

LYMPHOID DIFFERENTIATION AND ORGANIZATION OF THE SPLEEN IN THE LIZARD, *CALOTES VERSICOLOR*

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ABSTRACT

In *Calotes versicolor*, splenic primordium appears at stage 30 as a protuberance of mesenchymal cells from the dorsal mesentery. Lymphopoiesis is initiated at stage 40, followed by an increase in the lymphoid population of the rudiment during the successive stages of development. In the adult, splenic pulp is poorly demarcated into white pulp and red pulp. The former is in the form of closely arranged lymphoid follicles and the latter is highly restricted to narrow strands of blood spaces. A comparative study on the lymphoid organization of the spleen of a few other species of reptiles was also carried out.

INTRODUCTION

THERE is a lack of detailed information about the ontogeny and cyto-architecture of lymphoid tissues in various groups of reptiles. Among the representative reptiles studied, lymphoid tissue is reported in the spleen of snapping turtle (Sidky and Auerbach, 1968), tuatara (Marchalonis *et al.*, 1969) and lizards (Kanakambika, 1971; Wetherall and Turner, 1972). Our studies on the immune response of the lizard, *Calotes versicolor*, indicated that spleen is the principal site of antibody formation (Kanakambika, 1971; Kanakambika and Muthukkaruppan, 1972 *a*, 1972 *b*, 1972 *c*). While splenectomy completely abolishes antibody production to sheep erythrocytes (Kanakambika and Muthukkaruppan, 1972 *c*) and bovine serum albumen (Kanakambika, unpublished) the allograft response was totally unaffected (Manickavel, 1972).

Earlier studies available on the development of the spleen in snake (Danschakoff, 1916) and in the gecko, *Gymnodactylus* (Evans, 1934) are restricted to the origin and pattern of vascularization of the spleen rudiments.

Even though a number of studies have been made to elucidate the various types of immune response in different groups of reptiles (*cf.* Cohen, 1971), no attempt has been made to describe the development and differentiation of lymphoid structures in reptiles. The present report is a part of our comprehensive study on the development of immunity in reptiles.

MATERIALS AND METHODS

More than 100 embryos belonging to various developmental stages (28-42) and adult lizards of *Calotes versicolor* were used in this study. Details regarding the maintenance of the lizards, incubation of the eggs and staging of the embryos were already described (Muthukkaruppan *et al.*, 1970). Embryos were dissected out in Phosphate Buffered Saline (PBS pH 7.2). After identifying the embryonic stage of development, the trunk region of the embryo was fixed in Bouin's fluid, serially sectioned at 6μ and stained with haematoxylin and eosin. To identify the various cell types of the embryonic spleen, imprints were also prepared from stages 39 and 40 and differentiated with Wright's stain.

Spleen from adult lizard, *Calotes versicolor* as well as from four other species of reptiles, namely, *Lessemys punctatus* (turtle), *Tropidonotus* (snake), *Hemidactylus brooki* and *Mabuya carinata* (lizards) were sectioned at 6μ and stained with haematoxylin and eosin. Haematoxylin Van Gieson stain was used to distinguish the distribution of collagen fibres in the spleen. In some cases, sections were impregnated with silver for the demonstration of reticular fibres, following the procedures outlined by Bielshowsky Addison, 1950). For detailed morphological study of different cell types, imprints were taken from spleen fragments and these preparations were rapidly air-dried and stained with Wright's. The procedures for immunization of lizards with sheep erythrocytes (SRBC) have been described elsewhere (Kanakambika and Muthukkaruppan, 1972 *b*).

RESULTS

Normal development of the spleen in Calotes versicolor.—The spleen primordium appears at stage 30 as a small protuberance, consisting of loose mesenchymal cells and extends into the body cavity from the mesentery close to the dorsal pancreatic rudiment (Fig. 1). The outer margin of the rudiment is well-defined. During the subsequent stages of development there is further enlargement of the rudiment due to the increase in the number of mesenchymal cells and blood spaces (Fig. 2). The rudiment becomes well-

oriented and attains its definite shape during stages 34 to 35. The entire organ can be easily separated from its surrounding tissues at stage 35. It becomes more compact with various cell types from stage 38 onwards.

