

## Morphology, cytochemical staining, and ultrastructural characteristics of the blood cells of the giant lizard of El Hierro (*Gallotia simonyi*)

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### Abstract

The object of this study was to examine the erythrocytes, leukocytes and thrombocytes of the giant lizard of El Hierro (*Gallotia simonyi*) by light and electron (TEM) microscopy, and cytochemical staining. Smears were prepared from blood from the ventral coccygeal vein of 10 healthy adult lizards (five males and five females) from the Giant Lizard of El Hierro Reproduction and Research Centre, Canary Islands, Spain. The cytochemical stains used were: benzidine peroxidase (BP), chloroacetate esterase (CAE), alpha-naphthyl acetate esterase (ANAE), acid phosphatase (AP), periodic acid-Schiff (PAS), toluidine blue (TB) and May-Grünwald-Giemsa (MGG). Electron microscopy was also performed on all samples. Heterophils had granules that were heterogeneous in both size and electron density, and stained with BP, PAS and ANAE. Eosinophil granules were homogeneously electron-dense and stained for AP, CAE and ANAE. Basophils had both highly and moderately electron-dense granules, and stained with TB and ANAE. Azurophil granules were of low electron-density and stained for AP, CAE and ANAE. Azurophil cytoplasm was vacuolated on TEM. The cytoplasm of lymphocytes contained many ribosomes and was positive for AP. Monocytes had a large nucleus and a vacuolated cytoplasm but did not stain by any of the cytochemical methods used. Thrombocytes had a relatively large nucleus but little cytoplasm; they did not stain cytochemically.

The blood cells of the giant lizards of El Hierro differ from those of other members of the Order *Squamata* both morphologically and cytochemically. The variation in cytochemical responses in the blood of reptiles makes it necessary to study species individually if meaningful clinical decisions are to be made.

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**Keywords:** Giant lizard of El Hierro; *Gallotia simonyi*; Blood cells; TEM; Cytochemical staining

### 1. Introduction

The giant lizard of El Hierro (*Gallotia simonyi*) is one of the most endangered reptiles in the world, with

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a wild population of less than 400 animals and a captive population of 300 (Pérez-Mellado et al., 1999). The conservation programme for this species includes efforts to ascertain the normal blood values and other physiological parameters in order to prevent disease. In reptiles, circulating blood cells can be grouped into erythrocytes, leucocytes and thrombocytes. Although erythrocytes, thrombocytes, eosinophils, basophils, lymphocytes and monocytes are morphologically similar amongst reptiles, there are notable species differences in heterophils (LeBlanc et al., 2000) and azurophils (Raskin, 2000). Heterophils vary in number and in their nuclear lobules. Monocytes and azurophils are considered to be the same by some (Raskin, 2000; Roskopf, 2000) but different by others (Ellman, 1997). Azurophils are common cells in the Squamata (lizards and snakes) and Crocodylia (caimans, crocodiles and gavials) but are seen only occasionally in chelonians (turtles and tortoises) (Anderson et al., 1997).

Here we report the light microscopic, cytochemical and ultrastructural features of the blood cells of healthy El Hierro Giant Lizards. This information is essential for the recognition of changes in the blood of diseased lizards, identification of inflammatory cells in damaged tissues and an understanding of the role of blood cells in resisting infection.

## 2. Materials and methods

### 2.1. Animals

Samples were taken on El Hierro Island (lat. 18° 0'W and long. 27° 40'N) in the Canary Islands (Spain). Ten lizards (five males and five females each over 10 years old) from the Giant Lizard of El Hierro Reproduction and Research Centre were used. When the samples were taken, each lizard was healthy and eating normally. Their diet has been described before and is similar to their diet in the wild (Orrit et al., 1999).

### 2.2. Samples

Each lizard was restrained manually, a 0.6 × 25 mm needle was placed in the ventral coccygeal vein and 0.2 ml of blood was withdrawn. Smears were made immediately and air-dried, and blood for electron microscopy was placed in a tube containing lithium heparin (Tapval, Madrid, Spain).

