To the Graduate Council:

I am submitting herewith a dissertation written by Obaid Muhesen Faroon entitled "A Quantitative Evaluation of the Development and Anatomy of the Ovine Spleen and the Hematology and Histopathology of the Ovine Spleen Exposed to Hypoxia." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Animal Science.

Robert W. Henry, Major Professor

We have read this dissertation
and recommend its acceptance:

Accepted for the Council:

Vice Provost
and Dean of The Graduate School

A QUANTITATIVE EVALUATION OF THE DEVELOPMENT AND ANATOMY OF THE OVINE SPLEEN AND THE HEMATOLOGY AND HISTOPATHOLOGY OF THE OVINE SPLEEN EXPOSED TO HYPOXIA

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ABSTRACT

The spleen is an important hematological and immunological organ; however, results of studies are not available on the spleen of the sheep. Therefore, this experiment was designed to study the normal development and structure of the sheep spleen and the effect of hypobaric hypoxia on splenic volume and cellular structure, and blood elements.

The normal development and microanatomy of the spleen of the sheep were studied in 8-age-groups: prenatal; 1-day; 1-, 3-, and 6-weeks; 4- and 5-months; and 2-years of age. Morphometrical data were gathered and tested by statistical analysis of variance. The effects of exposure to hypoxia for 15 days on the spleen and peripheral blood were evaluated in 4-month-old sheep. The morphology of the spleen and the changes in the peripheral blood after exposure to hypoxia were studied and compared with the spleen of sheep kept at normal atmospheric pressure.

The developmental findings in the 8-age-groups were: 1) splenic-weight per body-weight ratio increased from prenatal to 3-weeks of age, then declined; 2) germinal centers were observed after 3-weeks of age; 3) white pulp increased with age and reached its maximum development by

4-months of age; 4) capsule and trabeculae increased in thickness with age; and 5) no megakaryocytes were observed in any age groups.

Histological findings in the spleen of hypoxic sheep were: 1) a significant increase in red pulp volume; 2) multiple nuclei in reticular cells; and 3) an increased thickness of the capsule of the spleen. The hematopoietic results after exposure to hypoxia were: 1) a decrease in platelet sizes; 2) an increase in red blood cell numbers and packed cell volumes; and 3) an initial increase followed by a decrease in platelet numbers. The effects of hypoxia on body growth and splenic weight were: 1) decreased body weight and 2) splenic weight.

The increase in white pulp compartments reflects an increase in the immunity of the animal until the fourth month of life. Hypoxia studies lead to the conclusion that stem cell competition between the megakaryocytic and erythroid cell lines is responsible for the decrease in platelet numbers.

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The spleen is an important hematological and immunological organ and the largest organ of the lymphatic system. It is triangular with rounded angles, and is covered by a serous membrane and capsule. In the sheep its base is dorsal and attached to the left crus of the diaphragm. The parietal surface is convex and the visceral surface is concave and attached to the dorsum of the atrium of the rumen and caudoventral to the diaphragm. It ranges from 12 to 15 centimeters in length and courses obliquely from the ventral end of the last rib to the middle of the tenth intercostal space on the left side. Its width is 7.5 to 10 centimeters (Getty, 1975).

The capsule of the spleen is continuous with the trabeculae which extend into the parenchyma. The trabeculae and capsule consist of smooth muscle cells and collagenous and elastic connective tissue elements. The thickness of the capsule and distribution of its smooth muscle shows species variation (thinnest in carnivores to thickest in the equine species) (Dellmann and Brown, 1981).

The parenchyma consists of white and red pulp. The red pulp contains venous sinuses and splenic cords. It lies between the trabeculae and the white pulp (Banks, 1986). The white pulp is formed of a sheath of lymphatic tissue which surrounds efferent blood vessels (Junqueira and Carneiro, 1980).

The cellular constituents, vascular communications, and histological structure of the spleen have long been subjects of interest much of the earlier studies have been reviewed by Fliedner et al. (1964).

THE MICROANATOMY OF THE SPLEEN

The thick **capsule** of the spleen is overlain by a serous membrane. It has ill-defined layers of connective tissue and smooth muscle whose total thickness and a relative amount of smooth muscle has species variation (Dellmann and Brown, 1981). From the capsule, dense connective tissue **trabeculae** extend into the interior of the organ and anastomose, dividing the spleen into incomplete compartments (Copenhaver et. al, 1978). The fibroblasts are large, flat, branching cells which appear fusiform or spindle-shaped and produce the fibers. The cytoplasm contains rough endoplasmic reticulum and cylindrical mitochondria. Its nucleus is oval or elongated and contains one or two nucleoli (Lesson and

Lesson, 1981). The smooth muscle cells are elongated, tapered, and vary in size. The cytoplasm contains mitochondria, glycogen and small amounts of rough endoplasmic reticulum, and the nucleus is centrally located (Ham and Cormack, 1979).

The **splenic artery**, which enters the hilus of the spleen, is one of the three primary branches of the celiac artery (Getty, 1975). It branches into trabecular arteries which course in the trabeculae. Upon leaving the trabeculae, the new branches are called the arteries of the white pulp. The accumulation of lymphocytes and reticular cells around these arteries is called the white pulp. As the artery of the white pulp passes through the lymph nodule it is called the artery of the lymph nodule or central artery. In the nodule, the central artery gives off several branches which end in the lymphatic nodules and others which terminate in the marginal zone. As the central artery leaves the nodule, it branches into penicillar arterioles (Videbaek et al., 1982) which have three segments: 1) pulp arterioles, 2) sheathed (ellipsoidal) arterioles, 3) terminal capillaries (Dellmann and Brown, 1981). The ellipsoidal arteriolar sheath is composed of lymphocytes, macrophages, reticular cells and fibers. The macrophages often contain phagocytized red blood cells within their cytoplasm. In

the cat, the endothelial cells of the ellipsoidal sheath are surrounded by an incomplete basement membrane (Hatae, 1978), and the endothelial cell filaments are of two diameters (100A° and 60A°). The organization of sheathed arterioles shows species variation, being more prominent in the dog than in the mouse. The endothelial cells of sheathed arterioles of the dog are tall and surrounded by a complete basement membrane (Sakuma, 1968).

The sinuses of the red pulp are a network of multiple vascular channels lined by a basement membrane and reticular cells (Weiss, 1962, 1963). The endothelium of sinusoids and venules varies from oval to elongated with well-defined cellular membranes. The endothelial cells contain numerous mitochondria particularly in the smaller vessels. The cytoplasm of endothelial cells is rich in granules, vacuoles, and cytoskeletal filaments (Weiss, 1962). The nucleus is spindle shaped, contains a large nucleolus, and loosely-arranged nuclear chromatin (Grouls and Helpap, 1982).

What happens to the terminal capillaries has been a controversial topic for the past 90 years (Peck and Hoerr, 1951). Three methods of capillary termination have been discussed. 1) Open circulation, the terminal capillaries open into the splenic cords. For instance in the cat, mouse, pig, ox and horse, the pulp venules originated as

open-ended vessels in the reticular mesh work of the splenic cords, near the trabeculae and drained into trabecular veins (Blue and Weiss, 1981; Banks, 1986). 2) Closed circulation, the terminal arterial capillaries end in the splenic sinuses. From here, the blood drains into venules and into trabecular veins. This type of circulation presumably is found in the dog and man (Banks, 1986); however, both closed and open circulation has been reported in the spleen of man (Kashimura and Fujita, 1987). 3) In rats, both open and closed circulation were observed. The terminal arterial capillaries open into both the splenic cords and sinuses (Chen, 1986).

The bulk of the spleen is made of reticulum, which is a three-dimensional web or spongy network of cells. Weiss (1973) named these constituent cells, reticular cells, and the network the reticulum. Reticular cells can differentiate into fibroblasts that have increased quantities of rough endoplasmic reticulum. When stained with periodic acid-Schiff, reticular fibers of the adult spleen were seen as strands of compact ground substance or matrix (Weiss, 1973). An increase in the amount of reticulum coincides with an increase in fetal size. In early development of the spleen, the reticular cells have an irregular contour with little branching. In older human fetuses, reticular cells are characterized by

slender processes of more than 10 μm in length, and more junctional complexes were found in 57-mm (CRL) human fetuses (Weiss, 1973).

Reticular cells have been classified according to function (phagocytizing and non-phagocytizing cells). Non-phagocytizing reticular cells vary in size, and their oval to fusiform nuclei are pale and poor in chromatin. The nucleus often contains two large nucleoli. Phagocytized material is a characteristic feature of the phagocytizing reticular cells. Typical phagocytized material is expelled erythroblast nuclei (Grouls and Helpap, 1982). Galindo and Imaeda (1962) classified reticular cells as fixed reticular cells, type A and fixed reticular cells, type B. The type A fixed reticular cell had elongated, fusiform extensions, was smaller, and was found primarily in the white pulp. It had an elongated, indented nucleus with uniform chromatin which contained relatively large nucleoli. The nuclei of fixed reticular cells, type B, were larger than type A and were slightly elongated with a smooth nuclear envelope.

A third classification by Roberts and Latta (1964) divided the reticular cells into three types. Type I, thought to be a primitive and multipotential connective tissue cell, had an unindented nuclear margin and large nucleoli. Type II had darker cytoplasm with numerous RNA

granules, a slightly roughened nuclear envelope, and less prominent nucleoli. Type III had dark cytoplasm which contained increased amounts of RNA and was in contact with the basement membrane of the sinuses by long cytoplasmic processes. The nucleus had clumped chromatin and an irregular nuclear membrane.

During developmental phases, continuous change and remodeling of the underlying reticular framework occurs. At birth, only a few immature reticular cells and reticuloblasts are visible around the small vessels which are destined to become the arteries of the lymph nodules. These cells are characterized by electron-lucent karyoplasm and cytoplasm. The cytoplasm contains increased amounts of rough endoplasmic reticulum and numerous ribosomes (Wiersbowsky et al., 1982).

At three to five days of age, the reticular network of the periarterial zone consists of undifferentiated or non-phagocytic reticular cells (Hoefsmit 1975, 1980) and fibroblastic reticular cells that are closely associated with collagen and resemble fibroblasts (Lennert, 1978, cited by Wiersbowsky et al., 1982). Seven to ten days after birth, an increased number of immigrating T-lymphocytes localize around the artery of the lymph nodule and are tightly packed.

The **white pulp** selectively removes lymphocytes from the blood (Gowans, 1962; Porter et al., 1962), supports mitotic division of resident lymphocytes (Kindred, 1940; Billingham, 1962), and is a site of phagocytosis by free macrophages and fixed phagocytic reticular cells. Lymphatic nodules are observed in various forms. The splenic white pulp is similar to the white pulp of lymph nodes and other lymphatic tissues, and it is thymus dependent for both immunological power and as a source of T-lymphocytes (Miller, 1962; Waksman et al., 1962).

The white pulp forms a dense sheath of lymphoid tissue around branches of the trabecular arteries which are destined to become the arteries of the white pulp and arteries of the lymph nodule. The arteries of the lymph nodule branch forming a plexus of slender arteries which supply the white pulp and have no smooth muscle in their walls.

The white pulp is divided into periarterial lymphatic sheaths and lymph nodules (primary and secondary) (Klemperer, 1938; Dellmann and Brown, 1981; Banks, 1986). Each periarterial lymphatic sheath surrounds the artery of the lymph nodule. The periarterial lymphatic sheaths may be enlarged, making up most of the white pulp as in rabbits and rats and not have well-developed lymph nodules and germinal centers (Weiss, 1964) or smaller well-defined

sheaths may be present. The sheath is primarily composed of lymphocytes and a reticular network formed by reticular cells and fibers. Other cells (plasma cells and macrophages) also lie in the network.

Lymph nodules (secondary or primary) are aggregations of lymphocytes located around the artery of the lymph nodule (central artery), which is usually an arteriole (Leeson and Leeson, 1981). The most characteristic histological appearance of a secondary nodule is an aggregation of cells with a central zone of pale-staining cells and an outer dark staining zone of closely packed smaller cells. This central zone was named the germinal center by Flemming in 1885, cited by Fliedner et al. (1964). Flemming observed the high mitotic activity in the germinal center and felt this area was actively forming lymphocytes. The outer darker area is called the corona. In man, germinal centers are not found during uterine life or during the first three weeks after birth. Even with antigenic challenge, no germinal centers were observed by three weeks of age. However, germinal centers developed rapidly after three weeks of age (Barzanji and Emery, 1976, 1978). The absence of germinal centers does not indicate that the spleen is inactive at this period of life (VanFurth et al., 1965; Silverstein and Lukes, 1962).

Comparative histological studies of the spleen of several mammalian species reveal the presence of the clear zone, marginal zone, (Altschul and Hummason, 1947) surrounding the lymph nodule which contains diffuse reticular tissue (Snook, 1950). The marginal zone of the spleen receives terminal segments of the arteriolar plexus from the artery of the nodule (MacNeal et al., 1927; MacNeal, 1929; Weiss, 1963; Moore et al., 1964; Snook, 1980). This arrangement indicates an important intermediate vascular channel within the spleen (MacNeal et al., 1927). Phagocytic reticular cells are concentrated in the marginal zone, and lymphocytic migration from the blood occurs in both the internal and external layers of the marginal zone (Ford and Smith, 1979; Pabst and Geisler, 1981). Macrophages located in the marginal zone provide an effective means of filtration of the various blood elements, in particular red blood cells after their destruction (Weiss, 1962; Moore et al., 1964).

The predominant cell of the white pulp of the spleen is the small lymphocyte. A moderate amount of ribosomes are found in their cytoplasm; however, it has little endoplasmic reticulum (Weiss, 1964). A large nucleus with condensed nuclear chromatin fills most of the cytoplasm, and often a centrally located nucleolus can be observed

(Grouls and Helpap, 1982). Lymphoblasts are found throughout the white pulp and can be differentiated from small lymphocytes by their more abundant cytoplasm which contains a few ribosomes, scant profiles of rough endoplasmic reticulum, large vesicles, large mitochondria with prominent cristae, and a large nucleus (Galindo and Imaeda, 1962). Small, medium, and large lymphocytes are found in the white pulp and are differentiated on the basis of their size.

Plasma cells are observed in the spleen. In the frog (Xenopus laevis), plasma cells are numerous in the white pulp of the spleen and are seen at various stages of maturation. Few plasma cells, however, are seen in the red pulp (Obara et al., 1982).

Macrophages are large cells and an irregular shape. The oval, irregularly shaped nucleus has less condensed, marginated chromatin than lymphocytes. Moderate amounts of elongated, branching rough endoplasmic reticulum are found in the cytoplasmic matrix. The large mitochondria of the cytoplasm contain with well-developed cristae and phagocytic substances (Galindo and Imaeda, 1962).

The **red pulp** is the portion of the spleen found between the trabeculae and white pulp. It contains all of the circulating blood elements. It has a loose texture which is red in color. The framework of the red pulp is

composed of reticulum and reticular cells (Leeson and Leeson, 1981). The red pulp is subdivided into two portions (splenic cords and venous sinuses). The pulp cords are composed of a network of reticular fibers which originate from reticuloendothelial cells and are located between the sinuses (Dellmann and Brown, 1981). These cells have Fc for IgG receptors on their surface and participate in opsonic adherence and phagocytosis (Videbaek et al., 1982). However, the red pulp in the gerbil lacks venous sinuses (Hayes, 1973).

Electron microscopic studies of the rabbit spleen verify the contents of the red pulp (reticulum, reticular cells, and blood elements). The splenic cords communicate through pores with the sinuses. The pores are gaps in the sinus walls which have no basement membrane present. The framework for the sinuses is formed by cytoplasmic projections of the reticular cells (Roberts and Latta, 1964).

Immature granulopoietic cells and expelled free or phagocytized erythroblast nuclei have been noted by researchers, however, the immature sinusoidal system and the immature reticular framework are not prominent. The spleen of the newborn rat has an abundance of immature hematopoietic cells, particularly erythropoietic cells.

In the rat, hemopoietic activity was observed on the 5th and 10th day of postnatal life (Grouls and Helpap, 1982).

Megakaryocytes have been observed in the prenatal and early postnatal spleen. Howell (1890) introduced the term "megakaryocyte" when he used it to define a giant cell in the bone marrow. Since then many investigators have introduced additional information on megakaryocytes in the spleen (Rothermal, 1930). Megakaryocytopoiesis and megakaryocyte morphology in the rat spleen have been documented by McFadden (1967) and Grouls and Helpap (1980). With recent advances in electron microscopy, the ultrastructure of megakaryocytes has been described in the spleen (Yamada, 1957), in the liver (Sorenson, 1963; Paone, 1975), and in bone marrow (Rebuck and Monto, 1957). In the rat's late fetal and early postnatal life, the spleen is considered to be a major site for platelet production (Grouls and Helpap, 1982). The highest concentration of splenic megakaryocytes was observed on the 14th day of postnatal life in rats (Grouls and Helpap, 1980), but splenic megakaryocytopoiesis in the rat decreased with an increase in age. Furthermore, they reported that the life span of platelets is the same in hypersplenism or in normal or splenectomized rats. These findings lead to the conclusion that the spleen has an effect on platelet production rather than on platelet

destruction. However, how the spleen exerts its influence on the peripheral platelet number has not been clarified. Some investigators postulate that a splenic humoral factor acts as a negative feedback on platelet production (Rolovic and Baldini, 1970). Others believe general circulation and splenic platelet pools are in equilibrium (Tanum et al., 1984). In man, two-thirds of the platelet mass circulates and one-third is in the splenic platelet pool with a continual exchange between these compartments. However, in splenomegaly, 50-90% of the total platelet mass is concentrated in the spleen. This phenomenon has been interpreted as platelet production being controlled by the rate of platelet destruction and not by the concentration in the blood (Aster, 1966).

MORPHOMETRIC AND QUANTITATIVE STUDIES

Morphometry is defined as the measurement of structure and was founded on fundamental principles prescribed in mathematical equations (Weibel, 1969; Rohr et al., 1976). Stereology, a subdivision of morphometry, is a procedure for estimating three-dimensional parameters of a structure from measurements made on two-dimensional sections through that structure. Stereology provides estimates of surface area, volume, number of structures, and other parameters that may be derived from simple

counting procedures (Freere 1966). In biology and medicine, quantitative microscopy is the practice of stereology on two dimensional structures obtained using the microscope. When viewing surfaces with transmission electron microscopy, scanning electron microscopy, or an optical microscope, only two planes of three dimensional materials can be seen (Nicholson, 1978). Although stereological measurements have been used for many years in other fields of science, its application in biology was limited until recently. It is now widely used in interdisciplinary areas of research, especially in experimental pathology and developmental anatomy. In addition, it allows physiological and biochemical data to be correlated with morphological data by using morphometrical procedures (Rohr et al., 1976).

Only a few quantitative studies have been conducted on the lymphatic system, particularly the spleen. Postnatal development of lymphatic tissue in young calves was studied by Schultz et al. (1973) and Heilman and Steinbach (1978), cited by Lubis et al. (1982). Lymph node-weight and body-weight ratios were highest in fetuses or young animals, and then the ratio decreased (somewhat irregularly) with age (Lubis et al., 1982). Germinal centers were absent in lymph nodes of premature and newborn infants. However, germinal centers appeared

during the first year, and the number of centers was highest at this age (Luscieti et al., 1980). In the spleen of man, germinal center numbers increase rapidly three weeks after birth (Barzanji et al., 1976). In ducks, the red pulp is relatively prominent at hatching because of the scant development of the white pulp. Almost no periellipsoidal lymphoid tissue was observed, while small areas of periarterial and perivenous lymphoid tissue were present. However, the periellipsoidal lymphoid tissue was noticeably developed at three weeks after hatching and was estimated to be a principal element of the white pulp throughout postnatal life (Hashimoto and Sugimura, 1977).

In clinical and pathological studies, it is important to estimate the volume of the spleen in addition to the volumes of its component parts. Splenomegaly accompanies certain hematological diseases (leukemias, malignant lymphomas, myelofibrosis, and polycythemia vera). Splenomegaly is also found in infectious diseases of various etiologies (viruses, protozoa, other parasites, hereditary conditions such as hemolytic anemia, and inhibited venous return or congestive spleen (Stutte and Heusermann, 1972; Videbaek et al., 1982). Splenomegaly is due to hyperplasia of normal structures or to localized or diffuse infiltration of cellular elements. Hyperplasia of

the white pulp accompanies many infectious and immunological diseases. Thrombocytopenic purpura and lymphoproliferative disease are the most frequently observed examples. Hyperplasia of red pulp is observed in hemolytic anemia and in hereditary spherocytosis; however, hyperplasia of the red pulp occurs to a lesser extent in immune hemolytic conditions (Stutte and Heusermann, 1972; Jensen, 1986a). Malignant lymphoma and Hodgkin's disease are focal infiltration processes that lead to splenomegaly (Videbaek et al., 1982; Jensen 1986a, 1986b).

HYPOXIA

The study of hypoxia is important clinically. Hypoxia (reduced oxygen tension) in body tissues is common in patients with shock, heart failure, myocardial infarction, and pulmonary embolism. Hypoxia is an added stress in these patients and most likely limits circulatory adaptation (Heistad and Abboud, 1980). Space travel and sea diving also have the potential for altering tissue oxygen tension.

Hypoxia produces varied responses by the body. Hypoxia usually produces an increase in red blood cell number, packed cell volume, and concentration of hemoglobin. Hypoxia of longer duration causes a different hematological response than hypoxia of short duration.

For example, the platelet count is biphasic (Jackson and Edwards, 1977) with the initial platelet count increased, followed by a decrease in the count after 6 days (McDonald et al., 1986).

Short duration hypoxia (1-3 days) was shown to increase incorporation of radioisotopes into platelets of mice (Cooper and Cooper, 1977; Shreiner and Levin, 1976) and rats (Jackson and Edwards, 1977), to increase megakaryocyte precursor cell [small acetylcholinesterase positive (SAChE+)] count (McDonald et al., 1986), to increase megakaryocyte size (Jackson and Edwards, 1977), and thus leading to an increase in platelet count (Cooper and Cooper, 1972; Jackson and Edwards, 1977; McDonald, 1978a; McDonald et al., 1978; Shreiner and Levin, 1976). Short term hypoxia of 24 hour duration had no effect on the percentage of ^{35}S incorporation into platelets of mice (McDonald et al., 1978). Shreiner and Levin (1976) observed only a slight increase in platelet counts after 24 hours of exposure to hypoxia. However, McDonald et al. (1986) noticed a significant increase in the platelet count after 3 days of hypoxia. The increase in platelet number was not due to the splenic release of platelets. These increases in platelet numbers cannot be explained by action of a humoral factor since 3 to 4 days are required for thrombopoietin to stimulate immature megakaryocytes

(McDonald et al., 1978). The increase in the number of circulating platelets by hypoxia probably represents an increase in the shedding of platelets by megakaryocytes because of the stress associated with hypoxia (McDonald et al., 1978).

Hypoxia of longer duration (chronic hypoxia) leads to decreased platelet counts in rats (Jackson and Edwards, 1977) and in mice (Birks et al., 1975; Cooper and Cooper, 1977; Langdon and McDonald, 1977) and to a decrease in platelet volume (McDonald et al., 1978). After six days of hypoxia in mice, Shreiner and Levin (1976) and McDonald et al. (1986) observed a significant drop in the platelet count after the initial elevation; however, ^{75}SeM (selenomethionine) isotope incorporation into platelets had decreased to normal levels. It is surprising to have thrombocytopenia with normal platelet production. This decrease in platelet count is thought to be a result of a decrease in platelet production (Cooper and Cooper, 1977), probably caused by stem cell competition between the megakaryocytic and the erythroid lines in the bone marrow (Langdon and McDonald, 1977).

Decreased platelet numbers have been associated with reduced numbers of megakaryocyte precursor cells (SAChE+) (McDonald et al., 1986), decreased isotope incorporation into platelets (Cooper and Cooper, 1977; Langdon and

McDonald, 1977; McDonald et al., 1978), and reduced numbers of megakaryocytes (Jackson and Edwards, 1977; McDonald, 1978). It has been suggested that thrombocytopenia induced by hypoxia cannot be explained entirely by expanding blood volumes (McDonald et al., 1978) or by excess sequestration of platelets by the spleen (Birks et al., 1975; Langdon and McDonald, 1977). Even with a reduction of marrow megakaryocytes, platelet life span remains normal in hypoxic rats (Jackson and Edward, 1977) suggesting that thrombocytopenia is closely linked to decreased platelet production by megakaryocytes.

Decreased platelet volume (25%) has been observed with an increase in platelet age (Detwiler et al., 1962). McDonald et al. (1964) observed that younger platelets were significantly larger than old platelets.

A negative correlation between red blood cell counts and platelet numbers has been documented both experimentally (Birks et al., 1975; Choi and Simone, 1971, 1973; Choi et al., 1974; Langdon and McDonald, 1977; McDonald et al., 1986) and clinically (Gross et al., 1964). The mechanism that controls thrombocytopoiesis is different from that which regulates erythropoiesis (DeGabriele and Penington, 1967). Studies on iron-deficiency anemia and anemia induced by both transfusion of anti-erythrocyte serum and exchange transfusion of

erythrocyte-poor, platelet-rich plasma produced an increased platelet count in rats with inhibited erythroid differentiation (Jackson et al., 1974).

Researchers believe that reduced platelet counts are caused by stem-cell competition between the megakaryocytic and the erythroid cell lines in the bone marrow (Langdon and McDonald, 1977). Changes in the concentration of thrombopoietin or thrombocytopoiesis-stimulating factors (TSF) have been shown not to be a factor in platelet count reduction (McDonald et al., 1979). However, these tests added support to the stem-cell competition theory. McDonald et al. (1979) exposed mice to short-term hypoxia (24 hours) followed by 2 to 3 days at ambient O_2 levels. Incorporation of ^{35}S-sodium sulphate isotope into platelets and platelet numbers were significantly reduced. Significant differences in plasma thrombocytopoiesis-stimulating factor activity between normal mice and mice treated with hypoxia were not detected, suggesting that hypoxia had no effect on thrombopoietin production in mice.

Some of the effects of hypoxia on other peripheral blood elements are increases in red blood cell count (VanLiere and Stickney, 1963), packed cell volume (Lord and Murphy, 1973), and hemoglobin amount (DeGowin et al., 1962). Adaptation to high-altitude hypoxia produces an

increase in the total red blood cell count (Bert, 1882, cited by Kendall et al., 1985). Viault (1892), cited by Moffatt et al. (1964), reported an increase of 2 x 10^6 cells/mm^3 in his own red cell count after a three week trip in the Andes and an increase in erythrocyte counts in guinea pigs. Moreover, an increase in reticulocyte counts was observed in man and the rabbit by Barcroft et al. (1925) during his journey to the Cerro de Pasco (4,390 meters).

In animals exposed to hypoxia equivalent to 6,096 m, Grant and Hudson (1969) showed that the eosinophils in the peripheral blood were increased while the marrow eosinophils underwent severe depletion, particularly the compartments of late band and segmented forms. However, basophils and neutrophils underwent a less dramatic reduction. In secondary hypoxia (5200 m), there was a significant decrease in marrow lymphocyte counts.

Meyer et al. (1935) exposed rats and guinea pigs to low atmospheric pressure. They found a leukocytosis and concluded that leukocytes were discharged from the spleen, liver, and lung under these atmospheric conditions. No consistent changes were found in the differential blood count. Cress et al. (1943) subjected rats to hypobaric, anoxic, and hemic hypoxia. They observed an increase in leukocyte counts in the peripheral blood and concluded

that hypoxia caused an excitation of the sympathetic-adrenal system. They assumed that adrenaline exerts its effect on the bone marrow directly.

Hurtado et al. (1945) investigated white blood cell counts in Peruvian natives who lived at high altitudes. They found a nonsignificant increase in the total number of leukocytes in chronic hypoxia.

Studies by Fisher and Langston (1967) showed that lowered oxygen tension increased erythropoietin titers in the blood, but no change in erythropoietin was observed if kidneys were perfused with blood of normal oxygen tension.

Splenectomized C_3H mice placed in hypoxia chambers show decreased platelet counts and decreased platelet production when compared to splenectomized ambient air control mice (Langdon and McDonald, 1977). Mice exposed to hypoxia and given RAMPS injections exhibited marked inhibition of platelet production. Studies on platelet counts are contradictory. Some studies show a reduction in the platelet count in mice (Shreiner and Levin, 1973; Birks et al., 1975), in rats (DeGabriele and Penington, 1967), and in the Rhesus monkey (Garvey et al., 1975). Other studies report no significant changes (Evatt et al., 1975).

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II. LIGHT MICROSCOPY OF THE DEVELOPING OVINE SPLEEN

INTRODUCTION

The spleen of the sheep is a triangular-shaped organ located in the abdominal cavity. Its base is dorsal and attached to the left crus of the diaphragm. The parietal surface of the spleen is convex, whereas the visceral surface is concave and attached to the left craniodorsal surface of the atrium of the rumen. It ranges from 12 to 15 cm in length and courses obliquely from the ventral end of the last rib to the middle of the tenth intercostal space. Its greatest width is 7.5 to 10 cm (Getty, 1975).

A serous covered capsule surrounds the spleen, and trabeculae continuous with the capsule extend into the parenchyma. The capsule and trabeculae consist of smooth muscle cells and collagenous and elastic connective tissue elements (Leeson and Leeson, 1981; Banks, 1986). The thickness of the capsule and the distribution of its smooth muscle varies from species to species (thickest in the horse, intermediate in ruminants, and thinnest in carnivores) (Dellmann and Brown, 1981).

The parenchyma of the spleen is composed of red and white pulp. The red pulp, which lies between the trabeculae and the white pulp, contains venous sinuses and splenic cords (Dellmann and Brown, 1981; Banks, 1986).

The splenic (Billroth) cords consist of a network of reticular cells and fibers with macrophages and circulating blood elements (Kessel and Kardon, 1979). In contrast, the white pulp is formed of a sheath of lymphatic tissue which surrounds efferent blood vessels (Junqueira and Carneiro, 1980).