Numerous blood capillaries and blood spaces containing erythrocytes are observed among the mesenchymal cells of the rudiment from stage 30 onwards. The sinusoidal nature of the spleen becomes prominent during stages 33 and 34 accompanied by widespread distribution of erythrocytes. The spleen rudiment at stage 35 is primarily erythroid in nature.

A limited number of granulocytes is seen at the time of appearance of the rudiment at stage 30. Groups of granulocytes are frequently found distributed outside the rudiment at its junction with the pancreas. There is a pronounced increase in granulocytic differentiation from stage 36 onwards and a definite population of granulocytes exists in the spleen by stage 38 (Fig. 3). The majority of the granulocytes are eosinophilic in nature, their granules being spindle-shaped.

Small lymphocytes are recognized in the spleen rudiment at stage 40, suggesting the initiation of lymphopoiesis (Fig. 4). Several small lymphocytes and granulocytes are the constant cellular components of the rudiment at this stage (Fig. 5). Subsequently there occurs a pronounced increase in lymphoid differentiation resulting in the formation of 'lymphoid foci' at stage 42, the last stage of embryonic development. The latter are represented as groups of small lymphocytes, but differentiation of typical lymphoid follicles is not evident (Fig. 6). At the time of hatching the spleen contains 0.8 million lymphoid cells.

Morphology of the spleen.—In *Calotes versicolor* the spleen is attached to the anterior end of the pancreas and situated towards the left side of the stomach. It is roughly elongated or oval in shape. The size of the organ varies widely, its length ranging from 0.8 to 2.0 mm and width from 0.1 to 1.5 mm. The colour of the spleen is usually dark brown, often varying from reddish-brown to black.

The spleen is a lymphoid organ composed of an outer thin capsule and an inner splenic pulp. The capsule consists of collagenous fibres. The trabeculae are the branching and anastomosing continuations of the capsule, penetrating sporadically throughout the splenic pulp and often enclose small blood vessels. A mesh work of reticular fibres also extends throughout the splenic pulp.

In the spleen, the white pulp is represented by a number of lymphoid follicles: each follicle consists of reticular area (L) with a central arteriole and surrounding darkly stained lymphoid area (D). These follicles are arranged so closely that they are confluent with each other, thereby forming a continuous phase of lymphoid tissue throughout the splenic pulp (Figs. 7, 8). The red pulp is not distinctly seen and is represented only by narrow strands of blood spaces (N) found discontinuously distributed between the adjacent lymphoid follicles (Fig. 7). The sinusoidal system of the red pulp is very much restricted. However, after immunization with SRBC, such narrow strands of blood spaces develop prominently, thereby demarcating the lymphoid follicles clearly. Macrophages, as shown by the uptake of carbon particles are located around the rim of the follicles. Germinal centres are not observed in the spleen, even after repeated immunization with sheep erythrocytes.

The cellular component of the spleen consists primarily of small lymphocytes, with a scattering of medium and large lymphocytes, macrophages, granulocytes, monocytes and erythrocytes, as revealed by the examination of imprint or smear preparations. The white cell count of the spleen varies considerably, ranging from 2 to 28 million white cells per spleen with an average of 13 million.

