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## Haematopoiesis and a new mechanism for the release of mature blood cells from the bone marrow into the circulation in snakes (Ophidia)

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**Abstract** This is the first description of haematopoiesis in snakes. Studies were carried out on the following species belonging to Ophidia: *Bothrops jararaca*, *Bothrops jararacus*, *Waglerophis merremii*, *Elaphe taeniura taeniura*, *Boa constrictor*, and *Python reticulatus*. Smears of the peripheral blood and histological preparations from the vertebrae, ribs, liver, and spleen were studied under a light and electron microscope. Myeloid cells were present in the following locations in the vertebrae: the neural spine, zygoapophysial processes, floor of the neural canal, lacunae in the bodies of vertebrae and also inside the ribs. Although the vascular system was well developed, especially around the ribs, vessels inside the marrow cavities were scarce, both in the ribs and elsewhere where haematopoiesis was found. Venous sinuses were well developed in the vertebrae and in the rib regions from their costal head towards the middle area. They consisted of one layer of fine endothelial cells. Mature cells in the process of migration into the general circulation were only sporadically encountered when venous sinuses were studied on perfusion-fixed specimens. In contrast, almost every sinus venosus contained protrusions directed towards the lumen, filled mostly with mature and immature blood cells. Various stages of their formation were seen in the cross sections of venous sinuses ranging from small, newly formed to large, elongated ones, filled with many fully developed and some maturing blood

cells. In many cases the apices of the protrusions were ruptured, and mature blood cells, as well as a few immature ones, were seen in their vicinity. This observation led us to a new hypothesis that blood cells are released from the extravascular space into the lumen of venous sinuses. In snakes, these cells are released into the systemic circulation mainly via the rupture of protrusions filled with mature blood cells and, to a lesser degree, by transcytosis as known in mammals. In the spleens from young specimens, 1–2 foci of haematopoiesis were encountered where lymphopoiesis predominated. Haematopoiesis was not detected in the liver.

**Keywords** Haematopoiesis · Lymphopoiesis · Blood cells · Blood-cell release · Ophidia

### Introduction

Haematopoiesis is a highly complicated and variable process. Also the processes that regulate blood cell egress into the systemic circulation are complex and not fully understood. In order to understand this process, further studies in the animal orders lower than mammals, such as reptiles, could yield simpler and easier data regarding these processes. Moreover, it is evident that in this respect, among Reptilia, snakes are the least-studied group. The process of haematopoiesis among the reptilian class has been studied in certain lizards – *Lacerta hispanica* (Zapata et al. 1981), *Cordylus vittifer* (Pienaar 1962), *Lacerta muralis* (Taïb-Cazal 1973), *Agama stellio* (Efrati et al. 1970) – and in a turtle *Gopherus agessizii* (Garner et al. 1996). Lymphopoiesis has been studied in snakes (Chapman and Conklin 1935; Kotani 1959). However, there are no studies to our knowledge on haematopoiesis in snakes. Frye (1978) gave a description of cells obtained from the vertebral column and also in his *Atlas of Reptilian Care* (intended for veterinarians) (Frye and Frye 1991) described a method of obtaining marrow tissue from large and smaller snakes, but without a detailed description of that tissue. Until now the majority

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of researchers, starting with Mandl (1839) and Gulliver (1840, 1875), have only occasionally been interested in the quantitative studies on the peripheral blood cells of reptilian species, including snakes (Bergman 1957; Hutton 1958; Saint-Giron 1961; Binyon and Twigg 1965; Saint-Giron and Saint-Giron 1969; Wirth 1972; Otis 1973; McMahan and Hamer 1975; Board et al. 1977; Al-Badry and Nuzhy 1983; Pati and Thapliyal 1984; Troiano et al. 1997, 1999), or in describing the morphology of various blood cells at both the light and electron microscopy level (Kélenyi and Németh 1969; Montali 1988; Sano-Martins et al. 1994; Spadacci-Morena et al. 1998; Alleman et al. 1999). Excellent work on that subject has been done by Pienaar (1962), Sypek and Borysenko (1988) and Frye and Frye (1991). The issue of haematopoiesis in snakes will require many more studies directed towards ontogenetic development and the impact of changing seasons of the year. Saint Girons (1970) was only partially right when she stated that there is no blood-marrow barrier in snakes, as inferred from the presence of immature cells in the peripheral circulation.