### 2.3. Light microscopy

Air-dried smears were stained with May–Grünwald–Giemsa (MGG) stain for differential leukocyte counts

and description of erythrocytes, thrombocytes and leucocytes. Leucocytes were categorized as: heterophils, eosinophils, basophils, azurophils, lymphocytes and monocytes. Smears were also stained with the following: benzidine peroxidase (BP), chloroacetate esterase (CAE), alpha-naphthyl acetate esterase (ANAE), acid phosphatase (AP), periodic acid-Schiff (PAS) and toluidine blue (TB) as described by Jain (1986) (Woessner et al., 1991).

### 2.4. Electron microscopy

The blood from heparinized tubes was centrifuged and the plasma discarded. The buffy-coat was separated and fixed in 2.5% glutaraldehyde in 0.1 M, pH 7.2, sodium cacodylate buffer (TAAB Laboratories, England, UK) for 4 h at 4 °C. The cells were then washed three times in sodium cacodylate buffer and stored in fresh buffer at 4 °C until processed for electron microscopy by conventional methods. The identification of blood cells was based on nuclear appearance and the relative number, size, shape and distribution of granules.

## 3. Results

Each cell type is described below and illustrated in the figures. The cytochemical results are summarized in Table 1.

### 3.1. Erythrocytes

The erythrocytes were oval, consistent in size and shape, and had centrally located nuclei. They did not stain selectively by any of the cytochemical methods. Their nuclei had a characteristic pattern of peripherally located heterochromatin, and the cytoplasm was uniformly electron-dense (Figs. 1(a) and 3(a)).

### 3.2. Thrombocytes

The nucleus of thrombocytes was similar in size to the erythrocyte nucleus. The cytoplasm showed occasional pseudopodia and, on some occasions, formed only a minute halo around the nucleus; it was more extensive in other thrombocytes. Thrombocytes did not stain with any of the cytochemical stains used. Electron microscopy revealed vacuoles, small membrane-bound granules and pseudopodia. Numerous mitochondria were present, this being the cell type in which these organelles were most abundant. The nucleus-to-cytoplasm ratio was very high, and the nucleus contained obvious heterochromatin, located peripherally (Figs. 1(h) and 3(h)).



Table 1  
Leukocyte response to different stains in the El Hierro giant lizard (*Gallotia simonyi*)

Stain	Lymphocyte	Monocyte	Heterophil	Azurophil	Eosinophil	Basophil
PAS	–	–	+/-	–	–	–
TB	–	–	–	–	+	–
ANAE	–	–	+	+	+	+
CAE	–	–	–	+/-	+	–
BP	–	–	+	–	–	–
AP	+/-	–	–	+	+	–

Staining was scored as negative (–), few positive cells ( $\pm$ ) and moderate to strong (+).