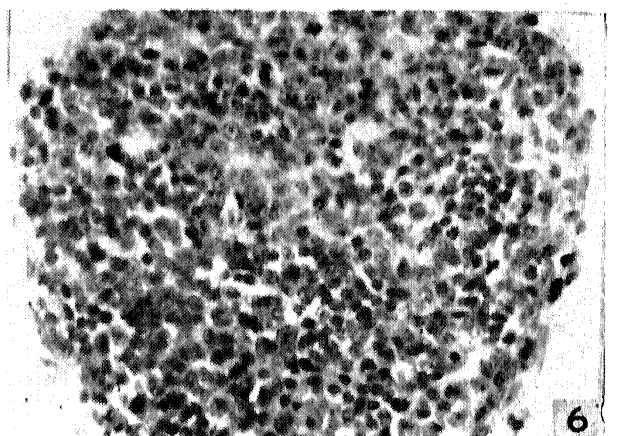
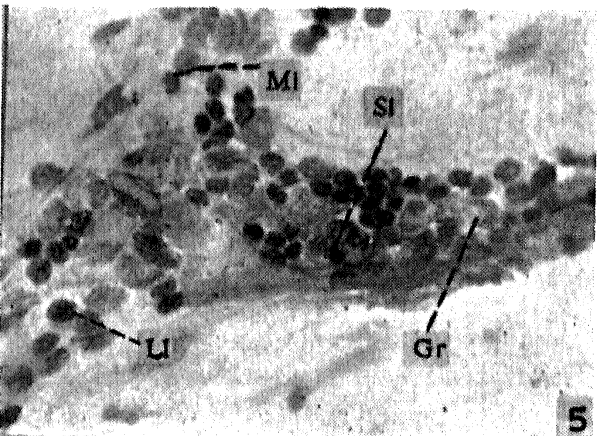
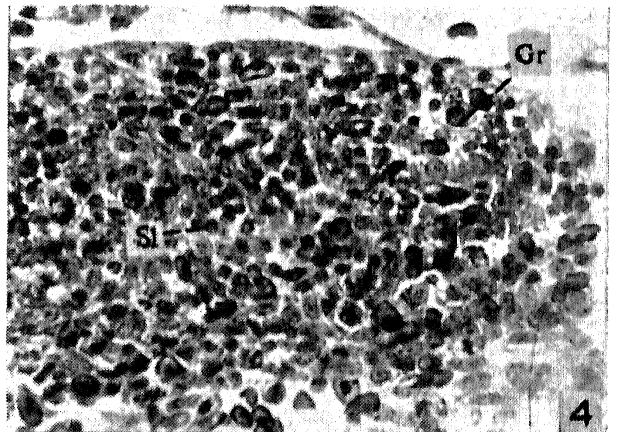
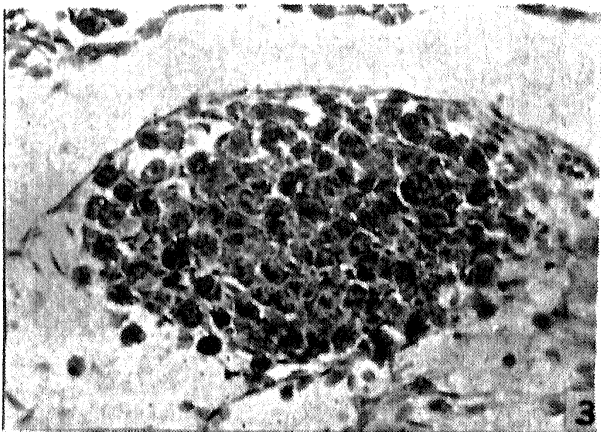
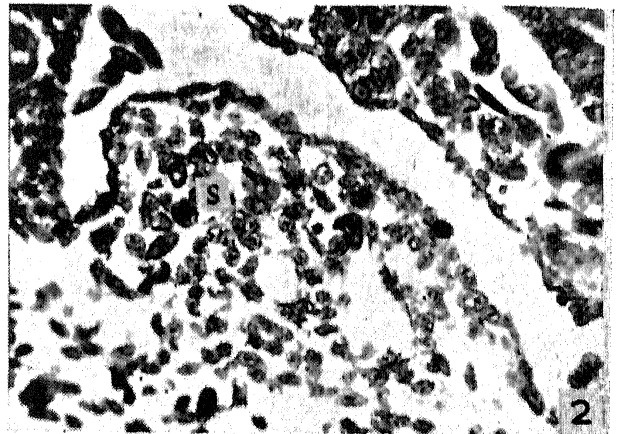
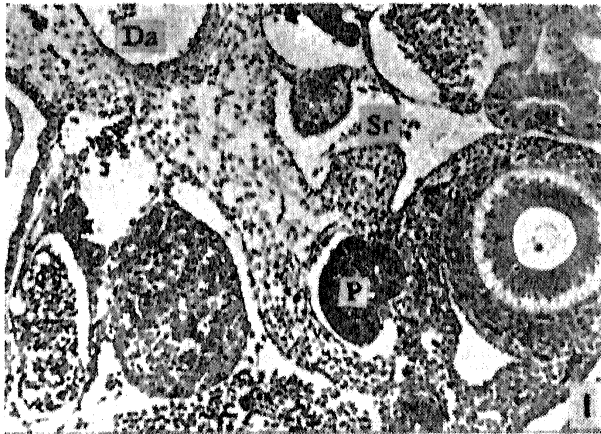
In the other two species of lizards, *Mabuya* and *Hemidactylus* (Fig. 10) the organization of the splenic pulp is very much similar to that of *Calotes*.