Since the earliest morphological and quantitative descriptions of blood cells (Gulliver 1840, 1842) there has been little information regarding the location and structure of haematopoietic tissues in snakes. Interesting papers do exist, describing in particular the problem of hemosomes (Spadacci-Morena et al. 1989); however, these papers do not refer to where haematopoiesis occurs. A brief report concerning this issue can be found only in a treatise by Frye (1978). Also, Frye and Frye (1991) in the first volume of an atlas described how bone-marrow samples could be obtained, but did not discuss marrow structure in detail with regard to peripheral blood cells. Moreover, there was little mention of the various sites where haematopoiesis could occur. Therefore, the aim of the present study was to determine the possible anatomical locations of haematopoiesis as a model for future studies regarding the problem of blood-cell egress from marrow into the general circulation.

## Materials and methods

Studies were carried out on the following species: *Bothrops jararaca* (Wied),  $n=4$ ; *Bothrops jararacusu* (Lacerda),  $n=4$ ; *Waglerophis merremii* (Romano et Hoge),  $n=2$ , all living in the wild, caught in the São Paulo area; *Elaphe teaniura taeniura* (Cope),  $n=12$ ; *Boa constrictor* L.,  $n=5$ ; and *Python reticulatus* (Schneider),  $n=4$  from Cracow Zoo. Animals were anaesthetised with an s.c. injection of thiopental, and 20 U of heparin was injected into the exposed heart. They were then perfused with a fixative containing 1.5% glutaraldehyde, 1% paraformaldehyde, in 0.75% NaCl. Samples of liver, spleen and kidney were removed from each specimen and immersed in fresh Schaffer's fixative (1 part of 35% formol and 2 parts of 80% ethyl alcohol) for 24 h. Marrow plugs were removed from the marrow cavity located in the ribs and fixed separately. Whole dissected bones (ribs and vertebrae) were decalcified in 10% buffered EDTA. All tissue samples were routinely processed in paraffin or methylmethacrylate. Tissue embedded in paraffin was cut into 5- to 7- $\mu$ m-thick sections and stained with the following methods: Goldner, Movat, H&E, and Giemsa.

Smears of the peripheral blood and marrow were fixed in methyl alcohol or formaldehyde vapour and stained according to Wright, Giemsa, or Leischman using phosphate buffers, pH 6.4–7.5. The percentage of immature cells present in smears was calculated. For electron microscopy, tissues were fixed in Karnovsky's fixative and routinely processed in Epon. Ultrathin sections were double stained with lead citrate and uranyl acetate. Semi-thin sections approximately 1–2  $\mu$ m thick were cut and stained with an alkaline solution of azur and methylene blue according to Richardson.

## Results

### Vertebral column

Haematopoietic marrow was found in cross sections of vertebrae from the following locations:

1. The dorsal part of vertebrae (depending on the animal's age) in the neural spine (Fig. 1, ns) (haemal spine after Chapman and Concklin 1935). In young specimens there were 2–3 lacunae, while in older ones up to 5 were observed.
2. At both ends of the neural arch (Fig. 1, na) in well-developed paired transverse processes (Fig. 1, na, za) where large marrow lacunae could be easily seen.
3. The ridge of the anterior wall of the vertebral canal – comprising two layers, namely a bony and cartilaginous one (endoosteum, pia, and dura mater not taken into account).