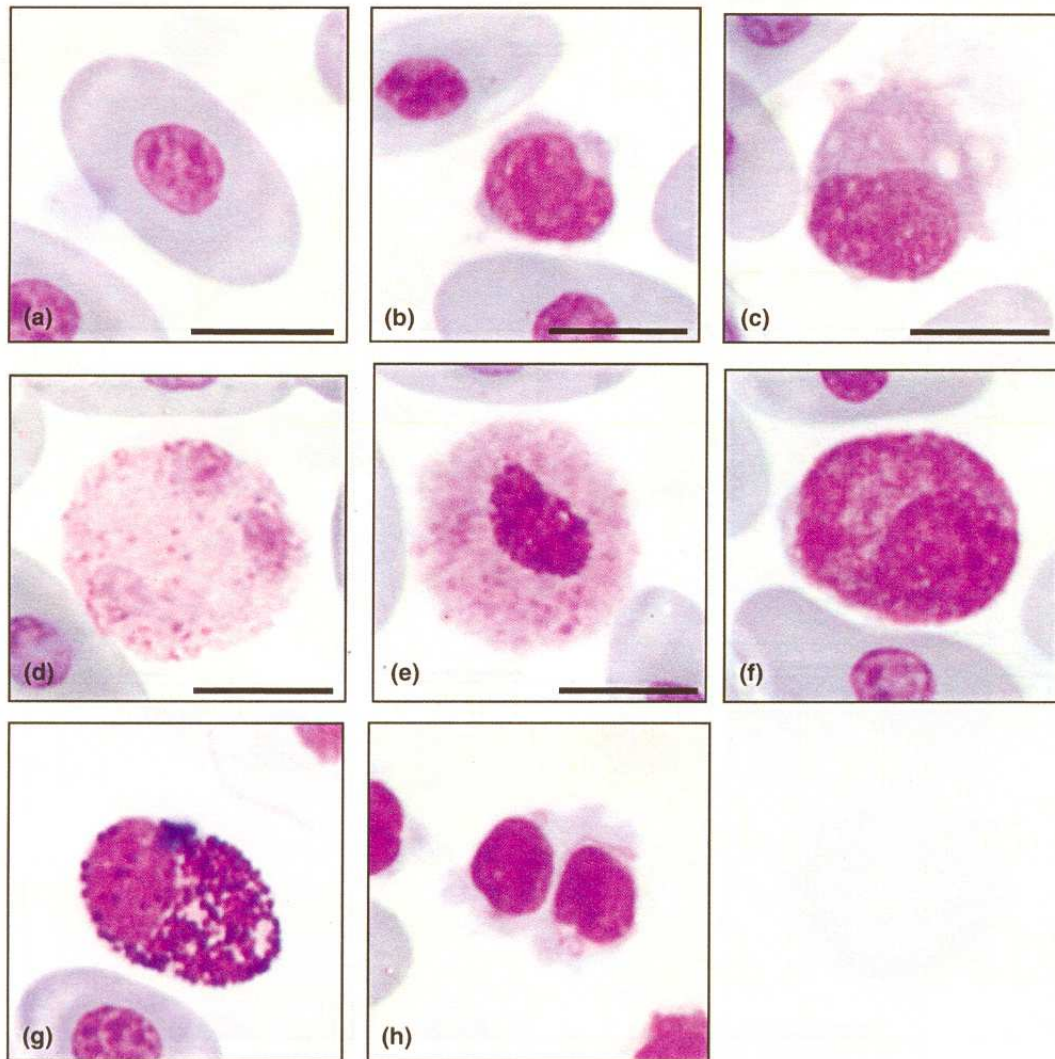


Fig. 1. Light micrographs of blood cells of the El Hierro giant lizard (*Gallotia simonyi*) stained with May–Grünwald–Giemsa stain. (a) Erythrocyte, (b) lymphocyte, (c) monocyte, (d) heterophil, (e) azurophil, (f) eosinophil, (g) basophil, (h) thrombocytes. Bar = 10  $\mu$ m.

### 3.3. Leucocytes

#### 3.3.1. Heterophils

The heterophils were large cells with an irregular nucleus with two to five lobes. The heterogeneous nature of the granules meant that the staining of the cytoplasm was very variable both within and between cells. The granules were often refringent, a unique feature of this cell type. Heterophils stained with BP, PAS and ANAE but not with the other cytochemical stains. This cell is easily identified given the characteristic shape of its nucleus and the granule distribution.

Some degranulated heterophils were seen in the blood smears of three lizards. On electron microscopy heterophils show many heterogeneous granules that differed in shape, size and electron density. The lobed shape of the nucleus means that its outline was very unpredictable when seen in section (Figs. 1(d), 2(e)–(g) and 3(d)).

#### 3.3.2. Eosinophils

These had a compact appearance that was more homogeneous than the heterophil, with a nucleus with few lobes (two at most). With MGG staining, the