In the turtle, distinct red pulp and white pulp are clearly delineated. The white pulp is represented by a number of lymphoid follicles and each follicle is an aggregation of lymphoid cells enclosing a layer of reticular cells with an arteriole in the centre (Fig. 11). Between the lymphoid follicles, is present the extensively developed red pulp of the spleen, enclosing blood sinuses and myeloid cells.

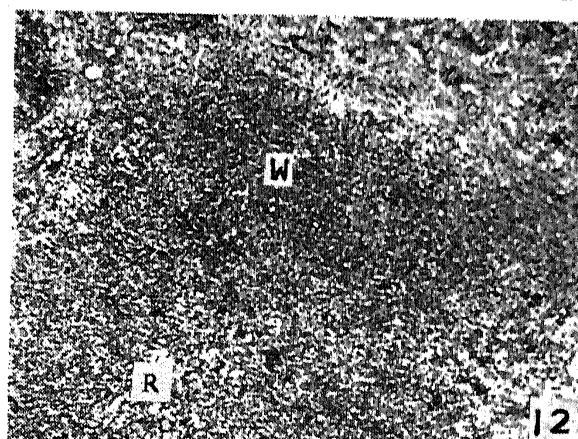
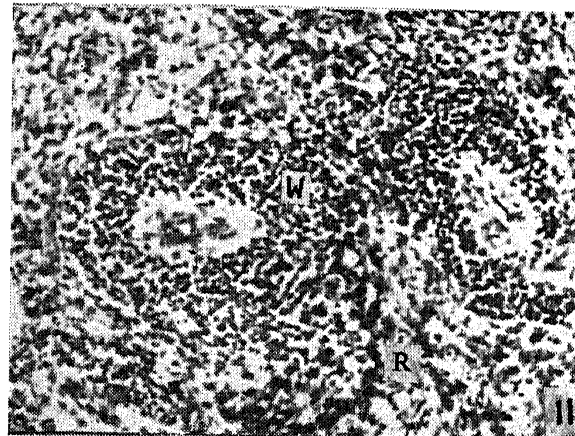
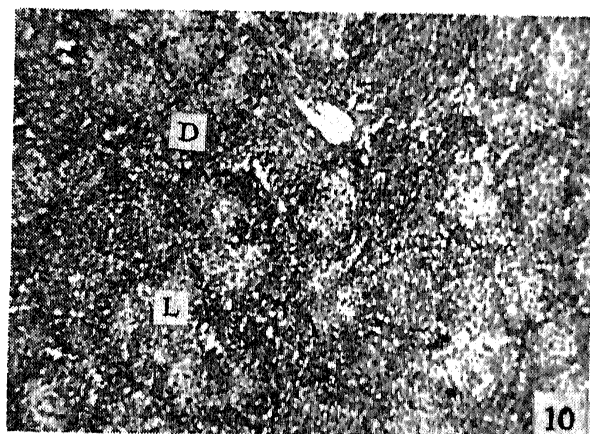
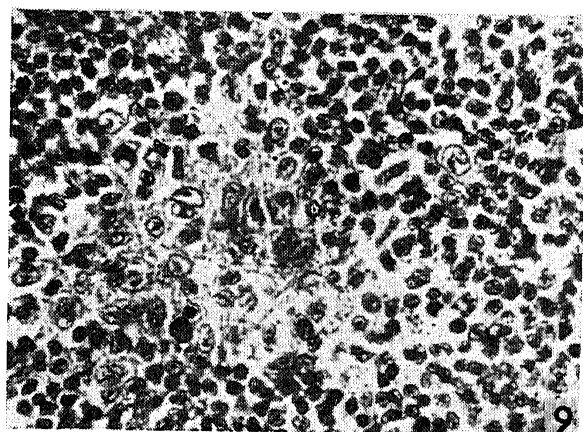
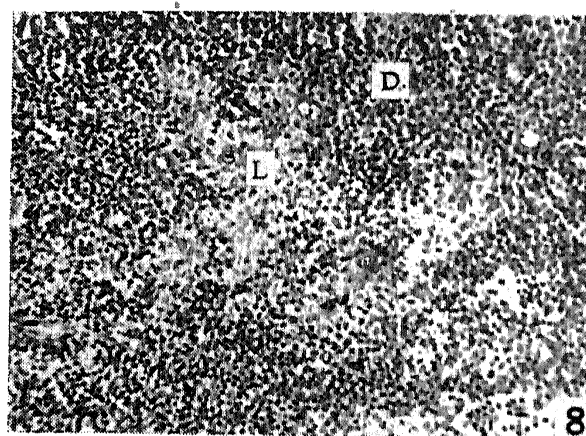
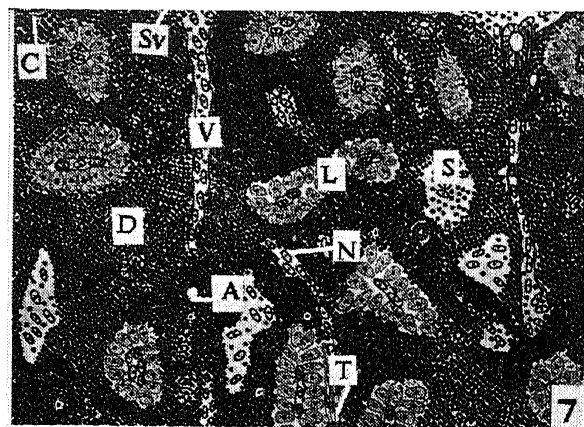
In the snake the white pulp (W) is in the form of distinct lymphoid nodules in spleen (Fig. 12). The red pulp (R) is well developed and easily distinguishable from the white pulp. The reticular area as reported in the spleen of *Calotes* is absent.