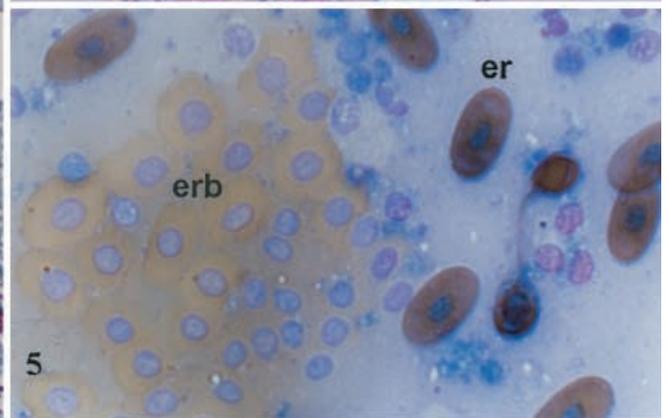
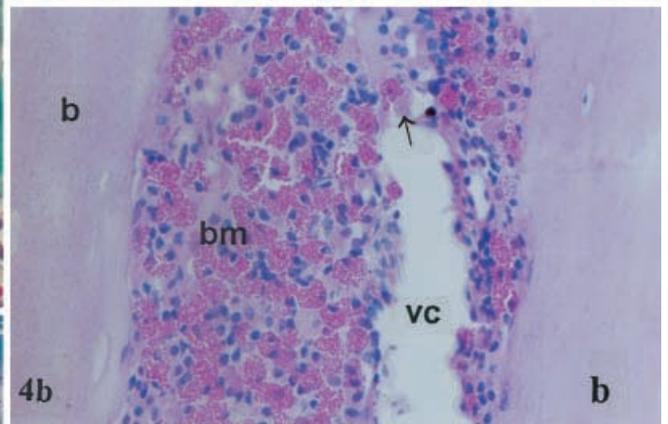
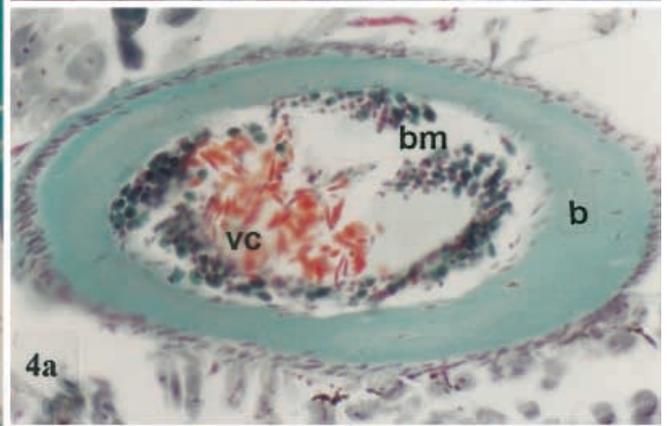
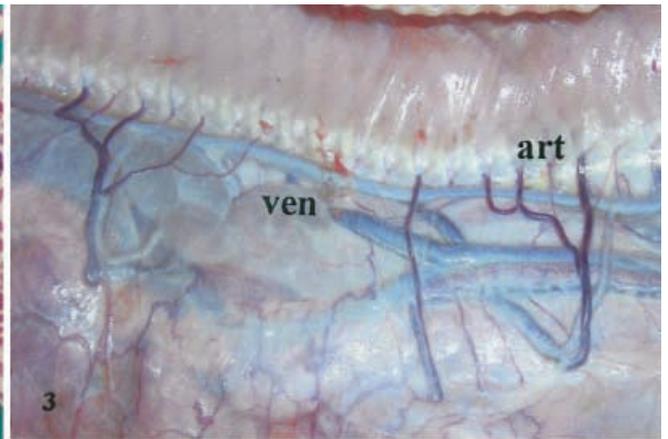
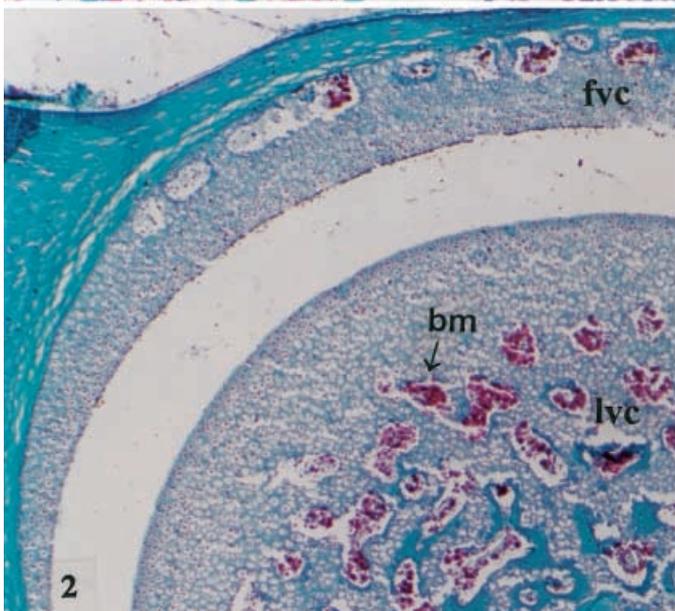
**Fig. 1** Cross section through the vertebrae from the trunk part of the spine. In the neural spine (ns) there are two lacunae in which marrow is located (just barely seen here). On the left side of a neural arch (na), the postzygapophysial process (za) is filled with bone marrow. In the ventrally oriented hypapophysis (h) bone marrow is present in the thoracic vertebrae (arrow). In the vertebral centre (lvc) numerous lacunae are filled with bone marrow (bm). Stained according to the Goldner method,  $\times 50$

