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THE PHAGOCYTIC FUNCTION OF REGENERATED SPLENIC TISSUE

by

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ABBREVIATIONS USED IN THE TEXT

^{51}Cr	Chromium-51
^{99m}Tc	Technetium-99m
APC	Antigen presenting cell
AT	Autotransplanted
CON	Control
DAB	3'3'-Diaminobenzidine
Dh	Dominant hemimelia
FDC	Follicular dendritic reticulum cell
HRP	Horse radish peroxidase
IDC	Interdigitating reticulum cell
IES	Interendothelial slits
IVC	Inferior vena cava
MZ	Marginal zone
NK	Natural killer
OPSI	Overwhelming postsplenectomy infection
PALS	Periarteriolar sheath
RBC	Red blood cells
Rh	Rhesus
RP	Red pulp
SHAM	Sham operated controls
SRBC	Sheep red blood cells
TIE	Splenic artery ligated
WP	White pulp

Abstract

The phagocytic function of regenerated splenic tissue has been studied to determine its potential for protection against overwhelming post-splenectomy infection.

An *in vivo* model was established in rats. Splenic autotransplantation was performed and the ability of the regenerated splenic tissue to phagocytose a radiolabelled colloid was investigated and compared to normal controls and spleens which had been rendered avascular by ligation. Autotransplanted and devascularised spleens regenerated and increased in weight over the following 3 months, and did not further increase in weight over the next 3 months. Technetium-99m stannous fluoride colloid was injected *i.v.* into the rats which were allowed 3 to 15 months to recover after surgery, colloid was measured in splenic tissue in all surgical groups. Both forms of regenerated splenic tissue demonstrated only 10% of uptake when compared to normal controls. When the uptake per gram of splenic tissue was calculated to allow for differences in splenic weight, the regenerated spleens recorded only 20% of normal splenic clearance. Autoradiographic and *i.v.* colloidal carbon studies isolated uptake of colloid to the inner layer of the marginal zone. Digitiser measurement of the size of the marginal zone in all groups demonstrated significant reduction of this compartment in regenerated splenic tissue and a direct correlation between the amount of marginal zone and the phagocytosis of radiolabelled colloid was identified.

Phagocytosis by regenerated and normal splenic tissue of rat red blood cells coated with rabbit anti-rat red blood cell IgG was studied. Normal splenic tissue cleared 10 times more red cells but on a per gram basis there was no significant difference. Autoradiographic studies identified the red pulp as the region for uptake of the red cells. Digitiser measurement of this compartment demonstrated a significant increase of this compartment in regenerated splenic tissue and a direct correlation between the amount of red pulp and the phagocytosis of radiolabelled red cells was identified.

Histological examination of regenerated splenic tissue demonstrated a significant reduction in the amount of white pulp and marginal zone. In addition, a central arteriole was often absent in those areas of white pulp that were present.

The vascular supply of autotransplanted spleens was studied using methyl methacrylate corrosion casting. The vasculature of the transplants were abnormal with dilation of the red pulp cords. Areas of white pulp and marginal zone were rare and the capillary system traversing these was also abnormally dilated. Central arterioles were rarely found.

The marginal zone was studied using immunohistochemical stains. It was abnormal with the thin inner layer of $\mu^+\delta^+$ B lymphocytes and outer layer of $\mu^+\delta^-$ B lymphocytes invariably replaced by a narrow, single layer of $\mu^+\delta^-$ cells. A significant reduction in the T cell region within regenerated spleens was also noted with white pulp reconstituted by predominantly B cells.

These studies on the phagocytic function of regenerated splenic tissue indicate that this form of spleen has markedly reduced phagocytic function due to abnormal cellular regeneration with disorganisation of the lymphoid compartments, principally impaired white pulp and marginal zone reconstitution in association with an abnormal blood supply.

Declaration

The thesis contains no material which has been accepted for the award of any other degree or diploma in any University and that, to the best of my knowledge and belief, the thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis. I consent to the thesis being made available for photocopying and loan if accepted for the award of the degree.

Mark Clayer

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Publications

The following publications have arisen from the studies reported in this thesis.

Clayer M.T.

"The ultrastructure of splenic transplants"

Invited Chapter in "Spleen Transplantation" Eds Liu, Pachter & Seufert

Springer-Verlag (In press)

Clayer M.T., Drew P.A., Leong A.S-Y., Jamieson G.G.

"Fc-receptor mediated phagocytosis in regenerated splenic tissue"

Clinical and Experimental Immunology 97: 242-47 (1994)

Clayer M.T., Drew P.A., Leong A.S-Y., Jamieson G.G.

"Vascular supply of splenic autotransplants"

Journal of Surgical Research 53: 475-84 (1992)

Clayer M.T., Drew P.A., Leong A.S-Y., Jamieson G.G.

"Antibody responses following splenectomy: Implications for the timing of prophylactic vaccination"

Australian and New Zealand Journal of Surgery 62: 142-6 (1992)

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"Phagocytic function of devascularised splenic tissue"

Australian and New Zealand Journal of Surgery 59: 653-658 (1989)

CHAPTER 1

Introduction

The spleen has been shown since the 1970s to have a valuable role in the defence against infection. This was first suggested in 1952 when splenectomised children were reported as being at increased risk of bacterial infection. A specific syndrome was identified which may occur after splenectomy, and which has been described as consisting of 2 phases, an initial phase of general malaise which is then followed by rapid progression to overwhelming septic shock that was refractory to conventional treatment. This syndrome was called overwhelming post-splenectomy infection (OPSI). The reporting of OPSI led to renewed interest in the spleen, revealing it to be important in protection against infection. It has been shown to phagocytose antigen to which there is a low antibody concentration, transport and process it in the marginal zone, periarteriolar sheath and germinal centres where a rapid immunoglobulin response is made, predominantly IgM. It addition it produces a polypeptide (tuftsin) that enhances neutrophil phagocytosis. It has been proposed that the loss of these functions are the most significant deficits resulting from asplenia which predispose the individual to OPSI. Consequently, conservation of the spleen is now regarded by most as the treatment of choice in cases of splenic trauma, especially in children. In those cases where splenectomy is unavoidable due to massive destruction of splenic parenchyma, autotransplantation of splenic fragments to the omentum, or splenic devascularisation by splenic artery ligation, have been suggested. Both these techniques have been performed in human and animal models and shown to result in the growth of viable tissue which macroscopically resembles spleen.

Early studies demonstrated that the splenic tissue that regenerated following these 2 procedures was capable of clearing colloids, removing senescent or abnormal red blood cells from the blood stream and decreasing the percentage of vacuolated (pitted) red cells in the circulation. In the light of these early reports, this splenic tissue was described as "regenerated" and was believed to confer all the protective functions of normal splenic tissue (Tavassoli *et al*, 1973; Schwartz *et al,* 1978; Patel *et al,* 1982; Malangoni *et al,* 1985; Westermann and Pabst,1986; Christensen *et al,* 1986). In later investigations it has been demonstrated that the rapid IgM response of normal splenic tissue is not maintained by the "regenerated" splenic tissue (Drew *et al,* 1984). It is not known if the ability to phagocytose antigen (or bacteria) opsonised with low concentrations of antibody is restored, and there is no convincing evidence that regenerated splenic tissue can protect the host against OPSI. If this phagocytic function of

the normal spleen is retained, then it may provide a useful protective function early in an infection in a patient who, because of splenectomy, has an impaired antibody response to the microbe.

The aim of this thesis was to compare the phagocytic function of "regenerated" autotransplanted splenic tissue to normal spleen and correlate this to the anatomy. Phagocytosis of colloids was studied using stannous fluoride and carbon colloids. The ability to phagocytose a particle with a low concentration of opsonising IgG (hence simulating an antigen to which there is low background antibody levels) was also measured. The ability to clear an antigen will be related to the circulation of the blood within the tissue, consequently, the microvascular system was studied using corrosion casting and scanning electron microscopy to further investigate the regenerated splenic tissue. The regions within the spleen in which the colloid and IgG opsonised particles were phagocytosed, and the cellular components of these regions, were identified using autoradiography, immunoperoxidase and conventional histological techniques.

CHAPTER 2

The Structure and Function of the Spleen: a Review

2.1 Historical perspective

2.2 The embryological development of the spleen

2.3 The structure of the spleen

2.4 The function of the spleen

2.5 The immunological changes due to asplenia

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2.7 The value of splenic repair or restoration

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2.2 Historical perspective

The earliest known description of the spleen is attributed to Aristotle who is quoted as saying that the spleen was not essential to life. In contrast, Galen believed that it probably removed the humors derived from the liver or blood and purified blood going to the stomach. In the first century AD, Pliny stated that the spleen hindered running and had a role in the production of laughter. The belief that the spleen produced laughter was also found in Babylonian writings in the second to sixth centuries AD. In the twelfth century, Maimonides questioned "the production of laughter but re-emphasised the role of purification of the blood". Despite this early interest, it was not until splenectomy was performed in large numbers that the roles of the spleen were first scientifically investigated.

The first documented splenectomy, reported by Fioravanti in 1549, was performed by Zaccarelli, a surgeon from Palermo, who excised the spleen from a woman suffering from (presumed) splenomegaly. The first splenectomy for trauma, reported by Cruger in 1684, was performed by Matthias in 1678 following evisceration of the spleen through a flank wound. He ligated the vascular pedicle and excised the organ 3 days later.