DISCUSSION

During the development of the lizard, *Calotes versicolor*, the splenic primordium appearing as a protuberance of mesenchymal cells at stage 30, develops into a profusely vascularized erythroid structure at stage 35. It



FIGS. 1-6



Figs. 7-12

becomes predominantly granulocytic during stages 36 to 38 and the lymphopoiesis is evident in the rudiment from stage 40 onwards. Such a sequence of cellular events is in general agreement with the earlier findings of Thiel and Downey (1921) in pig, of Ono (1930) in man, of Auerbach (1965) in mouse, of Delaney and Ebert (1962) in chick and of Evans (1934) in the lizard, *Gymnodactylus kotschy*. The similarity is further extended to the relative time or stage (outward appearance of embryos) and the position of origin of the splenic primordium among different vertebrate groups.

During the development of the lizard, *Calotes*, the epithelio-mesenchymal rudiment of thymus (stage 34) differentiates into a lymphoid organ at stage 37 (Muthukkaruppan, unpublished). This is followed by the appearance of lymphocytes in the spleen at stage 40. This sequence of lymphoid differentiation in the embryo compares well with the observations of Auerbach (1965) in mouse.

The spleen in late embryonic stages of *Calotes* contains a considerable number of lymphocytes, confirming the earlier description of Evans (1934) in another lizard, *Gymnodactylus kotschy*. The functional nature of these lymphocytes may be correlated with our earlier findings on the immunocompetence of hatchling lizards. This has been demonstrated in terms of the development of haemolysin antibody-producing cells in the spleen (Kanakambika and Muthukkaruppan, 1972 a), and rejection of skin allografts (Manickavel, 1972) in hatchling lizards.

The present studies indicate that there is wide variation in the lymphoid organization of the spleen in different reptiles. In the lizards and turtles the reticular area forms part of the white pulp; while such reticular tissue is absent in the spleen of the snake, *Tropidonotus* and in *Sphenodon* (Marchalonis *et al.*, 1969). The red pulp is highly restricted in the spleen of lizards, while there is extensive development of this tissue in the spleen of turtle and snake.