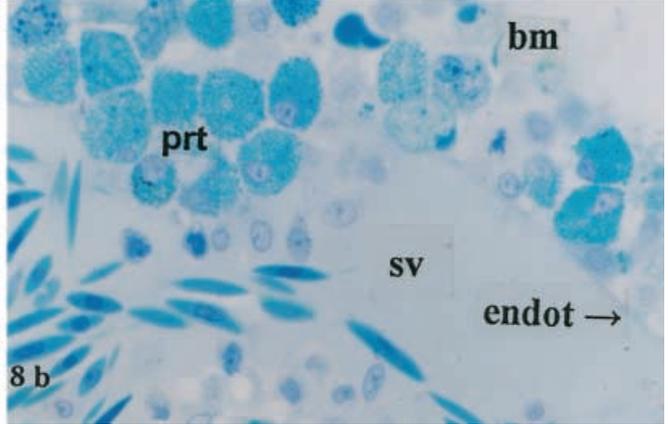
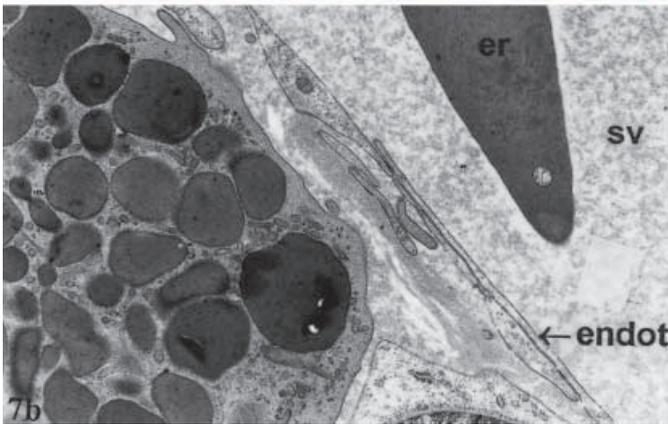
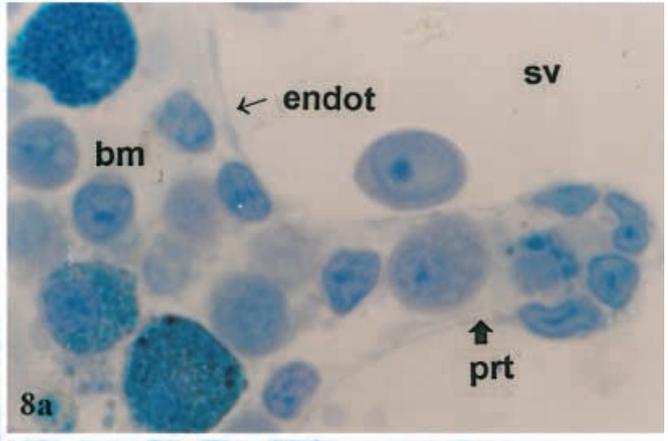
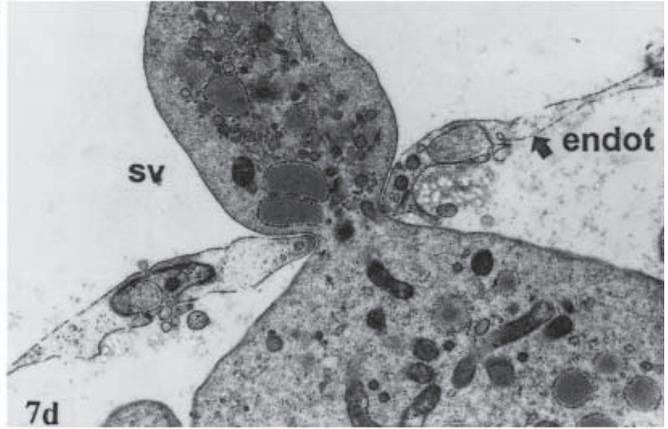
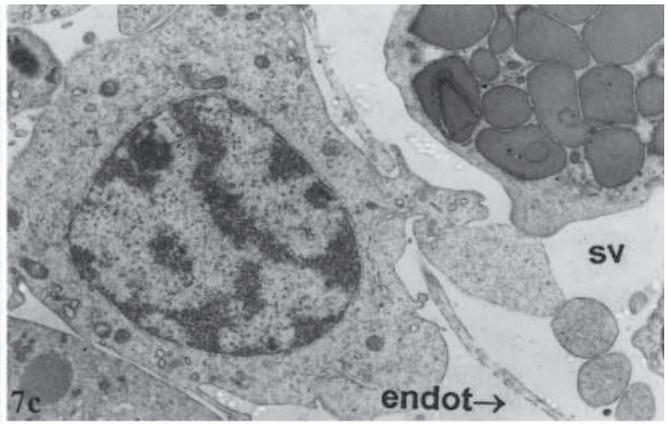
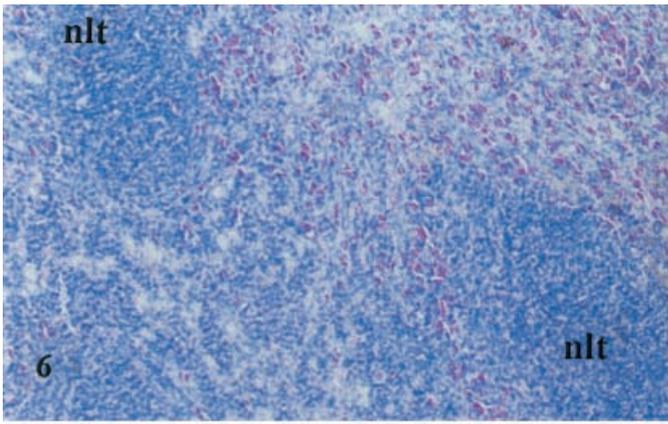
**Fig. 2** In the lower part the cross section through the vertebral centre has numerous lacunae (lvc) filled with bone marrow (bm). Lacunae occur in the cartilage part and central bony part of that vertebral centre. Above the vertebral centre the arch-shaped "floor" of the neural canal is formed by two layers, the lower cartilaginous one and the upper bony one. In the lower part the lacunae are filled with myeloid tissue (fvc). Goldner staining method,  $\times 100$