Partial splenectomy was reported by Rosetti who, in 1590, described the excision of a pole of the spleen which was protruding through a wound. Partial splenectomy was also described in 1673 by Church, following evisceration secondary to trauma.

Total and partial splenectomies were performed in the seventeenth to nineteenth centuries, but the first attempts at splenic repair (splenorrhaphy) were reported in 1895, when Zikoff was credited with the first successful splenorrhaphy of a lacerated spleen. Following this, reports of successful splenorrhaphy were also presented by La Marchie (1897) and Mayo (1910). Dretzka (1930), reporting 3 cases of splenorrhaphy from the Detroit Hospital, described the use of mattress sutures for repair of capsular lacerations.

Splenectomy remained the treatment of choice for splenic trauma, because of the low mortality associated with the operation, and the belief that the spleen had no important role in patient health. This belief was expounded by Kocher in 1911 who stated that "Injuries of the spleen demand excision of the gland. No evil effects follow its removal, while the danger of haemorrhage is effectively stopped".

The concept that the spleen may provide some protection against infection was first scientifically studied by Morris and Bullock in 1919. They demonstrated that splenectomised

rats were more susceptible to rat plague and concluded that the spleen protected against infection. Their work was disputed on the basis of anecdotal human work and retrospective patient reviews (Moynihan, 1908; Bailey, 1927), but in these the period of follow up was short. Pfeiffer (1924) expressed concern that the literature on the subject was deficient in regards to the long term effects of splenectomy.

O'Donnell (1929) described a father and son who had splenectomy performed for hereditary spherocytosis and both died from "acute septicaemia, manifesting a lack of resistance to the disease". In 1952 King and Schumacker published their report of fatal septicaemia in 4 out of 5 infants who were splenectomised electively for congenital spherocytosis before the age of 6 months. Initially these poor outcomes were attributed to the underlying disease and not the splenectomy. In 1957, Smith published the first cases of severe infection following splenectomy for trauma. Ellis and Smith (1966) reported an increased incidence of sepsis in infants who were splenectomised before 12 months of age compared to children splenectomised later (5/10 compared to 3/129). The first deaths due to the syndrome of OPSI were reported in children in 1963 by Coler. Stossel (1970) is credited with being the first to describe OPSI in adults. There have been many further reports of OPSI following splenectomy for trauma to the spleen (Table 2.1).

Author	Number of patients	Number of OPSI	Incidence of OPSI
Singer (1973)	688	10	1.45%
O'Neal & McDonald (1981)	187	4	2.1%
Schwartz *et al* (1982)	101	1	1%
Vilde (1982)	688	4	0.6%
Pate *et al* (1985)	249	0	0%
Vichard & Dreyfus-Schmidt (1985)	228	4	2.2%
Di Cataldo *et al* (1987)	71	0	0%

Table 2.1 The reported incidence of OPSI following splenectomy for trauma.

The reports of OPSI identified that the spleen was an important organ involved in the protection against infection, and led to renewed interest in splenic preservation and methods for replacing splenic tissue in cases of irreparable splenic damage. Two methods of retaining splenic tissue, when repair is not possible, were described. These were splenic artery ligation and splenic autotransplantation.

Ligation of the splenic artery was first described by Senn in 1903 as a technique to reduce haemorrhage following laceration of the spleen. Although there was no documented increase in mortality, the procedure was not popular as it was felt to be more difficult to perform than splenectomy and splenectomy was not then known to be associated with any complications. The procedure was not described again until 1978 by Keramides.

Buchbinder and Liphoff (1939) observed viable splenic tissue in the peritoneal cavity following trauma to the spleen. They termed this macroscopically normal, ectopic splenic tissue "splenosis". The deliberate creation of splenosis by autotransplantation was first performed in rats by Tavassoli (1973) who described this as "regenerated splenic tissue". Studies in mice (Dickerman *et al*, 1979), rabbits (Stutte *et al*, 1974) and pigs (Pabst and Reilmann, 1980) followed.

Splenic autotransplantation in humans was reported by Benjamin (1978), followed by Patel (1981) and Seufert (1981). Patel described transplantation of thin slices of spleen to the omentum whereas Seufert minced the spleen and inserted it into a surgically created pocket of omentum.

2.2 The embryological development of the spleen

The spleen is derived from mesenchymal cells that form between the leaves of the dorsal mesogastrium in the sixth week of gestation. As the mesenchymal cells divide, they project into the greater sac. When the stomach rotates, the spleen is rotated to the left where its mesogastrium fuses with the peritoneum over the kidney. By the third to fourth month the mesenchymal cells condense to form trabeculae interspersed with irregular lacunae. Within these lacunae some mesenchymal cells remain free. In the fourth to fifth month the free mesenchymal cells divide rapidly and differentiate into erythroblasts, myeloblasts, megakaryocytes and lymphoblasts. The fixed mesenchyme forms the capsule and connective tissue framework from which reticular fibres are produced. The pulp cords develop between

this connective tissue framework. The arteries terminate by opening freely into the splenic tissue where the pulp cords are developing.

After the fifth month, the spleen switches to production of erythroblasts only, which continues up to the eighth month. The white pulp is first seen after six months and develops around the ingrowing arterioles. The periarteriolar sheath develops first, followed by primary follicles and the marginal zone (Dijkstra and Döpp, 1983).

2.3 The structure of the spleen

2.3.1 Gross anatomy

The spleen is located in the left epigastrium under the ninth to eleventh ribs and weighs approximately 150 g in the adult human. It is shaped like the kidney (12 cm long by 6 cm wide by 3 cm thick), and on the concave aspect lies the hilum which faces medially. The anterior convexity is indented and remains under and closely applied to the ribs.

It is covered by peritoneum, except for the hilus into which the pancreatic tail often protrudes. The peritoneum forms 3 folds which attach the spleen to the diaphragm, transverse colon and stomach (phrenicolienal, lienocolic and gastrolienal ligaments respectively). The phrenicolienal ligament contains the large vessels of the spleen (splenic artery and vein) as well as the left gastroepiploic artery. The gastrolienal ligament contains the short gastric arteries which arise from the splenic artery prior to its entrance into the splenic hilum.

The splenic artery arises as the largest branch of the coeliac trunk and makes a tortuous passage to the spleen, dorsal to the pancreas at its cranial border. The left gastroepiploic artery arises from the splenic artery, 2 - 6 cm from the hilum. At this point, the splenic artery may divide into 2 branches. At the hilum, the splenic artery (or its terminal branches) divides into 4 or 5 branches which enter the spleen separately, 2 or 3 arteries per pole of the spleen. From the upper terminal branch, the short gastric arteries arise. Once within the spleen, the terminal vascular branches rarely anastomose (anastomoses occur in 10% of human spleens).

The veins form within the splenic parenchyma, either as direct continuations of the arterioles or from draining red pulp cords. The splenic vein is formed by the union of venules from the splenic segments and passes behind the splenic artery. It then runs below the artery, dorsal to the pancreas where it receives the short gastric veins, the left gastroepiploic vein,

pancreatic and duodenal veins and the inferior mesenteric vein. Behind the pancreatic head it joins the superior mesenteric vein to form the portal vein.

The nerve supply of the spleen arises from the coeliac plexus and travels within the wall of the artery. These nerves are sympathetic in origin, but their function is not known. It is not known if the spleen has a parasympathetic nerve supply.

Lymph drains to nodes in the hilum, which drain to retropancreatic nodes and then to the cisterna chyli at the level of the first lumbar vertebra.

2.3.2 Microscopic anatomy

The spleen is enclosed in a dense fibromuscular capsule from which trabeculae penetrate the parenchyma. The capsule and trabeculae form the stroma. Between the trabeculae is a reticular network called the pulp. The pulp is divided into red and white pulp and a newly recognised distinct zone called the marginal or mantle zone.

The capsule is 1 - 2 mm thick in the human and consists of connective tissue, elastin and smooth muscle. The trabeculae arise from the capsule and contain the larger arteries and veins (trabecular arteries and veins). The trabecular arteries branch within the trabeculae to give off arterioles (approximately 200 μm diameter) which leave the trabeculae and enter the pulp where they are ensheathed by lymphocytes to form Malpighian corpuscles (white pulp). This arteriole is known as the central or follicular arteriole and gives off smaller penicillar (brush) arterioles before it terminates into nodular capillaries. The penicillar arteriole leaves the PALS and may have a fusiform swelling of its wall, termed an ellipsoid (found in man but not in rats). The ellipsoid consists of an inner layer of endothelium surrounded by large pale cells and a basement membrane. The penicillar arteriole then terminates into a husk capillary (approximate diameter of 5 μm) which may either empty into the red pulp cords (open circulation) or directly into a venule (closed circulation). Branches of the penicillar arterioles also drain directly into the marginal sinus via the marginal zone which surrounds the white pulp (Groom, 1988). The nodular capillaries pursue a radial course through the white pulp (frequently anastomosing) and terminate by opening into the perifollicular space (marginal sinus in the rat).

The red pulp cords consist of vascular channels in which cells of the reticular network (pulp macrophages and dendritic reticulum cells) lie and are exposed directly to the blood. The pulp cords connect to venous sinuses via pores (interendothelial slits) between the elongated

endothelial cells that line the sinuses. These pores are approximately 3 μm in diameter, permitting only flexible cells to pass from the pulp to the sinuses. The sinuses are wide, thin walled and lined by elongated endothelial cells. These cells are specialised and may be capable of phagocytosis themselves (Buckley *et al*, 1987). The sinuses form venules which drain into trabecular veins that follow the arteries.