At birth the red pulp is the predominate tissue of the spleen. The white pulp compartments develop at different postnatal time periods (Williams and Nossal, 1966; Pettersen and Rose, 1968). In 1-day-old rats a few lymphocytes were observed surrounding small arterioles; at 9 days of age the marginal zone was first observed; and at 2 weeks fully developed thymus-dependent areas were identified (Veerman, 1975). In man even with infection, the splenic germinal centers were first seen at three weeks of age (Barzanji and Emery, 1978; Barzanji et al., 1979).

In man, splenic weight develops in a linear pattern with age until birth (Barzanji and Emery, 1979). In rats, the weight of the spleen increases in a linear pattern between 4 and 30 months of age. Fewer cells, however, can be recovered from 30-month-old spleens even though spleens of old animals are heavier than those of young animals (Cheung and Nadakavukaren, 1983).

In spite of the fact that the sheep has been used as a model for human diseases, no extensive investigation has been performed on the developmental changes in the spleen of the sheep. The sheep is a nice research model because of its intermediate size, availability, and cost. This study was conducted to clarify the structural and the quantitative changes that occur in the spleen with advancing age.

MATERIALS AND METHODS

Thirty-three mixed breed sheep representing eight age groups [prepartum (143rd day of gestation), n = 6; one day, n = 4, one week, n = 4; three weeks, n = 6; six weeks, n = 3; four months, n = 3, five months, n = 4; and two years of age, n = 3] were used in these studies. Spleens were surgically removed after infiltration of 5 - 15 ml of 2% lidocaine hydrochloride into the proposed line of incision (1-2 cm caudal to the left last rib). The splenic artery and vein were ligated, and the spleen was removed. The spleens were weighed and volumes determined by the fluid displacement method. Histological samples were taken from the dorsal, middle, and ventral portions of each spleen, fixed in 10% buffered formalin, dehydrated in a graded ethanol series, and embedded in JB-4 plastic (Polysciences, Inc., Warrington, PA) (Appendix A). Three

micron sections were cut, mounted on glass slides and stained with hematoxylin and eosin. The microanatomy of each specimen was examined with the aid of an Olympus compound microscope equipped for bright-field light. Photomicrographs were taken with an Olympus C-35A camera in conjunction with the microscope.

The general linear model of regression analysis from Statistical Analysis System (SAS) software in conjunction with an IBM 3081 computer were used to conduct statistical analysis.

RESULTS

Microanatomy

Prepartum: The capsule and trabeculae were well-organized with several layers of smooth muscle cells and fibroblasts embedded in collagenous fibers. The red pulp occupied the major portion of the parenchyma of the spleen. Large venous spaces were present, but they contained no cellular elements. The splenic cords consisted of a network of reticular cells and fibers dispersed between the sinuses of the red pulp. Peripheral blood elements (basophils, eosinophils, lymphocytes, monocytes, and neutrophils) and macrophages were observed. No megakaryocytes were noted. The white pulp was limited to small lymphatic sheaths around the major blood vessels

(Figure 2-1). Each sheath consisted of a few cellular layers composed primarily of lymphocytes between reticular cells. No splenic nodules were observed.

One Day: The capsule and trabeculae were similar to those observed in the prepartum spleen. No remarkable changes were observed in the red pulp. However, the large venous sinuses of the red pulp were less numerous than those of the prepartum spleen. Similar numbers of peripheral blood elements and macrophages were observed. However, no megakaryocytes were observed. In earliest postnatal life, the white pulp consists of periarterial lymphatic sheaths formed of several layers of small lymphocytes (Figure 2-2). While similar in structure to the sheaths observed in the prepartum spleen, these are more numerous at this time. However, no splenic nodules were observed.

One Week: The capsule and trabeculae appeared increased in thickness when compared to prepartum and 1-day-old spleens. The red-pulp region maintained its dominance in the field. The venous sinuses were further reduced in number, and some were filled with peripheral blood elements (Figure 2-3), but no megakaryocytes were seen. Large and well-organized periarterial sheaths of lymphocytes were observed in the white pulp, but well-developed splenic nodules were not found.

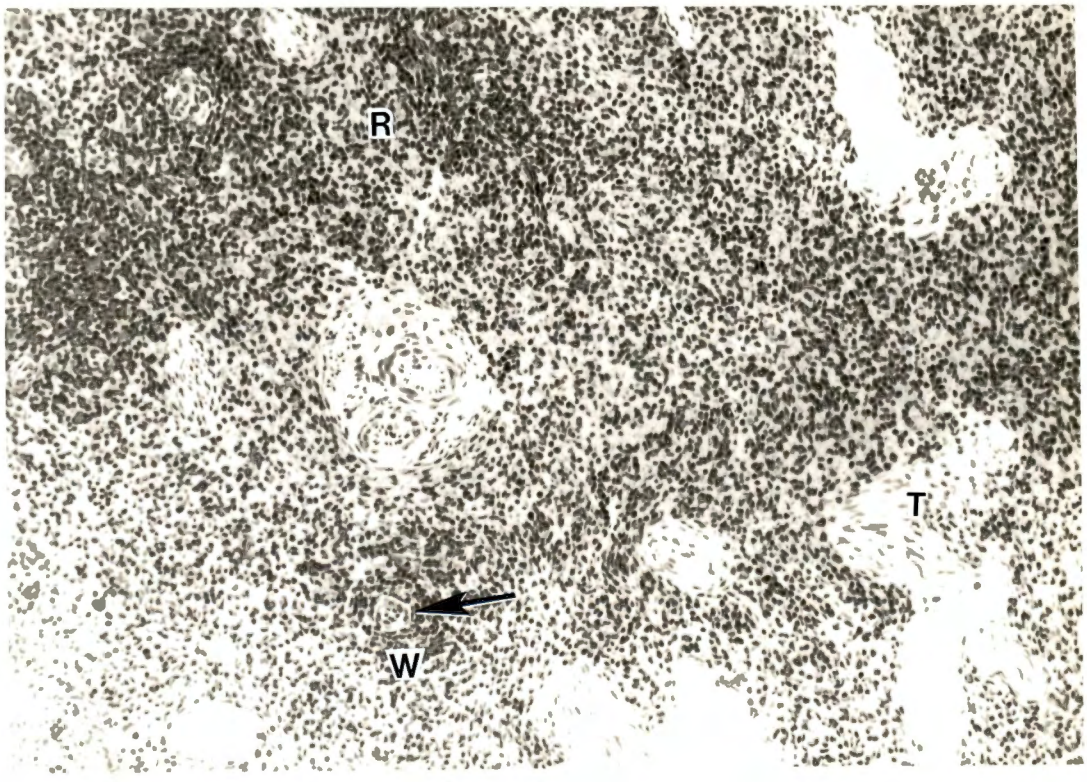

Figure 2-1 Light micrograph of prepartum sheep spleen. The red pulp (R) is well developed, and the trabecular system (T) can be observed. The white pulp (W) consists of a few cell layers (periarterial sheath) surrounding the artery of the white pulp (arrow). Stained with hematoxylin and eosin. X132

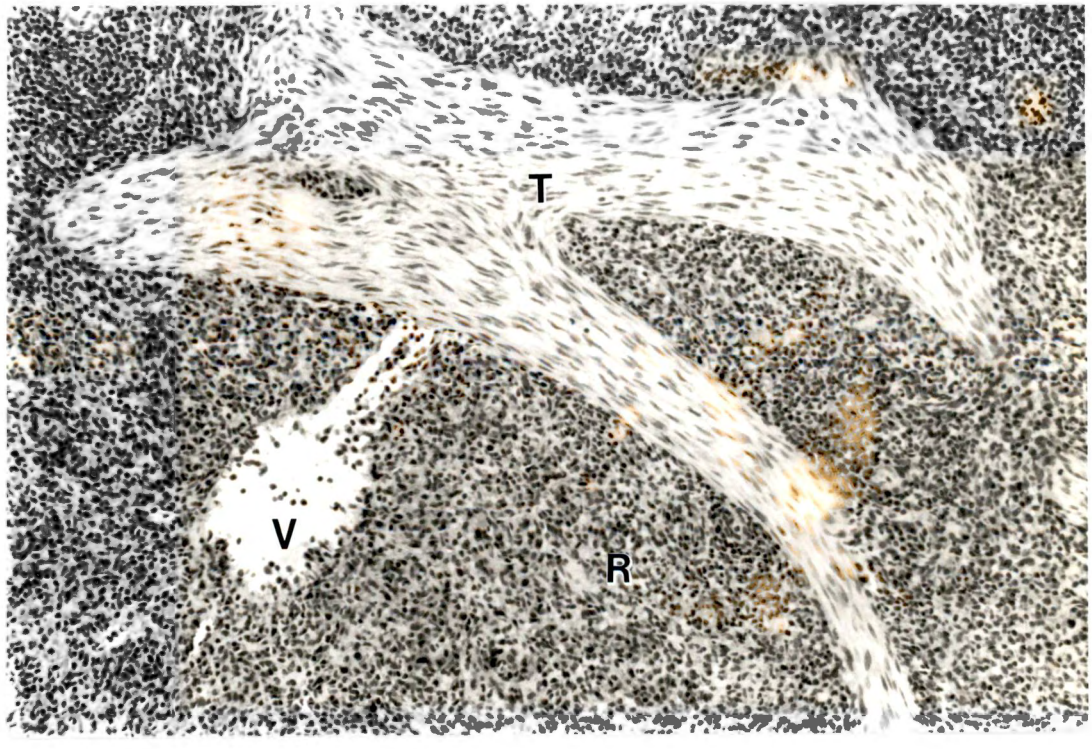

Figure 2-3 Light micrograph of 1-week-old sheep spleen. The venous sinus (V) contains blood cells. Trabecula (T), Red pulp (R). Stained with hematoxylin and eosin. X132.

Three weeks: When compared with those of less mature spleens, the capsule and trabeculae were observed to be slightly thicker. The spleen was now highly cellular in nature. The sinuses of the red pulp were filled with peripheral blood elements, but no megakaryocytes were observed. In the white pulp, the lymphoid tissue had developed large and compact nodules. Each nodule was formed of large, pale-staining lymphocytes with large rounded nuclei containing a prominent nucleolus. However, distinct and well-developed germinal centers were not recognized.

Six Weeks: The capsule and trabeculae were larger and consisted of multiple layers of smooth muscle fibers, collagenous fibers, and associated fibroblasts. Occasionally one or two muscle cells were observed surrounded by the red pulp which was filled with cellular elements. Again, no megakaryocytes were observed. The white pulp was fully developed and represented a significant portion (about 23%) of the splenic parenchyma. Large lymphatic nodules this time with well-developed germinal centers were observed. The cells of the pale-staining germinal centers were primarily large lymphoblasts with large, pale-staining nuclei or mitotic figures. The corona or cortex surrounded each germinal center and was a cuff of small lymphocytes with darkly

stained pachychromatic nuclei. The lymphatic nodules were surrounded by reticular cells and marginal sinuses and contained paracentrally located arterioles.

Four Months: The capsule and trabeculae were slightly thicker than in the 6-week-old sheep, but the red pulp appeared less cellular. All peripheral blood elements were observed in the red pulp, but no megakaryocytes were observed. The white pulp with its well defined germinal centers has not changed.

Five Months: The capsule and trabeculae were slightly thicker and the red pulp was less cellular than that of the 4-month-old spleen. Peripheral blood cells and macrophages were observed; however, no megakaryocytes were observed. The white pulp was dominated by large, mature lymph nodules each with a germinal center (Figure 2-4).

Two Year: The capsule and trabecular system were the thickest of all ages studied and about twice as thick as seen in the peripartum group. The sinusoidal system of the red pulp appeared dilated and splenic cords appeared less cellular than any of the other groups studied (Figure 2-5). The macrophages of the sinusoidal system were filled with hemosiderin pigments. Peripheral blood cells were observed but as at other time points no

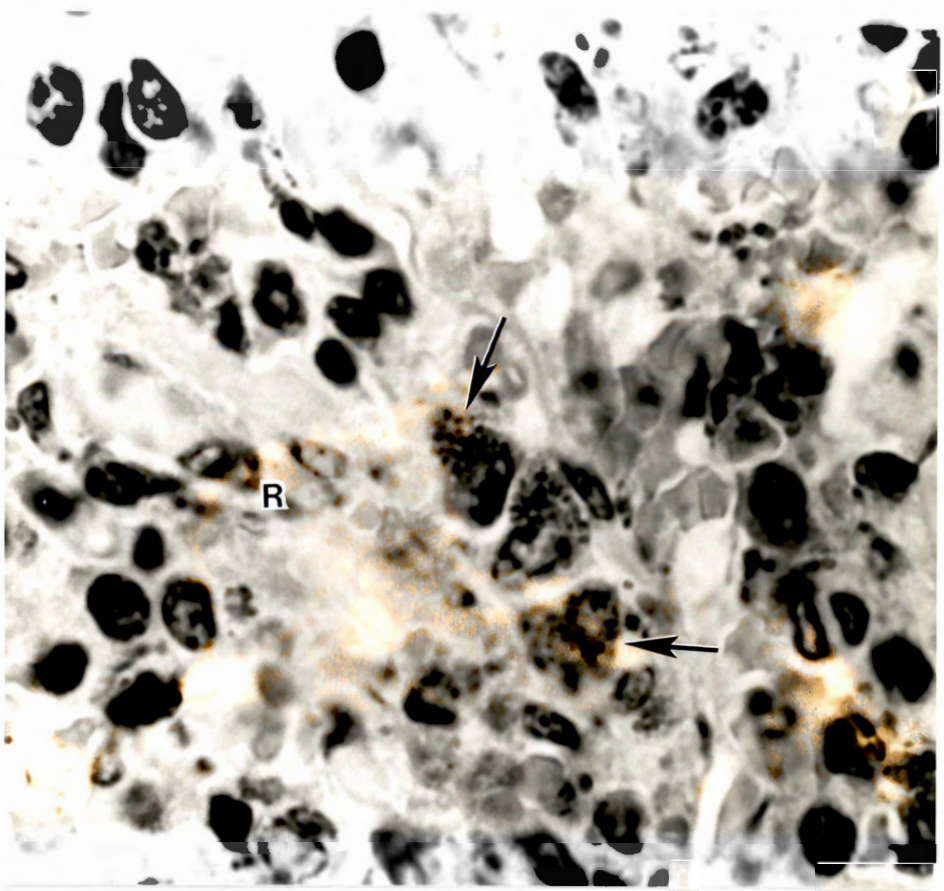

Figure 2-5 Light micrograph of 2-year-old sheep spleen. Note the decreased cellularity in the red pulp (R). The macrophages contain hemosiderin pigments (arrows). Stained with hematoxylin and eosin. X330.

megakaryocytes were observed. The white pulp in contrast had small lymph nodules with less cellular germinal centers.

Quantitative Studies

A summary of weight and volume data is presented in Table 2-1. The spleen of the sheep progressively increased in weight and volume with age. At birth the spleen did not undergo reduction in volume. A linear relationship was observed between the splenic weight and animal weight (Figure 2-6). Linear regression analysis of these data results in the model:

animal weight = a (splenic weight) + b

where: a = 633.144 and b = -1.1615;

n = 33, r^2 = 0.935, s = 7.934.

Plots of the splenic-weight to body-weight ratios (Figure 2-7) and splenic-volume to body-weight ratios (Figures 2-8) show the highest ratios in 3-week-old animals.

Table 2-1 **Summary table for changes in mean body weight (MBW), mean splenic weight (MSW), splenic-weight/body-weight ratio (SW/BWR), and mean splenic volume (MSV) with advance in age of sheep.**

Age	No. of Animals	MBW[a] (kg)	MSW[a] (gm)	SW/BWR[a] x 100	MSV[a] (ml)
prepartum	6	3.6 ±1.2	4.1 ±1.8	0.11 ±0.02	4.1 ±1.7
one day	4	3.0 ±0.8	4.3 ±1.3	0.14 ±0.02	4.8 ±1.6
one week	4	4.4 ±1.5	8.6 ±2.7	0.20 ±0.02	8.6 ±2.8
three weeks	6	9.1 ±2.5	21.1 ±8.2	0.23 ±0.04	21.0 ±8.4
six weeks	3	12.2 ±1.2	24.0 ±2.5	0.20 ±0.01	23.3 ±2.5
four months	3	34.4 ±2.7	63.0 ±3.0	0.18 ±0.01	63.0 ±2.0
five months	4	45.0 ±5.7	76.5 ±12.2	0.17 ±0.02	75.9 ±10.2
two years	3	106.3 ±1.7	155.3 ±38.6	0.15 ±0.04	152.7 ±36.1

[a] ± standard deviation

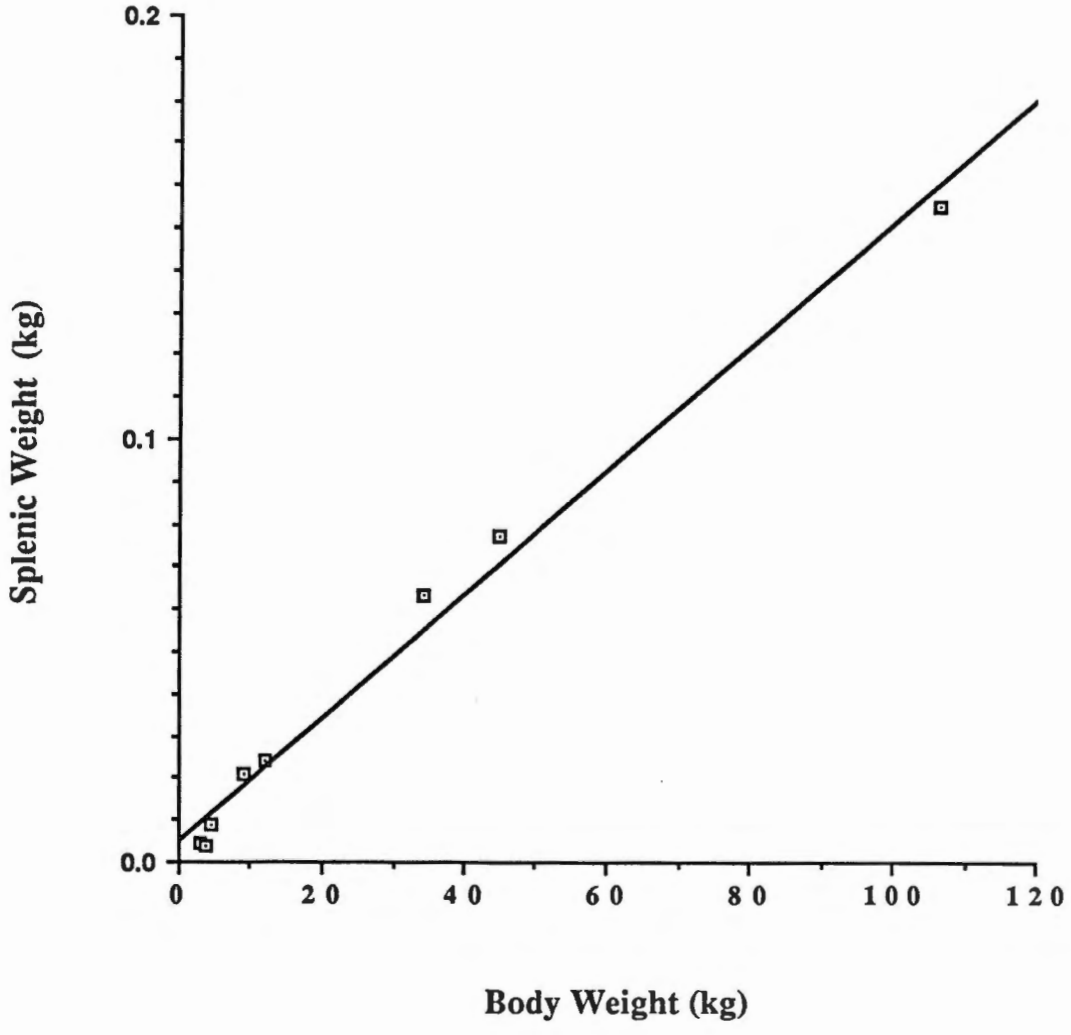

Figure 2-6 A linear regression plot of splenic-weight versus body-weight. Equation noted in text.

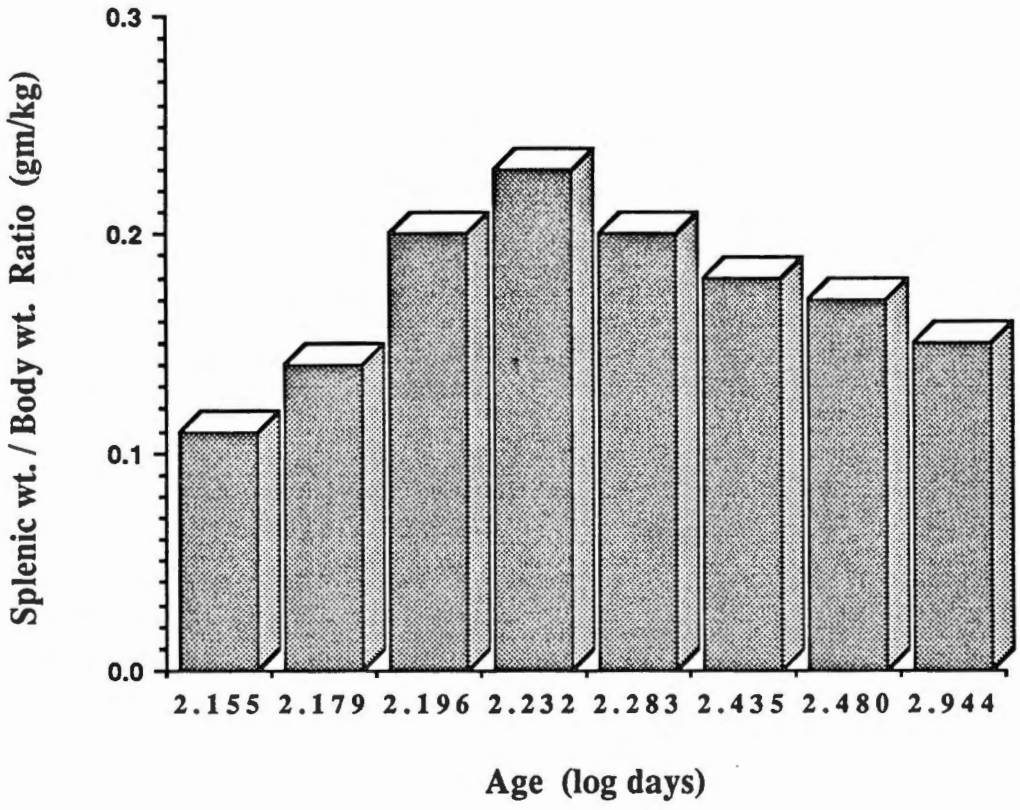

Figure 2-7 A histogram of splenic-weight to body-weight ratio versus age.

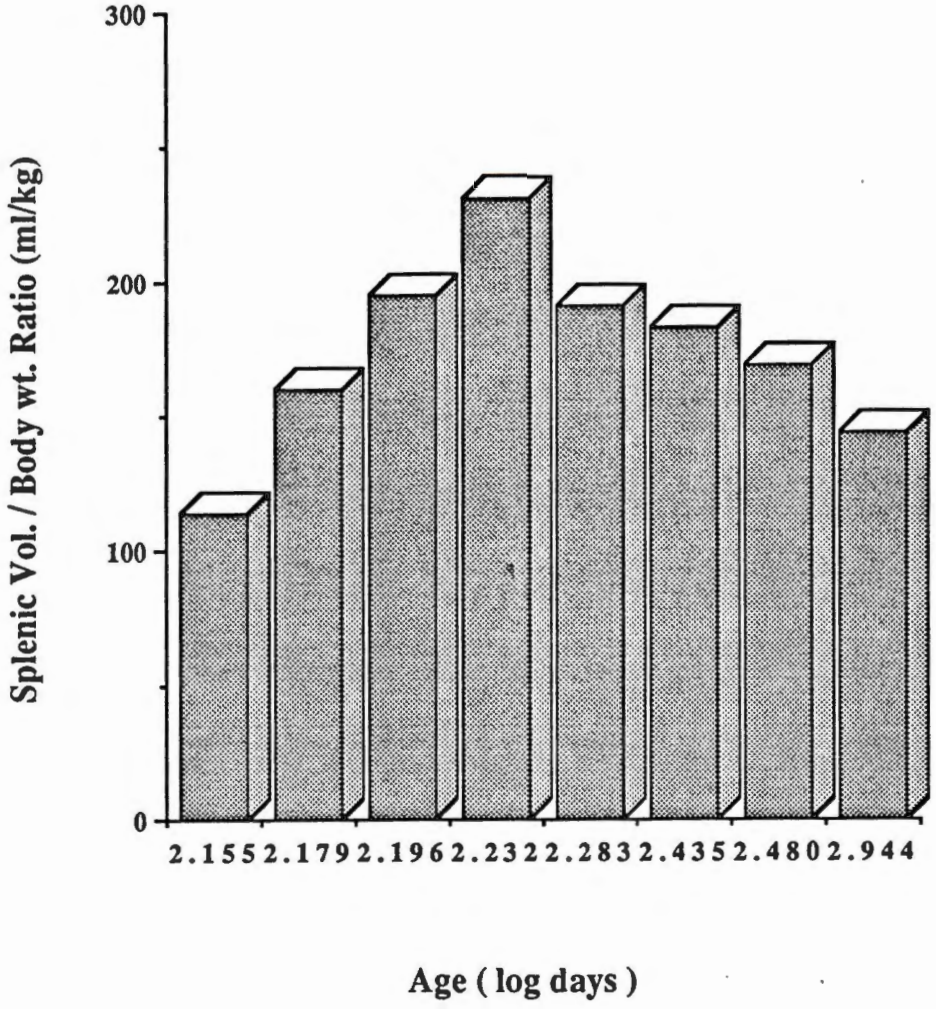

Figure 2-8 A histogram of splenic-volume to body-weight ratio versus age.

DISCUSSION

The capsule and trabeculae increased in their thickness progressively with age. This structural elaboration is due to an increase in both the muscular and connective tissue components. This increase in thickness is to support the increase in volume and weight of the developing spleen and to enhance the contractability of the spleen for ejection of large quantities of blood into the circulatory system. A correlation between the effectiveness of the release of blood and the quantity of smooth muscle in the trabeculae has been reported by vonHerrath (1935), cited by Hartwig and Hartwig (1985).

In the study reported here, splenic weight increased progressively with age (Table 2-1). However, when splenic-weight to body-weight ratios were plotted with age, the ratio increased from prepartum life (one week before birth) until three weeks of age, then the ratio declined (Figure 2-7). Hashimoto and Sugimura (1977) using splenic-weight to body-weight ratios in ducks, and Lubis et al. (1982) used lymph node-weight to body-weight ratios in cattle reported findings similar to this study. Barzanji and Emery (1979) reported for humans a steady increase in the splenic weight throughout gestation and until the fourth week of postpartum life. However, they did not compare splenic-weight to body-weight ratio with

age which appears a more appropriate method for evaluation of splenic development.

In the present study, no loss in splenic weight was observed following parturition. In contrast, Barzanji and Emery (1979) reported for humans a minor weight loss in the spleen directly following birth. This loss was explained by loss of blood from the sinuses rather than a loss in parenchymal lymphoid tissue. This suggests that the apparent decrease in sinus numbers in the one-day-old spleen, observed in this study, is probably artifactual because of expulsion of blood into the circulation and blood loss at parturition.

In the sheep, the cellularity of the spleen decreased after three weeks of life. This finding is consistent with the work of Cheung et al. (1981) and Cheung and Nadakavukaren (1983) on rats. They recovered less cellular elements from thirty-month-old than from four-month-old rat spleens. An age related decline in weight and lymphoid cellularity has been observed in gut-associated lymphoid tissue of mice (Chanana et al., 1973) and in the thymus of rats (Colley et al., 1970). While most of the lymphocytes in the young and neonatal spleens were T-lymphocytes, the proliferative activity of T-lymphocytes declines with age (Cheung et al., 1981) probably due to the decreased regulation by the thymus

(Luscieti et al., 1980). This suggests that the decline in lymphoid organ weight is primarily due to the decline of the T-cell system.

Germinal centers were not observed in the spleens of prepartum sheep nor during the first three weeks of postpartum life. The absence of germinal centers is similar to the results found by Barzanji and Emery (1979) in man. This does not suggest, however, that the spleen does not participate in immunological activity. Silverstein and Lukes (1962) and VanFurth et al. (1965) observed immunoglobulin-reacting cells in the spleen of early human fetuses.

Megakaryocytes were not observed in any age-groups examined in this study. In contrast, megakaryocytes were observed in the spleen of postnatal cats (Faroon and Henry, 1987) and rats (Grouls and Helpap, 1982). Absence of megakaryocytes in the sheep spleen is attributed to species variation. These results suggest that the spleen of the sheep does not play an active role in megakaryocyte and platelet production in the age groups examined.

In this investigation, small to moderate amounts of yellow to brown hemosiderin pigments were observed in the macrophages of the red pulp of the spleen. Jones and Hunts (1983) indicated that these pigments normally occur in the red pulp as a result of the break down of

hemoglobin. However, large quantities of hemosiderin pigments is a sign of passive chronic congestion due to cirrhosis of the spleen. However, histopathological study did not reveal any sign of cirrhosis of the spleens examined, therefore, the amounts of these pigments are considered to be within normal limits. The pigments were prominent because of the low cellularity of the red pulp of two-year-old spleens.

In summary, the thickness of the capsule and trabeculae increased progressively with age. The cellularity of the red pulp increased until three weeks of age and then declined thereafter. Peripheral blood cells were observed in the red pulp of the spleen, however, no megakaryocytes were observed in any of the age groups studied. The lymphoid sheath of the white pulp increased in thickness around the blood vessels with age. However, well defined germinal centers were not observed until three weeks of age. Less cellular splenic nodules and periarterial sheaths were observed in adult (two-year-old) sheep.