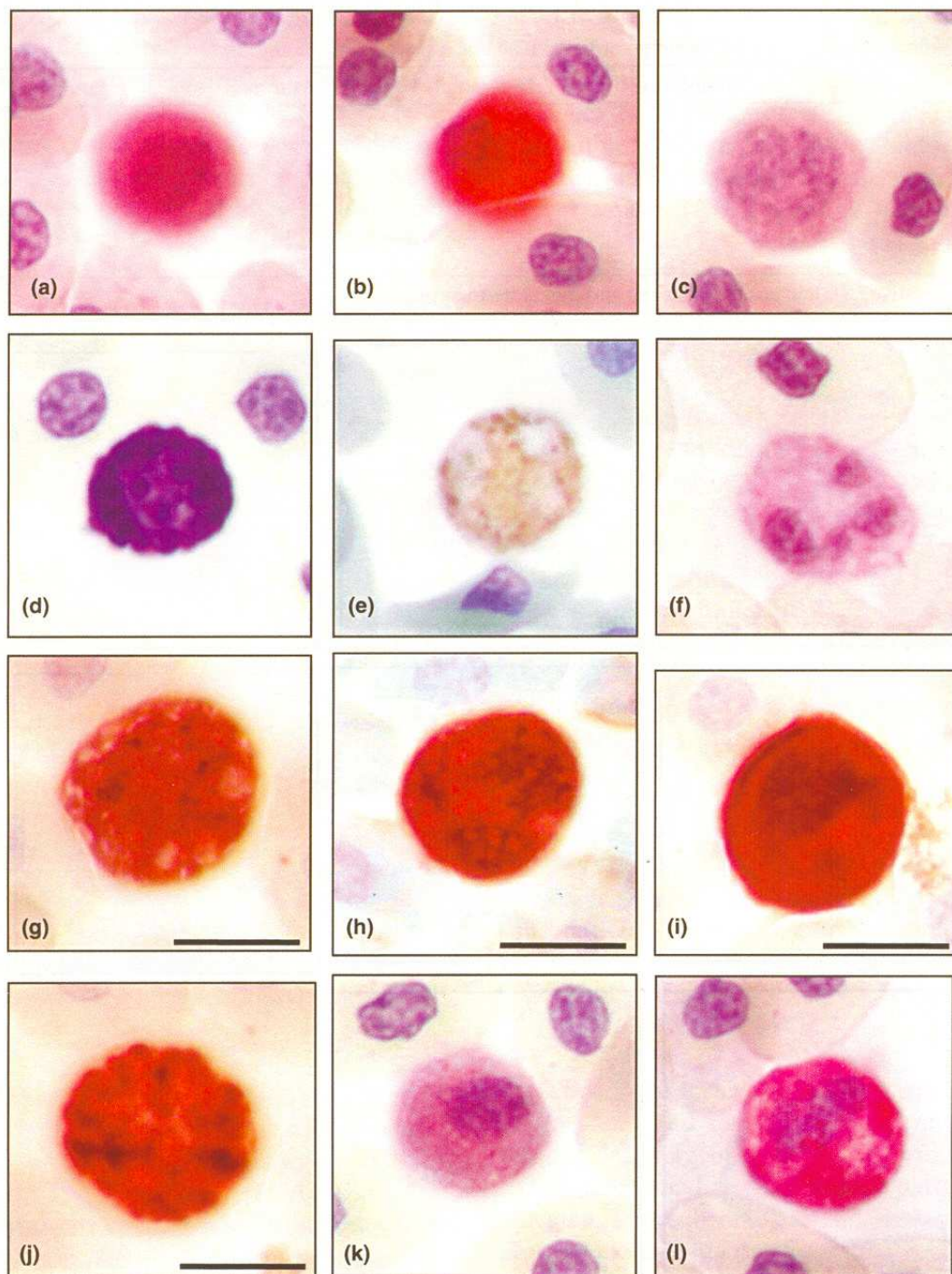


Fig. 2. Light micrographs of blood cells of the El Hierro giant lizard (*Gallotia simonyi*) stained as follows. Acid phosphatase: (a) azurophil, (b) eosinophil, (c) lymphocyte. Toluidine blue: (d) basophil. Benzidine peroxidase: (e) heterophil. Periodic acid-Schiff: (f) heterophil. Alpha-naphthyl acetate esterase: (g) heterophil, (h) azurophil, (i) eosinophil, (j) basophil. Chloroacetate esterase: (k) azurophil, (l) eosinophil. Bar = 10  $\mu$ m.

granules were round or oval, and strongly eosinophilic like the nucleus. On occasion, the nucleus was completely hidden by the granules. Staining was observed with the AP, CAE and ANAE cytochemical stains. Large, round, homogeneous granules with moderate electron density were observed by TEM (Figs. 1(f), 2(b), (i) and (l), and 3(f)).