Lymphatic vessels arise within the white pulp only, and drain to hilar nodes. Little is known of the lymphatic drainage of the spleen.

The parenchyma is divided into white pulp, red pulp and the marginal zone. The lymphocytes immediately surrounding the arteriole form the periarteriolar lymphoid sheath (PALS) of the white pulp. Periarteriolar lymphoid sheaths are composed predominantly of T cells (predominantly T-helper) and interdigitating reticulum cells (IDC). Germinal follicles/centres adjoin the PALS and are formed by B cells, macrophages (tingible body macrophages) and dendritic reticulum cells/follicular dendritic cells (FDC) (Dugan *et al*, 1986; Buckley *et al*, 1987).

At the periphery of the PALS, the marginal zone bridging channels may be found. They consist of concentrically arranged reticular cells and form a cylindrical sheath in association with coarse reticular fibres (Veerman and Van Ewijk, 1975; Kotani *et al*, 1985). Mixing of B and T cells and macrophages occurs at this point and the channels serve to link the MZ and the WP.

In the rat spleen, the white pulp and germinal follicles are surrounded by a layer of macrophages called marginal metallophils (so named because of their staining pattern with Marshall's silver-impregnation method) (Snook, 1964). These are bound by the marginal sinus around which lies the marginal zone (MZ). The marginal sinus consists of a series of anastomosing vascular spaces between the white pulp and marginal zone, mostly quite flattened and often interconnected by short vessels of capillary dimensions (Schmidt *et al,* 1985). It is supplied with blood from numerous capillaries that terminate from the central arteriole. The marginal sinus then drains to venous sinuses.

The MZ consists of 2 layers, an inner layer called the mantle zone (or lymphatic corona) and an outer layer called the marginal zone proper. The mantle zone contains T cells and small B cells. The marginal zone contains medium sized B cells, marginal zone macrophages and possibly T cells (Timens and Poppema, 1985).

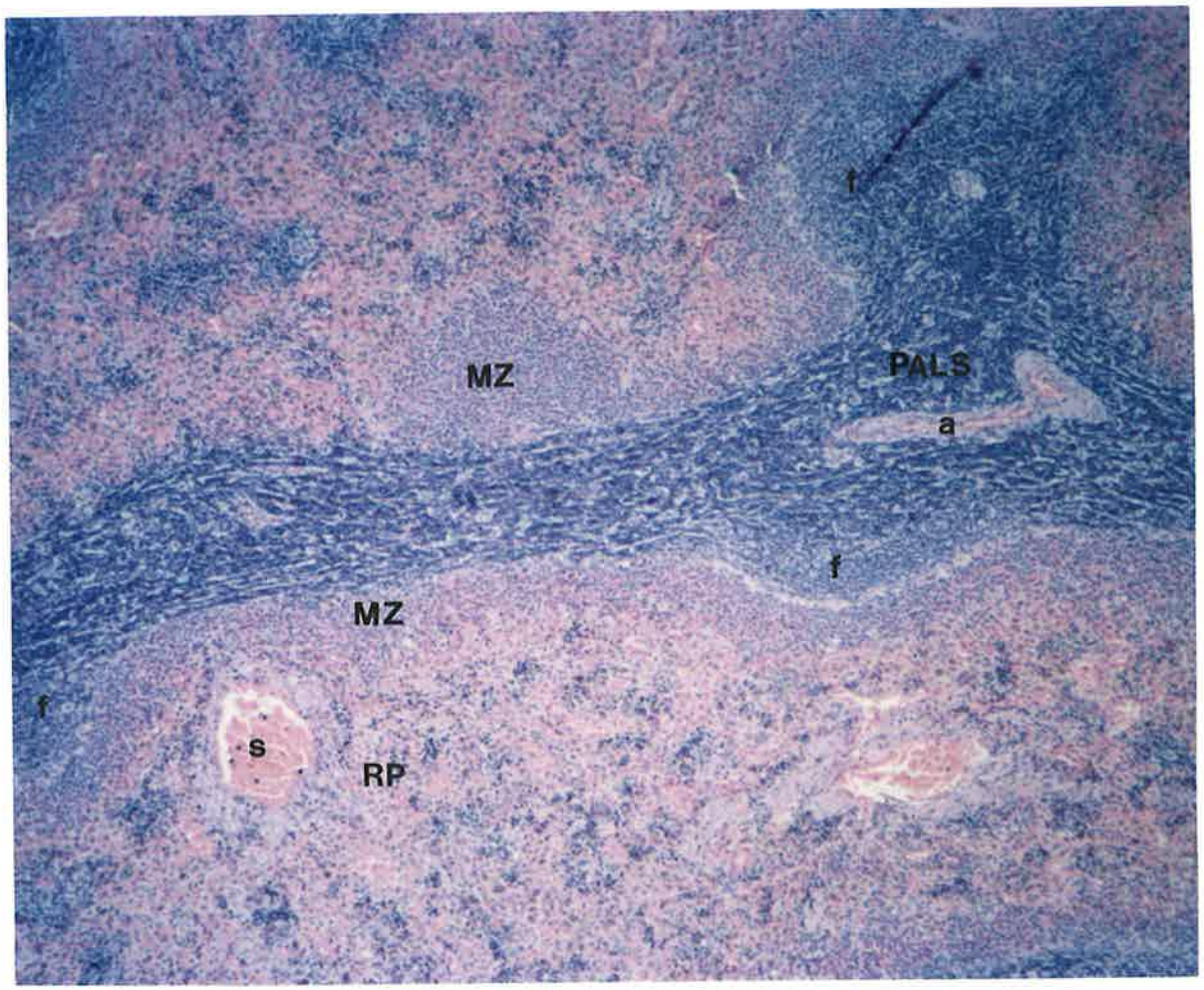

Figure 2.1 Low power view of normal splenic tissue. An area of WP contains a central arteriole (**a**), surrounded by the periarteriolar sheath (**PALS**) and a germinal follicle (**f**). Surrounding these is the marginal zone (**MZ**). The MZ is surrounded by the red pulp (**RP**) in which can be seen venous sinuses (**s**). (Haematoxylin and Eosin, x40)

The red pulp is composed of occasional T (predominantly T suppressor) and B cells, pulp macrophages and follicular dendritic cells in association with the B cells (Westermann *et al*, 1988) which line the pulp cords.

2.4 The function of the spleen

2.4.1 Filtration

The spleen is a filter through which 4% of the circulating blood volume passes every minute. The existence of narrow pores, the interendothelial slits, as the only pathway for blood to the venous side from the red pulp cords, permits just elastic, flexible cells to move from the cords. Those cells which are not flexible remain trapped within the pulp cords (McDonald *et al*, 1987). Macrophages lining the cords are able to phagocytose dead or altered cells and cellular debris. In this way, senescent or abnormal cells (*eg* spherocytes, cells containing intracellular parasites) are removed from the circulation (Seufert and Mitrou, 1986). The normal spleen removes approximately 20 g of erythrocytes daily.

In addition to this mechanical barrier, cells with membrane abnormalities may be phagocytosed more readily by the macrophages and reticulum cells (Lennert and Stutte, 1968). The loss of deformability, increased sphericity, loss of surface charge, inhibition of membrane sulphydryl groups and immaturity all enhance adherence to and sequestration by red pulp macrophages (Levesque and Groom, 1980).

The spleen is able to remove intracellular components of normal erythrocytes such as chromatin vestiges (Howell-Jolly bodies), Heinz bodies, and vacuoles (also called pits due to their appearance under phase microscopy) that may contain haemoglobin, ferritin, membrane or mitochondrial remnants. The normal erythrocyte is able to deform its membrane to pass through the narrow sinal pores, but the intracellular vestiges are not deformable and can not squeeze through and hence are trapped on the cordal side where they are avulsed from the cell, to be phagocytosed by the pulp macrophages (Groom, 1987; Rosse, 1987). This process has been called erythrocyte conditioning.

2.4.2 Storage

The spleen is a store for reticulocytes. It has been demonstrated that reticulocytes remain within the spleen for approximately 24 - 36 hours before they re-circulate (Groom,

1987; Rosse, 1987). The reticulocytes adhere to reticulum cells and macrophages in the reticular meshwork of the red pulp. It has been proposed that this may aid maturation of the immature red cell., macrophages and possibly granulocytes

Platelets are also stored by the spleen. A normal spleen holds approximately a third of all blood platelets (Aster, 1966). These platelets, although stored, are exchangeable with the circulation and can be released if needed. Factor VIII may also be stored and the spleen may be involved in its synthesis. The products of the clotting cascade are removed by the spleen.

The observation that granulocytopaenia is a common late problem following splenectomy suggests that the spleen may also be a store for granulocytes (Christensen *et al*, 1978).

2.4.3 Phagocytic and antigen processing and presentation

The spleen contains unique macrophage sub populations, each in different anatomical zones. These are follicular or tingible body macrophages, marginal metallophils, marginal zone macrophages and pulp macrophages (Buckley *et al*, 1987; Matsuno *et al*, 1989). Although these cells share certain antigenic characteristics, they also have distinct and unique antigens and receptors. In particular, pulp and MZ macrophages bear Fc receptors whereas follicular macrophages do not. In contrast, MZ macrophages have a significant quantity of receptors for the third component of complement (C3) whereas pulp macrophages have low amounts (less than 10% of these cells have C3 receptors) and follicular macrophages have none (Buckley *et al*, 1987).