**Fig. 3** The arrangement of arteries (art) and veins (ven) originating from the aorta and posterior vena cava supplying the ribs and vertebrae

**Fig. 4 a** Cross section through a rib below the costal head showing dark-coloured haematopoietic tissue (bm). In the centre the vena centralis (vc) is filled with erythrocytes. Goldner staining,  $\times 250$ . **b** Longitudinal section through a rib, also below the costal head. The bone marrow is dominated by both immature and mature heterophils. On the upper part of the vena centralis (vc) the sinus venosus (sv) can be seen (arrow). H&E,  $\times 250$

**Fig. 5** Replica from the spleen of a young specimen of *Bothrops jararaca* [erythroblastic islet (erb) and erythrocytes (er)]. Giemsa staining, benzidine reaction for the presence of haemoglobin; immersion magnification,  $\times 1000$





4. In the distal part of the cartilaginous layer; there were numerous lacunae filled with haematopoietic tissue (Fig. 2, fvc).
5. Bodies of vertebral centrum, especially in young specimens, where lacunae were detected both in the cartilage and in the central bony part with myeloid tissue (Fig. 2, lvc).
6. A medullar cavity was also found in the ventrally oriented hypapophysis, present in the thoracic vertebrae (Fig. 1h). In mature specimens of *Python* and *Boa* lacunae in the bodies of vertebrae were restricted to two narrow lateral strands of fat tissue. In these species haematopoiesis was restricted to the ribs. In the case of *Bothrops*, *Wagleropsis*, and *Elaphe*, mixed haematopoietic tissue was present in all haematopoietic lacunae, with either erythropoietic tissue or granulocyto-thrombocytic tissue being predominant in some places.

### Ribs

The skeletal muscles surrounding the ribs are extensively vascularised (Fig. 3). In all the ribs studied from the representative samples of *Ophidia* the marrow cavity was filled with the haematopoietic tissue (Fig. 4, bm). This tissue is supplied with blood by an artery (arteria nutritiva) that bifurcates into an arteriole (one or a few, depending on the age and size of the specimen) and extends to the distal regions of the ribs. The central artery and vein (Fig. 4a, vc) run longitudinally through the centre or near the wall of the rib. It is extremely rare to find venous sinuses in the distal part of a rib, especially in young specimens. The proximal part of the rib has one large sinus venosus or a few smaller ones and sparse arterioles where granulopoiesis, via heterophils, was believed to occur (Fig. 4b, bm).

