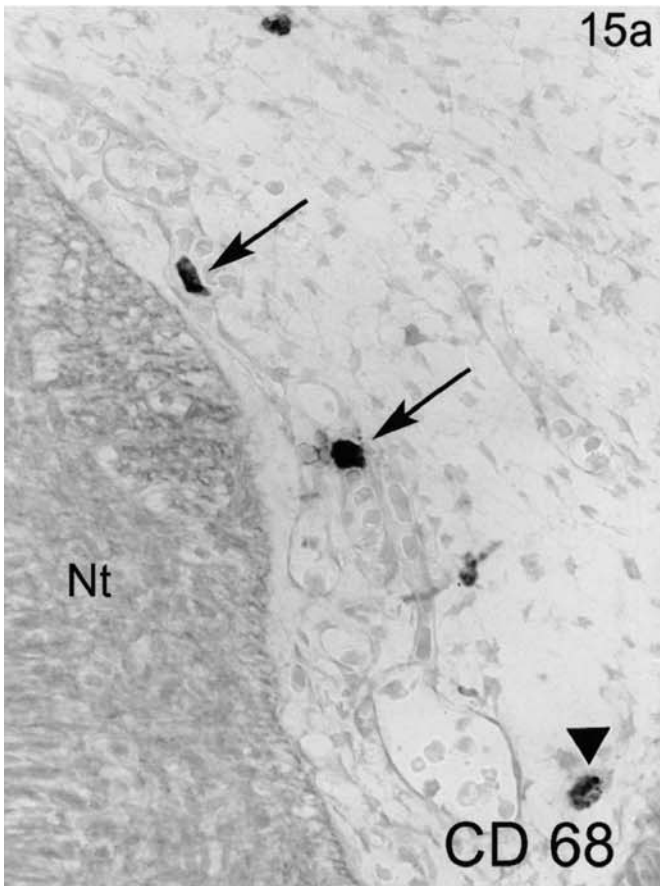
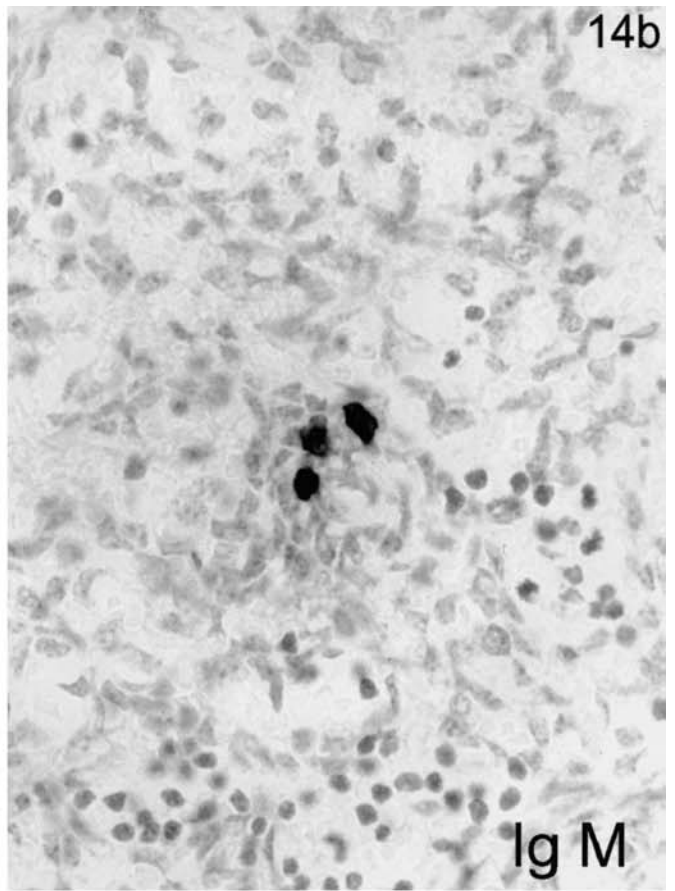
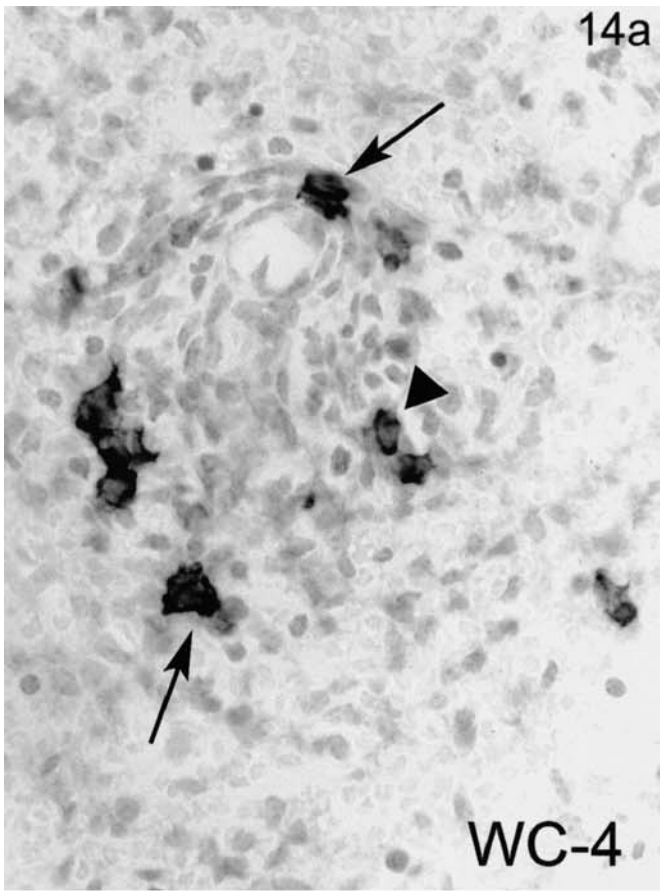