When the blood enters the spleen, it must pass through the white pulp (via the central arteriole) before it enters into sinuses or pulp cords. This ensures prolonged exposure of any circulating antigen to marginal metallophils, marginal zone and pulp macrophages due to the sluggish blood flow through these regions. It has been shown in animal models that macrophages of the MZ and RP and metallophils are responsible for phagocytosis within the spleen and may act as antigen presenting cells (the role of follicular macrophages remains to be defined) (Bell, 1984; Kotani *et al* , 1985). Matsumo *et al* (1986) found that in mice and rats MZ macrophages removed almost all injected labelled polysaccharide. Others have found that colloidal particles localise to macrophages in the MZ and RP (Nossal *et al*, 1966; Burke and Simon, 1970). The mechanism by which colloidal particles are removed has not been

described. Antibody mediated phagocytosis has not been studied in as great depth despite reports that the spleen is the most efficient organ for the removal of antigen with low levels of opsonins on its surface (Schulkind *et al*, 1967; Dickerman, 1976). The proposed explanation for this is that splenic macrophages in the MZ and RP have high numbers of Fc receptors on their cell membranes (in contrast to the Kupffer cells of the liver which have only a low concentration of these receptors) (Buckley *et al*, 1987). In the host that has been previously exposed to the antigen, higher circulating levels of IgG and IgM will be present, which will facilitate the binding of complement. The Kupffer cells have C3 receptors and so the liver becomes the most efficient organ for phagocytosis, as the particles will be coated with both antibody and complement.

In addition to phagocytosis of antigen, the MZ macrophages appear to act as antigen presenting cells (APC) also. It has been shown that antigen that is trapped in the MZ is transported to the germinal centres in the WP (Mitchell, 1972), where IgM is rapidly produced (Sullivan *et al*, 1978). This rapid production of IgM directed against the phagocytosed antigen facilitates hepatic clearance of the remaining circulating antigen. Colloid-containing macrophages have been shown to migrate not only to the germinal centres in the MZ (sometimes via the PALS) but also to the RP where they coalesce (Kotani *et al*, 1985). The marginal metallophil may have a role in phagocytosis of thymus-independent type-2 antigen and presentation to antibody forming cells (Matsumo *et al*, 1989). In addition to macrophages, the B lymphocytes in the MZ are able to present antigen (Greaves, 1970; Mitchell, 1972; Liu *et al,* 1988). Reticulum cells (IDC and FDC) have been implicated in antigen trapping and possibly antigen presentation also, but not phagocytosis.

In summary, colloidal phagocytosis, followed by migration to germinal centres and presentation of antigen appears predominantly to be a function of MZ macrophages. Phagocytosis mediated by low concentrations of IgG occurs predominantly in the spleen, but the cell type responsible or site of phagocytosis is unknown. Phagocytosis of IgM coated particles is mediated predominantly by the liver. The spleen therefore has 2 phagocytic roles in the host, phagocytosis with clearance of antigen, and phagocytosis, antigen transport and presentation to antibody forming cells.

2.4.4 Production of opsonins

Opsonins are antibodies that bind to an antigen and enhance its phagocytosis. The spleen produces opsonins, notably IgM by cells in the germinal follicle (Ellis and Smith, 1966; Bohnsack and Brown, 1986). The follicle consists of B cells surrounded by T helper and suppressor cells. Antigen is transported through the T cell region to the B cell region where IgM is produced. Thymus-independent antigen appears to be handled differently with the antigen localised to the outer PALS and MZ in association with B cells. The spleen plays a major role in antibody production in response to antigen presented *i.v.* (Rowley, 1950; Jenkin and Rowley, 1961; Lozzio and Wargon, 1974; Sullivan *et al*, 1978), and animal studies have demonstrated that plasma cells appear first in the spleen in response to *i.v.* antigen (Christensen *et al*, 1978). There is also evidence that antigen presented *i.p.* requires the spleen for optimal antibody response, although subcutaneously administered antigen does not appear to be dependent (Caplan *et al*, 1983). The regulation of the class of antibody produced, in particular the switching from IgM production to IgG, is regulated by cells in the spleen (Sullivan *et al*, 1978).

Fibronectin may act as an opsonin (Owens and Cimono, 1982). While the predominant site of synthesis is reported to be the liver (Owens and Cimono, 1982), the plasma levels fall following splenectomy, suggesting that the spleen plays a role in regulation of its hepatic synthesis (Hashimoto *et al*, 1983). The spleen is a site of synthesis of properdin (Corry *et al*, 1979), which molecule, by activating the alternate complement pathway, may promote phagocytosis.

2.4.5 Effect of the spleen on other cells

The spleen may have a role in the maturation or conditioning of cells other than erythrocytes. It contains many T-suppressor cells and is a site of T - B cell interaction (Sampson *et al*, 1975). Memory T cell production occurs in the spleen, especially in response to polysaccharide antigens (Bohnsack and Brown, 1985; Seufert and Mitrou, 1986).

It also appears to influence macrophage function. Peritoneal macrophages had reduced phagocytic function and impaired antigen presenting capacity following splenectomy (Ron *et al*, 1981). The splenic influence on alveolar macrophage function is less clear. While Shennib *et al* (1983) reported reduced phagocytic function by pulmonary alveolar macrophages against

pneumococcus type III following splenectomy, this was not found by Lau *et al* (1983). It has been suggested that a phase of monocyte differentiation may take place in the spleen. Natural killer cell activity is modified by the spleen and following splenectomy, NK cell cytotoxicity is reduced (Gill *et al*, 1985).

In addition to its direct influence on cell function, the spleen also produces an enzyme, endocarboxydase, which cleaves a tetrapeptide from IgG molecules. The spleen is the only source of this enzyme and the tetrapeptide formed is called tuftsin. Tuftsin binds to neutrophils and macrophages to enhance motility and phagocytosis (Najjar and Nishiska, 1968).

2.4.6 Summary

The spleen has 3 broad categories of function; it acts as a mechanical filter, it stores and possibly conditions blood elements before returning them to the circulation and it is a site for phagocytosis, antigen presentation, opsonin formation and stimulation of other immunocompetent cells.

2.5 The immunological changes due to asplenia

2.5.1 Filtration

With asplenia, the normal filtration function of the spleen is lost, resulting in an increase in the number of atypical erythrocytes in the peripheral blood, including pitted cells, cells with inclusions, siderocytes, acanthocytes and leptocytes (Robertson *et al,* 1981). An increase in the number of senescent cells also occurs. These cells have reduced membrane flexibility and therefore have difficulty in deforming to pass through capillaries, resulting in slower capillary passage time (Robertson *et al*, 1981). This resistance to deformation produces a rise in viscosity.

2.5.2 Storage

In studies using congenitally asplenic mice (heterozygous for dominant hemimelia, Dh), granulo-, lympho- and mono-cytosis in the peripheral blood were reported, as well as hyperplasia of granulocytes and lymphocytes in the bone marrow (Lozzio, 1972). In humans, granulocytosis also develops following splenectomy, with an early increase in the neutrophil count. This increase lasts for several months only, but at 1 year, lymphocytosis, monocytosis

and eosinophilia may still persist (Durig *et al,* 1984). The lymphocytosis is predominantly due to an increase in the number of T lymphocytes (Crosby, 1963; Ellison and Fabri 1983; Seufert and Mitrou, 1986).

Thrombocytosis also develops in 45% of humans following splenectomy, and usually rises to levels that are 30% above normal (Ellison and Fabri, 1983). The rise in platelets is due to increased production with release of immature thrombocytes, not an increase in life-span (Laufer *et al*, 1978).

2.5.3 Phagocytosis

The effect of the absence of the spleen on the ability to clear bacteria from the blood stream is not clear. A number of studies have investigated the clearance of antigen from the blood circulation, but because the concentration of natural or background antibody is never reported, the interpretation of the results is not possible, and the conclusions have been conflicting. It has been clearly shown that the greater the circulating antibody to an antigen, the more efficient the clearance, and the more the liver is responsible for clearance (Yousaf *et al,* 1986). In addition, during clearance of bacteria more antibody is synthesised, which further enhances phagocytosis, and the rate of this synthesis is a variable that may be altered following splenectomy (Ellis & Smith, 1966).

In humans, the clearance of particulate matter is depressed in the absence of the spleen. Using Rh positive autologous erythrocytes as the antigen and opsonising these with known concentrations of IgG anti-D antibody (thus avoiding the problems associated with variable background serum antibody concentration), Yousaf *et al* (1986) demonstrated that splenectomy resulted in significantly reduced clearance of the labelled autologous erythrocytes from the circulation when compared to patients with spleens. When higher concentrations of IgG antibody were mixed with the erythrocytes, the hepatic clearance increased.

In splenectomised mice the clearance of an *i.v.* bacterial challenge has been reported to be reduced resulting in higher mortality when compared to eusplenic controls (Coil *et al*, 1978; Likhite *et al*, 1978; Vega *et al*, 1981). A reduction in the clearance of bacteria from the circulation has also been demonstrated following splenectomy in rats (Leung *et al*, 1972; Schwartz *et al*, 1978; Cooney *et al*, 1979; Gullstrand *et al*, 1982; Dawes *et al*, 1984; Christensen *et al*, 1986), guinea pigs (Brown *et al*, 1981), pigs (Hosea *et al*, 1981) and rabbits

(Schulkind *et al*, 1967). The clearance of *i.p* or aerosol administered antigen has also been reported to be reduced in splenectomized rats (Patel *et al*, 1982; Scher *et al*, 1982; Malangoni *et al*, 1985; Herbert *et al*, 1983).