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III. MORPHOMETRY AND QUANTIFICATION OF THE OVINE SPLEEN

INTRODUCTION

Few quantitative studies have been conducted on the lymphatic system, particularly on the spleen. Postnatal development of lymphatic tissue in calves was studied by Schultz et al. (1973) and Heilman and Steinbach (1978), cited by Lubis et al. (1982). Lymph node-weight and body-weight ratios were highest in fetuses or young animals, and then the ratio decreased with age (Lubis et al., 1982). Germinal centers were absent in lymph nodes of premature and newborn infants. However, germinal centers appeared during the first year, and the number of centers was highest at this age (Luscieti et al., 1980). In the spleen of man, germinal center numbers increase rapidly in the three weeks after birth (Barzanji et al., 1976). In ducks, the red pulp is relatively prominent at hatching because of the scant development of white pulp elements. Almost no periellipsoidal lymphoid tissue was observed, while small areas of periarterial and perivenous lymphoid tissue were present. However, the periellipsoidal lymphoid tissue was noticeably developed at three weeks after hatching and was estimated to be a major component of the white pulp throughout postnatal life (Hashimoto and Sugimura 1977).

In clinical and pathological studies, it is important to estimate the volume of the spleen as well as the volume of its components. Splenomegaly accompanies certain hematological diseases, including leukemia, malignant

lymphoma, myelofibrosis, and polycythemia vera. Splenomegaly is also found in infectious diseases of various etiologies, such as viruses, protozoa, other parasites, hereditary conditions such as hemolytic anemia, and inhibited venous return or congestive spleen (Stutte and Heusermann, 1972; Videbaek et al., 1982). Splenomegaly is due to hyperplasia of normal structures or to localized or diffused infiltration of cellular elements. Hyperplasia of the white pulp accompanies many infectious and immunological diseases. Thrombocytopenic purpura and lymphoproliferative disease are the most frequently observed examples. Hyperplasia of the red pulp is observed in hemolytic anemia and in hereditary spherocytosis. However, hyperplasia of the red pulp occurs to a lesser extent in immune hemolytic conditions. Malignant lymphoma and Hodgkin's disease are focal infiltration processes that lead to splenomegaly (Videbaek 1982; Jensen 1986a, 1986b).

In patients with thalassemia, heredity spherocytosis, and heredity elliptocytosis anemias, it was hard to distinguish morphologically between their spleens. However, quantitative microscopic data offers a great opportunity to understand the role of the spleen in these three anemias (Bosman and Cavaliere, 1967).

In health and disease or under various physiological changes of an organ, it is important to find the volume fraction of a structure within an organ. For instance, the volume ratio of white to red pulp in the normal spleen differs from the ratios found in the spleens of patients

with leukemia, polycythemia, portal hypertension, and severe hemorrhage. Sometimes investigators would like to compare the volume of malignant tissue to that of the original parenchyma (Elias, 1971). Morphometry of normal tissue ratios can help answer this question.

The objective of this study was to quantify the structural development of the spleen in sheep from prepartum to adulthood. These data will serve as a basis for correlation with developing splenic function and a normal data base for comparing splenic abnormalities.

MATERIALS AND METHODS

Spleens were collected from three mixed breed sheep in each of eight age groups (prepartum, 1 day, 1 week, 3 weeks, 6 weeks, 4 months, 5 months, and 2 years). Splenic volume was determined by fluid displacement. Random samples were taken from the dorsal, middle, and ventral regions of each spleen, fixed in 10% buffered formalin, and processed for methacrylate embedment (JB4 plastic, Polysciences, Inc., Warrington, PA) (Appendix A). Five-micron-thick sections were prepared, mounted on slides, and stained with hematoxylin-eosin.

For quantification a 100-point eye-piece reticule was placed on the microscope and the tissues were examined at 100X magnification. Point counts were performed on red pulp, white pulp, capsule and trabeculae, and large blood vessels. The number of points that fell on each component was counted. Total-point counts were determined by summing the points falling on each substructure in the

spleen (Appendix B). The volume fraction of each component was estimated by dividing the points that fell on that component by the total number of points counted over all sections per spleen (Appendix C). The absolute volume of a given component was calculated as the fractional volume of that component multiplied by the splenic volume.

The thickness of the capsule was measured on projected images of slides using a video overlay onto the X-Y digitizing tablet of a Ziess Videoplan Image Analysis System.

General linear model (GLM) analysis of variance and the t-test were used in these experiments to analyze the data.

RESULTS

Development

The results of the morphometric analysis are shown in Tables 3-1 and 3-2. Splenic volume increased progressively with age. The white pulp fractional volume increased progressively with age and reached maximum development in four-month-old lambs ($P<0.05$); then white pulp fractional volume declined with advancing age. Histological examination revealed the decrease in white pulp fractional volume was accompanied by decreased white pulp cellularity in the adult animals (Figures 3-1, 3-2).

TABLE 3-1 **Volumetric parameters of splenic red pulp, white pulp, trabeculae, blood vessels, and capsular thickness.**

Age	Mean Splenic Volume (ml)	Mean Red Pulp Volume Fraction (%)	Mean White Pulp Volume Fraction (%)	Mean Trabecular Volume Fraction (%)	Mean Blood Vessel Fraction (%)	Mean Capsule Thickness (μm)
prepartum	4.1 ± 1.7	71.0 ± 6.3	15.0 ± 6.3	10.8 ± 2.2	3.2 ± 2.0	87.7 ± 13.4
one day	4.8 ± 1.6	71.0 ± 7.0	17.0 ± 6.8	10.0 ± 4.0	2.0 ± 1.0	122.9 ± 38.5
one week	8.6 ± 2.8	72.0 ± 3.1	17.0 ± 3.3	10.0 ± 2.1	1.0 ± 0.5	124.2 ± 34.0
three weeks	21.4 ± 8.4	73.0 ± 3.6	17.0 ± 4.0	9.0 ± 2.2	1.0 ± 0.6	179.9 ± 31.0
six weeks	23.3 ± 2.5	69.0 ± 7.0	23.0 ± 10.0	7.0 ± 3.3	1.0 ± 1.0	244.0 ± 51.7
four months	63.0 ± 2.0	58.0 ± 4.0	32.0 ± 5.0	8.0 ± 3.0	2.0 ± 1.0	215.9 ± 56.5
five months	75.9 ± 10.2	63.0 ± 5.2	25.0 ± 7.0	10.0 ± 4.1	2.0 ± 0.9	207.8 ± 37.6
two years	152.7 ± 36.1	69.0 ± 9.0	14.0 ± 9.4	15.0 ± 8.0	2.0 ± 1.2	480.7 ± 162.1

TABLE 3-2 The absolute volumes of red pulp, white pulp, trabeculae & capsule, and blood vessels

Age	Absolute Blood Vessel Volume (ml)	Absolute Red Pulp Volume (ml)	Absolute White Pulp Volume (ml)	Absolute Trabecular & Capsular Volume (ml)
prepartum	0.1	2.9	0.6	0.4
one day	0.1	3.4	0.8	0.5
one week	0.1	6.2	1.5	0.9
three weeks	0.2	15.7	3.5	2.0
six weeks	0.3	16.1	5.3	1.6
four months	1.1	36.7	19.0	5.3
five months	1.4	48.0	19.9	7.4
two years	2.8	105.0	21.4	23.4

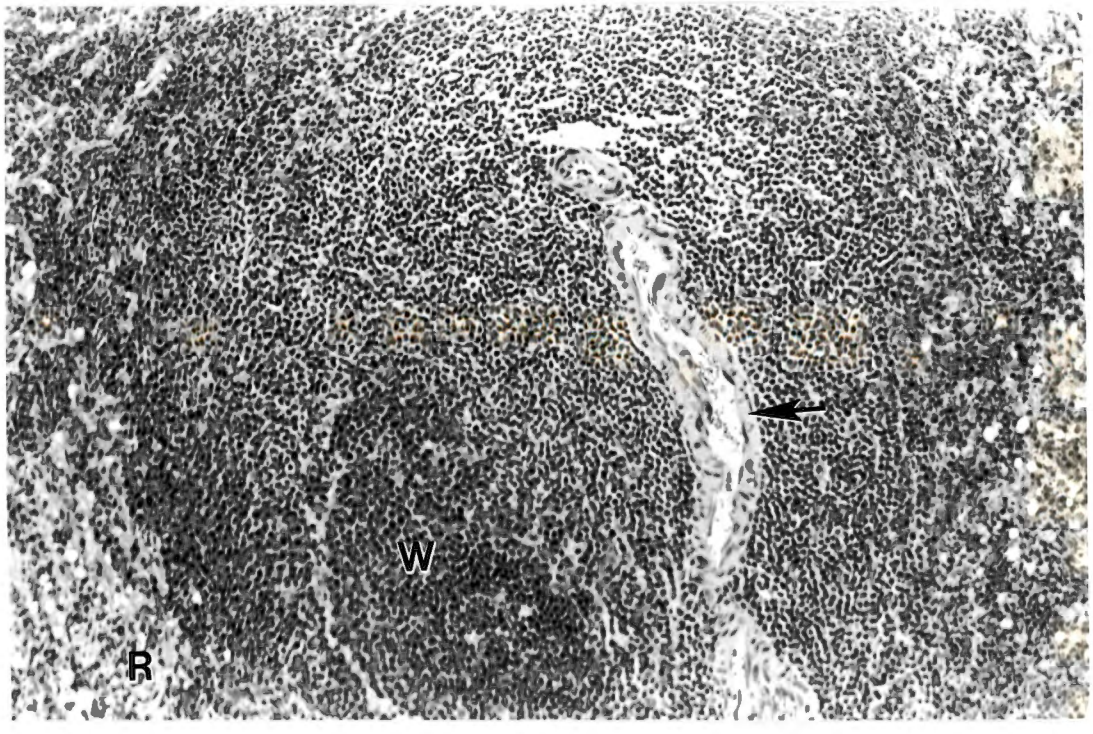

Figure 3-1 Light micrograph of a splenic nodule of the white pulp (W) with its central artery (arrow) of the 4-month-old sheep spleen. Red pulp (R). Hematoxylin and eosin stain. X132.

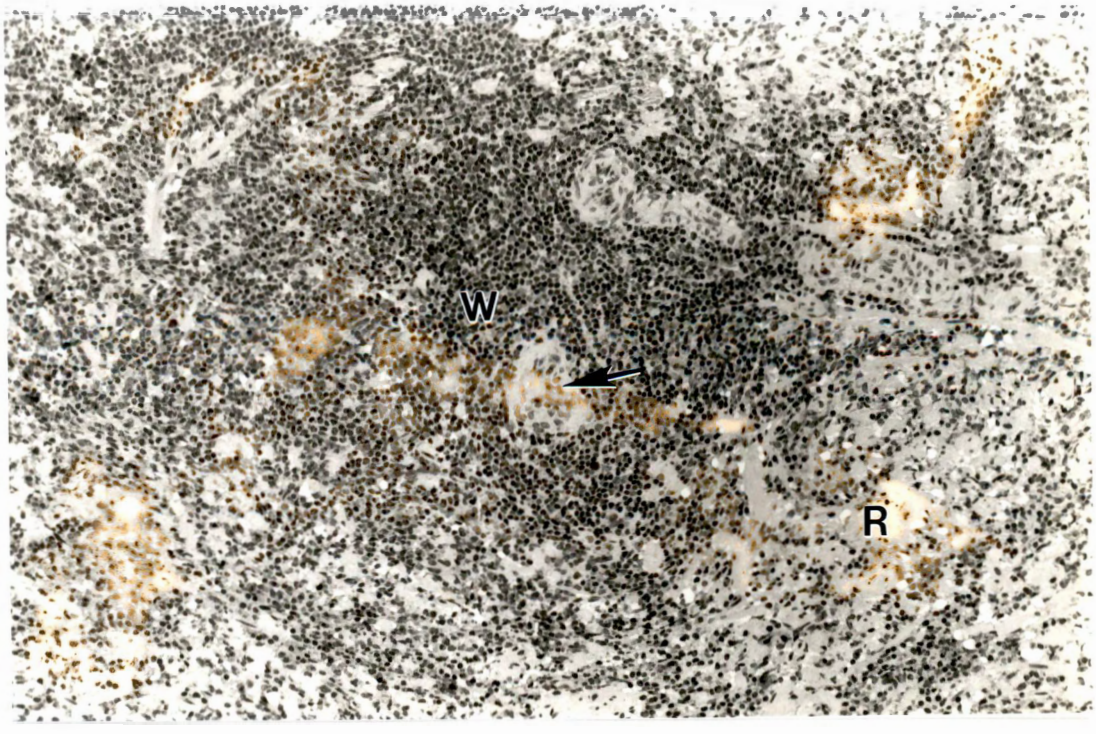

Figure 3-2 Light micrograph of a splenic nodule of the white pulp (W) with its central artery (arrow) of the 2-year-old sheep spleen. The nodule is much smaller than that of the four-month-old sheep. Red pulp (R). Hematoxylin and eosin stain. X132.

Red pulp was the dominant component in all spleens examined in this study. It occupied 71% of the volume of the spleen in prepartum animals. However, the fractional volume of the red pulp decreased significantly ($P<0.001$) with advancing age through four months of age.

The volume fraction of trabeculae increased progressively with age ($P<0.05$). The maximum value was found in adult sheep. Blood vessel fractional volume showed no significant changes ($P>0.05$) during the age periods of this investigation. The thickness of the capsule reached its maximum value of 480 μm at two years of age. The absolute volume of blood vessels, red pulp, white pulp, capsule, and trabeculae increased significantly ($P<0.05$) with age (Table 3-2).

DISCUSSION

In this investigation, the rapid growth of lymphoid tissue in the spleen occurred from prepartum until four months of age in the sheep and then subsequently declined. This phenomenon has been observed also in man. In this investigation the maximum development of the white pulp was reached around four months of age; however, Lubis et al. (1982) stated that the lymphoid tissue and Peyer's patches reached the highest value at six months in bovine species. This discrepancy between our finding and that of

Lubis et al. (1982) probably is due to species variation or the type and location of the lymphoid organ.

Banks (1986) classified the spleen of the ruminant as an intermediate form between the storage (large amounts of smooth muscle and trabeculae) and defensive (a high percentage of lymphatic tissue with a decreased percentage of capsule and trabeculae) types of the spleen. In this investigation, the red pulp, capsule and trabeculae of the spleen formed more than two thirds of the volume of the spleen. As a result,it is suggested that the spleen of the sheep fits better into the storage type, rather than the intermediate type as classified by Banks (1986).

The progressive decrease in red pulp fractional volume from prepartum until adulthood can be explained by the expansion of fractional volumes of the white pulp and trabeculae at the expense of red pulp fractional volume.

The trabecular system of the sheep spleen was found to be well-developed in the prepartum and one day after birth. This is clearly earlier than that reported in rat spleens, where the trabecular system develops after birth. The thickest trabecular system was observed in the adult animals. The increase in thickness was due to an increase in both smooth muscle cells and connective tissue elements. The thick trabeculae and capsule increase support to the architecture of the large spleen and

increase the spleen's capacity to squeeze blood from its sinusoidal reservoir system when it is needed.

The absolute volume of the splenic components (blood vessels, red pulp, white pulp, capsule, and trabeculae) increased. This probably is due to the fact that splenic volume increased progressively with age.

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IV. ULTRASTRUCTURE OF THE ADULT OVINE SPLEEN

INTRODUCTION

The spleen is the largest lymphatic organ. It consists of a connective tissue and smooth muscle-containing capsule and incomplete trabeculae as well as a highly vascular parenchyma (Dellmann and Brown, 1981) The parenchyma is subdivided into two structural regions, white pulp and red pulp. The capsule consists mainly of smooth muscle cells, connective tissue fibers and fibroblasts. The inner surface of the capsule is lined by endothelial cells, while the external surface of the capsule is lined by mesothelial cells (Burke and Simon, 1970). Trabeculae composed of smooth muscle cells, collagen, and elastic fibers extend from the capsule into the parenchyma (Dellmann and Brown, 1981).

The white pulp forms a sheath of dense lymphatic tissue around extra-trabecular branches of trabecular arteries. These branches are the arteries of the white pulp and arteries of the lymph nodule (central arteries).

The white pulp is divided into periarterial lymphatic sheaths and lymph nodules (primary or secondary) (Klemperer, 1938; Dellmann and Brown, 1981; Banks, 1986). Lymph nodules are denser accumulations of lymphocytes and are located around the artery of the lymph nodule (central

artery), which is usually an arteriole (Leeson and Leeson, 1976). Each periarterial lymphatic sheath coaxially surrounds the artery of the lymph nodule. The periarterial lymphatic sheaths may be enlarged, making up the majority of the white pulp as in rabbits and rats and lack well-developed lymph nodules and germinal centers (Weiss, 1964) or the sheaths may be smaller well-defined units. The sheath is composed predominantly of lymphocytes and a reticular network formed by branched reticular cells, extracellular fibers, and other cells (plasma cells, macrophages) that lie in the reticular network.

Lymph nodules are accumulations of lymphoid tissue around the artery of the lymph nodule. Secondary lymph nodules are divided into a central germinal center and an outer corona. In man, germinal centers are not found during uterine life or during the first three weeks of postnatal life. Even with antigenic challenge, the spleen has developed no germinal centers by three weeks of age. However, after three weeks of age, germinal centers develop rapidly (Barzanji and Emery, 1978). The absence of germinal centers is not an indication that the spleen is inactive in this period of life (VanFurth et al., 1965; Silverstein and Lukes, 1962).

The marginal zone, identified in several mammalian species, is a clear zone surrounding the secondary nodule (Altschul and Hummason, 1947; Blue and Weiss, 1981a; Saitoh et al., 1982; Sasou et al., 1986). It consists of a dense reticular network forming irregular vascular spaces (marginal sinuses) which intercommunicate (Snook, 1950; Weiss, 1974). Several investigators report that the marginal zone of the spleen receives the terminal segments of the arteriolar plexus which arise from the artery of the nodule (MacNeal et al., 1927; MacNeal, 1929; Weiss, 1963; Moore et al., 1964; Snook, 1980).

Small lymphocytes are numerous and are the predominant cell in the white pulp of the spleen. However, plasma cells, reticular cells, platelets, red blood cells and macrophages are also observed (Galindo and Imaeda, 1962; Obara et al., 1982; Tablin and Weiss, 1983).

Reticular cells synthesize the reticular network (Weiss, 1973). When stained with periodic acid-Schiff, reticular fibers of the adult spleen are seen as strands of compact ground substance or matrix (Weiss, 1973). Reticular cells have been classified according to function (phagocytizing and non-phagocytizing cells). Non-phagocytizing reticular cells vary in size. Phagocytized material is a characteristic feature of the cytoplasm of the phagocytic type cell. Typical phagocytized material

is expelled erythroblast nuclei (Grouls and Helpap, 1982). The nucleus is oval to fusiform, pale, poor in chromatin, and often contains two large nucleoli.

The ellipsoidal arteriolar sheath is composed of lymphocytes, macrophages, reticular cells, and fibers. In the cat, the filaments of the endothelial cells of the ellipsoidal sheath are of two diameters (100A° and 60A°). The endothelial cells are surrounded by an incomplete basement membrane (Hatae, 1978). Species-related differences have been reported in the organization of sheathed arterioles. This sheath is more prominent in the dog than in the mouse. In the dog, the endothelial cells of sheathed arterioles are tall and surrounded by a complete basement membrane (Sakuma, 1968). The endothelium of sinusoids and venules ranges from oval to elongated in shape with well-defined cellular membranes. The nucleus is spindle shaped with a large nucleolus. The nuclear chromatin is loosely arranged (Grouls and Helpap, 1982).

The red pulp is the area localized between the white pulp and trabeculae. It contains all elements of the circulating blood, is loose in texture and is red in color. The framework of the red pulp is reticulum and reticular cells (Leeson and Leeson, 1976). The red pulp is divided into splenic cords and venous sinuses. The

splenic cords are compact cellular elements located between the sinuses (Dellmann and Brown, 1981). The pulp cords are composed of a network of reticular fibers originating from reticuloendothelial cells. These cells have Fc for IgG receptors on their surface and participate in opsonic adherence and phagocytosis (Videbaek et al., 1982). Other cellular elements (red blood cells, neutrophils and eosinophils are found in the cords.

Electron microscopic studies on the rabbit spleen confirmed the composition of the red pulp: reticular cells, reticulum, and blood elements. The splenic cords communicate with the sinuses of the red pulp through pores. These pores represent breaks in sinus walls with no basement membrane present. The sinuses also intercommunicate. The cytoplasmic projections of the reticular cells formed a framework for the sinuses (Roberts and Latta, 1964). The red pulp in the gerbil, however, lacked venous sinuses (Hayes, 1973).

The venous sinuses of the red pulp were described by Weiss (1962, 1963) as a system of multiple vascular channels lined by endothelial cells, basement membrane and reticular cells. The endothelial cells contain numerous mitochondria particularly in the smaller vessels. The

cytoplasm of endothelial cells is rich in granules, vacuoles, and cytoskeletal filaments (Weiss, 1962).

The microcirculation of the spleen has remained a subject of controversy for the past century. Three theories have been proposed: 1) The open theory indicates that the arterial capillaries open directly into splenic cords and then the blood flows into the venous sinuses; 2) The closed theory indicates that the arterial capillaries open directly into the venous sinuses; and 3) The third theory indicates that both open and closed circulation exist (Chen, 1978). Banks (1986) reported that the closed theory exists in man, the rat and the dog, while open circulation is a characteristic of the mouse, cat, ox, horse and pig. However, Kashimura and Fujita (1987) found that both open and closed circulation exist in man.

MATERIALS AND METHODS

Spleens were surgically removed from three 2-year-old sheep after infiltration of 15 ml of 2% lidocaine hydrochloride into the proposed line of incision (1-2 cm caudal to the left last rib). The splenic artery and vein were ligated and the spleens were removed. Tissue samples were collected from the dorsal, middle, and ventral portions of each spleen. Minute pieces of each spleen

were fixed in 3% glutaraldehyde in 0.1M sodium cacodylate buffer (pH 7.4) for 2 hours. Fixed specimens were then washed three times for 10 minutes each with a sodium cacodylate buffer (0.1M, pH 7.4) prior to postfixation in 1% OsO_4 at 4°C for 90 minutes. The postfixed tissue was dehydrated in a graded ethanol series, cleared in propylene oxide, and embedded in Epon 812. Thick sections were cut, mounted on glass slides, stained with toluidine blue, and were used for thin section orientation. Silver colored thin sections (70 - 80 nm) were prepared with a LKB ultramicrotome, mounted on copper grids, and stained with uranyl acetate and lead citrate. Specimens were viewed with a Phillips model 201 electron microscope operated at 70 kv, and the ultrastructure of the spleen was recorded.

RESULTS

Histologically, the spleen of the sheep consists of a capsule, trabeculae, white pulp, and red pulp.

Capsule

The splenic capsule consisted primarily of fibroblasts, smooth muscle fibers, and collagen fibers (Figure 4-1). Each fibroblast was elongated and spindle-shaped with a large nucleus bordered by a nuclear envelope of an irregular thin margin of heterochromatin. There was

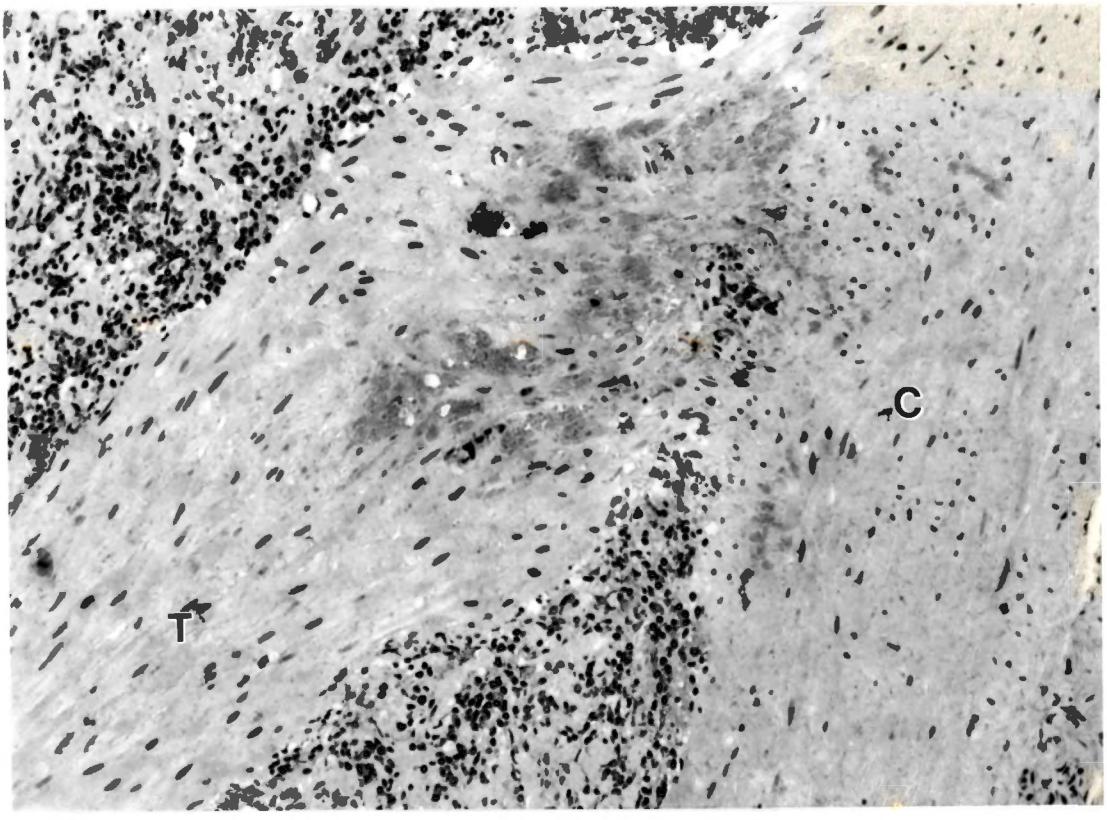

Figure 4-1 Light micrograph of capsule (C) and trabecula (T) of the spleen of the sheep. Hematoxylin and eosin stain. X160

usually a small centrally located nucleolus. The cytoplasm contained well-developed rough endoplasmic reticulum and bundles of tropocollagen.

The smooth muscle cells associated with the capsule and trabeculae were fusiform and surrounded by a thin basal lamina. Heterochromatin formed a thin band near the nuclear envelope, while the euchromatin occupied the center of the nucleus. The nucleus usually contained at least one nucleolus.

The outer surface of the capsule was covered by a single layer of flattened mesothelial cells. The cytoplasm was thin and attenuated on either side of the nucleus and contained a few cytoplasmic granules. The nuclei were oval to rounded, centrally located, and bulged toward the free surface of the spleen. The inner surface was lined by flattened endothelial cells that separated the capsule from the subcapsular sinus.

Trabecula

Trabeculae of varied thicknesses arose from the inner surface of the capsule and extended well into the parenchyma of the spleen. The predominant cells of the trabeculae, like the capsule, were smooth muscle fibers and fibroblasts. On cross-section, the collagen fibers appeared to be concentrated in the center of the trabeculae (Figure 4-2), while the cellular elements were

arranged around the periphery. Variable amounts of collagen fibers were also observed between the cellular elements.

Nonmyelinated nerve fiber bundles, separated from the sinus by thin, cytoplasmic extensions of endothelial cells, were seen in the trabecular system (Figure 4-3). Each bundle of several fibers was separated from the others by collagenous septa. All fiber bundles were separated by connective tissue septa. The diameter range of the axons was 0.85-1.27 μm. The fibers contained granular materials and small, rounded mitochondria.