Fig. 14a, b Embryo 60 dpi. **a** WC-4-IR. **b** IgM-IR. **a** Two populations of WC-4-IR cells can be identified in the embryonic liver: one fraction is large with irregular outer contours (*arrows*), the other smaller and of ovoid shape (*arrowhead*). **b** Cells of the smaller, ovoid fraction are also positive for IgM and most likely represent pre-B cells. $\times 580$

Fig. 15a, b Embryo 34 dpi, CD 68-IR. **a** Primitive macrophages are found in intravascular (*arrows*) and extravascular (*arrowhead*) localisation in the perineural mesenchyme. **b** In the liver, macrophages are numerous in the parenchymal strands, but are also located within the sinusoids. $\times 360$

most probably representing primitive Kupffer cells. CD 68 is also suited for the identification of an intravascular macrophage population and extravascular tissue macrophages in the bovine embryo, corroborating findings in adult cattle (Bertram 1986; Ackermann et al. 1994). Thus, CD 68 constitutes a reliable marker for bovine macrophages from the embryonic period through to the adult state. It may be used in the study of embryonic/fetal haematopoiesis for the detection of haematopoietic foci, and in developmental studies of different tissue macrophage populations, for example, Kupffer cells in the liver, or, at later developmental stages, alveolar macrophages in the lung.

Acknowledgements The authors greatly appreciate the excellent technical assistance of Mrs. I. Hees, Mrs. M. Schimmel, Mrs. U. Schmitt and Mrs. E. Stauber, and the skilful photographic work of Mrs. A. Zenker and Mr. T. Maurer. We thank Mrs. C. Ross-Cavanna for linguistic help. We are especially grateful to H. Hikono, National Institute of Animal Health, 3-1-1 Kannondai, Tsukuba, Ibaraki, 305, Japan, for his kind donation of anti-c-kit (bk1-6) antibodies. The monoclonal antibody M1/70.15.11.5.2 developed by T.A. Springer was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242, USA. This research was financially supported by DFG grant Wr 7/10-1.

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