The congenitally asplenic mice (Dh/+) have reduced extra-splenic antigen trapping with the lymph nodes shown to have reduced phagocytic function (Borek, 1976).

2.5.4 Production of opsonins

The IgM antibody response to *i.v.* administered sheep red blood cells (SRBC) is reduced by 75% in congenitally asplenic mice (Dh/+) (Lozzio and Wargon, 1974), as was total antibody production. However, the responsewas found to be 5 times greater than in neonatally splenectomised mice. The levels of IgG_2 antibody were significantly reduced in both but IgG_1 was normal. A higher proportion of IgG antibody was produced compared to IgM. This finding was also demonstrated when lipopolysaccharide (LPS) was used. This alteration in antibody production was found to be in part due to altered T-B cell collaboration with defective T helper cell activity (Battisto *et al*, 1970).

Rowley (1950) reported that asplenic patients were unable to form antibody in response to intravenously injected SRBC. The serum antibody response to antigen injected subcutaneously has been reported as either normal (Ammann *et al*, 1977; Caplan *et al*, 1983; Karup Pedersen *et al*, 1983; Oldfield *et al*, 1985), or reduced (Giebink *et al*, 1980; Hosea *et al*, 1981; Kiroff *et al*, 1985).

2.5.5 Effect of the spleen on other cell types

Wound healing in asplenic patients may be delayed, which has been suggested as due to an alteration in the normal T helper: T suppressor cell ratio in the wound, with a relative increase in the T suppressor cells (Klaue *et al,* 1979). A lack of fibronectin has also been postulated to contribute to wound healing difficulties (Nagelschmidt *et al*, 1987).

The tetrapeptide tuftsin is absent in patients who have been splenectomised, in the absence of splenosis (Francke and Neu, 1981; Orda *et al*, 1981).

2.5.6 Overwhelming post splenectomy infection

The major role of the spleen in protecting against infection appears to be the phagocytosis of organisms that have low concentrations of circulating antibody directed against them. Antigens from these organisms are then processed within the spleen, where they stimulate antibody producing cells to synthesise specific antibody against the organism, which then enables other phagocytic organs to take part in clearance. Therefore the spleen is critically important for protection when the host has low circulating levels of antibody directed against a virulent, rapidly dividing, blood-borne pathogen. As children are less likely to have experienced prior exposure to an organism and therefore, less likely to mount a secondary response, it would be expected that they are at greater risk of developing OPSI. This has been found in retrospective studies and the syndrome of OPSI was first documented in children (Coler, 1963).

The risk of OPSI decreases as the age at the time of splenectomy increases. Children splenectomised under the age of 10 have been reported as having a risk of OPSI as high as 4.1%, while in adults the risk is significantly lower and has been cited as between 0.2% and 0.9% (Robinette and Fraumeni, 1977; O'Neal and McDonald, 1981; Ellison and Fabri, 1983; Aitkin, 1987).

Patients are more likely to develop OPSI within the first few years after splenectomy. In several reports, 20% of cases of OPSI have occurred in the first 6 months post-splenectomy and 66% by 2 years (Singer, 1973; West and Grosfeld, 1985). Late infection has been reported, with 1 case occurring 31 years after splenectomy (Zarrabi and Rosner, 1984).

The underlying indication for splenectomy has also been reported to influence outcome. Splenectomy following trauma is associated with the lowest risk of OPSI, incidental splenectomy and splenectomy for malignant disease or haematological disorders are associated with the greatest risk (O'Neal and McDonald, 1981; Schwartz *et al*, 1982; Di Cataldo *et al,* 1987). The reason why splenectomy due to trauma is associated with less risk of OPSI is not clear. It has been postulated that rupture of the spleen enhances the development of splenosis, due to fragmentation of the spleen by the traumatic event, and that the splenosis may provide some protection against OPSI (Singer, 1973; Pearson *et al*, 1978). In elective procedures fragmentation of the spleen and hence splenosis are less likely to occur.

The syndrome is usually caused by encapsulated bacteria (Dickerman, 1976; Bullen and Losowsky, 1979; Francke and Neu, 1981; Ellison and Fabri, 1983), especially pneumococcus which is reported as being responsible for 50-60% of cases. *Neisseria meningitidis*, *Haemophilus influenzae* and *Eschericia coli* are reported as being the causative organism in another 30% of cases (Singer, 1973). Other bacteria reported to produce OPSI include β-haemolytic *Streptococcus*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Salmonella* species and *Bacteroides* species (Ellison and Fabri, 1983; Cooper and Williamson, 1984; Peters, 1984; West and Grosfeld, 1985). Other organisms less commonly associated with OPSI include viruses, fungi, protozoa and *Mycobacteria* (Peters, 1984).

The initial presentation of OPSI frequently consists of general malaise with flu-like symptoms, a raised temperature and peripheral flushing. This may be present for 24 hours. Often no focus of infection can be found. The clinical picture rapidly progresses to one of septic shock. Maldistribution of blood occurs with vasodilation and vasoconstriction, and capillary plugging from aggregated leucocytes and then oedema can ensue. Peripheral vasoconstriction may cause gangrene (Perkins *et al*, 1988). Lactic acidosis develops with associated tachypnoea. Pulmonary hypertension may develop resulting in hypoxia in association with systemic hypotension. Disseminated intravascular coagulation is common, as is multi-organ failure. Waterhouse-Friderichsen's syndrome is frequently reported (Peters, 1984). Death often occurs within 24-36 hours and the mortality rate is 100% if untreated. The infection is often refractory to conventional, appropriate antimicrobial treatment.

In cases of OPSI in which pneumococcus is the causative bacteria, unusually high numbers of bacteria, often more than 10^6 per ml, may be found in the peripheral blood. Infections caused by the less common microbes (*e.g.*, viruses, fungi) are associated with a better prognosis (Peters, 1984).

Treatment consists of immediate fluid and electrolyte replacement, institution of antibiotic therapy (penicillin and gentamicin) and administration of dexamethasone. Acetaminophen or hypothermia may be used to lower the temperature (Peters, 1984). The mortality rate with this treatment is 50% (Peters, 1984).

Although OPSI is a rare complication following splenectomy, it has a poor prognosis when it does occur. This has led to support for splenic conservation in preference to total splenectomy wherever possible.

2.5.7 Comparison between human and animal spleens

Two classifications of the spleen have been described, one based on the vascular structure as described by Billroth (1862), and a more recent classification based on both structure and function (Hartwig and Hartwig, 1985).

Billroth allocated spleens into 1 of 2 groups (sinusal or non-sinusal) based on the presence of venous sinuses in the red pulp, with non-sinusal spleens having primordial veins. Animals with sinusal spleens included rats, dogs and man, those with non-sinusal spleens including mice, pigs, cats and horses.

Hartwig and Hartwig (1985) further refined this classification. He defined 3 different types of spleen based on histological and functional characteristics: storage, metabolic or immunological. The storage spleen is primarily a red cell storage organ and consists of a big spleen with large arteries and veins, small amounts of white pulp and a capsule and trabeculae containing much smooth muscle. This allows the spleen to store up to 300 mls of blood that is available for immediate return to the circulation on demand. The large amount of smooth muscle within the organ wall provides a mechanism for brisk volume replacement, usually in response to stress. Animals that have a storage spleen include elks, chamois, sheep, goats, pigs and elephants.

The immunological type of spleen has a capsule and trabeculae composed of connective tissue, a smaller organ weight and relatively large amounts of white pulp. Animals included in this group are humans, other primates and rodents. The metabolic spleen has both storage and immunological functions, in addition to the ability to accumulate biologically active substances (*ie.* ferritin). Animals included in this group include dogs, cats and horses (Hartwig and Hartwig, 1985).

The rodent spleen has been described as the closest histologically to the human spleen (with the exception of primates), and has therefore been used as the model for investigation of splenic function.

Similarities between rodent and human spleens have also been shown in functional studies. Schiffman (1981) compared humoral responses of mice and humans against pneumococcal polysaccharide. He found that the mouse spleen had a more significant role in anti-polysaccharide antibody production than the human spleen, but an intact spleen was

necessary for optimal response by both. Rozing *et al* (1978) reported a reduction in the concentration of IgM in the serum of both species following splenectomy.

Other studies have demonstrated that splenectomy in rats produces similar changes to those found in humans. Splenectomised rats have reduced concentrations of opsonins in the serum, and impaired antibody response to protein and polysaccharide antigens administered *i.v.* (Schwartz *et al*, 1977; Church *et al*, 1981; Barringer *et al*, 1982). In addition, clearance from the bloodstream of pneumococci has been shown to be reduced, and mortality of the host increased, following injection of live organisms, compared to eusplenic controls (Schwartz *et al*, 1978; Cooney *et al*, 1979; Offenbartl *et al*, 1984; Christensen *et al*, 1986). The similarities between splenectomised rats and humans have led to the rat being the most widely used animal model for studies of immunological function following splenectomy.

2.6 Restorative techniques in splenic surgery

2.6.1 Conservative management

The conservative management of suspected splenic trauma has been suggested by a number of authors (Douglas and Simpson, 1971; Ein *et al*, 1978; Hunter *et al*, 1984), particularly in children (Buntain and Gould, 1985; Touloukian, 1985). Howman-Giles *et al* (1978) reported successful recovery from trauma with conservative management in 60% of 49 children, and Touloukian (1985) reported a success rate of over 95% for conservative management of splenic trauma. This success in children is probably due to the differences between the adult and child's spleen. The spleen of the child has a higher content of smooth muscle and contraction of these fibres has been shown to arrest bleeding from splenic lacerations, especially if the tear is transverse (Upadhyaya *et al*, 1971).