### Spleen and liver

Lymphopoiesis occurred in the spleen of mature specimens of *Boa* and *Python* (Fig. 6) but there were no signs of haemopoiesis, myelopoiesis, or thrombocytopoiesis. In *Bothrops jararaca*, and *B. jararacusu* small, sparse, and inconspicuous erythropoietic foci were seen (Fig. 5, erb). These were not mentioned in either of the published monographs on the lymphatic system in snakes (Chapman and Conklin 1935; Kotani 1959). The presence of erythropoietic foci most probably depends on several factors such as time of year, age, and physiological status (Tischendorf 1985). In addition, the spleen is dispensable for haematopoiesis in snakes and in mature specimens there was no haematopoiesis in the liver.

Mechanisms underlying the release of blood cells from the bone marrow into the systemic circulation

### The vertebral column

Walls of marrow sinuses are formed by a very fine structure composed of a single layer of endothelial cells (Fig. 7a, endot). In EM pictures they imbricate each other (Fig. 7b), but no basal lamina was found. In the marrow fixed using both methods, i.e. perfusion fixation and immersion fixation, cells in the process of transit through the wall were rarely encountered (Fig. 7c, d). In observations carried out under light and electron microscopes, protrusions of various size were seen in the lumen of marrow sinuses. In small protrusions undifferentiated cells dominated, while in larger ones (Fig. 8a) mostly mature cells accumulated in their apices (Fig. 8b). However, ruptured apices of protrusions were observed in perfusion fixed tissues, and above them, in the sinus lumen, a population of both mature and immature blood cells was seen (Fig. 9a, b, prt o).