White Pulp

The white pulp, randomly distributed in the parenchyma, consisted of two major portions, the periarterial lymphatic sheath and the lymphatic nodule. The periarterial lymphatic sheath consisted of a highly cellular zone around small arterial blood vessels (Figure 4-4). The lymph nodule was an enlargement of the white pulp around the arterial blood vessel which was normally paracentrally located (Figure 4-5). The main elements of the white pulp were reticular cells which formed a network around the artery of the white pulp with small to medium-sized lymphocytes trapped in the reticular web (Figure 4-6). Macrophages were in close contact with lymphocytes

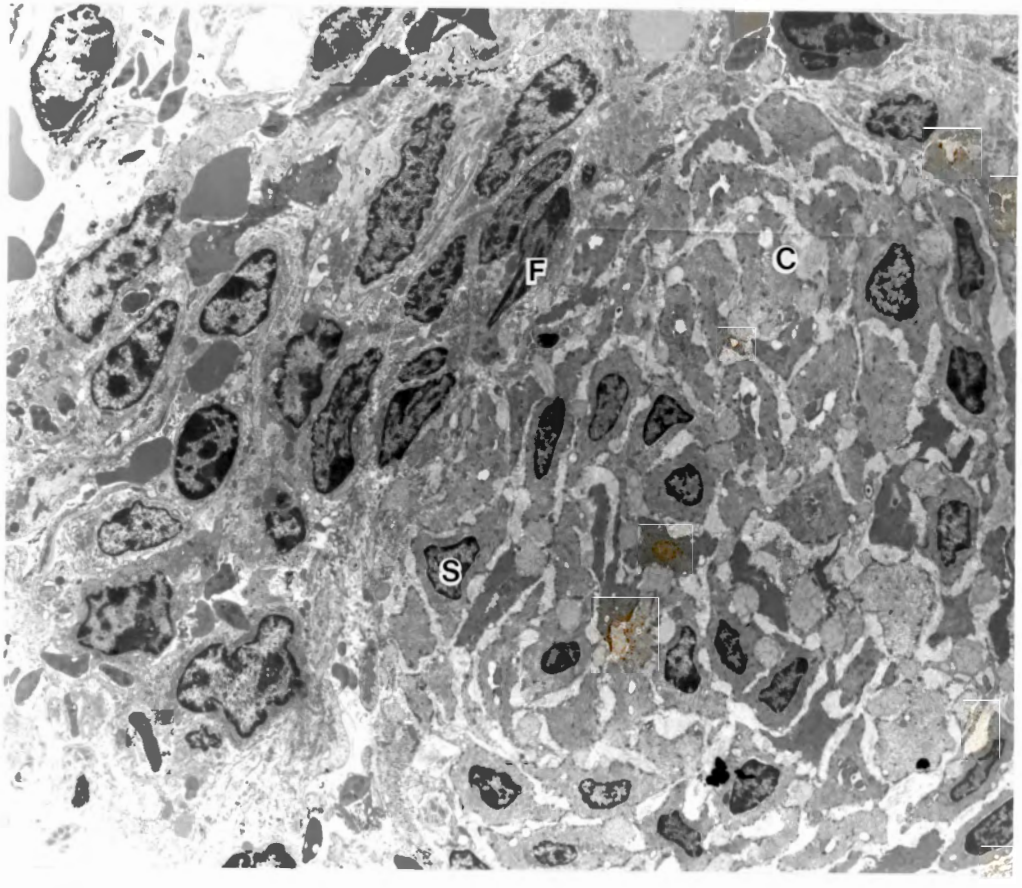

Figure 4-2 Electron micrograph of cross-section of a trabecula. Note smooth muscle cells (S), fibroblasts (F) and collagenous fibers (C). Stained with uranyl acetate and lead citrate. X3700

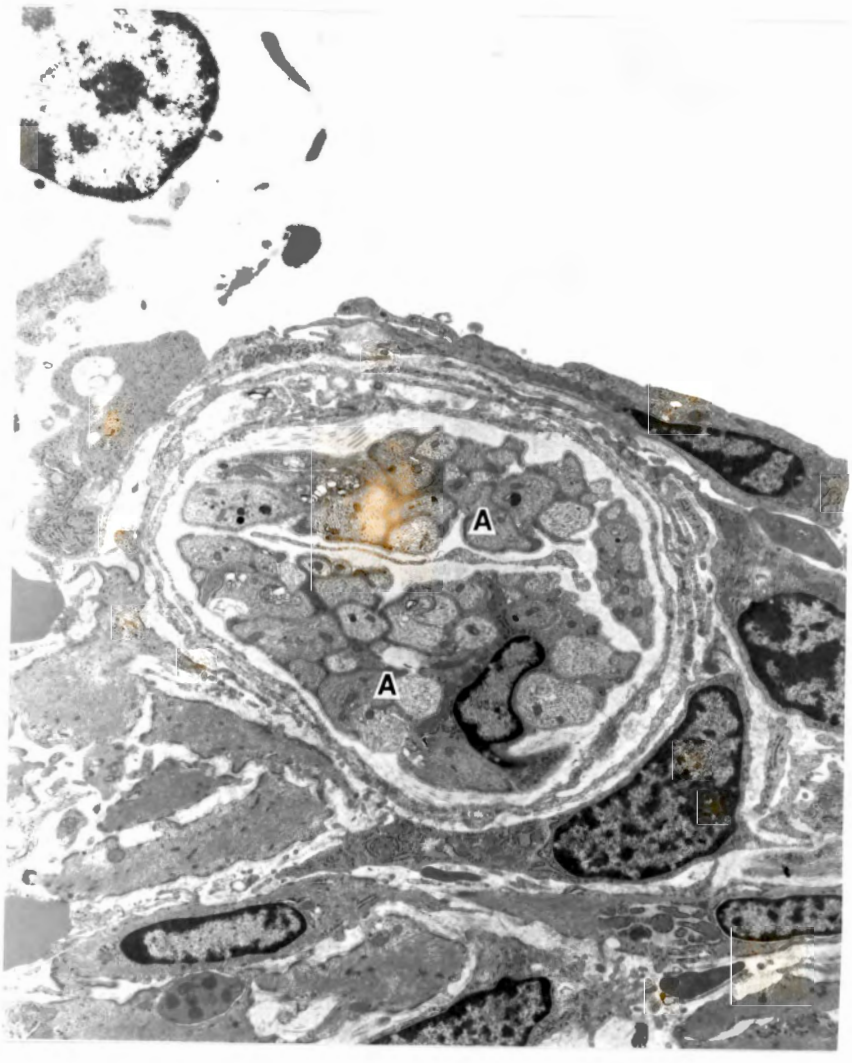

Figure 4-3 Electron micrograph of a cross-section of an nonmyelinated nerve bundle in a trabecula with numerous fibers (A). It is separated from the red pulp sinus by a thin wall. Stained with uranyl acetate and lead citrate. X9000

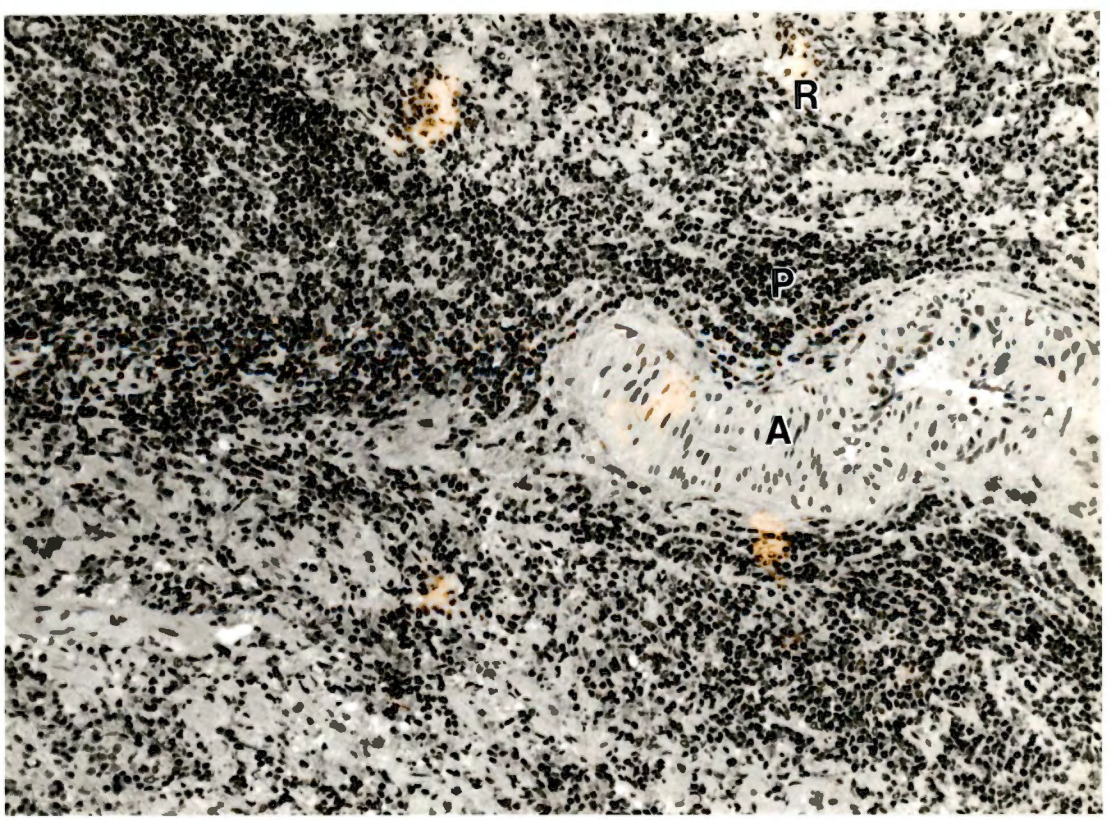

Figure 4-4 Light micrograph of a periarterial lymphatic sheath (P) of the white pulp. Note the aggregation of lymphoid tissue around the artery (A) of the white pulp. The red pulp (R) is less cellular. Hematoxylin and eosin stain. X160.

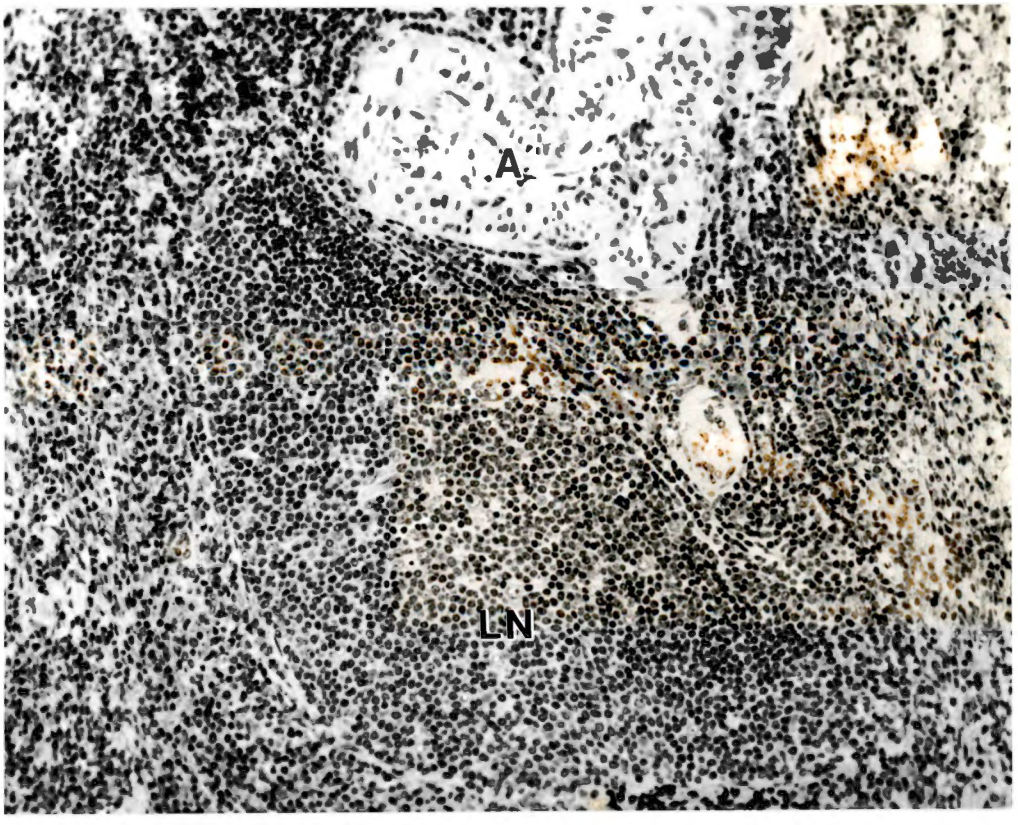

Figure 4-5 Light micrograph of lymph nodule (LN) of the white pulp. The arterial blood vessel (A) is located paracentrally. Hematoxylin and eosin stain. X160

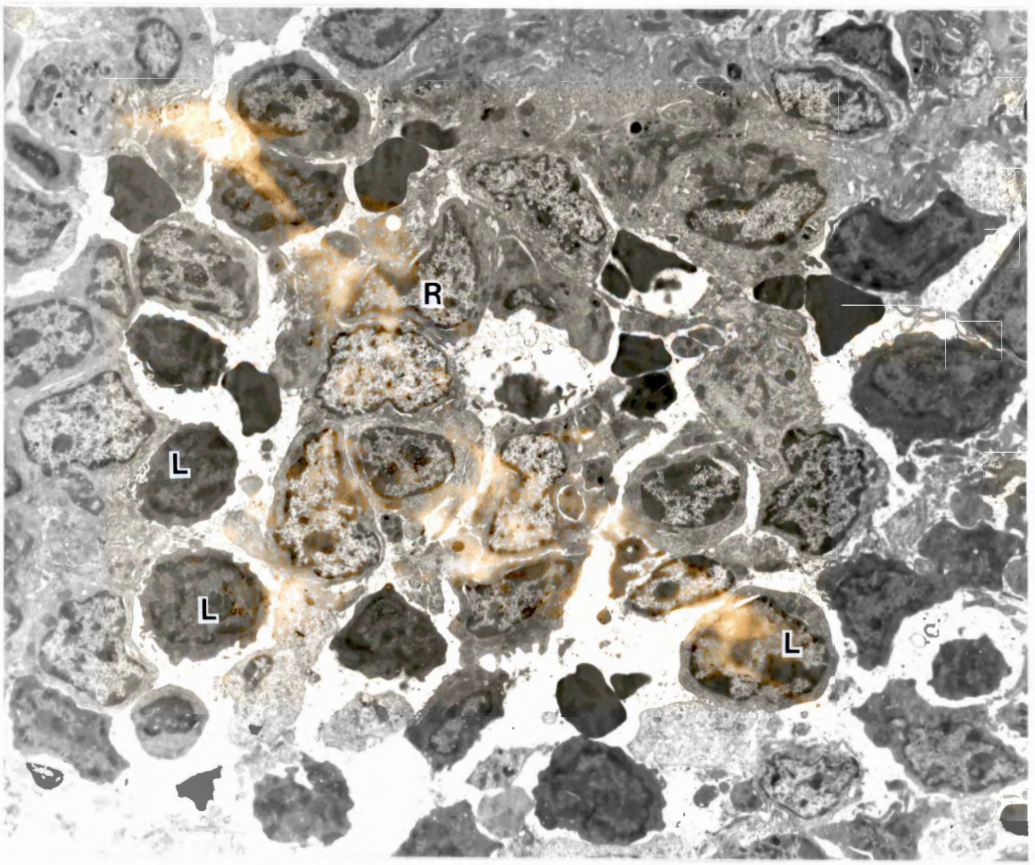

Figure 4-6 Electron micrograph of the white pulp of the spleen of the sheep. The lymphocytes (L) and reticular cells (R) are the prominent cellular constituents. Stained with uranyl acetate and lead citrate. X3400

and reticular cells. However, other cellular elements such as plasma cells, granulocytes, macrophages, red blood cells and platelets were also seen. The arterial wall consisted of endothelial cells, which bulged into the arterial lumen, and a few layers of smooth muscle cells.

Small lymphocytes with a high ratio of nucleoplasm to cytoplasm were the predominate cells of the white pulp (Figure 4-7). The heterochromatin density of the rounded nuclei was highest near the nuclear envelope and diminished gradually toward the center, where a single prominent nucleolus was located. The cytoplasm contained a few rounded mitochondria with prominent cristae, but few other organelles. Lymphocytes were occasionally observed in close relationship with macrophages.

Reticular cell nucleoplasm-to-cytoplasmic ratio was less than that of lymphocytes (Figure 4-8). There were moderate amounts of rough endoplasmic reticulum and mitochondria of various shapes and sizes.

The cytoplasm extended in a three-dimensional pattern forming the characteristic processes of the reticular cell. The nuclei had one or two prominent nucleoli and were generally elongated to oval in shape, irregularly marginated, and often deeply indented. The chromatin was less condensed than that of lymphocytes and was

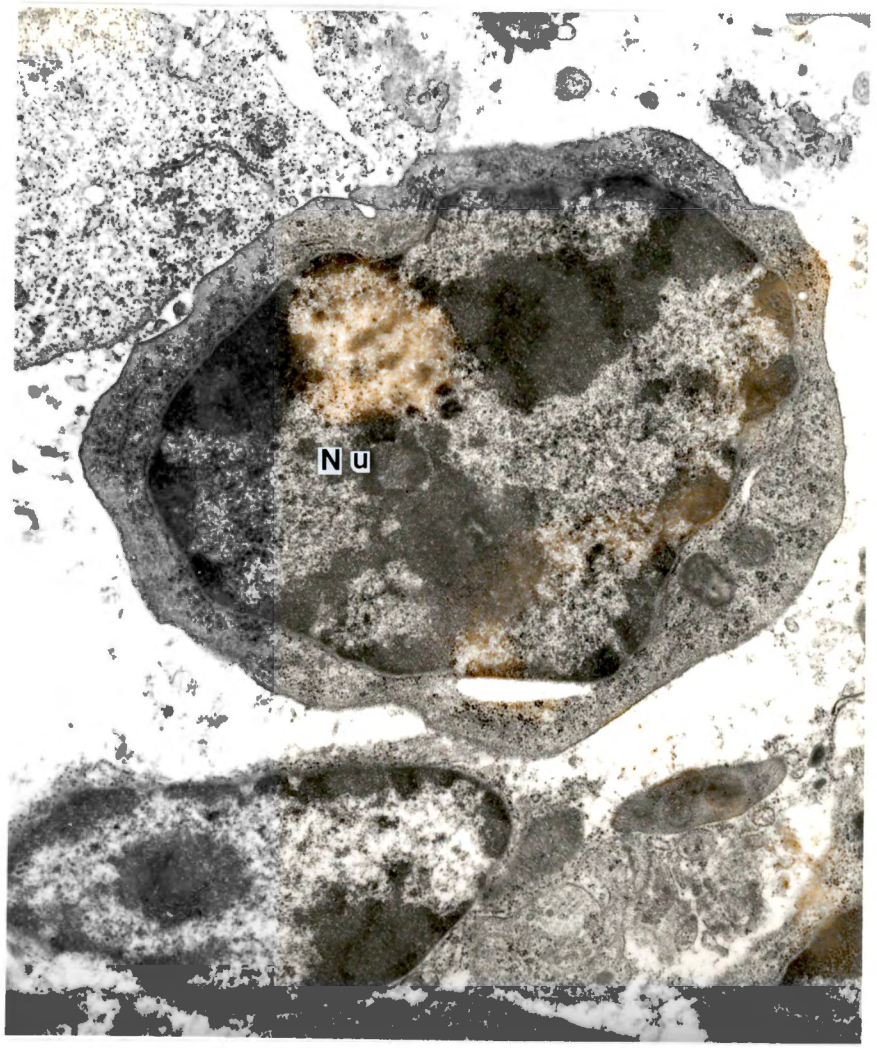

Figure 4-7 Electron micrograph of a small lymphocyte in a sinus of the red pulp. Note the high nucleoplasm-to-cytoplasm ratio. Usually, one nucleolus (Nu) is eccentrically located. The cytoplasm contains a few cytoplasmic granules. Stained with uranyl acetate and lead citrate. X11670

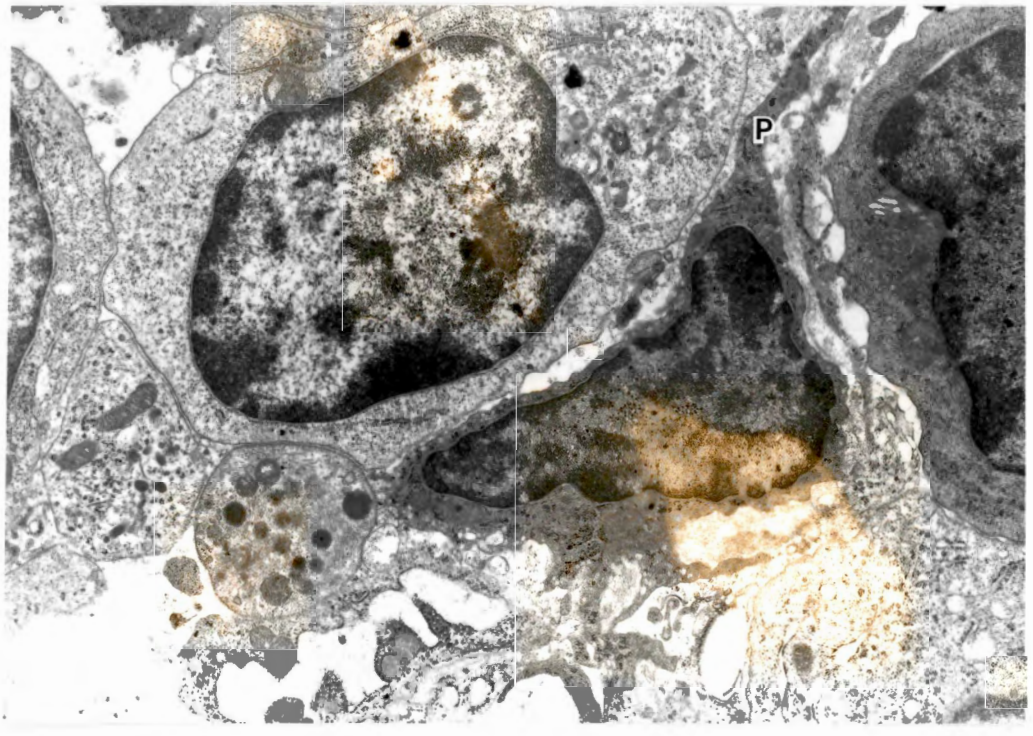

Figure 4-8 Electron micrograph of a reticular cell (RC). Note its elongated cytoplasmic process (p) extending between other cellular elements. Stained with uranyl acetate and lead citrate. X12300

aggregated. Some reticular cells were arranged concentrically around blood vessels.

Plasma cells were usually seen in the peripheral zone of the white pulp. The cytoplasm was dominated by the abundant rough endoplasmic reticulum (Figure 4-9). A few oval-to-rounded mitochondria were observed. The nucleus was large and located eccentrically in the cytoplasm. The dark nuclear heterochromatin was clumped close to the nuclear envelope. The nuclear euchromatin was centrally located and radiated out toward the nuclear envelope.

Red Pulp

The red pulp had two major divisions, the sinuses and the cords (Figures 4-10, 4-11). The cords consisted of free cells such as lymphocytes, plasma cells, red blood cells, platelets, and macrophages all trapped in a network of reticular cells and fibers. Some sinuses were collapsed, while others formed large spaces containing red blood cells, lymphocytes, basophils, and plasma cells. The sinus wall was composed of a single layer of endothelial cells, basement membrane and reticular cell processes. The endothelial cells were large, spindle-shaped cells running parallel to the longitudinal axis of the sinus. Their nuclei bulged toward the sinus lumen. The nucleus usually had one pointed end, and chromatin

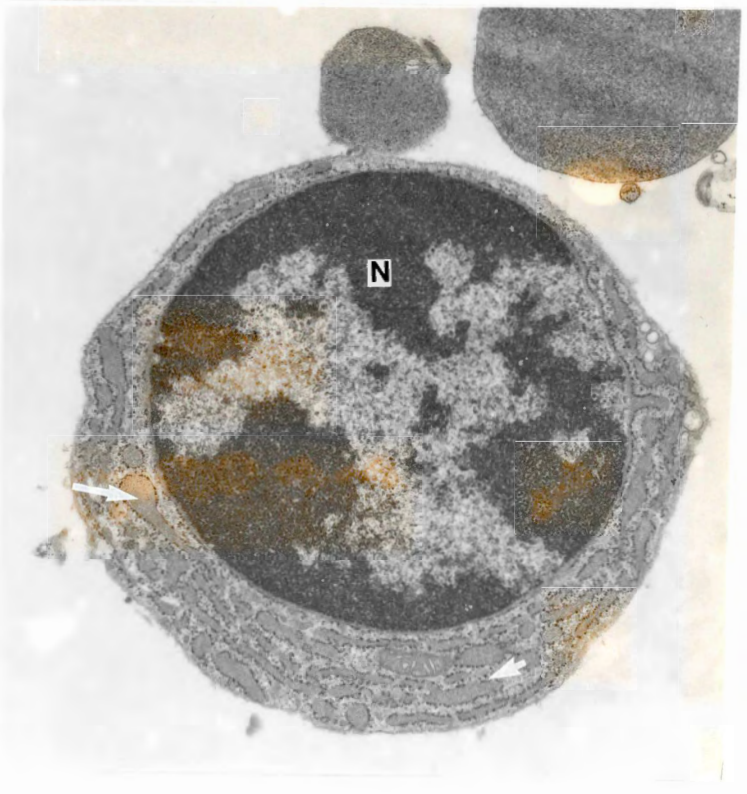

Figure 4-9 Electron micrograph of a plasma cell. Note the rounded, eccentrically-located nucleus (N). The cytoplasm contains large quantities of rough endoplasmic reticulum (arrows). Stained with uranyl acetate and lead citrate. X17500

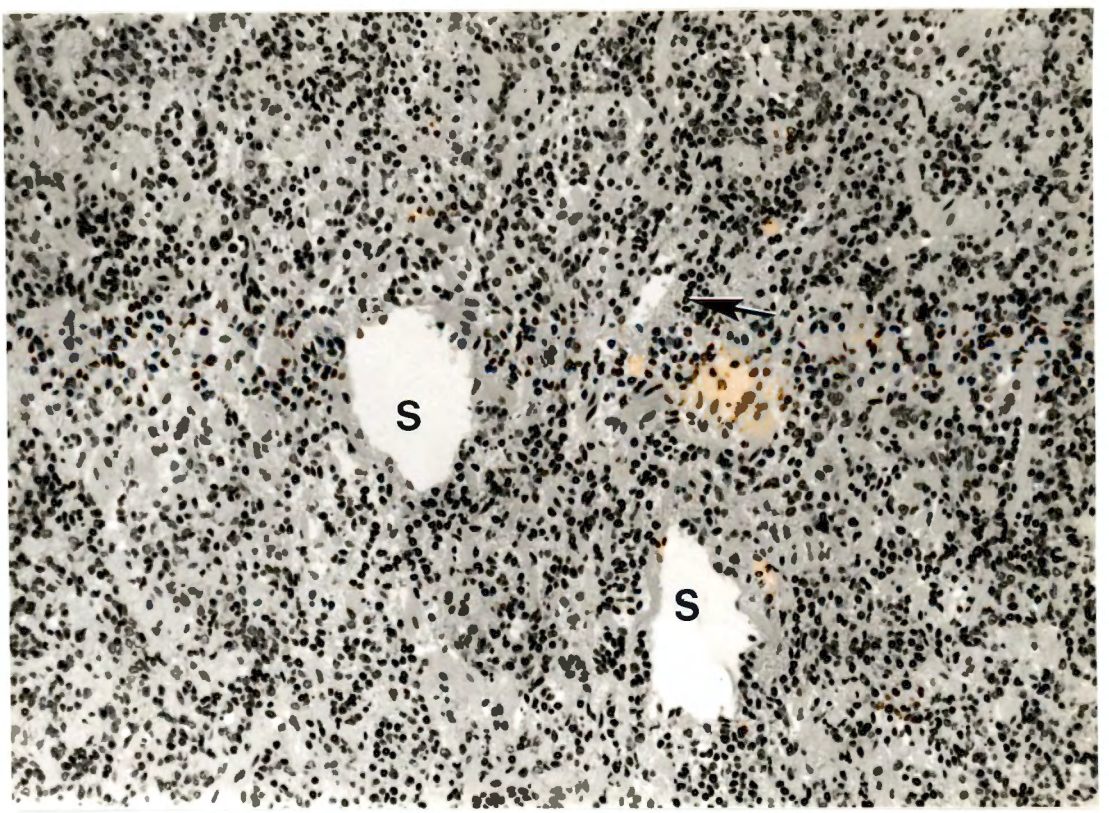

Figure 4-10 Light micrograph of the red pulp of the spleen of the sheep. Note the venous sinuses (S) and splenic cords (arrow). Hematoxylin and eosin stain. X160.

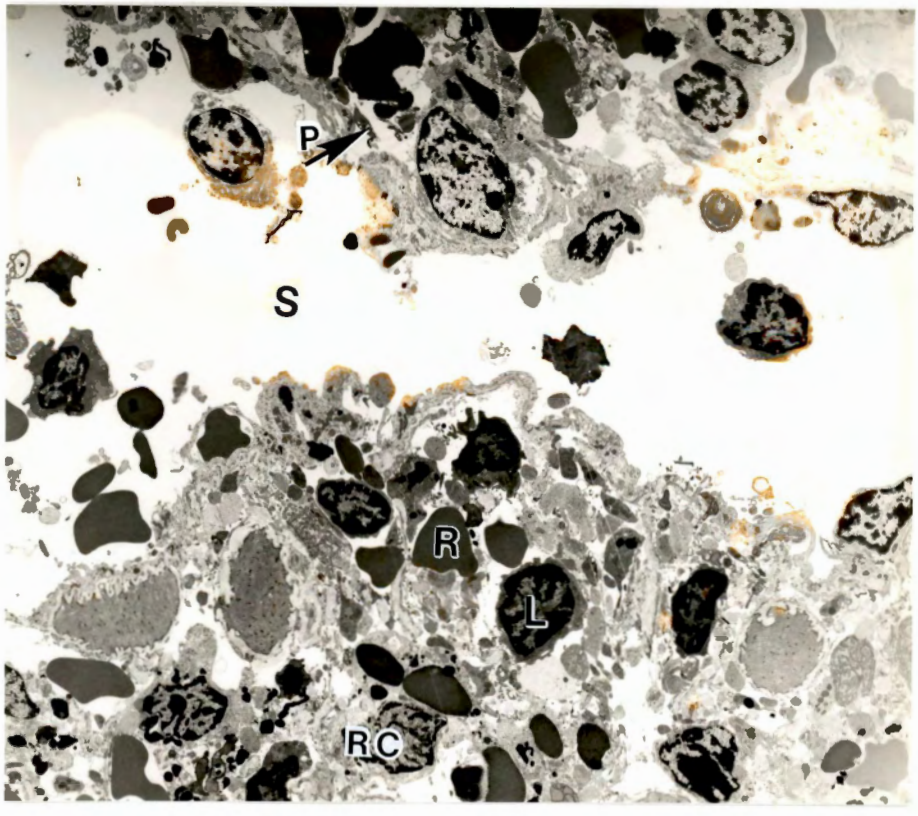

Figure 4-11 Electron micrograph of the red pulp. The sinus (S) walls are lined by endothelial cells. The splenic cords contain lymphocytes (L), reticular cells (RC), red blood cells (R), and platelets (P). Stained with uranyl acetate and lead citrate. X2600

formed thin bands along the margin of the nuclear envelope. The sinusoidal wall, supported by a discontinuous basement membrane, contained numerous large pores. The basement membrane was lightly stained and composed of fine granular materials (Figure 4-12). Lymphocytes were attached to endothelial cells and as the lymphocytes passed through the pores, their cytoplasm and nucleus were constricted (Figure 4-13). Terminal arterial capillaries with numerous slits in their walls opened into the sinusoidal spaces (Figure 4-14).

The neutrophil was a large cell about 10 μm in diameter. The cytoplasm contained granules of various shapes and sizes that also differed in electron density. The nucleus was segmented, and up to three lobules were observed. Heterochromatin made up the major part of the nuclear chromatin, while euchromatin formed patchy areas usually seen in the central area of the nucleus.