A major drawback to conservative management is the risk of delayed rupture. The figures quoted for its incidence vary markedly. Ein *et al* (1978) reported it only twice in 37 consecutive cases. Olsen and Polley (1977) quoted rates of 0.3-1.0% and this has been supported by other authors (Burlatzky *et al*, 1980). In contrast, however, Aitken quoted rates for delayed rupture following conservative management of up to 33%. Whether these represent true delayed ruptures or missed diagnosis is not known.

Type 1	Localised capsular disruption or subcapsular haematoma
Type 2	Single or multiple capsular and parenchymal disruptions, transverse or longitudinal, not extending into the hilum or involving major vessels.
Type 3	Deep fractures, single or multiple, transverse or longitudinal, extending into the hilum and involving major segmental blood vessels.
Type 4	Spleen completely shattered or fragmented, or separated from its normal blood supply at the pedicle

Table 2.2 The grading of splenic trauma (Upadhyaya and Simpson, 1968)

In animal studies Dulchavsky *et al* (1987) developed models of splenic trauma in the pig and dog. A type 2 injury (as defined by the criteria shown in Table 2.2) was inflicted and the spleen was either managed by splenorrhaphy or allowed to cease bleeding spontaneously. In dogs, the splenic scars that developed following management by observation alone were significantly weaker than normal splenic tissue after 3 weeks, but by six weeks there was no difference. In pigs (which have a higher smooth muscle content in the spleen, as described earlier) there was no difference at any time point. The animals which underwent splenorrhaphy had significantly stronger splenic scars than both the conservatively managed and normal spleen groups.

The recommendation for patients who are managed conservatively is bed rest for 1-2 weeks with regular clinical examination and the availability of CT scanning to detect bleeding. Should delayed rupture occur it usually presents within 2 weeks after the initial injury. After a period which is symptom free, the patient presents with sudden abdominal pain (sometimes referred to the left shoulder), nausea and vomiting. Management is via operative intervention (Peters, 1984).

Although the merits of non-operative management of the adult remain controversial, it has been recommended as the most appropriate management for children with suspected splenic trauma if they are haemodynamically stable on admission (Cooper and Williamson, 1984; Splenic injury study group, 1987).

2.6.2 Splenic repair

2.6.2.1 Splenorrhaphy

If a type I injury of the spleen exists (see Table 2.2), the injury may be left to heal without further intervention. Buntain and Gould (1985) reported that some type II injuries can also be managed by careful observation. However, all patients with type III and IV injuries require resuscitation and surgical exploration. In cases where other intra-abdominal injury is suspected, early laparotomy must be performed.

It has been suggested that all injuries other than massive destruction of the splenic parenchyma can be managed by splenorrhaphy (Seufert and Mitrou, 1986). In splenorrhaphy, the spleen is mobilised and the injuries assessed. All tissue of doubtful viability is debrided and blood clots removed. Haemostasis may be achieved by one of the following:

i) Application of omentum or topical haemostatic agents (*ie.* gelatin sponge, thrombin or microfibrillar collagen),or

ii) Direct suture of splenic parenchyma and capsule. This can be performed using simple or mattress sutures. Pledgets or teflon buttresses or omentum may also be incorporated into the suture to increase the grip of the suture without cutting through the soft parenchyma, or

iii) Wrapping of the spleen in omentum or polyglycolic acid mesh following suture, to tamponade any further bleeding.

As an adjunct to splenorrhaphy, the splenic artery may be ligated or clamped to reduce the arterial blood pressure and improve visibility. The collateral blood supply is sufficient to ensure the maintenance of splenic viability without significant necrosis (Sherman and Asch, 1978; Keramides *et al*, 1980; Warshaw, 1988). Fifteen cases of splenorrhaphy were reported by Ratner *et al* (1977), 8 by Burrington (1977) and 24 by Wetzig *et al* (1986), with no instances of delayed rupture.

2.6.2.2 Partial splenectomy

The segmental vascular supply of the spleen allows segmental resection where major parenchymal injury involves only 1 pole (in particular the lower pole). Ligation of the segmental artery demonstrates the area of spleen that is fed by that artery and should result in devascularisation of the traumatised area. Excision of the devascularised portion and ligation of tangentially running arteries is then performed. The cut surface is then closed as for splenorrhaphy.

2.6.3 Splenic replacement

2.6.3.1 Splenic autotransplantation

Splenic autotransplantation was first suggested following the demonstration of splenosis after traumatic rupture of the spleen. Although splenosis was reported in 1939, deliberate autotransplantation was only performed in the late 1970's. Benjamin *et al* reported 2 cases of splenic autotransplantation in children in 1978, but it was not until the early 1980's that this technique became popular. Two main methods have been reported. Patel (1981) advocated cutting the excised spleen into thin slices which are then sutured onto omentum. Seufert (1981) used an alternative method whereby the excised spleen is cut into small slices and then grated into pulp. The grated splenic material is then spread onto the omentum or into the omental bursa. Omentum is used to form a pocket around the splenic tissue. Other potential sites for splenic implantation include the posterior aspect of the anterior abdominal wall, in subcutaneous pockets and intramuscularly. Experimental evidence in animals suggest that the final weight of splenic tissue is significantly less when the spleen is not supplied by portal blood supply (Vega *et al*, 1981).

Following splenic autotransplantation, the spleen undergoes ischaemic necrosis with the exception of a layer 3 to 4 cells deep in the outer cortical zone, which is maintained by diffusion of nutrients and oxygen from the surrounding adhesions (Tavassoli *et al,* 1973; Moore *et al,* 1986). Red pulp cords reconstitute at 2 weeks following transplantation and areas of white pulp are seen surrounding arteries at 5 weeks. Multiple small vessels penetrate the capsule of the transplant. The central areas of the transplant are the last to be replaced by splenic tissue and the larger transplants usually remain fibrotic centrally.

2.6.3.2 Splenic arterial ligation

In humans, ligation of the splenic artery close to the spleen does not result in splenic infarction (Cooper and Williamson, 1984; Taylor and Woodward, 1987; Warshaw, 1988). The spleen can be ligated above the body of the pancreas without splenic infarction as determined by CT or isotope scans. This is believed to be due to the collateral blood supply provided by the short gastric and gastro-epiploic arteries which maintain the spleen in the absence of the splenic artery. Platelet and reticulocyte counts may be temporarily raised, however, which may indicate transient disturbance of some splenic functions.

In animals that do not have a well established collateral blood supply (such as the rat and mouse), splenic artery ligation is followed by total splenic infarction (Wolf, 1982) or subtotal infarction if the short gastric arteries are present (Witte *et al*, 1976). The late histological picture of the spleen following this procedure resembles splenosis. In mice, Wolf (1982) reported a surviving peripheral rim of six to ten cell layers deep from which the regeneration occurs in a centripetal direction. He postulated that the peritoneal fluid and adhesions provide the nutrition and oxygen to maintain the peripheral rim of viable cells.

2.7 The value of splenic repair or restoration

2.7.1 Studies following splenic repair

In human studies, splenorrhaphy has not been associated with subsequent OPSI. The number of pitted red cells and serum antibody levels remain within normal limits (Traub *et al*, 1987), and the uptake of isotopes post-operatively is normal (Traub and Perry, 1982).

Cooney *et al* (1979) and Church *et al* (1981) reported that the serum concentration of anti-pneumococcal antibody measured following *i.v.* administered pneumococci did not differ significantly between rats with 50% and those with 100% of their spleens. Animal studies suggest that a minimum of one third of normal splenic tissue, whose blood supply has not been disrupted, will provide adequate protection against OPSI (Witte *et al*, 1983). Malangoni *et al* (1985) demonstrated that preserving 25% of normal splenic tissue in rats resulted in significantly better survival following pneumococcal challenge when compared to splenectomised rats, but significantly worse than rats with 50% of their spleen. There was no significant difference in survival between rats with 50% of their spleen and unoperated rats. Van Wyck *et al* (1986) found that rats with 30% of their normal spleen left *in situ* survived

bacterial challenge better than splenectomised rats, but significantly worse than sham operated rats. This was also found by Bradshaw and Thomas (1982) and Alwmark *et al* (1983).

2.7.2 Studies following splenic replacement

The area of splenic replacement which has received the most attention recently is splenic autotransplantation. As mentioned previously, at least 2 techniques have been described in humans and, following either of these procedures, tissue regenerates which macroscopically resembles normal spleen. After initial parenchymal necrosis, new vessels grow in from the surrounding granulation tissue and the splenic tissue regenerates from a thin layer of viable cells which survive under the splenic capsule.

In humans, the presence of splenosis or splenic autotransplants have been shown to reduce the number of pitted red cells in the peripheral blood (Kiroff *et al*, 1983; Drew *et al*, 1984) and this reduction is directly proportional to the amount of splenic tissue present (Corazza *et al*, 1984). It has also been shown to normalise the serum concentration of tuftsin (Orda *et al*, 1981) and complement (Alvarez *et al*, 1987). In contrast, the increase in the level of T suppressor cells seen following splenectomy does not return to normal in the presence of splenosis or autotransplants (Durig *et al*, 1984). The concentration of IgM has been reported to be reduced (Drew *et al*, 1984) although this was not found by Alvarez *et al* (1985). Antibody formation in response to subcutaneous immunisation is significantly reduced (Kiroff *et al*, 1985).