### 3.3.3. Basophils

The cytoplasmic granules of basophils were strongly basophilic when stained with MGG. They stained with TB and ANAE. Highly heterogeneous

granules were seen on ultrastructure. Some of them were condensed and very electron-dense, while others were less intensely stained. There were also a high number of membrane-bound organelles (Figs. 1(g), 2(d) and (j), and 3(g)).

### 3.3.4. Azurophils

Stained with MGG, azurophils had a slightly spongy cytoplasm, with dispersed vacuoles of variable size and dispersed, azurophil granules. The granules were not as intensely stained as in other granulocytes. The nucleus was not lobated and its position was usually cen-

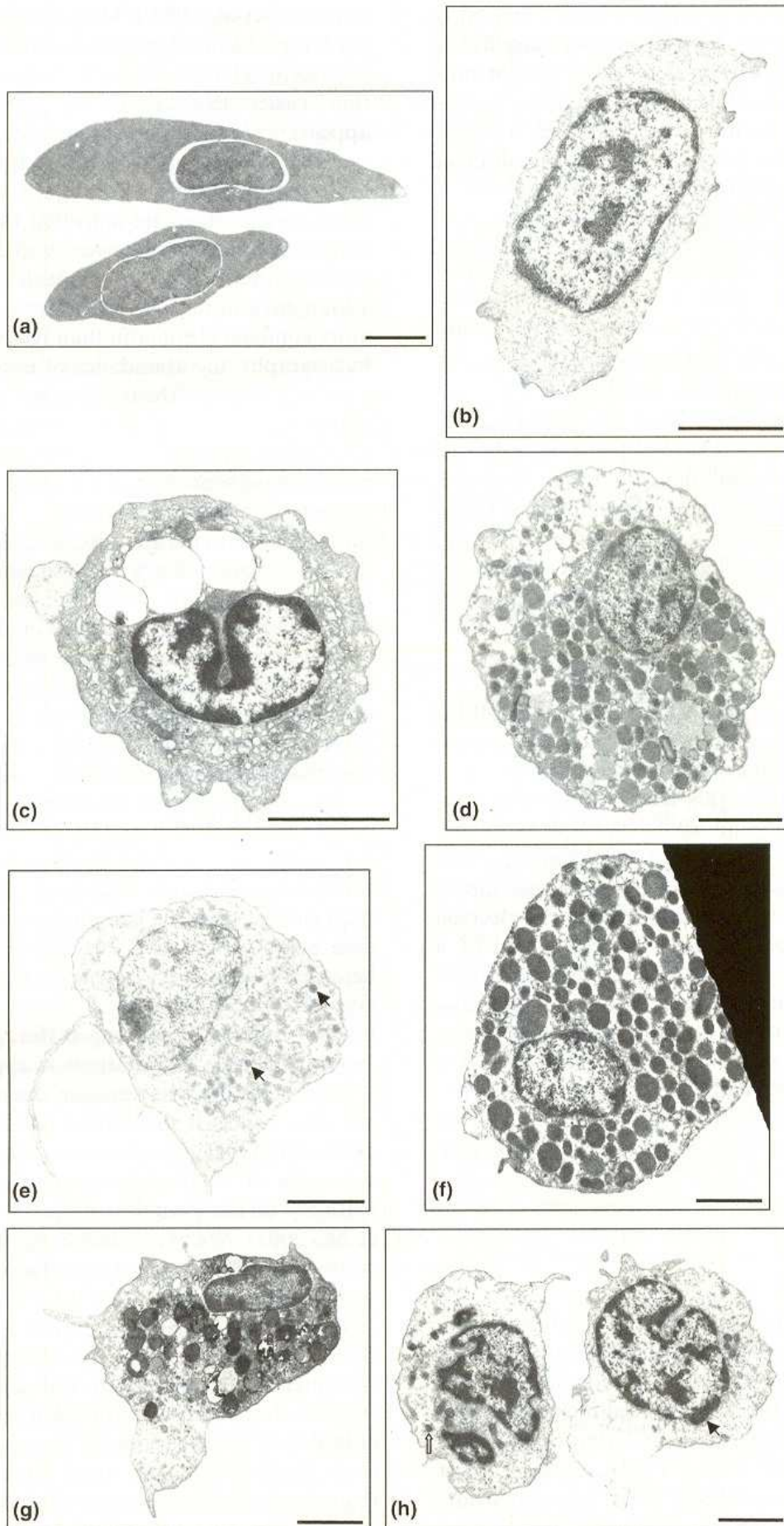


Fig. 3. Electronmicrographs of blood cells of the El Hierro giant lizard (*Gallotia simonyi*). (a) Erythrocyte, (b) lymphocyte, (c) monocyte, (d) heterophil, (e) azurophil (arrows show dispersed lysosomes), (f) eosinophil, (g) basophil, (h) thrombocytes (open arrow shows small membrane-bound granules, closed arrow shows heterochromatin located peripherally). Bar = 2 μm.



tral, unlike eosinophils or basophils where it tended to be peripheral. Azurophilic cells gave a positive response to AP, ANAE and CAE. The nucleus-to-cytoplasm ratio was rather low (ratio was 1/3 to 1/4) and similar to that of the eosinophils. At an ultrastructural level, a significant amount of eccentric heterochromatin was observed in the nucleus and the cytoplasm contained moderate numbers of organelles, among them mitochondria, Golgi membranes and lysosomes (Figs. 1(e), 2(a), (h) and (k), and 3(e)).

### 3.3.5. Monocytes

Monocytes had a large nucleus that was almost always indented. The proportion nucleus to cytoplasm was equal because there was a large amount of slightly basophilic cytoplasm. Vacuoles, of variable size and number, were present in all the monocytes observed. None of the cytochemical stains were effective. Ultrastructurally, the cytoplasm contained a few very large granules and vacuoles of differing sizes. There were also some ribosomes. The heterochromatin was dense and homogeneous (Figs. 1(c) and 3(c)).