The cytoplasm of the eosinophil was dominated by large specific granules that varied in shape and size. The mean of the major axis of the granules was 1 μm, while that of the minor axis was 0.25 μm. Most granules had a single, central crystalline band along the longitudinal axis; others, usually oval, had more than one band. The cytoplasm also contained non-specific granules, or lysosomes, that were scattered in groups, and a few small,

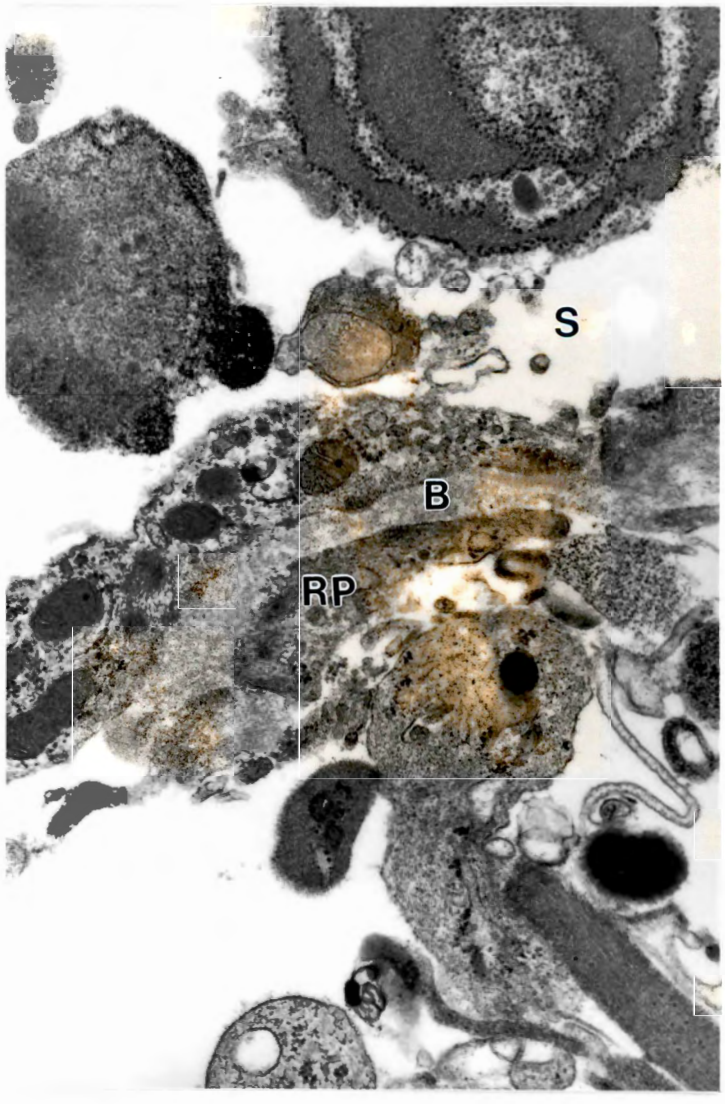

Figure 4-12 Electron micrograph of the sinus (S) wall. It consists of endothelial cells (E), supported by a discontinuous basal lamina (B) and reticular cell processes (RP). X25000

Figure 4-13 Electron micrograph of a red pulp sinus (S). Note the discontinuity of the wall of the sinus. A lymphocyte (L) is constricted while it passes through the sinusoidal pore (arrows). Stained with uranyl acetate and lead citrate. X5400

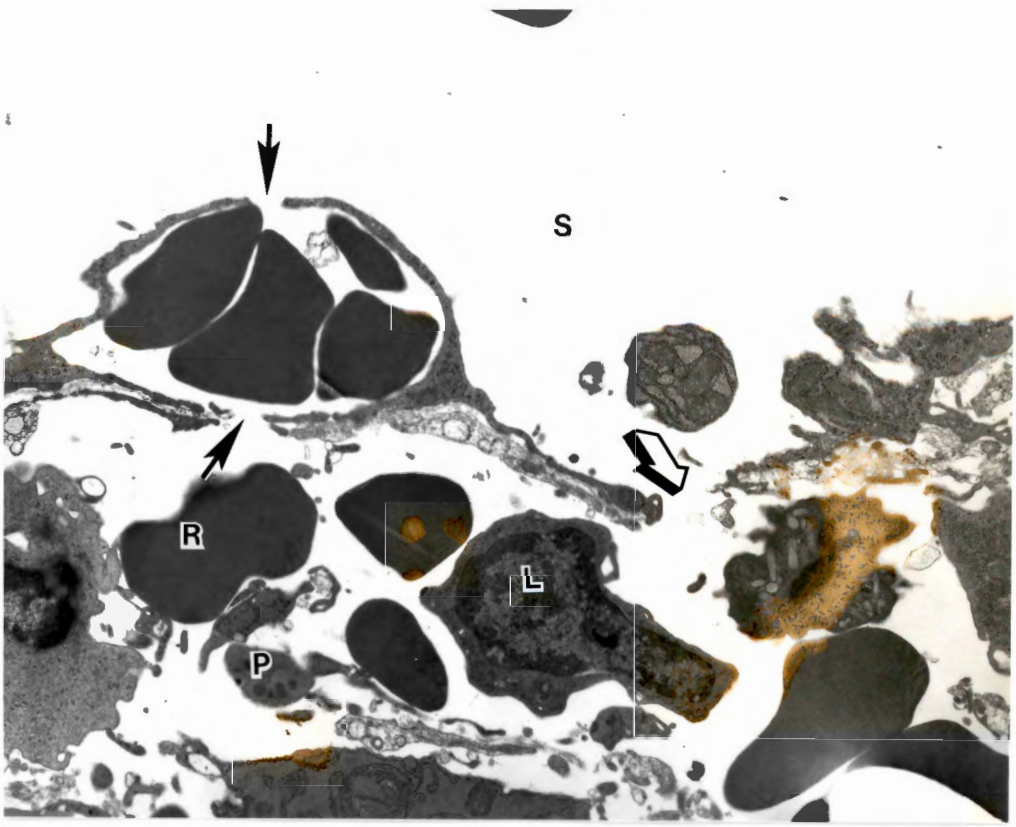

Figure 4-14 Electron micrograph of the red pulp of the spleen of the sheep. Note the terminal arterial capillary (TC) with slits (small arrows) in its wall. These slits open into the sinus (S) whose wall contains a pore (large arrow). Stained with uranyl acetate and lead citrate. X10000

round mitochondria. The eosinophil nucleus was large, segmented, and filling a significant proportion of the cell. The cell was often binucleated with condensed heterochromatin associated with the nuclear envelope. The euchromatin occupied the central area of the nucleus, but occasionally it reached the nuclear envelope.

DISCUSSION

Smooth muscle cells and fibroblasts were the main constituents of the capsule and trabeculae. The smooth muscle cells probably regulate the amount of stored blood within the spleen. Upon adrenergic stimulation, smooth muscle cells contract and force the blood out of the spleen into general circulation. The fibroblasts synthesize and release the precursors to the collagen of the capsule and trabeculae which preserves the architectural integrity of the organ. Blue and Weiss (1981c) reported that the inner surface of the capsule and trabeculae provide attachment for the reticular network which forms the supportive mechanism for the splenic architecture. The capsule and trabeculae were separated from the adjacent sinuses by a thin endothelial cell layer. This finding is similar to that of Burke and Simon (1970).

In this study, bundles of nonmyelinated nerve fibers were present in the trabecular system. Tablin and Weiss (1983) reported that these axons terminate on the reticular cells and are presumably sympathetic nerve fibers that regulate blood flow and storage in the spleen (Reilly, et al. 1979; Tablin and Weiss, 1983). Reilly et al. (1979) suggested that the adrenergic nerve endings in the white pulp influence the production of lymphocytes.

In the present study, the white pulp consisted of a periarterial lymphocytic infiltration around the arterial system. Lymphocytes were observed in contact with macrophages and the reticular cells that formed the framework of the white pulp. These lymphocytes are thought to have migrated from the blood via the marginal zone. The lymphocytes continue their migration from the periarterial or lymphoid sheath into the red pulp (Brelinska and Pilgrim, 1982). The close lymphocyte-macrophage association probably is important in immunological reactions (Lipsky and Rosenthal, 1975) or in the development of lymphocytes as shown <u>in vitro</u> by Garland and Owen (1978). Furthermore, the close association, observed in this study, between macrophages and reticular cells probably aids the interaction between these cells in the immune response (Kamperdijk, et al., 1982).

Pores were observed in the wall of the sinuses of the red pulp. They likely facilitate the passage of blood constituents and antigens between the circulation and the splenic cords. This allows continuous contact of foreign material with reticular cells which carry surface Fc receptors for IgG. These receptors presumably influence the ability for opsonic adherence of antigen (Videbaek et al., 1982) and subsequently clear the blood of particulates by phagocytosis by reticular cells. The openings in the sinus wall change in shape and size (from slits to round openings) with fluctuations in blood pressure (Fujita et al., 1985). Reticular cells play a major role in pore closure. Tablin and Weiss (1983) reported that reticular cells have terminals of nonmyelinated nerve fibers. Upon adrenergic stimulation, these reticular cells probably contract, closing the pores. In order to pass the narrow opening, as shown in Figure 4-12, erythrocytes and leukocytes need to constrict their cytoplasm and nuclei. The aged erythrocytes, which have lost flexibility and cannot pass through the narrow pores, are engulfed by macrophages in the splenic cords, and their iron is recycled (Weiss and Tavassoli, 1970; Chen and Weiss, 1973).

The removal of abnormal material in erythrocytes is an important function of the sinus pore of the spleen.

Following splenectomy, increased numbers of defective red blood cells, characterized by nuclear and cytoplasmic residue, are observed in the blood stream. During the migration of red blood cells through the splenic pores, erythrocytic solid bodies are squeezed out. Howell-Jolly bodies of reticulocytes and erythrocytes (Bessis et al., 1983) and plasmodia of malarial patients (Schnitzer et al., 1972, 1973) are removed by this mechanism.

In this study, the presence of the sinual pores and red blood cells in the splenic cords indicates the presence of an open microcirculation in the sheep spleen. Since in open circulation there is no direct communication between the terminal arterial blood vessels and the sinuses, the cellular elements of the blood have to pass through the cords to the slits in the wall of the sinus (Kashimura and Fujita, 1987). The observation of enlarged terminal arterial capillaries (Figure 4-13), which open into splenic sinuses, indicates the presence of closed microcirculation also. Blue and Weiss (1981b) reported similar observations in the spleen of the cat. Similarly, Dellmann and Brown (1981) stated that the terminal arterial blood capillary enlarged to form an ampulla which opened into the splenic sinus. The slits in the terminal arterial capillary (Figure 4-13) probably play an active role in the regulation of splenic blood flow. With an

increase in blood pressure, these slits probably open, resulting in the leakage of plasma and other materials into the surrounding tissue. These slits probably close by the contraction of reticular cells associated with the terminal arterial capillaries. Blue and Weiss (1981c) proposed that the contraction of the reticular cells directs blood flow (plasma and cells) to the readily accessible central venules. However, relaxation of these reticular cells leads to storage of large numbers of red blood cells, leukocytes, and platelets.

To further clarify the microcirculation in the spleen of the sheep, the use of resin casts with scanning electron microscopy (Murkami et al., 1973; Kashimura and Fujita, 1987), or microspheres (Chen, 1978) would be helpful.

Macrophages were observed near the pores of the sinuses and throughout the white pulp. Their cytoplasm contained inclusions, vacuoles, and lysosomes. Residual bodies were probably accumulations of engulfed aging cells and antigens in the spleen of older animals. The lysosomal system of macrophages contains hydrolytic enzymes which digest phagocytic materials (Milicevic et al., 1987).

In this investigation, numerous blood platelets were observed in the splenic cords of the red pulp. Similar

observations were reported in the spleen of the rabbit (Elgio, 1976). Weiss (1974) observed the close association of the platelets and reticular cells and fibers in the spleen of the rat. He proposed this as a method of the sequestration of the platelets in the spleen. The presence of platelets in this study suggests that the spleen acts as storage pool for platelets, which agrees with the observations of Hill-Zobel et al. (1986) on the human spleen. These platelets are released into the general circulation upon proper stimuli, while abnormal platelets are destroyed and phagocytized by spleen macrophages (Edwards and Simon, 1970).

In this investigation, variable sizes and shapes of neutrophilic granules were observed; Daems (1968) observed four types of granules in the human peripheral blood neutrophil. The variation in size and shape is likely due to the plane of section.

Other cellular elements (plasma cells, eosinophils and lymphocytes) were found to be similar in structure to those of other species.

In summary, the spleen of the sheep was composed of capsule and trabeculae, white pulp, and red pulp. The capsule and trabeculae had smooth muscle cells, fibroblasts, and collagen fibers and carried large blood vessels and nonmyelinated nerve fibers. The white pulp

had two major divisions, periarterial lymphatic sheaths and lymph nodules. The periarterial lymphatic sheath was a highly cellular zone around the small arterial blood vessels. The lymph nodule was an enlargement of the periarterial lymphatic sheath. Lymphocytes and reticular cells were the predominant cells of the white pulp; however, macrophages, plasma cells, and red blood cells were observed. The red pulp of the spleen was divided into splenic cords and sinuses. The splenic cords contained reticular cells, macrophages, and the blood cellular elements. The splenic sinuses were large spaces which contained peripheral blood elements. The sinus wall had a single layer of endothelial cells, a basement membrane, reticular cell processes, and large pores to facilitate cellular movement into and out of the sinus. The connection of the terminal arterial capillaries into the sinuses and the presence of large pores indicated the possibility of both closed and opened microcirculation in the spleen of the sheep.

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V. EFFECTS OF NEGATIVE-PRESSURE HYPOXIA ON THE OVINE SPLEEN

INTRODUCTION

Hypoxia produces a stress effect which limits circulatory adjustment (Heistad and Abboud, 1980) and probably is mediated through the adrenal secretion of corticosteroid (Yoffey et al., 1968) or by direct influence on hypothalamic-pituitary function (Gosney, 1986). Bert (1882), cited by Kendall et al. (1985), was the first to observe polycythemia in animals and people who live at high altitudes. Since then, numerous studies have been conducted on the effect of hypoxia on different body organs: myocardial cells (Lund and Tomanek, 1980); lungs (Reddy et al., 1986); body weight (Yoffey et al., 1966); and body temperature (Bonora and Gautier, 1987). Numerous investigations of hematopoietic organs or their cells include: bone marrow (Yoffey et al., 1965, 1966, 1967, 1968; Hudson, 1958); thymus (Kendall et al., 1985); reticular cells (Ben-Ishay and Yoffey,1971); eosinophils (Grant and Hudson, 1969); and megakaryocyte precursor cells (McDonald et al., 1986; Petursson and Chervenick, 1987).

Although the spleen is a significant hematopoietic organ in several mammalian species, the splenic response to hypoxia has not been thoroughly investigated. The

purpose of this study was to quantify the morphological changes in the spleen after exposure to negative-pressure hypoxia.

MATERIALS AND METHODS

Six mixed breed lambs, 3-1/2 months old and weighing an average of 31.8 kg, were used in this study. Three lambs served as a control and the remaining three were exposed to hypoxia for 15 days by enclosure in a negative-pressure chamber at 0.5 atmospheres of pressure. Following 1 hour of equilibration, the oxygen level was approximately 10% (Appendix D). The chamber temperature was maintained at 22°C to 23°C. The sheep were in the chamber for 23 hours each day and had free access to food and water. The chamber was decompressed slowly over a 1 hour period daily, and the sheep were removed from the chamber and allowed to exercise, eat, and drink for 1 hour at normal atmospheric pressures while the chamber was cleaned. Then sheep were returned to the chamber, and the pressure was slowly decreased over a 1 hour period to 0.5 atmospheres. The animals were monitored throughout the day and checked late at night for changes in their behavior that might indicate discomfort. Respiratory rate was monitored throughout the day. Wood chips were used to

absorb body excrements. Two high-speed vacuum pumps provided adequate air and moisture exchange.

The animals were kept in the chamber for 15 days and then sacrificed. The spleens were collected, weighed, measured for volume by fluid displacement, and processed for light microscopy. Samples were fixed in 10% buffered formalin, dehydrated in a graded ethanol series, and embedded in JB-4 plastic (Polysciences, Inc., Warrington, PA). Sections were stained with hematoxylin and eosin for light microscopy (Appendix A).

Sections of spleen were also fixed in 3% glutaraldehyde 0.1M sodium cacodylate buffer (pH 7.4) for electron microscopy. Specimens were then washed in sodium cacodylate buffer (0.1 M, pH 7.4) and postfixed in 1% OsO_4 at 4°C for 90 minutes. The tissue was dehydrated in a graded ethanol series, cleared in propylene oxide, and embedded in Epon 812 (Ernest F. Fullam, Inc., Latham, NY). Thick sections were stained with toluidine blue for orientation, and silver colored thin sections were prepared on a LKB ultramicrotome. Sections were then stained with uranyl acetate and lead citrate and were viewed with a Phillips electron microscope.

For the volumetric quantitation of splenic components, one 5μm thick-section from each of three randomly selected blocks per spleen was examined by light

microscopy at a magnification of 100X. A 100-point eye piece reticule was overlaid on each of approximately ten fields of view per section (Appendix B).

Sampling points over the red pulp, white pulp, capsule and trabeculae, and large blood vessels were tallied over microscopic fields per section. The volume fraction of each component was estimated by dividing the points that fell on the component by the total number of points on the section of the spleen (Appendix C).

The absolute volume of each component was calculated by multiplying the component volume fraction by splenic volume. A general linear models (GLM) procedure for analysis of variance and the t-test were used to analyze the data.

RESULTS

Histological examination of the spleens of the hypoxic group showed that the sinuses of the red pulp were extremely congested with red blood cells. Negative pressure hypoxia had only a slight effect on the white pulp compartments. Yellow to brown rounded pigments were observed in germinal centers, and the sinuses of the marginal zone were congested.

In control animals, each reticular cell contained one nucleus that was oval or elongated. The nucleus had

irregular margins with no deep indentations and contained moderate amounts of heterochromatin. The plasma cells contained large quantities of endoplasmic reticulum and few mitochondria. The heterochromatin was patchy and regularly distributed close to the nuclear envelop.

In hypoxia, some reticular cells had several nuclei (Figures 5-1, 5-2). Electron microscopic investigation revealed that these nuclei were oval to fusiform in shape, were variable in size, and had several deep indentations. They were hyperplastic and contained large amounts of euchromatin (Figures 5-3, 5-4). Usually, one large nucleolus was seen in each reticular cell nucleus. the cytoplasm appeared to have a slight increase in rough endoplasmic reticulum. Different shapes of plasma cells with highly vacuolated cytoplasm were observed. A few contained two large nuclei, usually with a large nucleolus in each, (Figure 5-5), and the heterochromatin was clumped. The rough endoplasmic reticulum was slightly distended in most cells but was extremely dilated or dispersed as small fragments in some. A few small mitochondria with complete cristae were seen (Figure 5-6).

Results of the morphometric analysis are shown in Table 5-1. A significant difference in body weight was found in hypoxic animals compared to the control animals ($P<0.0229$). The control sheep increased in weight by 15%

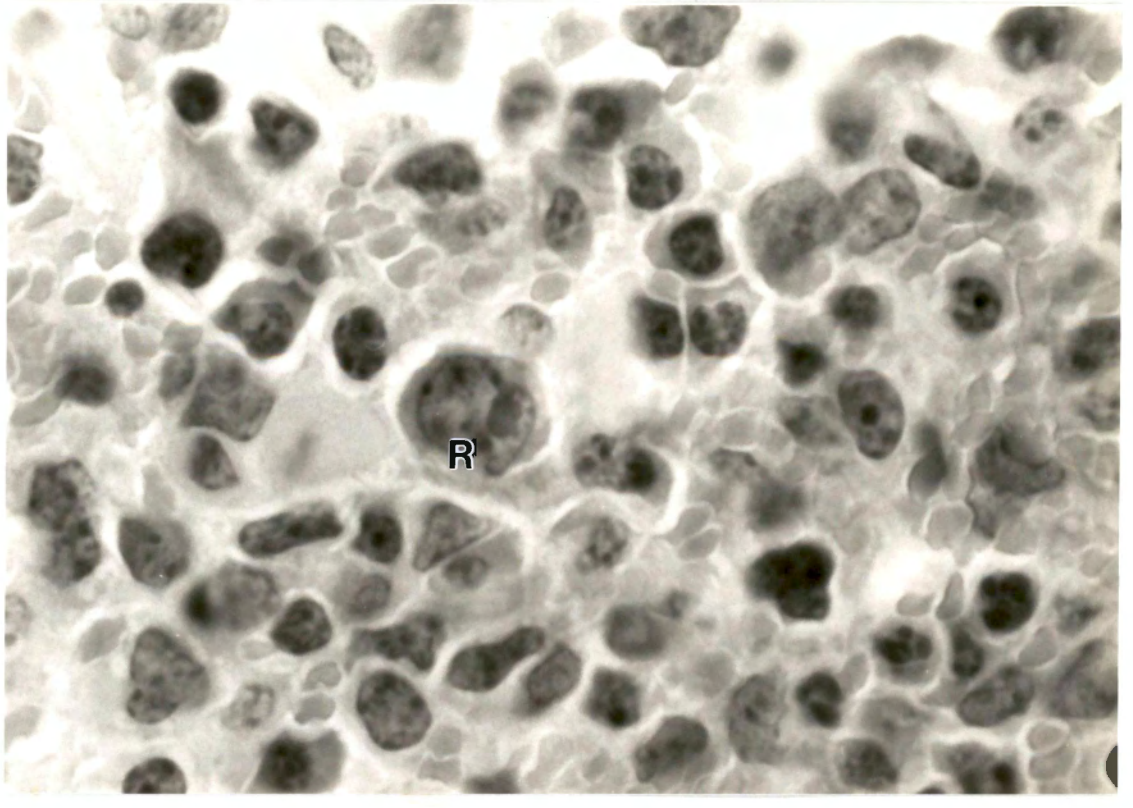

Figure 5-1 Light micrograph of sheep spleen after 15 days of hypoxia. Reticular cell (R) with constricted nucleus. Stained with hematoxylin and eosin. X 1,320.

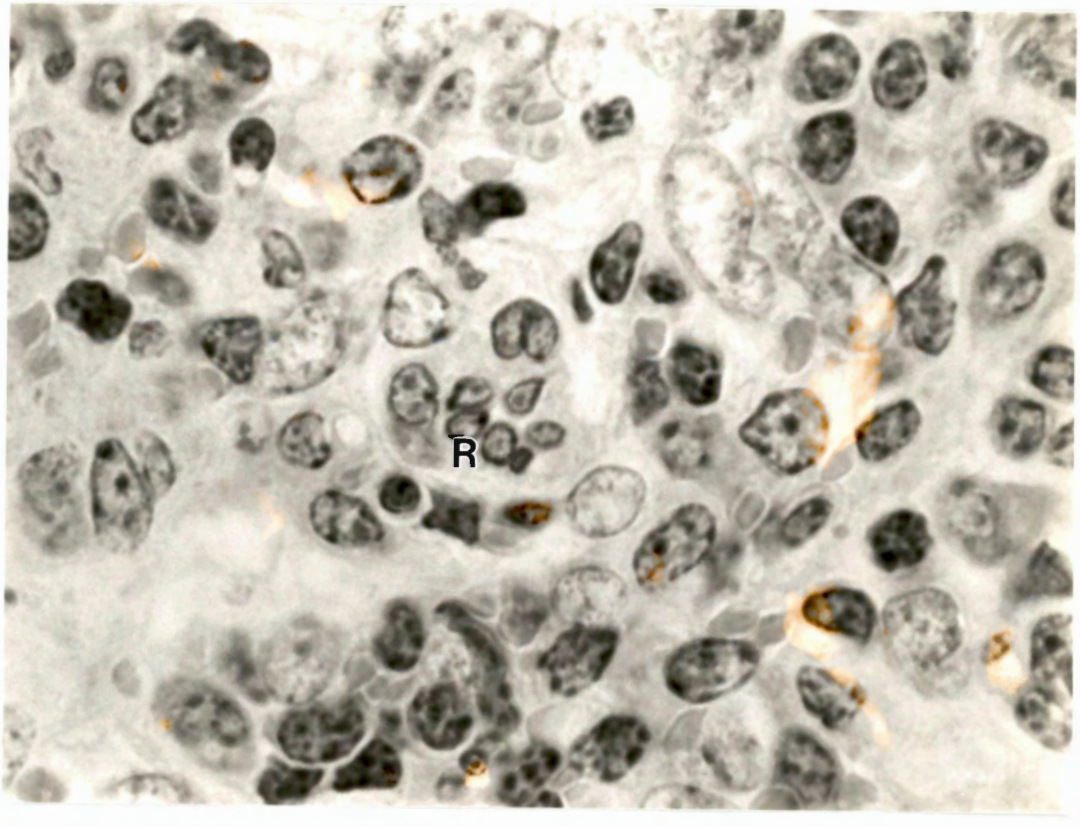

Figure 5-2 Light micrograph of sheep spleen after 15 days of hypoxia. Reticular cell (R) with multiple nuclei. Stained with hematoxylin and eosin. X 1,320.

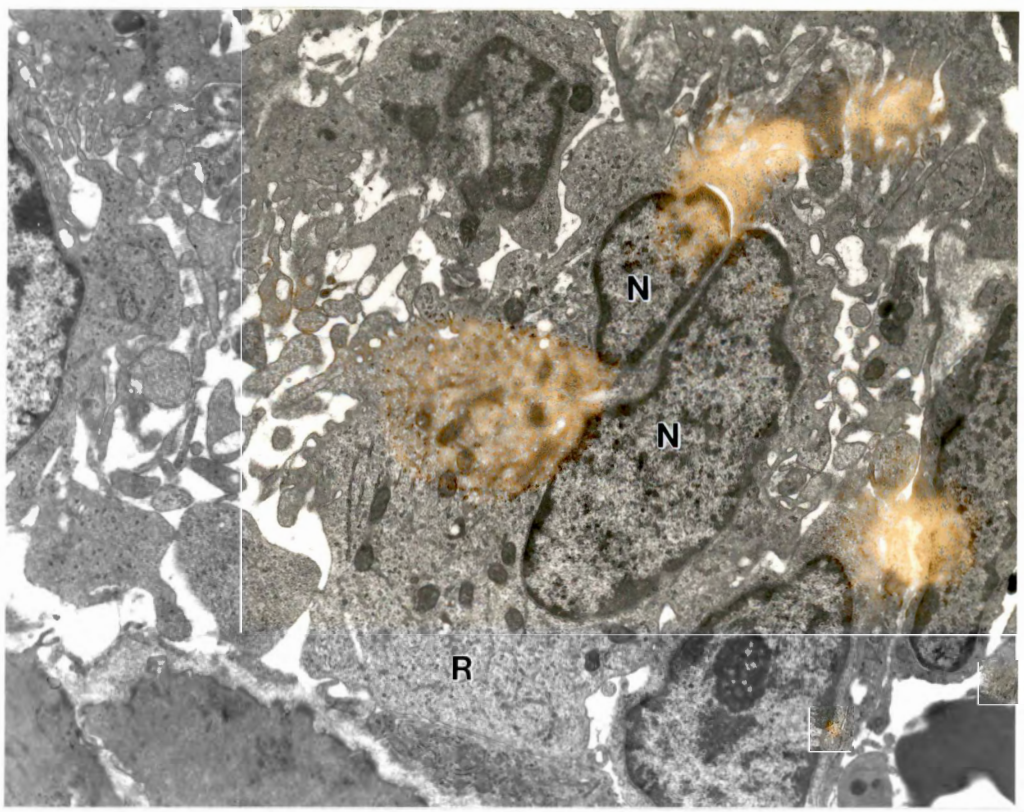

Figure 5-3 Electron micrograph of a reticular cell (R) with two nuclei (N). Stained with uranyl acetate and lead citrate. X 8,040.

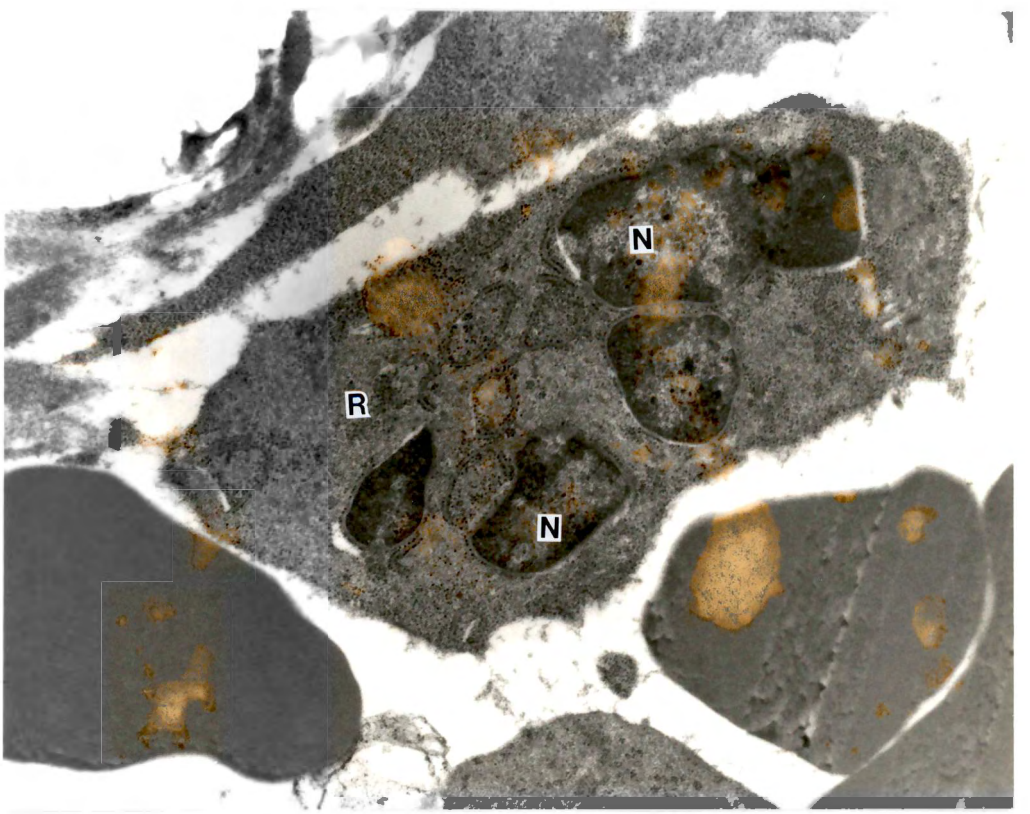

Figure 5-4 Electron micrograph of a reticular cell (R) with multiple nuclei (N). Amounts of rough endoplasmic reticulum are slightly increased. Stained with uranyl acetate and lead citrate. X 15,000.