Experimental studies in animals (predominantly rats) suggest that the response to *i.v.* administered antigen remains impaired. Church *et al* (1981) reported that the antibody response to sheep red blood cells was significantly worse in autotransplanted rats compared to eusplenic or partially splenectomised rats. Rats with only 25% of splenic tissue left *in situ* had better antibody responses than autotransplanted rats. Animal studies also have clearly shown that autotransplantation does not protect against OPSI, although it may delay death (Schwartz *et al*, 1978; Cooney, Swanson *et al*, 1979; Alwmark *et al*, 1983; Malangoni *et al*, 1985; Van Wyck *et al*, 1986). The reason for this apparent lack of protection remains unknown. Witte *et al* (1983) reported that following autotransplantation the blood supply to the regenerated spleen was abnormal, being derived from arteries which penetrated the capsule rather than via large

hilar arteries, and suggested that this abnormal blood supply limited the ability of the transplant to clear bacteria.

Anecdotal human reports suggest that splenosis or autotransplants may not provide adequate protection to prevent OPSI (Gopel and Bisno, 1977; Rice and James, 1980; Moore *et al*, 1983). Based on these reports, it is unreasonable to regard splenosis or splenic autotransplantation as a "born again spleen" as referred to by Pearson in 1978. The risk of OPSI may well remain (although it may be decreased), and extra measures must be taken to prevent its occurrence, especially in children and the immunocompromised asplenic patient.

There have been few, similar studies following splenic artery ligation. Witte *et al* (1976) ligated the splenic artery in rats (with preservation of the short gastric arteries) and noted a reduction in splenic mass due to infarction, but no sustained alterations in haematological parameters (haematocrit, white cell, platelet or reticulocyte count). Keramidas *et al* (1980) have reported that in dogs the collateral blood supply is able to maintain the spleen and permit the taking up of a radiolabelled isotope. The morphology was found to be normal although the splenic mass was reduced. They also described the successful treatment of 2 children by ligation of the splenic artery. Buyukunal *et al* (1987) reported ligation of the splenic artery in 14 cases of type 3 (see Table 2.2) splenic injury in children and stated that scintigraphic and haematological variables were normal at a 3 month follow up.

2.8 Additional measures to protect against infection

In those cases where splenectomy has been performed or inadequate splenic mass remains (as in splenic autotransplantation or partial splenectomy leaving less than a third of the spleen), alternative measures must be taken to protect against OPSI. Prophylactic penicillin has been advocated (Ammann *et al*, 1978; Dickerman *et al*, 1979), but problems with compliance and the emergence of resistant microbes have occurred. It is now recommended that children splenectomised before the age of 5, and the immunocompromised, be placed on prophylactic penicillin for the first 3 to 5 years after splenectomy, and until at least 7 years of age (Francke and Neu, 1983).

Pneumococci are the causative organisms in approximately 50% of cases of OPSI and consequently pneumococcal vaccination has been advocated for all asplenic patients (with the exception of children under the age of 2 who do not make an adequate response to the vaccine).

Meningococcal and *Haemophilus* vaccines are now available and it has been suggested that these should also be administered. This would provide vaccination against up to 80% of causative organisms (Sherman, 1979; Dickerman, 1981; Cooper and Williamson, 1984; Peters, 1984; Perkins *et al*; 1988). If splenectomy is planned as an elective procedure, vaccination should be performed 1 to 2 months prior to surgery to enhance the antibody response (Clayer *et al,* 1992).

The patient, their relatives and family medical practioner should be advised of the risk of OPSI. They should be aware of the trivial nature of the prodromal symptoms, and antibiotics should be commenced for all minor ailments.

CHAPTER 3

Materials and Methods

3.1 The experimental animals

Porton outbred male rats, obtained from the Central Animal House, the University of Adelaide, were used for all experiments. They were housed in air-conditioned accommodation and fed on a diet of rat chow and water *ad libitum*. They were allowed at least 7 days acclimatisation before surgical or experimental procedures were performed. A female New Zealand white rabbit was obtained from the same source for preparation of antibodies to rat RBC.

3.2 Surgical procedures

The rats were allocated into 6 groups; unoperated (CON), sham autotransplantation (SHAM), splenic devascularisation (TIE), splenectomy with splenic autotransplantation (AT), splenectomy (SPX) or hemisplenectomy (HS). SHAM rats were anaesthetised using halothane-nitrous oxide inhalant and the spleen was exposed via a midline incision. After exposure, the spleen was mobilised and brought to the wound surface. It was then replaced in the left upper quadrant of the abdomen and the wound was closed in 2 layers with 4/0 silk. Splenic devascularisation was performed as per sham transplantation, however, following mobilisation of the spleen on its vascular pedicle, a deep cut was made on the costal surface longitudinally (to mimic a type 3 injury) and the splenic vessels were clamped (including any short gastric arteries, if present). The vessels were then ligated with 4/0 silk close to the hilum and the devascularised spleen was replaced in the abdomen. The abdomen was closed as for sham operations. This operation was performed to provide a model of autotransplanted tissue that was left *in situ*.

Splenic autotransplantation was also performed via a midline incision as described by Livingstone *et al* (1983). The spleen was mobilised and brought to the surface. The vasculature was clamped, the spleen excised and the vessels ligated. A piece of spleen weighing between 150 and 225 mg was excised and sutured onto the mesentery of the terminal ileum with 4/0 chromic catgut. The abdomen was closed as described previously.

Splenectomy was performed in the same manner as the autotransplantation except that the spleen was discarded after removal. Hemisplenectomy was performed via a midline incision and the vessels supplying half of the spleen were ligated. The devascularised portion

was then excised and the raw surface of the spleen was closed with 4/0 chromic catgut mattress sutures. The rats were allowed a minimum of 2 weeks to recover from the operations.

3.3 Preparation of technetium stannous colloid

Stannous fluoride colloid, prepared by the method described by Schroth *et al* (1981), was labelled with 99m-pertechnetate (^{99m}Tc). Stannous fluoride (13 mg) and sodium fluoride (100 mg) were dissolved in 10 ml of water for injection. From this solution, 500 µl were removed and diluted with 5 ml of water for injection. This was filtered through a 0.22 micron filter (Millex-GS) to ensure sterility. A 1 ml aliquot of this solution was then removed and added to the ^{99m}Tc containing approximately 740 kBq of radioactivity, under sterile conditions. This was gently rotated for 1 hour before allocation into 100 µl aliquots. The aliquots were gently rotated until use, which was always within 3 hours of preparation.

3.4 Clearance of 99mTc stannous fluoride colloid

To determine the time over which clearance experiments should be conducted, 3 rats were anaesthetised with halothane-nitrous oxide inhalant and their spleens exposed via a midline incision. The spleen was mobilised, brought to the wound surface, and placed on lead shields with the vascular supply intact. An isotope localisation monitor (Model 235, D.A. Pitman Ltd) was used to measure the presence of ^{99m}Tc within the spleen. After baseline radiation levels were measured over 2 minutes, a 100 µl aliquot of the labelled stannous colloid was injected into the exposed inferior vena cava (IVC). Serial measurements of the radioactivity of the spleen were taken at 5 minute intervals for the first 30 minutes and then each 10 minutes up to 2 hours after injection. These experiments demonstrated that there was no increase in the radioactivity measured in the spleen after 25 minutes following the injection. Therefore, in all subsequent experiments, colloid was allowed to circulate for 30 minutes before the rat was killed.

The ability of normal spleens to phagocytose a colloid was tested in 5 CON rats and 5 SHAM rats. The rats were anaesthetised as described above, their IVC exposed and a 100 µl aliquot of ^{99m}Tc stannous colloid injected. The colloid was allowed to circulate for 30 minutes before the rat was killed and the spleen, liver, lungs, kidneys and 1 ml of blood were taken for

counting. A standard aliquot of colloid was kept to act as a reference for the total number of counts per minute injected.

To determine the specificity of clearance, in 5 rats uptake was blocked by injection of 2 ml of a 60 fold more concentrated solution of unlabelled colloid 30 minutes before the labelled colloid. Blood and organs were taken for counting 30 minutes after the injection of labelled colloid.

Having established the appropriate techniques for colloid administration and isotope measurement, experiments were conducted using CON, TIE and AT rats. The uptake of colloid was studied in TIE rats from 2 weeks to 15 months after splenic devascularisation, in AT rats from 3 to 15 months after transplantation and in CON rats of equivalent ages.

An aliquot (100 µl) of stannous fluoride colloid labelled with ^{99m}Tc was injected intravenously into TIE (n=29), AT (n=28) and CON (n=30) rats. It was allowed to circulate for 30 minutes before the rat was killed and its spleen, liver, lungs, kidneys and blood were taken for counting. At completion of the experiment, the spleen was fixed in buffered formalin for histological examination.

3.5 Preparation of colloidal carbon

Colloidal carbon was prepared as described by Jenkin and Rowley (1961). A 1% solution of gelatin was prepared in 0.9% saline. Indian ink (Pelikan) was then diluted 1:1 in the 1% gelatin solution.

3.6 Clearance of colloidal carbon

The histological site of uptake of colloidal carbon within the spleens was investigated. The carbon suspension was warmed to 37°C and injected *i.v.* in a dose of 1 mg/kg. The carbon was allowed to circulate for 5 minutes before the rat was killed. The spleen was then excised and prepared for histological examination.