### 3.3.6. Lymphocytes

The lymphocytes were very variable in size in all the lizards examined, ranging from as large as a monocyte to as small as a thrombocyte. Normally, lymphocytes were larger than thrombocytes with a more abundant basophilic cytoplasm. Lymphocytes had a denser chromatin pattern than thrombocytes. Cytochemically, they only gave a slightly positive response to acid phosphate staining. Pseudopodia were always evident on electron microscopy. The cytoplasm was completely filled by a homogeneous mesh of free ribosomes. There were few mitochondria though it was possible to find the occasional lymphocyte with one or two. The nucleus was round in appearance with slight indentations and the chromatin was as condensed as that of thrombocytes, and slightly more condensed than in granulocytes or thrombocytes. Nucleoli were not observed (Figs 1(b), 2(c), 3(b)).

## 4. Discussion

The erythrocytes of the giant lizard of El Hierro were similar to those of other reptiles (Mader, 2000). Erythrocyte abnormalities are linked to nutritional status (Campbell, 1998), and also include viral inclusions, haemoglobin cysts, haemoparasites (Clark et al., 2001; Martínez-Silvestre et al., 2001). They are also subject to osmotic insults, both in life and in the laboratory during preparation (Troiano et al., 2000).

The thrombocytes in the giant lizards in our study were difficult to differentiate from the lymphocytes, as is the case in chelonians (Alleman et al., 1992) and ophidians

(Alleman et al., 1999). Only in certain species, such as the King Cobra (*Ophiophagus hannah*), in which thrombocytes are characteristically elongated, is cell differentiation easier (Salakij et al., 2002). Although size, appearance and stain response are similar in both cell types, there are some differences that may be helpful. In general, thrombocytes are slightly smaller than lymphocytes, though there are activated thrombocytes or small lymphocytes that could give rise to doubt. Thrombocytes are very similar in size to the nucleus of the erythrocytes, and slightly more basophilic. Thrombocytes have denser, more compact chromatin than lymphocytes. In electron-micrographs, the abundance of mitochondria in thrombocytes allows them to be distinguished from lymphocytes, which, in the resting state, do not have an activated energy source (Wheater et al., 1987). The thrombocytes have some vacuoles that are not observed in inactivated circulating lymphocytes. PAS did not stain thrombocytes in the giant lizards; in this respect, they differ from diamondback rattlesnakes (*Crotalus adamanteus*; Alleman et al., 1999), but are similar to alligators (*Alligator mississippiensis*; Mateo et al., 1984).