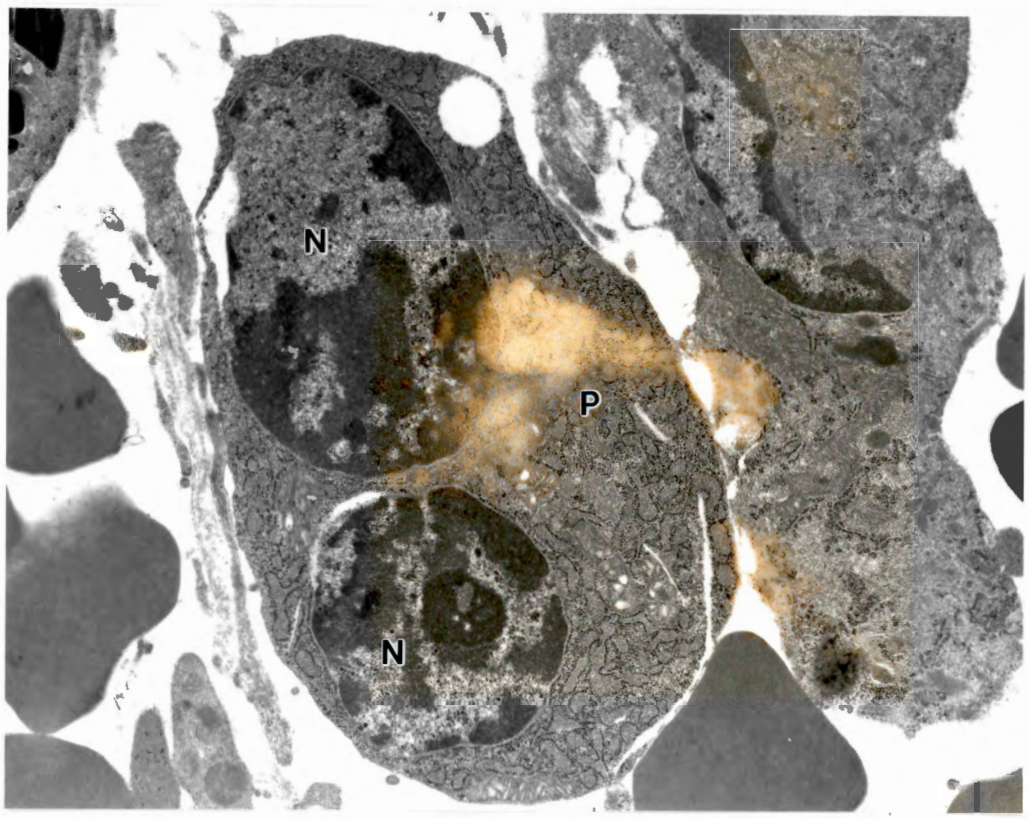

Figure 5-5 Electron micrograph of plasma cell (P) in red pulp sinus. Notice that the cell has two nuclei (N). Stained with uranyl acetate and lead citrate. X 11,250.

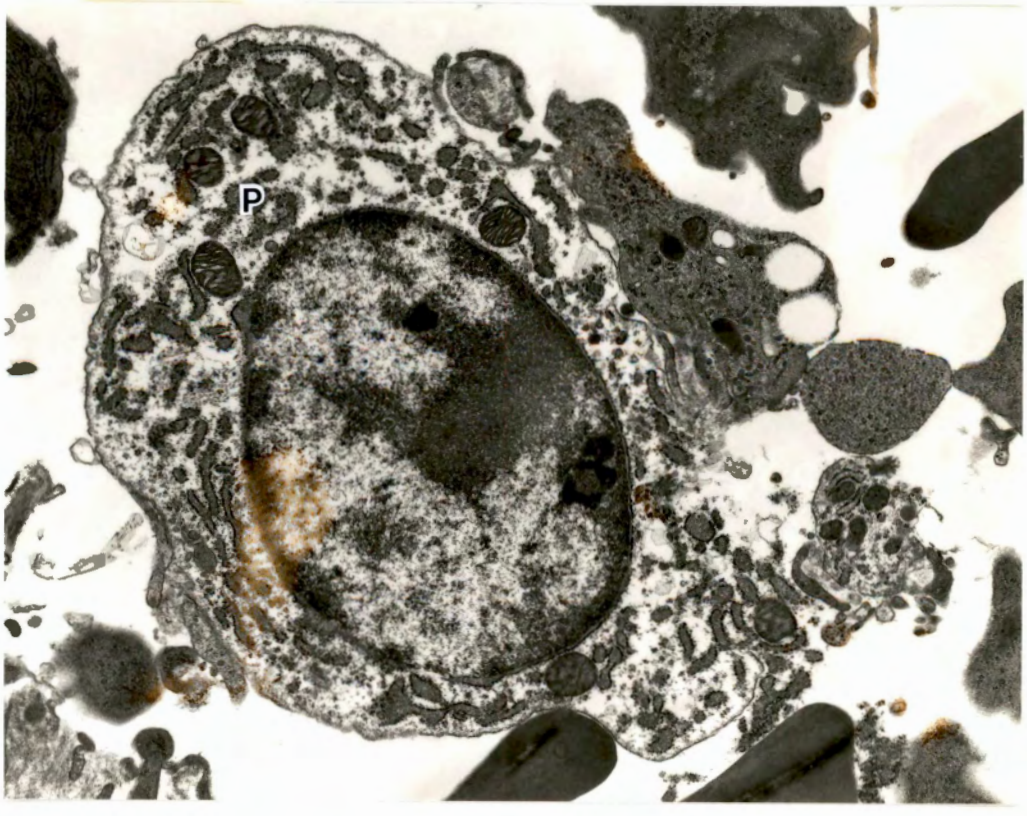

Figure 5.6 Electron micrograph of plasma cell (P). Notice, the few fragmented profiles of rough endoplasmic reticulum, vacuolated cytoplasm, and few rounded to oval mitochondria. Stained with uranyl acetate and lead citrate. X 14,300.

TABLE 5-1. Means of Morphometric Analysis of Spleens of Normal Baric and Hypoxia-Treated Sheep

Variable	Normal	Hypoxia	Significance Level
Animal Weight (kg)			
Day 0 -	33.1 ±1.0	32.4 ±3.9	P > 0.755
Day 15 -	37.8 ±2.7	30.7 ±1.9	P < 0.0229
Splenic Weight (gm)	63.0 ±3.0	51.8 ±1.8	P < 0.005
Splenic Volume (ml)	63.0 ±2.0	50.0 ±0.0	P < 0.005
Red Pulp			
A. Volume (%)	58.3 ±4.0	66.2 ±8.5	P < 0.05
B. Absolute Volume (ml)	36.7 ±2.5	33.1 ±4.2	P < 0.05
White Pulp			
A. Volume (%)	31.6 ±4.7	25.3 ±7.8	P > 0.05
B. Absolute Volume (ml)	19.9 ±2.9	12.6 ±3.9	P < 0.05
Trabecular			
A. Volume (%)	8.2 ±2.6	6.4 ±3.1	P > 0.05
B. Absolute Volume (ml)	5.1 ±1.6	3.2 ±1.5	P < 0.05
Blood Vessel			
A. Volume (%)	1.7 ±1.0	1.8 ±1.2	P > 0.05
B. Absolute Volume (ml)	1.0 ±0.6	0.9 ±0.6	P > 0.05
Capsular Thickness (μm)	215.8 ±56.5	284.5 ±91.2	P < 0.005

over the 15 day experimental period, while the hypoxic sheep had a 4% decrease. Furthermore, splenic volume and weight were significantly reduced ($P<0.005$, $P<0.005$). The fractional volume of red pulp and splenic capsular thickness increased significantly ($P<0.05$, $P<0.005$) following hypoxia; however, the volume fractions of white pulp, trabeculae, or blood vessels did not change ($P>0.05$, $P>0.05$, $P>0.05$). Absolute volume of red pulp, white pulp, and trabeculae were significantly decreased ($P<0.05$). However, no significant change in absolute volume of blood vessels was observed.

DISCUSSION

In this experiment, sheep were kept in a hypobaric chamber that simulated an altitude of 4,878 meters. A slight reduction in body weight (4%) occurred during the 15 days of hypoxia, which is similar to the findings of Yoffey et al. (1966) and Kendall et al. (1985). Campbell (1934), cited by Kendall et al. (1985), reported cessation of growth, progressive weight loss, and death of animals from chronic heart failure. Therefore, it is probable that the differences in the weight of the hypoxic sheep and the normal sheep in the present experiment were due to a disturbance and/or cessation of growth. Body weight of the hypoxic sheep was significantly less ($P<0.0229$) when

compared to that of the control sheep, which had a 15% increase in body weight.

The hypoxic sheep suffered a significant loss in splenic volume and weight after 15 days of hypoxia. The red pulp fractional volume was significantly increased, but there was no reduction in the white pulp fractional volume. In contrast, Yoffey et al. (1968) reported an increase in the weight of the spleen of guinea pigs after seven days of exposure to hypoxia. They explained the increase in the splenic weight by an increase in the amount of the lymphoid tissue and splenic pulp. These discrepancies are likely due to species and age variations, since the spleen in higher mammals ceases hematopoietic activity before birth (Seifert and Mark, 1985). Also, variation of length of exposure could possibly produce varied results. The sheep may have had an increase in lymphoid tissue early in hypoxia followed by a subsequent reduction. Birks et al. (1975) reported an initial increase in splenic weight through 5 days of hypoxia; thereafter, the spleen lost weight until the end of 12 days of hypoxia.

The spleen of the sheep showed no evidence of erythropoiesis. However, it was surprising that Yoffey et al. (1968) found only minimal signs of erythropoiesis in the spleen of guinea pigs after hypoxia, even though the

spleen of rodents has been shown to be a site of hematopoiesis after hypoxia (Petursson and Chervenick, 1987) and in the postnatal period (McFadden, 1967; Grouls and Helpap, 1982; Seifert and Mark, 1985).

In our morphometrical analysis, the significant increase of the red pulp fractional volume in the hypoxic group was probably due to the increase in the number of the circulating red blood cells. The increased blood volume leads to dilitation of the red pulp sinuses.

In this experiment, an increase in the capsular thickness was detected. This increase is possibly due to an increase in the smooth muscle cell and/or collagen fibers. An increase in capsular thickness is possibly in response to expelling the higher-viscosity blood resulting from hypoxia and not postmortem contracture artifact.

The structural changes in reticular cells occur mainly in the nucleus and to a lesser extent in the cytoplasm. The reticular cells become binucleated and multinucleated. Similarly, binucleated and multinucleated type 2 epithelial reticular cells were reported in the thymus of hypoxic mice (Kendall et al., 1985). Kendall et al. (1985) concluded that changes seen in epithelial reticular cells may be associated with their production of hormones. These hormones are necessary for maturation and differentiation of thymocytes when the thymus is depleted

of lymphocytes. In our experiment, the spleen was not depleted of its lymphoid tissue. Therefore, Kendall and coworker's conclusion seems unsatisfactory in explaining the binucleated and multinucleated reticular cells in the spleen since these cells (reticular and epithelial reticular) have a different embryonic origin and different function. However, we agree with Yoffey et al. (1968) who stated...

> The spleen itself does not produce large numbers of red cells, but it could still make an important contribution to the increased erythropoiesis by the production of stem cells which could then migrate through the blood stream and colonize the bone marrow.

Binucleated and multinucleated reticular cells found in this investigation possibly are the source of stem cells. Undifferentiated reticular cells were also found in the spleen by Weiss (1973) and Wiersbowsky et al. (1982). Roberts and Latta reported primitive and multipotential reticular cells (reticular cell, type I) in the spleen.

The observed fragmentation of rough endoplasmic reticulum, vacuolation of cytoplasm, and clumped heterochromatin in the plasma cells of sheep are most likely associated with destruction of the cell. Consequently, the remaining plasma cells have undergone hypertrophy of the rough endoplasmic reticulum and hyperplasia of the nuclei to compensate for the destruction of plasma cells. Possibly these variant

plasma cells were observed in different stages of maturation.

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VI. EFFECTS OF NEGATIVE-PRESSURE HYPOXIA ON OVINE BLOOD

INTRODUCTION

Clinical studies show that hypoxia (reduced oxygen tension in body tissues) is common in patients with shock, heart failure, myocardial infarction, and pulmonary embolism. In these patients, hypoxia is an additional stress and probably limits circulatory adaptation (Heistad and Abboud, 1980). Sea diving and space travel alter tissue oxygen tension. Therefore, the study of hypoxia is important clinically.

Generally, hypoxia produces an increase in red blood cell count, packed cell volume, and hemoglobin concentration. However, the body's response varies with the length of exposure to hypoxia. For example, the platelet count is biphasic (Jackson and Edwards, 1977) showing an initial increase in platelet count followed by a decline after 6 days (McDonald et al, 1986).

Hypoxia of short duration (1-3 days) was shown to increase radioisotope incorporation into platelets of rats (Jackson and Edwards, 1977) and mice (Cooper and Cooper, 1977; Shreiner and Levin, 1976), to increase [small acetylcholinesterase positive (SAChE+)] cell numbers (McDonald et al., 1986), to increase megakaryocyte size after three days of hypoxia (Jackson and Edwards, 1977),

and thus leading to increase platelet counts (Cooper and Cooper, 1972; Jackson and Edwards, 1977; McDonald, 1978; McDonald et al., 1978; Shreiner and Levin, 1976). Shreiner and Levin (1976) noticed only a slight increase in platelet counts after 24 hours of hypoxia. However, McDonald et al. (1986) observed a significant increase in the platelet count after three days of hypoxia. The increase in platelet count was not due to splenic release of platelets. These increases in platelet numbers cannot be explained by action of a humoral factor since three to four days are required for thrombopoietin to stimulate immature megakaryocytes (McDonald et al., 1978). The increase in the number of circulating platelets by hypoxia probably represents an increase in the shedding of platelets by megakaryocytes because of the stress associated with hypoxia (McDonald et al., 1978).

Hypoxia of longer duration (chronic hypoxia) leads to decreased platelet counts in mice (Birks et al., 1975; Cooper and Cooper, 1977; Langdon and McDonald, 1977) and rats (Jackson and Edwards, 1977) and to a decrease in platelet volume (McDonald et al., 1978). After six days of hypoxia in mice, Shreiner and Levin (1976) and McDonald et al. (1986) observed a significant drop in the platelet count after the initial elevation; however, ^{75}SeM (selenomethionine) isotope incorporation into platelets

had decreased to normal levels; it is surprising to have thrombocytopenia with normal platelet production. This decrease in platelet count was thought to be a result of decreased platelet production (Cooper and Cooper, 1977) probably caused by stem cell competition between the megakaryocytic and erythroid lines in the bone marrow (Langdon and McDonald, 1977).

Decreased platelet counts have been associated with reduced isotope incorporation into platelets (Cooper and Cooper, 1977; Langdon and McDonald, 1977), along with decreased numbers of megakaryocyte precursor cells (SAChE+) (McDonald et al., 1986) and decreased concentration of megakaryocytes (Jackson and Edwards, 1977; McDonald, 1978). It has been reported that thrombocytopenia induced by hypoxia cannot be explained entirely by expanding blood volumes (McDonald et al., 1978) or by excess splenic sequestration of platelets (Birks et al., 1975; Langdon and McDonald, 1977). The life span of platelets remains normal even with a reduction of marrow megakaryocytes in hypoxic rats (Jackson and Edward, 1977). These findings suggest that thrombocytopenia is closely linked to decreased platelet production by megakaryocytes.

Decreased platelet volume has been associated with an increase in the age of the platelet. Platelets decrease

by 25% in volume as they age (Detwiler et al., 1962). McDonald et al. (1964) reported that young platelets were significantly larger than older platelets.

A negative correlation between platelet counts and red blood cell counts has been documented clinically (Gross et al., 1964) and experimentally (Birks et al., 1975; Choi and Simone, 1971; 1973; Choi et al., 1974; Langdon and McDonald, 1977; McDonald et al., 1986). The mechanism that regulates thrombocytopoiesis is different from that which controls erythropoiesis (DeGabriele and Penington, 1967). Iron-deficiency anemia and anemia induced by both transfusion of anti-erythrocyte serum and exchange transfusion of erythrocyte-poor platelet-rich plasma resulted in increased platelet counts in rats whose erythroid differentiation was inhibited (Jackson et al., 1974).

Researchers believe that reduced platelet counts are caused by stem-cell competition between the megakaryocytic and the erythroid cell lines in the bone marrow (Langdon and McDonald, 1977). Changes in the concentration of thrombopoietin or thrombocytopoiesis-stimulating factors (TSF) were not a factor in platelet count reduction (McDonald et al., 1979). McDonald et al. (1979) exposed mice to short-term hypoxia (24 hours) followed by 2 to 3 days of ambient O_2 levels. Incorporation of ^{35}S-sodium

sulphate isotope into platelets and platelet numbers was significantly reduced. Significant differences in plasma thrombocytopoiesis-stimulating factor activity between normal mice and mice treated with hypoxia were not detected, suggesting that hypoxia had no effect on thrombopoietin production in mice.

The effects of hypoxia on other peripheral blood elements are increases in red blood cell count (VanLiere and Stickney, 1963), packed cell volume (Lord and Murphy, 1973), and hemoglobin amount (DeGowin et al., 1962). In animals exposed to hypoxia equivalent to 6,096 m, Grant and Hudson (1969) showed that the eosinophils in the peripheral blood were increased while the marrow eosinophils underwent severe depletion, particularly the compartments of late band and segmented forms. However, basophils and neutrophils underwent a less dramatic reduction. In secondary hypoxia (5200 m), there was a significant decrease in marrow lymphocyte counts.

Meyer et al. (1935) exposed rats and guinea pigs to low atmospheric pressures. They found a leukocytosis and concluded that leukocytes were discharged from the spleen, liver, and lung under altered atmospheric conditions. They found no consistent changes in the differential blood count. Cress et al. (1943) subjected rats to hypobaric, anoxic, and hemic hypoxia. They noticed an increase in

leukocyte counts in the peripheral blood and concluded that hypoxia caused an excitation of the sympathetic-adrenal system. They assumed that adrenaline exerts its effect on the bone marrow directly.

Hurtado et al. (1945) investigated the white blood cell count in Peruvian natives who lived at high altitudes. They found a nonsignificant increase in the total number of leukocytes in chronic hypoxia.

This investigation was conducted to study the effects of negative-pressure hypoxia on platelet counts and sizes and on the complete blood cell count in sheep a species not previously studied.

MATERIALS AND METHODS

Three mixed breed lambs (3-1/2 months old) were kept in a negative-pressure chamber and exposed to hypoxia for 15 days. Following 1 hour of equilibration, the oxygen level was held at approximately 10% (PO_2=76mm Hg) (Appendix D). The chamber temperature was maintained at 22°C to 23°C. The sheep were in the chamber for 23 hours each day and had access to food and water. The chamber was decompressed slowly over a one-hour period and the sheep were removed from the chamber and allowed to exercise, eat, and drink for one hour at normal atmospheric pressures. During this time the chamber was

cleaned. After the time of normal oxygen, the sheep were returned to the chamber and the vacuum slowly decreased over a one-hour period to 380 mm Hg. The animals were checked on throughout the day and late at night to monitor behavior that might indicate discomfort. Respiratory rate was recorded throughout the day. Wood chips were used to absorb body excrements. Two high-speed vacuum pumps facilitated continual air exchange through the relief valve (to maintain 380 mm Hg), thus providing adequate air and moisture exchange.

Blood was drawn twice (at 2 day intervals) from the jugular vein before exposure to hypoxia, and three times a week thereafter (a total of seven) to monitor changes in the blood while in the hypoxic atmosphere for the 15 day period. A complete blood cell count (red blood cell count, white blood cell count, hemoglobin, hematocrit, platelet count, and total protein), differential white blood cell count, and platelet volume were performed on each blood sample. Vacutainers containing sodium citrate (American Scientific Products, Inc., Stone Mountain, GA) were used to collect blood for platelet evaluation; for platelet sizing, the blood was centrifuged at 800 X g at 18°C for five minutes. The platelet-rich plasma supernatant fluid was removed, and 5 μl diluted into 10 ml of isotonic-buffered saline (1:2,000 dilution). Platelet

volume was determined using a Coulter Counter model ZM instrument set at a current (I) of 30 and 10mA, with a gain of 1, attenuation of 2, lower threshold = 0.2128 fl, upper threshold = 13.44 fl, with a 50μm diameter orifice tube. The counter was calibrated using 2.02 μm diameter latex particles. Data were analyzed via the Coulter Channelyzer^{R} 256 (Coulter Electronics, Inc., Hialeah, FL), an automated, self-contained unit intended to analyze particle size and distribution curves generated by Coulter Counter^{R} model ZM, and a Texas Instrument computer (Texas Instrument Inc., Austin, TX). Platelet counts were obtained by manual counting on a thin hemocytometer chamber using direct phase-contrast microscopy (Brecher and Cronkite, 1950). The following red blood cell indices were calculated: mean corpuscular volume (MCV); mean corpuscular hemoglobin (MCH); and mean corpuscular hemoglobin concentration (MCHC). The data were evaluated with the analysis of variance (GLM) and the Student-Newman-Keuls (SNK) tests.

RESULTS

Platelet volume decreased steadily from the first day of exposure to hypoxia through day 15 of hypoxia (Figure 6-1). A significant decrease ($P<0.0001$) in mean platelet volume was observed after 9 days of hypoxia. After 15

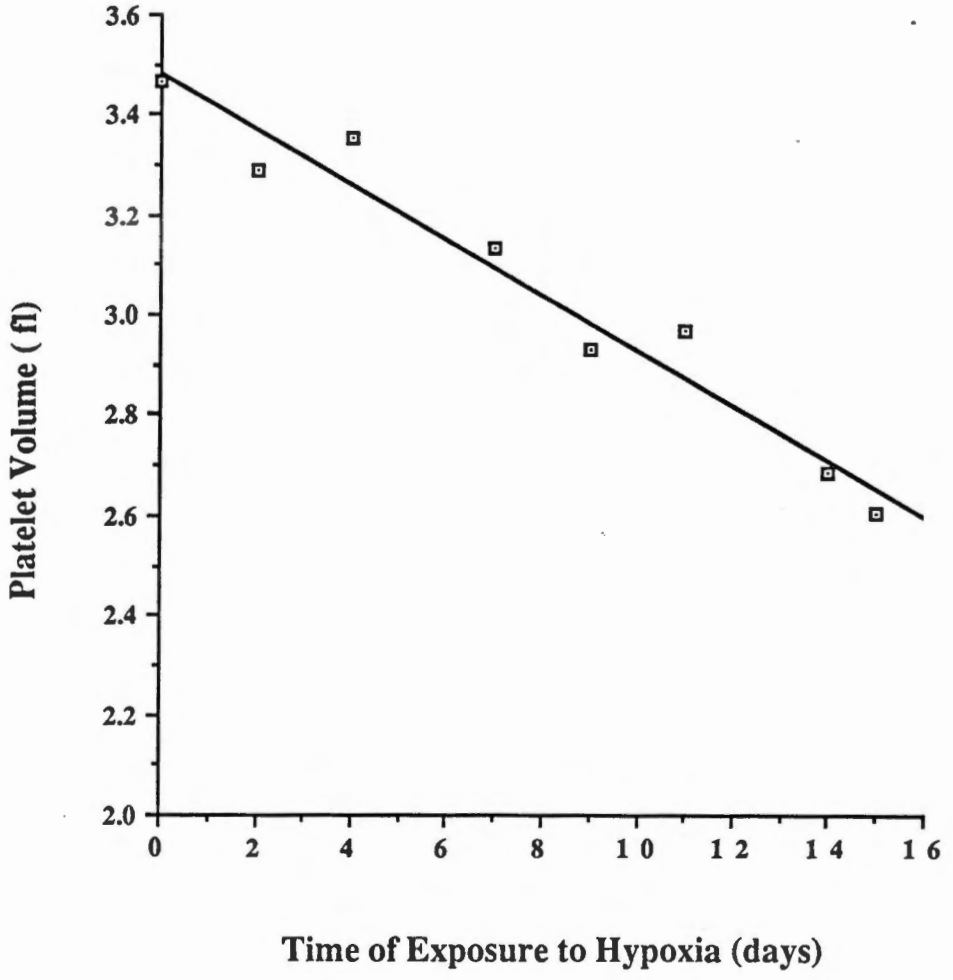

Figure 6-1 A linear regression plot of platelet volume in femtoliters (fl) versus exposure time to hypoxia. Each point represents the mean value for three animals except at day 11 which represents the mean of two animals. Platelet volumes decreased in a linear pattern with time of exposure to hypoxia ($P<0.0001$); $r^2 = 0.944$.

days of hypoxia, platelet volume had decreased by 26% (from 3.5 to 2.6 fl) (Tables 6-1, 6-2).

The platelet count decreased significantly ($P<0.0005$) and linearly with time of exposure to the hypoxic atmosphere ($r^2 = 0.751$) (Figure 6-2). The platelet count was decreased by 34% (from 751,111 to 496,000/μl) on the 15th day of hypoxia (Table 6-1).

During the 15-day period of hypoxia, the red blood cell count (Figure 6-3), packed cell volume (hematocrit) (Figure 6-4), and hemoglobin (Figure 6-5) increased significantly ($P<0.005$, $P<0.0001$, $P<0.0002$) and linearly. These increases were 22%, 31%, and 25%, respectively (Tables 6-1, 6-2) by day 15. A significant increase in packed cell volume began after day 7 of hypoxia (SNK test). After day 11 of hypoxia, a significant increase in hemoglobin concentration occurred (SNK test). No significant changes were observed in the red blood cell indixes (MCV, MCH, MCHC) (Table 6-3).

The relationship between platelet count and red blood cell numbers, packed cell volume, and the amount of hemoglobin was tested. As the red blood cell count increased, the platelet count decreased significantly ($P<0.002$) (Figure 6-6). A significant ($P<0.0004$) negative linear relationship between platelet count and packed cell volume was shown for the hypoxic sheep during the 15 days

TABLE 6.1 Mean platelet count/μl (PLTC), mean platelet volume in femtoliters (PLTV), % packed cell volume (PCV), and hemoglobin in grams/deciliter (HB) of control sheep and sheep after 15 days of hypoxia.

Treatment	PLTC ±SE	PLTV ±SE	PCV ±SE	HB ±SE
Control	751,111 ±114,762	3.5 ±0.2	33.3 ±2.4	12.4 ±0.5
15 days of hypoxia	496,000 ±135,030	2.6 ±0.1	43.5 ±0.8	15.5 ±0.5
Percent change	34	26	31	25
P Values	<0.01	<0.001	<0.0001	<0.0001

TABLE 6.2 Regression analysis of mean values: packed cell volume (PCV), platelet volume (PLTV), platelet count (PLTC), hemoglobin (HB), total protein (TP), red blood cell count (RBC), and white blood cell count (WBC) of sheep with varying times in a hypoxic environment.

Regression	P value	r^2	S	N
PCV X Time	<0.0001	0.957	1.830	8
PLTV X Time	<0.0001	0.944	0.079	8
PLTC X Time	<0.0005	0.751	0.567	8
HB X Time	<0.0002	0.818	0.498	8
TP X Time	<0.004	0.768	0.162	8
RBC X Time	<0.004	0.776	0.453	8
WBC X Time	<0.02	0.616	7.647	8
PLTC X PCV	<0.0004	0.889	0.377	8
PLTC X HB	<0.0002	0.910	0.340	8
PLTC X RBC	<0.002	0.814	48.939	8

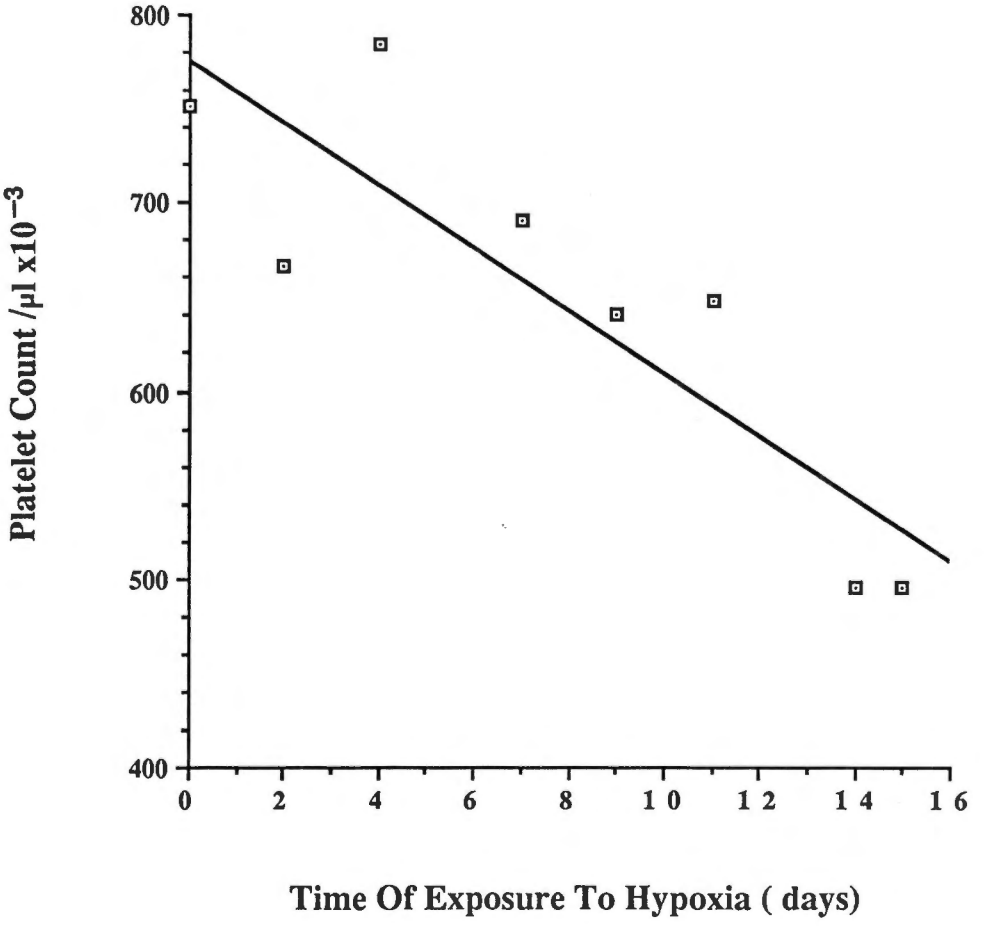

Figure 6-2 A linear regression plot of platelet count/μl versus exposure time to hypoxia. Each point represents the mean value for three animals, except at day 4 which represents the mean of two animals. A marked decrease ($P<0.0005$) in platelet count is observed with time of exposure; $r^2 = 0.751$.