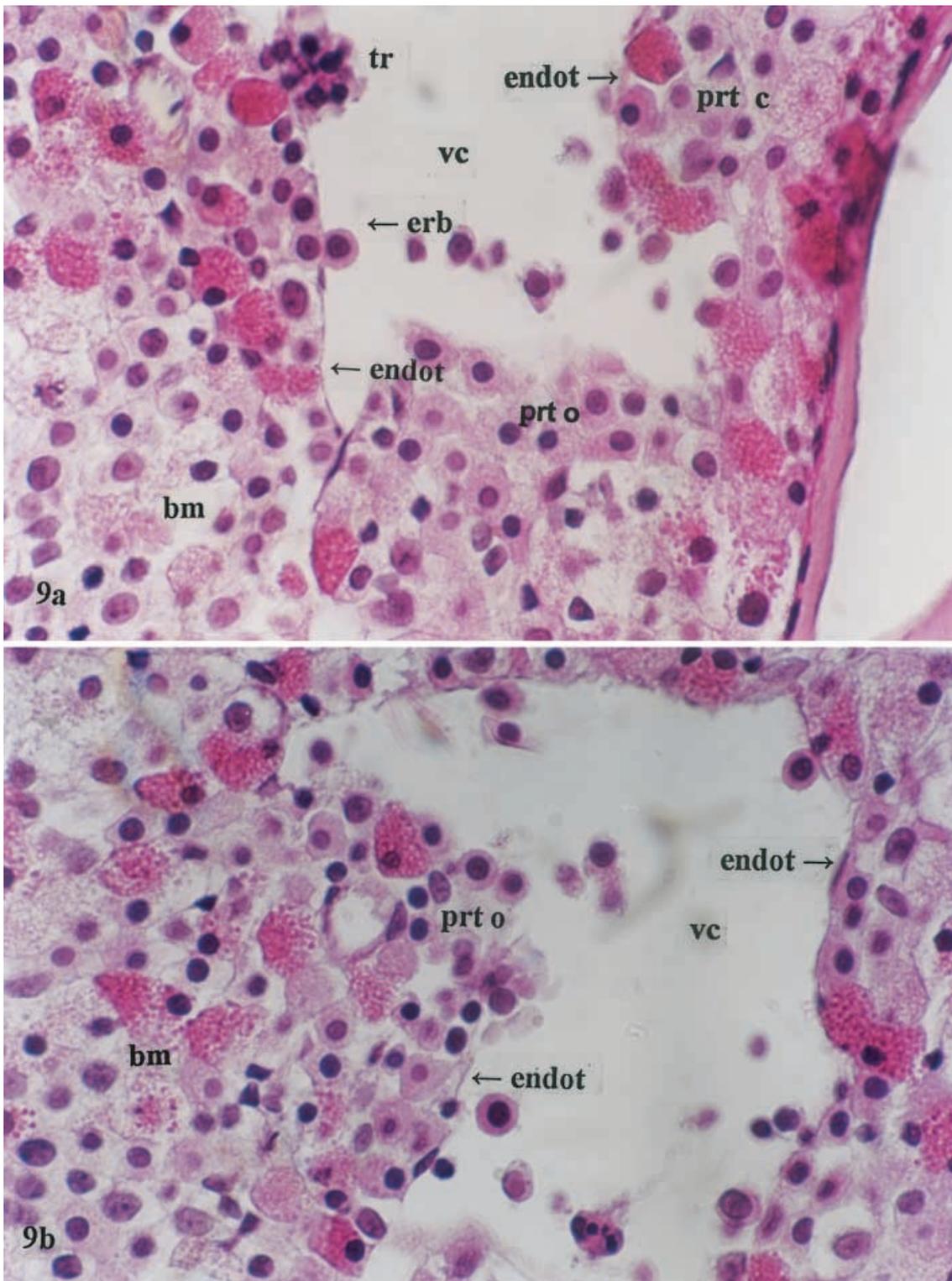
### Ribs

In all species studied, each rib, in its paravertebral part had well-developed venous sinuses with visible protrusions. Vascularisation of the marrow cavity was poorly developed between the middle of a rib arch and the distal region. In the cross sections of *Boa* and *Python* ribs arteria nutritiva and a few small arteries and venules with a very narrow lumen were observed, whereas in the remaining species studied, especially the young animals, there was usually only one small arteriole and 2–3 venules. The extravascular space was tightly filled with the myelopoietic tissue.

◀ **Fig. 6** The structure of the *Python* spleen. Dark-coloured nodular lymphoid tissue (*nld*) can be seen. Giemsa, ×200

**Fig. 7 a** Endothelial layer (*endot*) demarcating the sinus venosus (*sv*). On the extravascular side (*bm*) are cells belonging to the erythroid cell line adhering to the wall of sinus venosus. Semithin section, stained with azur/methylene blue. Immersion magnification. **b** Endothelial cells (*endot*) (lining cells) imbricating each other. Electron microscopy, ×14,000. **c** Young myeloid cell with a filopodium-like process penetrating through an endothelial cell (*endot*) into the sinus lumen (*sv*). Electron microscopy, ×11,200. **d** Young granulocytic leucocyte with filopodium-like process into the lumen of sinus venosus (*sv*). Endothelial layer (*endot*). Electron microscopy, ×14,000

**Fig. 8 a.** Protrusion (*prt*) directed towards the lumen of a sinus venosus (*sv*), at an early stage. Semithin section not yet filled with blood cells. Azur/methylene-blue staining. Immersion magnification. **b** Protrusion (*prt*) directed towards the lumen of a sinus venosus (*sv*) – a well-developed stage, filled with blood cells in various stages of development. Semithin section. Azur/methylene blue staining, ×400



**Fig. 9** **a** Large protrusions: closed (*prt c*) and opened (*prt o*) to the lumen of marrow sinus venosus. In its apical part there are erythroblasts that are being released into the lumen of the central sinus (*vc*). On the left side of the sinus are erythroblasts (*erb*) passing through the endothelium (*endot*) in a "traditional" way. A group of thrombocytes (*tr*) is also visible. Bone marrow (*bm*). Marrow isolated from a rib of *Bothrops jararaca*. Perfusion-fixed specimen. H&E staining. Immersion magnification. **b** On the left side of the central sinus venosus (*vc*) is an opened protrusion (*prt o*) emptying erythroblasts, thrombocytes, and heterophilic leucocytes. In the lower part of this protrusion and above it preserved endothelium of the central sinus (*endot*) can be seen. Marrow isolated from a rib of *Bothrops jararaca*. Perfusion-fixed specimen. H&E, immersion magnification

H&E staining. Immersion magnification. **b** On the left side of the central sinus venosus (*vc*) is an opened protrusion (*prt o*) emptying erythroblasts, thrombocytes, and heterophilic leucocytes. In the lower part of this protrusion and above it preserved endothelium of the central sinus (*endot*) can be seen. Marrow isolated from a rib of *Bothrops jararaca*. Perfusion-fixed specimen. H&E, immersion magnification