There is poor demarcation of the white pulp and red pulp in the spleen of *Calotes*, presumably because the latter is encroached by the densely packed lymphoid tissue, resulting in its confinement within the narrow-blood spaces. Thus in *Calotes versicolor* the white and red pulp are differentiated, but not distinctly delineated as seen in the spleen of snake, turtle or mammals. However well-defined red and white pulp are reported in the spleen of another species of lizard, *Tiliqua rugosa*, where the latter is represented as lymphoid aggregations surrounding arteries (Wetherall and Turner, 1972).

A notable feature of the spleen in *Calotes* is the absence of germinal centres in the immunized lizards, confirming the reports in other reptiles (*Sphenodon*, Marchalonis *et al.*, 1969 and *Tiliqua*, Wetherall and Turner, 1972). In fact this seems to be a common feature among lower vertebrates.

The striking differences in the lymphoid organization of the spleen of lizards from that of mammals are (1) the distribution of the lymphoid tissue in a continuous phase; (2) the predominant occurrence of the reticular area and (3) the poor representation of the red pulp. In the lizard *Calotes versicolor*, treated with appropriate doses of rabbit anti-*Calotes* thymocyte serum (RaCalTS) the darkly stained area is highly depleted of lymphoid cell population, without an apparent alternation in the red pulp (Muthukkaruppan and Kanakambika, unpublished). Therefore, it will be of interest to characterize the nature of thymus dependent area of the spleen, as described for mice (Parrott *et al.*, 1966).

The occurrence of reticular tissue within the white pulp of *Calotes* spleen is interesting in two respects: (1) during lymphoid regeneration of the RaCalTS treated spleen, the reticular area is filled with a number of medium lymphoid cells, followed by the appearance of small lymphocytes around this area, (2) While the reticular tissue within the lymphoid follicle occurs in all the three species of lizards studied, in the turtle, in the teleost *Tilapia mossambica* (Sailendri, unpublished), and in the chicken (Hoshi, 1972), it is found to be absent in snake, tuatara (Marchalonis *et al.*, 1969) and mammals. Therefore, further studies in other forms are required for understanding the phylogeny of splenic white pulp in vertebrates.

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EXPLANATION OF PLATES

PLATE II

- FIG. 1. Section of the embryo of stage 30 showing the spleen rudiment (Sr), pancreas (P) and dorsal aorta (Da), Haematoxylin-eosin, $\times 130$.
- FIG. 2. Enlarged view of the spleen rudiment at stage 33 showing the blood sinuses (S), Haematoxylin-eosin, $\times 500$.
- FIG. 3. Section of the spleen at stage 38 showing the abundance of granulocytes, Haematoxylin-eosin, $\times 500$.
- FIG. 4. Section of the spleen at stage 40 showing the presence of small lymphocytes (S1) and granulocytes (Gr). Haematoxylin and eosin, $\times 500$.

- FIG. 5. Smear preparation of the spleen at stage 40, showing the presence of small lymphocytes (S1), granulocytes (Gr), large lymphocytes (L1) and medium lymphocytes (M1), Wright's stain, $\times 500$.
- FIG. 6. Section of the spleen at stage 42 showing the lymphoid nature of the spleen, Haematoxylin-eosin, $\times 500$.

PLATE III

- FIG. 7. Diagrammatic representation of the longitudinal section of the spleen showing the distribution of capsule (C), darkly stained (D), and lightly stained (L) area, blood sinus (S), vein (V), artery (A), trabeculae (T), narrow strands of blood-spaces with erythrocytes (N), subcapsular vein (Sv).
- FIG. 8. Section of the spleen showing the distribution of darkly stained (D) and lightly stained (L) area, Haematoxylin-eosin, $\times 250$.
- FIG. 9. Enlarged view of the lightly stained area of Fig. 8 showing a blood capillary and reticular cells surrounded by lymphocytes, $\times 1,000$.
- FIG. 10. Section of the spleen of the gecko, *Hemidactylus*, showing the distribution of darkly stained (D) and lightly stained (L) areas, Haematoxylin-eosin, $\times 250$.
- FIG. 11. Section of the spleen of the turtle, *Lessemys*, showing the follicular arrangement of the lymphocytes (W). The surrounding area represents the red pulp (R). Haematoxylin-eosin, $\times 250$.
- FIG. 12. Section of the spleen of the snake, *Tropidonotus* showing the white pulp (W) and red pulp (R). Haematoxylin-eosin, $\times 250$.