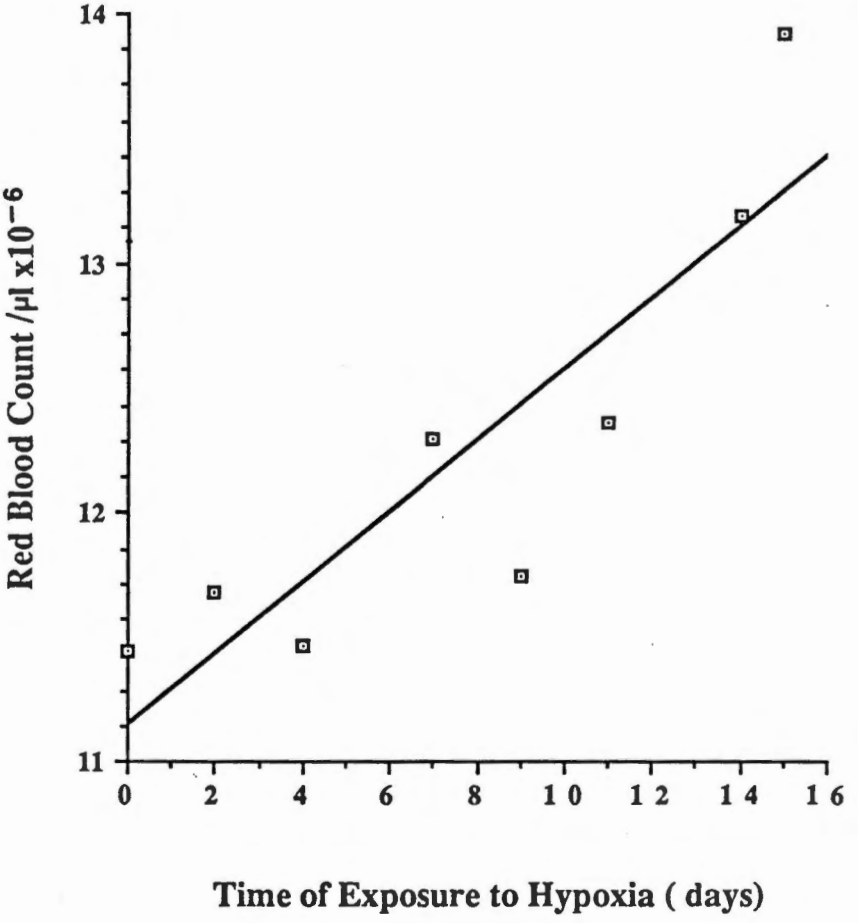

Figure 6-3 A linear regression plot of red blood cell count/µl versus time of exposure to hypoxia. Each point represents the mean for value of three sheep, except at day 7 which represents the mean of two animals. A significant increase with time ($P<0.0004$) in red blood cell count is shown; $r^2 = 0.776$.

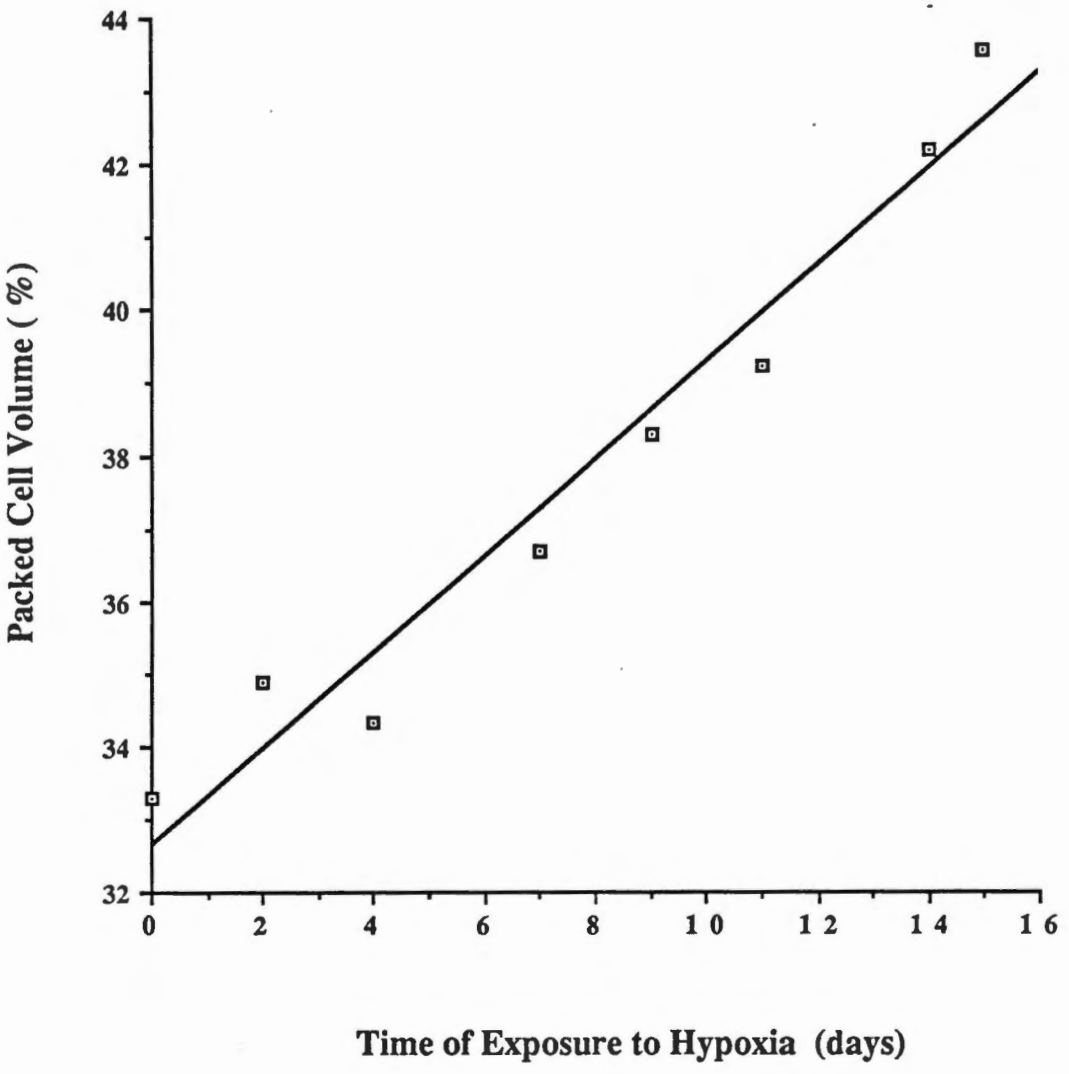

Figure 6-4 A linear regression plot of packed cell volume (hematocrit) % versus time of exposure to hypoxia. Each point represents the mean value for three animals. A significant increase ($P<0.0001$) in hematocrit was observed with time of exposure to hypoxia; $r^2 = 0.957$.

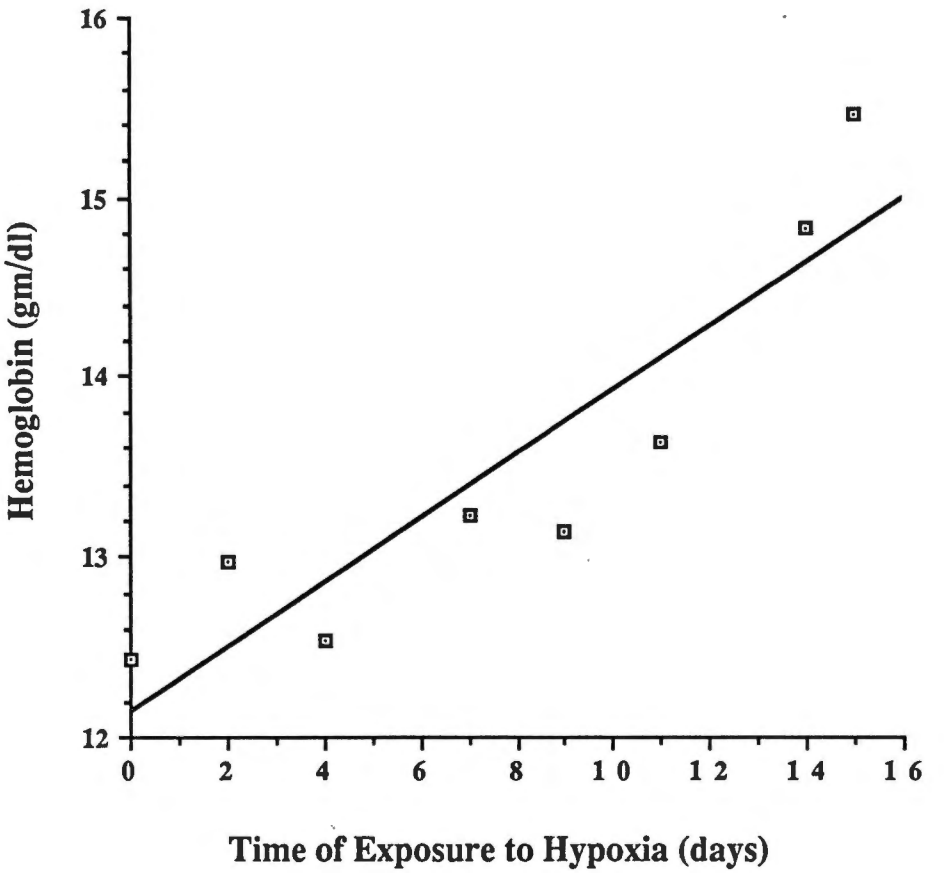

Figure 6-5 A linear regression plot of hemoglobin in grams/deciliter (gm/dl) versus time of exposure to hypoxia. Each point represents the mean value for three animals. A significant increase ($P<0.0002$) in hemoglobin concentration was observed with time of exposure to hypoxia; $r^2 = 0.818$.

TABLE 6.3 Effect of hypoxia on red blood cell indices: mean corpuscular volume in μ^3 (MCV), mean corpuscular hemoglobin in picograms (MCH), and mean corpuscular hemoglobin concentration % (MCHC).

Days of Hypoxia	MCV μ^3	MCH picograms	MCHC %
0 (control)	29 ±2	11 ±0.08	37 ±2
2	30 ±1	11 ±0.07	37 ±2
4	30 ±0.7	11 ±0.02	36.5 ±1
7	26 ±1	9 ±0.03	36 ±1
9	33 ±2	11 ±0.01	34 ±2
11	32 ±2	11 ±0.09	35 ±1
14	32 ±2	11 ±0.03	35 ±1
15	31 ±1	11 ±0.03	35.5 ±1

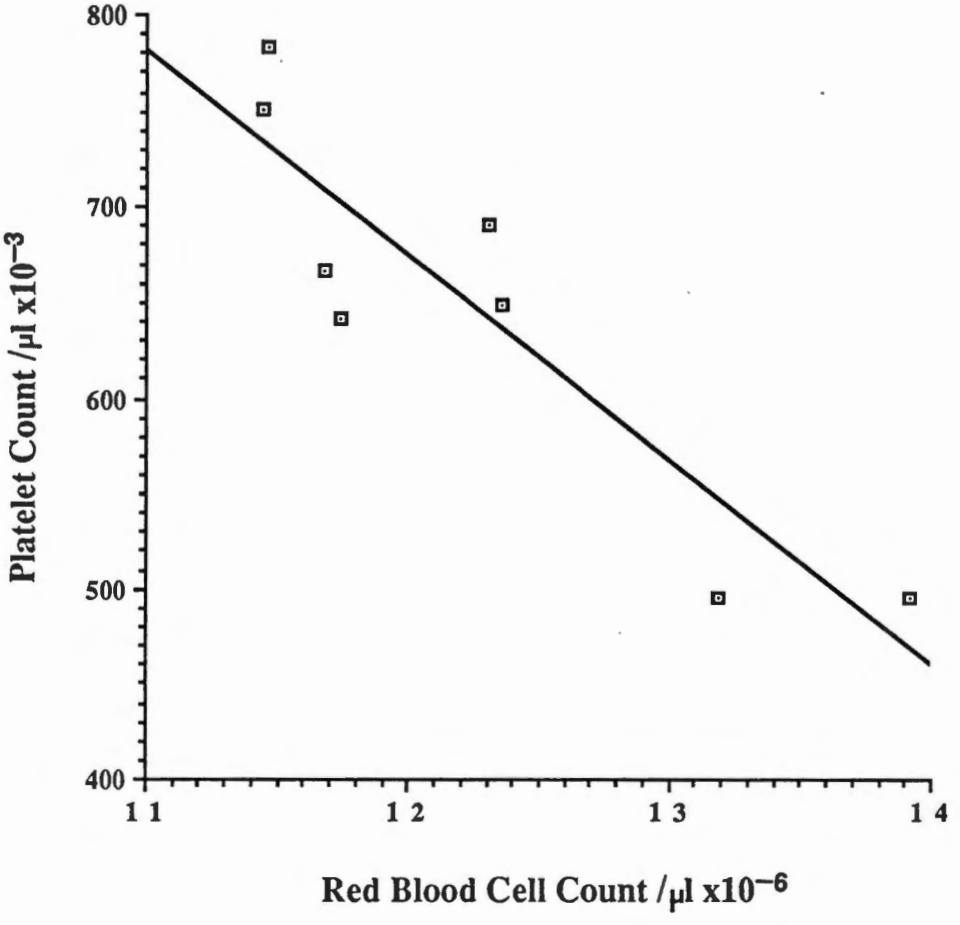

Figure 6-6 A linear regression plot of platelet count/µl versus red blood cell count/µl. A negative linear relationship was observed between platelet count and red blood cell count; $P<0.002$, $r^2 = 0.814$.

of hypoxia (Figure 6-7). The packed cell volume increased by 31%, while the platelet count dropped by 34% below normal control values (Table 6-1). As the amount of hemoglobin increased, the platelet count decreased significantly ($P<0.0002$) (Figure 6-8).

A significant ($P<0.004$) decline in total plasma protein (gm/dl) was shown when plotted against length of exposure to hypoxia (Figure 6-9, Table 6-2, Appendix E).

The white blood cell count per μl of blood increased significantly ($P<0.02$) with time of hypoxia. The differential count showed no significant changes in the leukocyte count (Table 6-4). However, there was a relative increase of neutrophils (from 32 to 42%) at the expense of lymphocytes. The percentage of monocytes was constant (approximately 2%) throughout the time of the experiment.

DISCUSSION

In this study, sheep were exposed to chronic hypoxia. The atmosphere in the chamber was maintained at 380 mm Hg. This is equivalent to an altitude of 4,878-5,488 m (Armstrong, 1939). This exposure of sheep to chronic hypobaric hypoxia (10% O_2) resulted in a decrease in platelet volume, platelet count, and in total plasma

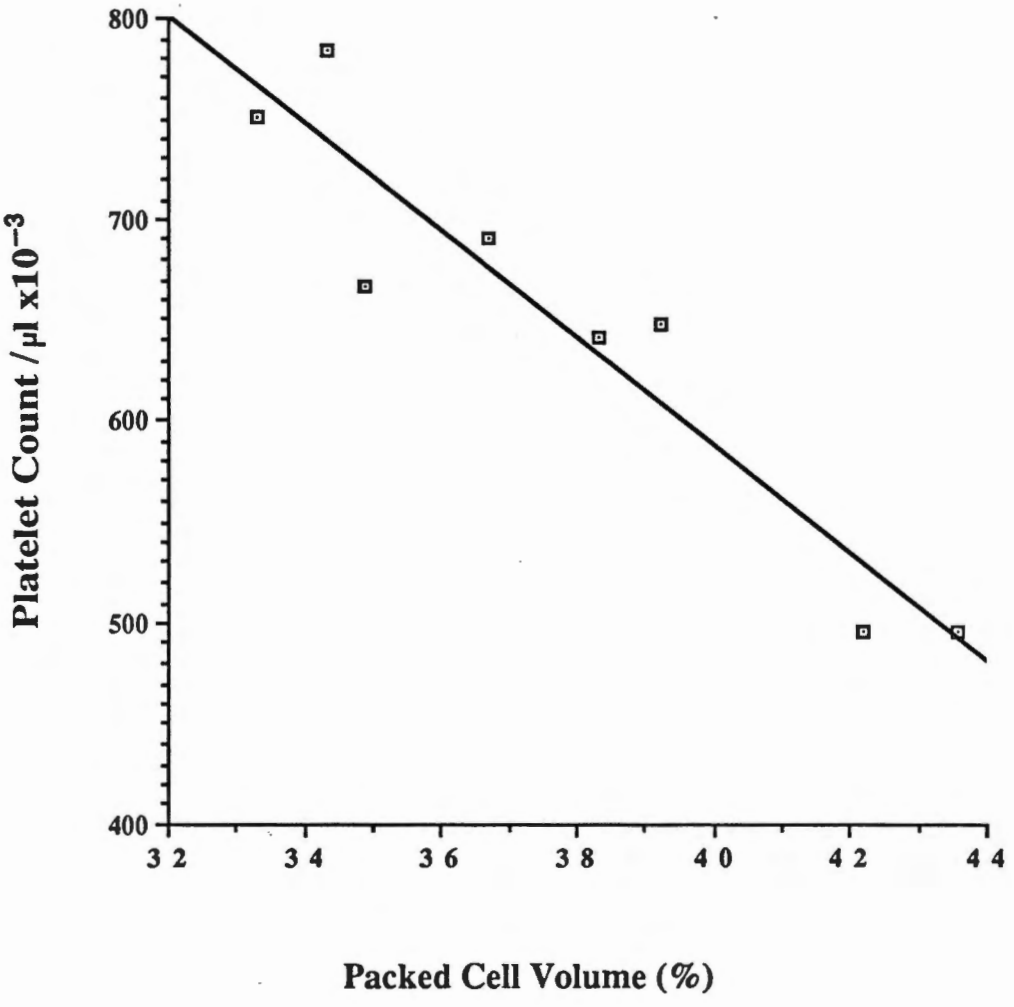

Figure 6-7. A linear regression plot of platelet count/µl versus packed cell volume %. A negative linear relationship was observed between platelet count and hematocrit; $P<0.0004$, $r^2 = 0.889$.

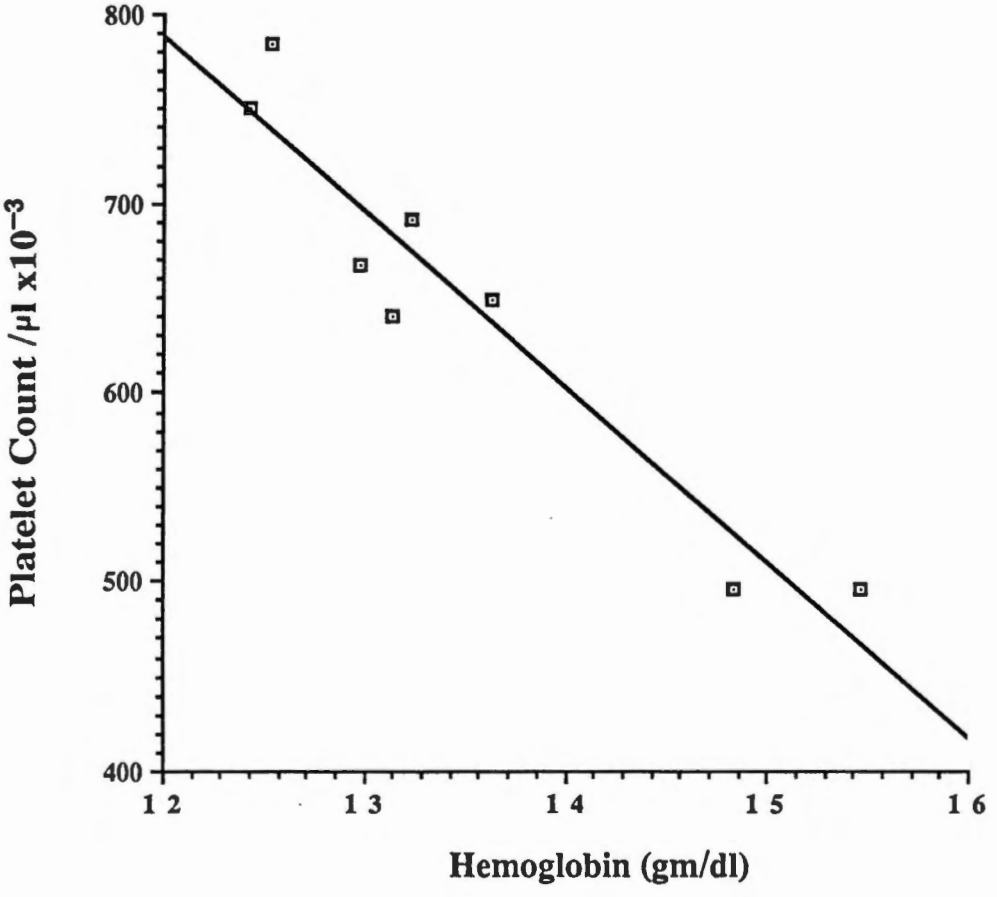

Figure 6-8. A linear regression plot of platelet count/µl versus hemoglobin in grams/deciliter (gm/dl). A negative linear relationship was observed between platelet count and hemoglobin; $P<0.0002$, $r^2 = 0.910$.

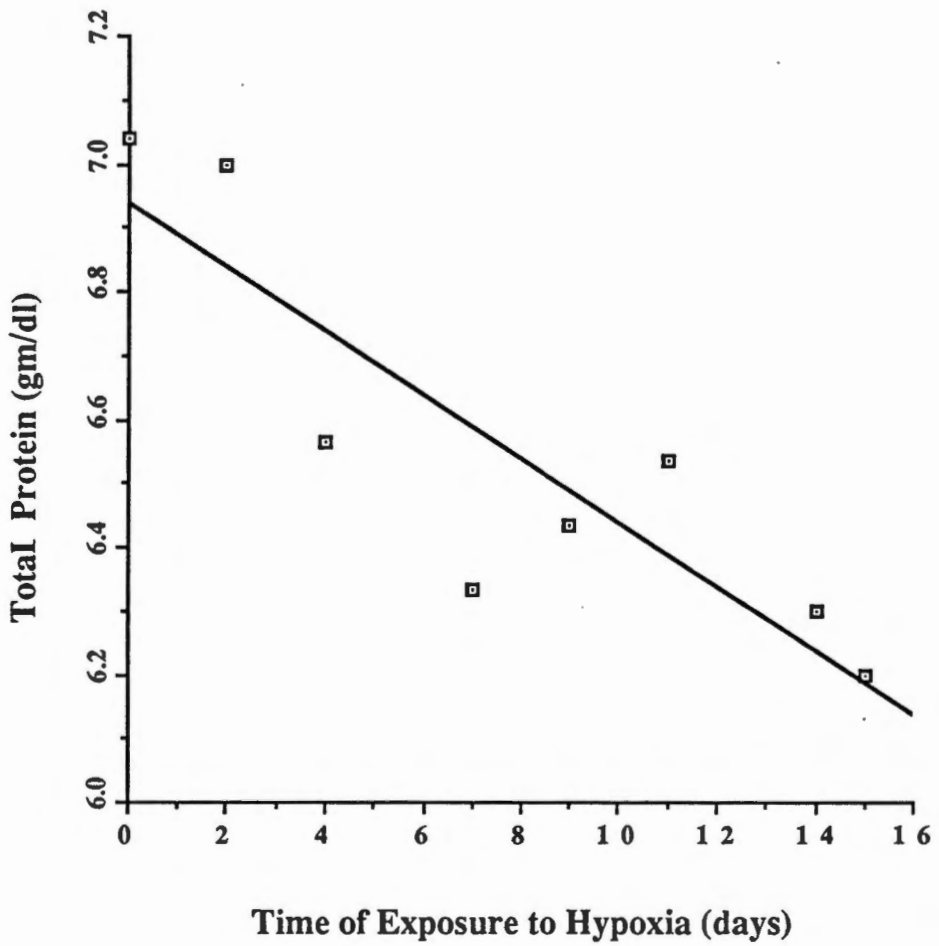

Figure 6-9 A linear regression plot of total protein in grams/deciliter (gm/dl) versus time of exposure to Hypoxia (Days). Each point represents the mean of three animals. A marked decrease in plasma protein ($P<0.004$) was observed; $r^2 = 0.768$.

TABLE 6.4 Effect of hypoxia on mean white blood cell count/μl X10^{-3} (WBC), mean % basophil (BASO), mean % eosinophil (EOS), mean % lymphocyte (LYMP), mean % monocyte (MONO), and mean % neutrophil (NEUT).

Days of Hypoxia	WBC	BASO	EOS	LYMP	MONO	NEUT
0 (control)	9.1 ±2.0	1	0	65 ±10	2 ±2	32 ±9
2	10.4 ±2.0	0	0	64 ±10	1 ±1	35 ±10
4	10.4 ±2.0	1	1	67 ±5	3 ±2	28 ±2
7	10.2 ±2.0	0	0	69 ±1	3 ±3	28 ±4
9	12.2 ±5.0	0	1	61 ±2	1 ±1	37 ±2
11	11.5 ±2.0	1	0	58 ±2	2 ±1	40 ±2
14	12.7 ±3.0	0	1	62 ±2	2 ±1	35 ±2
15	11.5 ±3.0	0	0	56 ±1	2 ±1	42 ±1

proteins, but an increase in red and white blood cell counts, packed cell volume, and hemoglobin.

A significant change in platelet volume was observed. The decrease in platelet volume, also reported in mice by McDonald et al. (1978) has been associated with an increase of platelet age (Detwiler et al., 1962; McDonald et al., 1964). Usually thrombocytopenia causes an increase in platelet volume, but in the sheep, hypoxia reduced platelet volume. This suggests that hypoxia interferes with platelet production. The decrease in platelet volume observed here would seem likely due to an increased ratio of aged-platelets resulting from a decrease in platelet production. The decrease in platelet production is likely a result of stem-cell competition.

The platelet counts of sheep, after 15 days of negative pressure hypoxia, were reduced to 66% of the normal value. This marked thrombocytopenia supports the findings of other researchers (Birks et al., 1975; Cooper and Cooper, 1977; Jackson and Edwards, 1977; Langdon and McDonald, 1977). Several explanations for the reduction in platelet count were found in the literature. Not all explanations were found to be fully satisfactory by all researchers in this field. Shortened platelet life span was rejected as an explanation by Jackson and Edwards (1977). Their experiments, using discontinuous hypobaric

hypoxia on rats for 11 days and labeling platelets with ^{51}Cr (Chromium), showed that platelet survival was normal. McDonald (1978) suggested as an unlikely explanation that platelet life span in mice may be shortened, as severe continuous hypoxia (dimethyl-silicone rubber membrane cages, enclosed for 14 days) produces more stress and hence higher levels of erythropoietin and the probable accumulation and/or release of more toxic materials. Shreiner and Levin (1973) reported no changes in ^{75}SeM labeled platelet production of mice enclosed in silicone-rubber membrane cages for 6 days. However, the mean platelet count was only 23% lower than normal. In contrast, Cooper and Cooper (1977) and Langdon and McDonald (1977) reported a decrease in platelet count which has been associated with reduced $^{35}Na_2SO_4$ isotope incorporation into platelets. The discrepancy is probably due to differing degrees of hypoxia, different mouse strains, and/or a difference in isotope uptake by platelets. ^{75}SeM probably attaches to the newly synthesized hemoglobin. As a result, a non-significant reduction in isotope incorporation was reported by Shreiner and Levin (1973).

Another interpretation of thrombocytopenia due to hypoxia was presented by Spivak et al. (1972). In the mouse, they concluded that erythropoietic stimulation led

to enlargement of the spleen and subsequently led to the extravagant sequestration of platelets by the spleen. However, this conclusion was not satisfactory to several authors (Birks, 1975; Langdon and McDonald, 1977). Langdon and McDonald (1977) found that splenectomized C_3H mice placed in hypoxia chambers showed decreased platelet counts and platelet production when compared with controls.

However, another possibility exists that the hematopoietic microenvironment may have been changed by the low 0_2 tension, which leads to an increased proliferation of the erythroid cell line. An increase in volume of erythroid cells leaves less space for the megakaryocytic cell line within the confined marrow space (McDonald et al., 1979). Stem cell competition causes reduction in platelet counts and an increase in red blood cell numbers. This proposal by McDonald et al. (1979) is probably the more acceptable explanation for the reduction in the platelet count. Higher levels of erythropoietin may be produced in response to hypoxia and could cancel the effect of thrombopoietin (McDonald et al., 1979).

The significant increase in red blood cell count, packed cell volume, and hemoglobin is similar to the findings of VanLiere and Stickney (1963), Lord and Murphy (1973), and DeGowin et al. (1962). The negative linear

relationship found between red blood cell count, packed cell volume and hemoglobin when plotted against platelet count was similar to the findings of Choi and Simone (1973). They reported thrombocytosis in the clinical conditions of chronic iron-deficiency anemia and acute anemia. Several explanations have been proposed to clarify the negative relationship between platelet count and red blood cell count, packed cell volume and hemoglobin. The increase in relative platelet numbers in rats could be a result of changes in blood volume, increased platelet life span, or increased platelet formation (Jackson et al., 1974). However, in experimental animals with hematocrits reduced to 25 percent of control, the results of blood volume estimation indicate that changes in blood volume could not be responsible for the increase in platelet counts of anemic animals (Jackson et al., 1974).