## Discussion

Our studies employing perfusion fixation have shown that there are considerable numbers of venous sinuses with a single layer of extremely delicate endothelial cells that disintegrate with traditional fixation methods. In the walls of venous sinuses (even in perfusion-fixed samples), cells captured during the process of migration from the haematopoietic space into the lumen of sinuses were seen extremely rarely. Endothelial cells clearly inhibit the passage of immature cells into the general circulation, although according to our estimate there were 18–22% in the blood. These values are especially higher in the lumen of venous sinuses where blood flow decreases. Here protrusions in various stages of development were found. In the apical parts of large protrusions were numerous mature cells. Frequently various types of blood cells were present around such a ruptured apex. There is no doubt that the opening of such a protrusion must have occurred on the well-known basis of interactions between specific membrane proteins present on mature blood cells and those on endothelial cells (Masek et al. 1994). Haemonectin plays a role in this process, especially in the case of granulopoiesis (Campbell et al. 1987). It is not known whether these factors play a similar role in reptiles.

This vascularisation has two important physiological functions. Firstly, muscles are necessary for body movement and therefore effective delivery of respiratory gases and nutrients and removal of metabolic end products are of importance. Secondly, these vessels directly penetrate the marrow cavity inside the ribs and vertebrae and permit the above-mentioned processes and, in addition, the uptake of mature and a few immature blood cells to occur.

This kind of structure suggested a slightly different mechanism of blood-cell egress. Proliferating myelopoietic cells from the lower part of a rib exerted pressure – a force called *vis a tergo* by Lichtman and Santillo (1986). This was directed towards the venous sinuses where they formed protrusions, which after rupture released blood cells into the bloodstream. This happened especially in the case of reticulocytes and erythrocytes (which are known to be incapable of diapedesis) located close to the sinus walls, which helped them enter into the sinus lumen. This resulted in the entrance of blood cells, being pushed by other maturing cells – *vis a tergo* – which also must have played a role in the opening of these protrusions.

Several papers on the subject of blood-cell egress from marrow into the general circulation were published between 1970 and 1990 (De Bruyn et al. 1971; Leblond 1973; Muto 1976; Tavassoli 1977; Lichtman et al. 1978; Chamberlain and Lichtman 1978; Tavassoli and Shaklai 1979; De Bruyn and Michelson 1979; Dabrowski et al. 1981; Petrides and Dittman 1990). However, since then, interest in the mechanism governing the release of mature blood cells from marrow into the general circulation

has waned. Nevertheless, there are still many uncertainties in this process that should be looked into. It seems that our theory is correct, but some supravital studies are needed. Campbell (1967) has published detailed studies on the structure of the walls of venous sinuses in chickens and pigeons. He proposed that in these species erythropoiesis takes place inside venous sinuses while heterophil myelocytes develop only in the extravascular spaces. Moreover, he stated that in contrast to the capillary endothelial cells, lining cells lack a basement membrane along their external surface. According to Campbell this favours the entrance of immature cells into the general circulation. However, in his studies no perfusion fixation was used, which could have resulted in the lack of a clear delineation of the endothelium. This could have led to the description of a sinus lumen being filled with maturing erythrocytes. In our material this would be equivalent to a large protrusion seen in the lumen of a sinus venosus.

An interesting stochastic model of the mechanism of blood-cell egress from marrow into the general circulation in mammals was outlined by Waugh et al. (1984). Calculations performed by them proved that in mammals the process of the flux of cells out of the haematopoietic space into the blood depends on several factors and thus is highly complicated. In these calculations they took into account values of the hydrostatic pressure, pore dimensions, and intrinsic cellular resistance to deformation on the egress process. However, in mammals only mature cells are allowed to get into the blood. In the snakes studied by us as representatives of Reptilia, located lower on the phylogenetic scale than mammals, the mechanism of cell egress relying on the rupture of protrusions is more primitive. This is indicated by the presence of many immature cells in the systemic circulation. It seems that such a process can be facilitated by the unusual structure of a particularly well-developed vascular system in the vicinity of the vertebral column recently described by Zippel et al. (2001).

We are aware that because of the phylogenetic distance separating reptiles from mammals, and differences in the structure of venous sinuses, there is some difficulty in accepting such an interpretation. Nevertheless, such boluses of cells entering the blood, by means of a rupture of a protrusion, may lead to a revision and new interpretation of this process. The histological structure and vascularisation of the studied snake spleens closely resembled that described in detail by Tanaka and Hirahara (1995) in *Elaphe climacophora*. In the spleens of all species studied by us the prevalence of the white pulp over the red pulp was also evident. The compact structure of the spleen limits the size of venous sinuses, resulting in their diameter being considerably smaller than in the bone marrow. That prevents the formation of protrusions, and perhaps this is why we did not observe them.

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