The absence of a response to cytochemical stains – except acid phosphatase – appears to be characteristic of lymphocytes. The large number of ribosomes in the cytoplasm of lymphocytes possibly accounts for their basophilic staining with Giemsa stains.

Heterophils are the main leucocytes of giant lizards (Martínez-Silvestre et al., 2002). They show a clear positive response to BP like the bearded lizard, *Acanthodracon vitticeps* (Tocidowski et al., 2001) although it has been claimed that the heterophils of reptiles lack peroxidase activity (Mateo et al., 1984). Some degranulated heterophils were seen in smears from our animals and, according to Alleman et al. (1999), this can be caused by poor fixation or prolonged storage in anti-coagulant. Neither of these explanations is applicable in our case. Ultrastructurally, the variable electron density of their granules makes it difficult to confuse heterophils with other cell types. Some snakes are said to have two sub-types of heterophils, depending on the degree of maturity of the granules (Alleman et al., 1999; Salakij et al., 2002). We were unable to recognize sufficiently consistent differences between heterophils to subdivide them in this way.

Eosinophils have round granules and an eccentric nucleus that are easy to recognize in lizard smears. Although the granules are not always very distinctly stained, they cannot be confused with the polymorphic granules of the heterophils. Even on electron microscopy, the granules were much more uniform in density than those in heterophils or the basophils. This feature seems to be common in reptiles (Frye, 1991).

Basophils were observed in all the lizards. Identification was easy owing to the large number of basophilic granules and the marginal nucleus which, on occasion,



could hardly be seen through the granules, a fact that makes this cell practically unmistakable (Frye, 1991). The granules stain with TB as in mammals (Raskin and Valenciano, 2000), chelonians (Alleman et al., 1992) and crocodylians (Mateo et al., 1984). They are also weakly positive for ANAE unlike the desert tortoise (Alleman et al., 1992) and diamondback rattle snake (Alleman et al., 1999). The appearance and high density of the granules on TEM accord with descriptions of other species (Salakij et al., 2002; Wheeler et al., 1987) though we also saw some low density granules that were not reported by others.

Azurophils are seen in small numbers in the blood of healthy lizards, and some authors (Alleman et al., 1999; Roskopf, 2000) suggest that they increase during infections, granulomatous conditions and protozoal blood parasitism (Salakij et al., 2002). The azurophils of the lizards stained with AP and ANAE, and were slightly positive to CAE. However, in the diamondback rattlesnake, they also stained with PAS and peroxidase (Alleman et al., 1999).

The monocytes of giant lizards can be clearly distinguished from other leucocytes by their nuclear morphology and high nucleus to cytoplasm ratio. As in other reptiles, the nucleus is large and indented and a number of large vacuoles can almost always be seen. Two kinds of monocytes have been reported in desert tortoises, one of them with azurophilic properties (Alleman et al., 1992). In our lizards, monocytes were readily distinguished from azurophils. Nevertheless, cytochemical staining was negative for all the techniques used, so there does not seem to be any very specific staining for this cell type. In this respect, giant lizards are like desert tortoises (Alleman et al., 1992) but differ from diamondback rattlesnakes, in which blood monocytes are rarely seen (Alleman et al., 1999). Ultrastructurally, the nucleus to cytoplasm ratio was much greater in monocytes than azurophils, and the monocyte cytoplasm was less rich in granules and vacuoles. The heterochromatin was more homogeneous and dense than in either azurophils or lymphocytes.

As mentioned above, distinguishing between lymphocytes and thrombocytes is difficult. In the giant lizards of El Hierro, lymphocytes have a more abundant cytoplasm but we were unable identify smooth endoplasmic reticulum or rudimentary Golgi apparatus (Wheeler et al., 1987). The lizards' lymphocytes did contain a large number of ribosomes, a fact that possibly gives them their basophilic cytoplasm.

In conclusion, the giant lizards of El Hierro differ from other reptiles, and from other members of the Order *Squamata*, in the morphology and cytochemistry their blood cells. This confirms that reptiles are a highly heterogeneous class in which it is difficult to draw inferences from one species to another. It is therefore important that the clinical characteristics of the blood cells of

each species is known, particularly if the future survival of the species is at issue.

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