The decreased oxygen tension in tissues possibly leads to increased levels of erythropoietin in the plasma. Erythropoietin stimulates stem cells to differentiate into the erythroid cell line, leading to decreased numbers of stem cells which can differentiate into the megakaryocytic cell line. This results in a reduced platelet count. The increase in hemoglobin is probably due to the increase of red blood cells. The increased amount of hemoglobin is

probably an adaptive response to the low oxygen environment, thus increasing the oxygen-carrying capacity of the blood. The increased packed cell volume is likely be due to the increase number of red blood cells.

In our investigation, the reduction in the total plasma protein can be explained by the stress caused by the hypoxic atmosphere. Insufficient oxygen leads to a decrease in the synthesis of various plasma proteins. Garvey et al. (1969) reported significant decreases in plasma levels of Factors V, VIII, IX and XI in monkeys subjected to hypobaric hypoxia.

The nonsignificant changes in the red blood cell indices (MCV, MCH, MCHC) indicated that no defects in erythropoiesis occurred upon exposure to hypoxia.

The increase of the peripheral blood leukocyte count that we observed may be due to the discharge of leukocytes from the bone marrow. This result supports the findings of Harris et al. (1966), who reported a decrease in bone marrow lymphocyte numbers during hypoxia. The increase of leukocyte numbers in the sheep was similar to investigations on humans at high altitudes (Hurtado, 1945) and on rats, guinea pigs (Meyer et al., 1935), and mice (Petursson and Chervenick, 1987) exposed to hypoxia.

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APPENDIXES

APPENDIX A

Tissue Processing for Plastic Sectioning:

The specimens were cut into 3 to 5 millimeter pieces and fixed in 10% phosphate buffered formalin, pH 7.0 (0.01 M) for 10 days. The specimens were then processed as follows:

A. Partial dehydration: The specimens were passed through a graded series of ethanol.

1. 50% ethanol: two changes, 10 minutes each.
2. 70% ethanol: two changes, 10 minutes each.
3. 95% ethanol: two changes, 10 minutes each.

B. Infiltration: The specimens were infiltrated with 100-ml JB-4 solution A and 0.9-gm catalyst. The infiltration was accomplished at room temperature with two to three changes, ranging from 40 to 50 minutes, of the catalyzed JB-4 solution A. The changes were continued until the samples became translucent and sank to the bottom of the container.

C. Embedding and Polymerization: The JB-4 mixture was polymerized at room temperature and prepared by the addition of one ml of JB-4 solution B to 40 ml of freshly prepared and catalyzed JB-4 solution A. All the mixtures were stirred thoroughly. The specimens were oriented and properly positioned in the plastic mold containing the JB-

4 mixture. A stub was attached to each specimen and allowed to polymerize at room temperature.

APPENDIX B

Procedure to estimate sufficient sampling:

To ensure satisfactory sampling, the mean value of the count derived from a sample at any one site (red pulp) must have a standard error (RSE) that is acceptable statistically ($P<0.025$). For instance, to ensure enough counting of the volume fraction of the red pulp, the standard error (RSE) should be less than 0.025 and was calculated as follows:

$$RSE = \sqrt{\frac{1 - V_V}{N}}$$

where:

N = the number of points that fell on the red pulp.

A preliminary study was conducted. To ensure sufficient sampling from each sheep, 2600 points were required.

APPENDIX C

Estimation of volume of different components of the spleen (volume fraction V_v)

$$V_v = \frac{\text{no. of points that fell on the component}}{\text{total no. of points that fell on the spleen section}}$$

APPENDIX D

Calculation of Oxygen Content:

$$\frac{\text{Atmospheric pressure}}{\text{Amount of pressure reduction}} = X$$

$$\frac{\text{Oxygen concentration of sea level air}}{X} = \%\ \text{oxygen at that pressure}$$

$$\frac{760\ \text{Hg mm}}{380\ \text{Hg mm}} = 2$$

$$\frac{20\%}{2} = 10\%$$

APPENDIX E

Effect of hypoxia on mean platelet volume in femtoliters (PLTV), mean platelet count/μl X10^{-5} (PLTC), mean hemoglobin grams/deciliter (HB), mean packed cell volume (PCV) %, mean red blood cell count/μl X10^{-6} (RBC), and mean total protein grams/deciliter (TP).

Days of Hypoxia	PLTV	PLTC	HB	PCV	RBC	TP
0 (control)	3.5 ±0.2	7.5 ±1.0	12.4 ±0.5	33.3 ±2.0	11.4 ±1.0	7.0 ±0.6
2	3.3 ±0.2	6.6 ±1.6	13.0 ±0.6	34.9 ±3.0	11.7 ±1.0	7.0 ±0.7
4	3.4 ±0.2	5.6 ±0.4	12.5 ±0.5	34.3 ±2.0	11.5 ±0.4	6.6 ±0.2
7	3.1 ±0.3	6.9 ±1.0	13.2 ±0.6	36.7 ±1.0	14.3 ±0.08	6.3 ±0.1
9	2.9 ±0.08	6.4 ±1.0	13.1 ±0.5	38.3 ±0.8	11.7 ±0.5	6.4 ±0.3
11	3.2 ±0.1	6.5 ±0.7	13.6 ±0.5	38.2 ±0.2	12.4 ±1.0	6.5 ±0.1
14	2.7 ±0.2	5.0 ±0.4	14.8 ±0.7	42.2 ±0.9	13.2 ±0.9	6.3 ±0.2
15	2.6	5.0	15.5	43.6	13.9	6.2

APPENDIX F

Data on animal age, animal weight (ANWT), splenic weight (SLWT), splenic volume (SESZ), and code for each animal.

OBS	AGE	ANWT	SLWT	SESZ	COD
1	PRENATAL	4.445	5.00	5.00	L4P
2	PRENATAL	4.899	6.00	6.00	L5P
3	PRENATAL	3.992	5.00	5.00	L6P
4	PRENATAL	3.993	4.90	4.90	L7P
5	PRENATAL	1.769	1.80	1.99	L13P
6	PRENATAL	2.359	2.00	2.00	L12P
7	1 DAY	2.200	2.60	2.60	L1SD1
8	1 DAY	3.980	5.78	6.50	L2SD1
9	1 DAY	3.190	4.40	5.00	L10D1
10	1 DAY	2.723	4.40	5.00	L14D1
11	1 WEEK	5.897	10.90	11.00	L11D7
12	1 WEEK	2.631	6.00	6.00	L16D7
13	1 WEEK	5.398	11.00	11.00	L17D7
14	1 WEEK	3.674	6.60	6.50	L18D7
15	3 WEEKS	12.247	30.00	30.00	L8D21
16	3 WEEKS	9.072	26.30	27.00	L9D21
17	3 WEEKS	9.525	21.50	24.00	L15D21
18	3 WEEKS	4.990	8.30	8.20	L19D21
19	3 WEEKS	10.886	25.80	25.00	L20D21
20	3 WEEKS	7.711	14.40	14.00	L21D21
21	6 WEEKS	12.600	24.00	23.00	L1W6
22	6 WEEKS	10.800	21.50	21.00	L2W6
23	6 WEEKS	13.050	26.50	26.00	L3W6
24	4 MONTHS	31.500	60.00	61.00	L1M4
25	4 MONTHS	34.650	63.00	63.00	L2M4
26	4 MONTHS	36.900	66.00	65.00	L3M4
27	5 MONTHS	43.091	70.00	71.50	616
28	5 MONTHS	52.617	93.00	91.00	606
29	5 MONTHS	45.359	65.00	69.00	620
30	5 MONTHS	39.009	78.00	72.00	618
31	2 YEARS	107.048	153.00	150.00	N
32	2 YEARS	104.326	195.00	190.00	Y
33	2 YEARS	107.501	118.00	118.00	X

APPENDIX G

Data collected on the number of points that fell on red pulp (RED), white pulp (WHITE), trabeculae (TRAB), and blood vessels (VESSEL).

ANIMAL	BLOCK	RED	WHITE	TRAB	VESSEL	AGE
1	1	655	154	75	16	PRENATAL
1	2	470	37	72	12	PRENATAL
1	3	397	27	47	29	PRENATAL
2	1	652	114	75	43	PRENATAL
2	2	339	107	45	9	PRENATAL
2	3	354	146	83	17	PRENATAL
3	1	713	151	125	16	PRENATAL
3	2	478	129	82	11	PRENATAL
3	3	377	67	69	39	PRENATAL
4	1	556	152	85	7	1 DAY
4	2	491	192	93	24	1 DAY
4	3	531	132	115	22	1 DAY
5	1	487	56	50	7	1 DAY
5	2	488	77	104	31	1 DAY
5	3	490	42	65	3	1 DAY
6	1	690	245	61	3	1 DAY
6	2	730	223	38	8	1 DAY
6	3	.	.	.	.	1 DAY
7	1	759	133	100	8	1 WEEK
7	2	667	188	136	9	1 WEEK
7	3	707	162	119	12	1 WEEK
8	1	737	149	102	12	1 WEEK
8	2	659	130	77	17	1 WEEK
8	3	755	122	115	8	1 WEEK
9	1	703	200	86	10	1 WEEK
9	2	692	222	67	19	1 WEEK
9	3	726	192	77	5	1 WEEK
10	1	746	158	89	10	3 WEEKS
10	2	754	163	83	0	3 WEEKS
10	3	688	241	62	9	3 WEEKS
11	1	755	167	67	12	3 WEEKS
11	2	637	168	81	14	3 WEEKS
11	3	703	164	129	4	3 WEEKS
12	1	751	143	102	4	3 WEEKS
12	2	784	105	100	10	3 WEEKS
12	3	609	159	115	16	3 WEEKS
13	1	634	297	57	12	6 WEEKS
13	2	761	169	35	35	6 WEEKS
13	3	587	363	32	16	6 WEEKS
14	1	552	318	118	12	6 WEEKS

ANIMAL	BLOCK	RED	WHITE	TRAB	VESSEL	AGE
14	2	739	185	73	3	6 WEEKS
14	3	621	210	62	7	6 WEEKS
15	1	598	270	24	8	6 WEEKS
15	2	812	73	109	5	6 WEEKS
15	3	757	122	96	25	6 WEEKS
16	1	595	300	80	25	4 MONTHS
16	2	531	357	101	11	4 MONTHS
16	3	614	260	113	13	4 MONTHS
17	1	565	345	45	15	4 MONTHS
17	2	595	322	45	38	4 MONTHS
17	3	615	299	61	25	4 MONTHS
18	1	525	388	79	8	4 MONTHS
18	2	646	242	104	8	4 MONTHS
18	3	550	327	111	12	4 MONTHS
19	1	617	305	56	22	5 MONTHS
19	2	655	120	97	28	5 MONTHS
19	3	640	200	143	17	5 MONTHS
20	1	621	296	54	29	5 MONTHS
20	2	673	254	64	9	5 MONTHS
20	3	563	282	145	10	5 MONTHS
21	1	493	200	95	12	5 MONTHS
21	2	567	348	52	33	5 MONTHS
21	3	670	175	146	9	5 MONTHS
22	1	695	66	220	19	2 YEARS
22	2	633	87	269	11	2 YEARS
22	3	805	29	143	23	2 YEARS
23	1	652	129	205	12	2 YEARS
23	2	720	115	149	16	2 YEARS
23	3	658	126	206	10	2 YEARS
24	1	522	330	100	48	2 YEARS
24	2	785	123	81	10	2 YEARS
24	3	722	255	10	13	2 YEARS

APPENDIX H

Data collected on the thickness (THICK) of the capsule of the spleen in μm.

OBS	AGE	ANIMAL	BLOCK	THICK
1	PRENATAL	1	1	104.920
2	PRENATAL	1	1	101.597
3	PRENATAL	1	1	81.737
4	PRENATAL	1	2	69.594
5	PRENATAL	1	2	73.860
6	PRENATAL	1	2	83.402
7	PRENATAL	1	3	76.151
8	PRENATAL	1	3	67.757
9	PRENATAL	1	3	84.574
10	PRENATAL	2	1	100.228
11	PRENATAL	2	1	103.526
12	PRENATAL	2	1	104.657
13	PRENATAL	2	2	80.588
14	PRENATAL	2	2	99.820
15	PRENATAL	2	2	79.887
16	PRENATAL	2	3	86.898
17	PRENATAL	2	3	81.336
18	PRENATAL	2	3	110.864
19	PRENATAL	3	1	105.717
20	PRENATAL	3	1	87.727
21	PRENATAL	3	1	104.227
22	PRENATAL	3	2	74.415
23	PRENATAL	3	2	65.805
24	PRENATAL	3	2	93.789
25	PRENATAL	3	3	79.329
26	PRENATAL	3	3	89.950
27	PRENATAL	3	3	75.500
28	1 DAY	1	1	89.063
29	1 DAY	1	1	95.550
30	1 DAY	1	1	151.886
31	1 DAY	1	2	79.372
32	1 DAY	1	2	69.790
33	1 DAY	1	2	56.590
34	1 DAY	1	3	126.938
35	1 DAY	1	3	123.772
36	1 DAY	1	3	112.757
37	1 DAY	2	1	134.576
38	1 DAY	2	1	130.294
39	1 DAY	2	1	141.413
40	1 DAY	2	2	92.533

OBS	AGE	ANIMAL	BLOCK	THICK
41	1 DAY	2	2	96.976
42	1 DAY	2	2	102.804
43	1 DAY	2	3	140.164
44	1 DAY	2	3	143.398
45	1 DAY	2	3	144.052
46	1 DAY	3	1	229.705
47	1 DAY	3	1	142.542
48	1 DAY	3	1	120.109
49	1 DAY	3	2	123.201
50	1 DAY	3	2	211.135
51	1 DAY	3	2	86.004
52	1 DAY	3	3	113.238
53	1 DAY	3	3	101.921
54	1 DAY	3	3	159.282
55	1 WEEK	1	1	72.637
56	1 WEEK	1	1	82.182
57	1 WEEK	1	1	82.239
58	1 WEEK	1	2	94.578
59	1 WEEK	1	2	104.235
60	1 WEEK	1	2	133.285
61	1 WEEK	1	3	76.589
62	1 WEEK	1	3	75.549
63	1 WEEK	1	3	104.995
64	1 WEEK	2	1	104.557
65	1 WEEK	2	1	114.222
66	1 WEEK	2	1	125.729
67	1 WEEK	2	2	113.155
68	1 WEEK	2	2	122.604
69	1 WEEK	2	2	86.136
70	1 WEEK	3	1	191.213
71	1 WEEK	3	1	177.965
72	1 WEEK	3	1	116.307
73	1 WEEK	3	2	138.254
74	1 WEEK	3	2	156.504
75	1 WEEK	3	2	167.568
76	1 WEEK	3	3	132.924
77	1 WEEK	3	3	156.317
78	1 WEEK	3	3	155.795
79	3 WEEKS	1	1	188.342
80	3 WEEKS	1	1	175.843

OBS	AGE	ANIMAL	BLOCK	THICK
81	3 WEEKS	1	1	210.461
82	3 WEEKS	1	2	176.041
83	3 WEEKS	1	2	210.616
84	3 WEEKS	1	2	239.372
85	3 WEEKS	1	3	115.845
86	3 WEEKS	1	3	131.716
87	3 WEEKS	1	3	107.280
88	3 WEEKS	2	1	163.329
89	3 WEEKS	2	1	186.615
90	3 WEEKS	2	1	171.551
91	3 WEEKS	2	2	212.373
92	3 WEEKS	2	2	187.416
93	3 WEEKS	2	2	175.770
94	3 WEEKS	2	3	205.011
95	3 WEEKS	2	3	189.390
96	3 WEEKS	2	3	196.632
97	3 WEEKS	3	1	225.981
98	3 WEEKS	3	1	180.134
99	3 WEEKS	3	1	169.290
100	3 WEEKS	3	2	159.584
101	3 WEEKS	3	2	169.286
102	3 WEEKS	3	2	166.919
103	3 WEEKS	3	3	156.827
104	3 WEEKS	3	3	220.090
105	3 WEEKS	3	3	166.311
106	6 WEEKS	1	1	240.741
107	6 WEEKS	1	1	212.317
108	6 WEEKS	1	1	300.000
109	6 WEEKS	1	2	300.000
110	6 WEEKS	1	2	281.718
111	6 WEEKS	1	2	294.238
112	6 WEEKS	1	3	256.675
113	6 WEEKS	1	3	206.074
114	6 WEEKS	1	3	196.454
115	6 WEEKS	2	1	203.548
116	6 WEEKS	2	1	223.285
117	6 WEEKS	2	1	192.294
118	6 WEEKS	2	2	317.851
119	6 WEEKS	2	2	222.702
120	6 WEEKS	2	2	284.179

OBS	AGE	ANIMAL	BLOCK	THICK
121	6 WEEKS	3	1	314.661
122	6 WEEKS	3	1	289.907
123	6 WEEKS	3	1	282.253
124	6 WEEKS	3	2	161.625
125	6 WEEKS	3	2	181.777
126	6 WEEKS	3	2	310.522
127	6 WEEKS	3	3	181.662
128	6 WEEKS	3	3	162.662
129	6 WEEKS	3	3	240.033
130	4 MONTHS	1	1	162.243
131	4 MONTHS	1	1	149.539
132	4 MONTHS	1	1	163.076
133	4 MONTHS	1	2	223.568
134	4 MONTHS	1	2	253.053
135	4 MONTHS	1	2	272.104
136	4 MONTHS	2	1	288.328
137	4 MONTHS	2	1	244.420
138	4 MONTHS	2	1	219.916
139	4 MONTHS	2	2	226.079
140	4 MONTHS	2	2	236.252
141	4 MONTHS	2	2	266.069
142	4 MONTHS	2	3	206.233
143	4 MONTHS	2	3	157.155
144	4 MONTHS	2	3	109.970
145	4 MONTHS	3	1	283.648
146	4 MONTHS	3	1	219.164
147	4 MONTHS	3	1	327.778
148	4 MONTHS	3	2	208.369
149	4 MONTHS	3	2	185.654
150	4 MONTHS	3	2	130.407
151	5 MONTHS	1	1	234.929
152	5 MONTHS	1	1	215.163
153	5 MONTHS	1	1	194.437
154	5 MONTHS	1	2	196.758
155	5 MONTHS	1	2	208.121
156	5 MONTHS	1	2	175.254
157	5 MONTHS	1	3	222.254
158	5 MONTHS	1	3	184.404
159	5 MONTHS	1	3	213.304
160	5 MONTHS	2	1	302.379

OBS	AGE	ANIMAL	BLOCK	THICK
161	5 MONTHS	2	1	231.962
162	5 MONTHS	2	1	219.467
163	5 MONTHS	2	2	203.729
164	5 MONTHS	2	2	231.595
165	5 MONTHS	2	2	235.233
166	5 MONTHS	2	3	272.373
167	5 MONTHS	2	3	250.446
168	5 MONTHS	2	3	252.106
169	5 MONTHS	3	1	173.341
170	5 MONTHS	3	1	159.043
171	5 MONTHS	3	1	159.647
172	5 MONTHS	3	2	213.678
173	5 MONTHS	3	2	187.130
174	5 MONTHS	3	2	181.019
175	5 MONTHS	3	3	193.221
176	5 MONTHS	3	3	170.405
177	5 MONTHS	3	3	129.421
178	2 YEARS	1	1	506.168
179	2 YEARS	1	1	592.161
180	2 YEARS	1	1	660.452
181	2 YEARS	1	2	457.191
182	2 YEARS	1	2	585.163
183	2 YEARS	1	2	673.623
184	2 YEARS	1	3	613.107
185	2 YEARS	1	3	408.660
186	2 YEARS	1	3	488.764
187	2 YEARS	2	1	620.860
188	2 YEARS	2	1	637.590
189	2 YEARS	2	1	441.754
190	2 YEARS	2	2	644.793
191	2 YEARS	2	2	683.665
192	2 YEARS	2	2	656.753
193	2 YEARS	2	3	631.631
194	2 YEARS	2	3	611.512
195	2 YEARS	2	3	426.324
196	2 YEARS	3	1	386.953
197	2 YEARS	3	1	408.831
198	2 YEARS	3	1	207.229
199	2 YEARS	3	2	215.430
200	2 YEARS	3	2	176.754
201	2 YEARS	3	2	326.429
202	2 YEARS	3	3	332.333
203	2 YEARS	3	3	364.649
204	2 YEARS	3	3	220.892

APPENDIX I

Data collected on the weight of normal and hypoxic sheep in kg, splenic weight in gm (SPWT) and splenic volume in ml (SPVOL).

Animal	Weight	Atmosphere	SPWT	SPVOL
1	34.9	Normal	60.0	61
2	26.3	Normal	63.0	63
3	34.5	Normal	66.0	65
4	30.3	Hypoxia	54.0	50
5	29.3	Hypoxia	50.5	50
6	33.3	Hypoxia	51.0	50

APPENDIX J

Data collected on the points that fell on the red pulp (RED), white pulp (WHITE), trabeculae (TRAB), and blood vessel (VESSEL) in the spleen of normal and hypoxic sheep.

OBS	SLIDE	RED	WHITE	TRAB	VESSEL	TRT
1	1	595	300	80	25	NORMAL
2	2	531	357	101	11	NORMAL
3	3	614	260	113	13	NORMAL
4	1	565	345	45	15	NORMAL
5	2	595	322	45	38	NORMAL
6	3	615	299	61	25	NORMAL
7	1	525	388	79	8	NORMAL
8	2	646	242	104	8	NORMAL
9	3	550	327	111	12	NORMAL
10	1	669	241	72	18	HYPOXIA
11	2	713	186	61	40	HYPOXIA
12	3	587	349	32	32	HYPOXIA
13	1	594	266	116	24	HYPOXIA
14	2	507	415	71	7	HYPOXIA
15	3	748	196	28	28	HYPOXIA
16	1	751	199	45	5	HYPOXIA
17	2	653	226	112	9	HYPOXIA
18	3	741	205	47	7	HYPOXIA

APPENDIX K

Data collected on the thickness (THICK) of the capsule of the spleen in μm of normal and hypoxic sheep.

OBS	TRT	ANIMAL	BLOCK	THICK
1	HYPOXIA	1	1	435.001
2	HYPOXIA	1	1	409.477
3	HYPOXIA	1	1	340.865
4	HYPOXIA	1	2	378.314
5	HYPOXIA	1	2	392.809
6	HYPOXIA	1	2	371.514
7	HYPOXIA	1	3	359.065
8	HYPOXIA	1	3	404.823
9	HYPOXIA	1	3	392.314
10	HYPOXIA	2	1	175.650
11	HYPOXIA	2	1	186.664
12	HYPOXIA	2	1	199.408
13	HYPOXIA	2	2	159.298
14	HYPOXIA	2	2	182.319
15	HYPOXIA	2	2	150.433
16	HYPOXIA	3	1	235.752
17	HYPOXIA	3	1	257.023
18	HYPOXIA	3	1	280.299
19	HYPOXIA	3	2	216.473
20	HYPOXIA	3	2	286.105
21	HYPOXIA	3	2	315.477
22	HYPOXIA	3	3	251.525
23	HYPOXIA	3	3	242.412
24	HYPOXIA	3	3	206.582
25	NORMAL	1	1	162.243
26	NORMAL	1	1	149.539
27	NORMAL	1	1	163.076
28	NORMAL	1	2	223.568
29	NORMAL	1	2	253.053
30	NORMAL	1	2	272.104
31	NORMAL	2	1	288.328
32	NORMAL	2	1	244.420
33	NORMAL	2	1	219.916
34	NORMAL	2	2	226.079
35	NORMAL	2	2	236.252
36	NORMAL	2	2	266.069
37	NORMAL	2	3	206.233
38	NORMAL	2	3	157.155
39	NORMAL	2	3	109.970
40	NORMAL	3	1	283.648
41	NORMAL	3	1	219.164
42	NORMAL	3	1	327.778
43	NORMAL	3	2	208.369
44	NORMAL	3	2	185.654
45	NORMAL	3	2	130.407

APPENDIX L

Data collected on the blood constituents of hypoxic sheep: platelet size (PLTS), platelet count (PLTN), hemoglobin (HB), packed cell volume (PCV), total protein (TP), white blood cells (WBC). Differential counts consisted of percentage of the following cellular elements neutrophil (SG), lymphocytes (LY), monocytes (MO), eosinophil (EO), and basophil (BS). Time of Collection: number (0) represents the collection before hypoxia and the other represents the days of collection after starting hypoxia.

PLTS	PLTN	HB	PCV	TP	RBC	WBC	SG	LY	MO	EO	BS	DT	AN	TPLTN	TRBC
3.64	695.0	13.3	38.0	8.0	11.82	7800	43	54	1	1	1	0	PRE	695000	11820000
3.35	742.5	12.1	34.0	7.2	10.80	8900	22	74	4	0	0	0	PRE	742500	10800000
3.09	500.0	13.6	38.5	8.1	12.32	9600	36	62	2	0	0	2	POST	500000	12320000
3.45	717.5	12.6	33.0	6.4	11.58	11400	31	67	1	0	1	0	PRE	717500	11580000
3.54	860.0	12.7	35.5	6.4	14.00	12400	22	77	1	0	0	0	PRE	860000	14000000
3.11	755.0	13.4	36.7	6.6	13.12	11800	24	76	0	0	0	2	POST	755000	13120000
3.10	810.0	13.1	36.0	6.4	11.82	9800	25	72	3	0	0	4	POST	810000	11820000
3.44	535.0	12.6	36.0	6.2	18.28	9900	22	70	7	0	0	7	POST	535000	18280000
2.92	545.0	12.7	38.7	6.1	11.22	11700	23	74	2	0	0	9	POST	545000	11220000
3.76	587.5	13.5	39.1	6.5	11.16	12300	54	42	2	0	2	11	POST	587500	11160000
2.37	530.0	15.7	43.2	6.1	13.30	15900	57	41	2	0	0	14	POST	530000	13300000
2.56	362.5	16.0	44.4	5.8	14.12	14100	52	47	1	0	0	15	POST	362500	14120000
3.14	847.5	12.2	31.6	6.8	10.48	8800	39	60	1	0	0	0	PRE	847500	10480000
3.46	955.0	12.4	31.5	7.2	11.94	8100	42	55	1	0	2	0	PRE	955000	11940000
3.50	845.0	12.1	31.4	6.3	10.62	12900	48	52	0	0	0	2	POST	845000	10620000
3.51	106.0	12.1	32.1	6.5	11.00	13600	29	67	1	2	0	4	POST	106000	11000000
3.14	842.5	13.3	36.1	6.3	12.36	13300	30	70	0	0	0	7	POST	842500	12360000
2.86	772.5	13.7	37.4	6.5	12.30	17500	62	37	0	1	0	9	POST	772500	12300000
2.88	720.0	14.2	39.1	6.4	13.12	13300	42	56	2	0	0	11	POST	720000	13120000
2.87	452.5	14.4	41.4	6.2	14.02	13200	20	73	3	4	0	14	POST	452500	14020000
2.72	492.5	15.4	42.8	5.7	14.33	13400	28	69	2	1	0	15	POST	492500	14330000
3.39	652.5	11.7	29.9	6.5	9.96	6700	39	61	0	0	0	0	PRE	652500	9960000
3.58	700.0	13.0	33.3	7.0	11.84	6600	19	81	0	0	0	0	PRE	700000	11840000
3.47	567.5	12.8	32.9	7.0	10.64	7300	32	67	1	0	0	2	POST	567500	10640000
3.45	757.5	12.4	35.0	6.8	11.56	7800	30	62	5	1	2	4	POST	757500	11560000
2.82	695.0	13.8	38.0	6.5	12.24	7500	31	68	1	0	0	7	POST	695000	12240000
3.02	605.0	13.0	38.8	6.7	11.70	7500	27	71	0	1	1	9	POST	605000	11700000
3.06	637.5	13.2	39.5	6.7	12.80	8800	24	75	1	0	0	11	POST	637500	12800000
2.81	505.0	14.4	42.0	6.6	12.24	8900	29	71	0	0	0	14	POST	505000	12240000
2.53	632.5	15.0	43.5	7.1	13.30	7000	44	53	3	0	0	15	POST	632500	13300000
3.64	590.0	11.9	33.0	7.9	10.56	11500	37	56	7	0	0	0	PRE	590000	10560000

APPENDIX M

Data collected on the temperature of the animal at the time of entry into the chamber (TEMP-In) and at the time of exit (Temp-out).

OBS	TEMP_OUT	TEMP_IN
1	103.8	103.4
2	103.7	103.2
3	105.0	104.2
4	103.9	103.1
5	103.0	103.0
6	102.8	103.0
7	103.1	103.5
8	103.8	103.6
9	103.8	103.5
10	103.8	103.4
11	103.9	103.9
12	103.8	103.5
13	104.0	103.5
14	103.9	103.4
15	103.0	103.0
16	103.0	103.0
17	103.0	103.4
18	102.5	102.5
19	103.2	103.0
20	103.9	103.2
21	104.0	103.0
22	103.8	103.2
23	103.4	103.2

VITA

Obaid Muhesen Faroon was born July 1, 1954 in Al-Diwanya. He graduated from Al-Diwanya High School in 1971. Following high school graduation, he enrolled in the University of Baghdad at Baghdad City. He received his Bachelor degree of Veterinary Medicine and Surgery on June 30, 1976. He joined the army for sixteen months, and was assigned to the Department of Clinical Virology. He was appointed as instructor in the Department of Veterinary Anatomy, School of Veterinary Medicine in 1978. He was awarded a scholarship from Baghdad University to pursue graduate study in the United States of America in 1981. He enrolled in Louisiana State University in the Department of Veterinary Anatomy in January, 1982 where he received a Master of Science degree in 1984. In January 1985 he received a graduate teaching assistantship at The University of Tennessee, Knoxville and began study toward a Doctor of Philosophy degree. He received his degree in June 1988 with a major in Veterinary Anatomy and a minor in Veterinary Pathology. The author is a member of the Phi Chapter of Phi Zeta, The American Association of Veterinary Anatomists, and The Electron Microscopy Society of America.