3.7 Preparation of anti-rat antibody

Anti-rat red blood cell antibody was obtained by hyperimmunising a rabbit. Blood, obtained from an anaesthetised rat by cardiac puncture, was anticoagulated in acid citrate dextrose (ACD) and centrifuged at 1880 rpm for 10 minutes. The packed cells were removed

and washed twice in Dulbecco's modified phosphate buffered saline (PBS) and 1 ml was then vortex mixed with 1 ml of Freund's incomplete adjuvant. The mixture was then injected subcutaneously into a New Zealand white rabbit on 3 occasions at monthly intervals. A week after the final immunisation, 50 mls of blood was taken from an ear vein of the rabbit. The blood was allowed to clot and serum collected.

The presence of antibody to rat RBC in the rabbit serum was determined by passive haemagglutination. Complement was inactivated by heating the serum in a water bath at 56°C for 30 minutes. Serial two-fold dilutions of 100 µl of the serum were made into 100 µl of Dulbecco's modified PBS in the wells of a microtitre tray. To each well was then added 100 µl of a 1% rat RBC suspension. The cells and serum dilution were then mixed by gentle agitation and allowed to settle overnight at 4°C.

3.8 Preparation of IgG from rabbit serum

The IgG was prepared from the hyperimmune rabbit serum using the octanoic acid fractionation as described by Steinbuch and Audran (1969). One ml of serum was added to 2 mls of 0.06M sodium acetate buffer (pH 4.0). The pH of this mixture was adjusted to 4.8 with 0.1M NaOH. To this was added 75 µl of octanoic acid in a dropwise fashion, resulting in production of a flocculate. The solution was filtered after 30 minutes of stirring and the filtrate was dialysed 3 times against Dulbecco's modified PBS (pH 7.2). The dialysate was then stored at 4°C with 0.01% azide and bovine serum albumin in a concentration of 1 mg per ml of dialysate. The titre of the dialysate was determined by passive haemagglutination.

3.9 Titration of *in vivo* opsonising activity of rabbit anti-rat RBC IgG antibody

The antibody concentration that resulted in the greatest splenic uptake and minimum hepatic clearance of opsonised autologous rat RBC *in vivo* was determined. The lowest titre of the rabbit IgG that produced haemagglutination was assigned the value of a 100% haemagglutinating dose.

Syngeneic rat blood was obtained by cardiac puncture. An aliquot of the washed cells (1 ml) was mixed with sodium chromate-51 (^{51}Cr), containing 2 mBq of radioactivity, for 30 minutes at room temperature, and then washed with saline to remove unbound radiolabel. Labelled cells were opsonised with one of four dilutions of the IgG: 100%, 50%, 25% or 10%

of an haemagglutinating dose. In addition, a sample of the labelled red cells were incubated with 0.9% saline alone. For opsonisation, packed, labelled RBC and the dilution of the IgG antibody (or saline for the preparation of unopsonised labelled RBC) were incubated at 37°C with continuous slow mixing for 30 minutes, washed twice in saline and re-suspended as a 50% packed cell solution.

The rat in which clearance was to be measured was anaesthetised using nembutal (60 mg/ml) *i.p.* (0.1 ml per 100 mg of body weight). The abdomen was opened with a midline incision and a 100 µl aliquot of the opsonised, radiolabelled RBC was injected into the IVC. The syringe was counted before and after injection and the amount of radioactivity injected determined by subtraction. In some of the rats blood samples were taken from the IVC at intermediate time points before they were killed. All rats were killed 3 hours after injection, and 3 ml of blood, the spleen, liver and lungs were harvested, weighed and the radioactivity in each counted. The counts in the blood measured the amount of radioactivity not removed from the circulation. The results are expressed as the percentage of the total counts injected, and the percentage of the total counts injected per gram of tissue (%cpm/g).

To measure the proportion of the radiolabel which remained bound to the RBC, 2 ml of the 3 ml blood sample taken from the rat at the time of harvest was anticoagulated in ACD centrifuged. The plasma and the packed cells were counted separately for radioactivity. To determine if background antibody to the syngeneic RBC was present in the recipient rat, 1 ml of the recipient's blood was collected and allowed to clot. The serum was collected and heated at 56°C for 30 minutes to inactivate complement., and then tested for haemagglutination with the donor rat's RBC.

3.10 Clearance of IgG coated RBC

Fifteen rats from each of the 5 groups were studied. The rats were anaesthetised (with halothane-nitrous oxide inhalant), their IVC exposed and a 100 µl aliquot of labelled, antibody coated 50% packed rat red cells were injected. Baseline samples of blood were taken 3 and 5 minutes after injection and the wound was closed. The rat was allowed to waken.

After 3 hours the rats were re-anaesthetised and their IVC exposed. A 2 ml sample of blood was removed and counted to determine the amount of radioactivity remaining in the blood. A 1 ml sample was anticoagulated in ACD and used to determine the percentage of

radiolabel remaining bound to the RBC, as described earlier. The spleen, liver and lungs were also excised, weighed and counted. The spleen was placed in buffered formalin prior to counting to enable subsequent histological examination.

3.11 Preparation of casting material

The casting material was prepared as described by Gannon (1981). Briefly, 120 ml of methyl methacrylate monomer was added to 2.4 g of 2,4-dichlorobenzoyl peroxide in 50% dibutylpthalate. This mixture was then exposed to UV light for approximately 85 minutes (until the viscosity was 3-4 centistoke at room temperature). The polymerised methacrylate was then stored at 4°C until use (which was always within 2 days of preparation). On the day of use, 2 ml of the polymer was added to 250 mg of benzoyl peroxide and 10 mg of waxylene dye. To this was added the catalyst composed of 7 ml of 2-hydroxy-propylmethacrylate and 0.4 ml of NN-dimethylaniline.

3.12 Preparation of perfusion fluid

Perfusion fluid was prepared by dissolving 9 g of NaCl, 50 g of polyvinylpyrolidone-40, 10,000 units of sodium heparin and 0.1 ml of papaverine in 1000 ml of distilled water (Gannon *et al,* 1982).

3.13 Vascular casting

Rats were anaesthetised as described as in Section 3.2 and the thoracic aorta was exposed via a left paramedian incision. It was cannulated and the proximal aorta, and the distal aorta proximal to the bifurcation, ligated. Perfusion fluid (at room temperature) was introduced into the cannula at a pressure of 120 mm Hg, which was monitored using an aneroid manometer to maintain a constant pressure. The IVC was divided at the diaphragm to allow blood and perfusion fluid to escape. To encourage filling of the spleen, the renal, common hepatic and mesenteric arteries were also ligated (for splenic autotransplants, the mesenteric arteries were not ligated). The perfusion was continued until the spleen or transplant blanched. A total of 27 ml of the casting medium was then infused via the thoracic cannula at a pressure of 240 mm Hg under manometric control. At completion of the infusion, the splenic vasculature was clamped and the methyl methacrylate allowed to polymerise.

3.14 Tissue digestion

On completion of polymerisation, the spleen or transplant was placed in a water bath at 37°C for 3 days to allow autolysis of parenchyma. The spleen was further digested at 37°C in 20% KOH. This was continued until all tissue had been digested from the vascular casts. The casts were then frozen in distilled water and freeze dried. The larger specimens were broken with forceps into smaller pieces, mounted onto stubs, spluttered, and examined using an Siemens autoscanner scanning electron microscope (ETEC Corp. Texas).

3.15 Histological examination

The spleen or regenerated splenic tissue was placed in buffered formalin after excision. It was then was dehydrated, fixed in paraffin and standard 5 μ sections were cut and stained with haematoxylin and eosin.

The areas of WP (PALS and germinal follicles), MZ and RP were measured in all AT and TIE and 5 CON spleens using a Hipad digitiser (Houston Instruments, Austin, Texas). The area of the RP was total area of the section less the area of the WP plus MZ. The percentage of the area of the section occupied by WP, MZ and RP were calculated. The relative amount of each compartment in the tissue was estimated by multiplying its percentage area by the weight of the tissue (Kumararatne *et al*, 1981).

The presence of a central arteriole in an area of white pulp was noted. In each section, all areas of white pulp (to a maximum of 50) were examined, and the number with a central arteriole was expressed as a percentage of the total.

3.16 Preparation of spleens for immunohistochemical staining

The splenic tissue from at least 5 rats in each group was collected for immunohistochemical staining. The spleen was removed, washed in Dulbeccos PBS and snap frozen in liquid nitrogen. It was then stored at -75°C until use. Cryostat sections were cut at 10 μm and allowed to dry at room temperature for 30 minutes.

3.17 Antibodies

The antibodies used in this study are shown in Table 3.1. The monoclonal antibodies W313 and OX6 were kindly donated by Dr G. Mayrhoffer, University of Adelaide. The rabbit

monoclonal antibody to rat IgD was kindly donated by Professor H. Bazin, University of Louvain, Belgium.

Antibody	Major reactivities	Source
W313	Pan T lymphocytes	Dr G Mayrhoffer
OX6	B lymphocytes; macrophages; IDCs; MHC class II	Dr G Mayrhoffer
anti-rat IgM	B lymphocytes	Janssen Biochim
anti-rat IgD	B lymphocytes	Prof H Bazin

Table 3.1. Monoclonal antibodies used to determine cell subpopulations in spleen sections

3.18 Immunohistochemical staining

After warming at room temperature for 30 minutes, freshly cut sections were fixed in 96% alcohol for 10 minutes. They were then washed 3 times in PBS and immersed in bovine serum albumin (1 mg/ml in distilled water). The section was then incubated in a moist chamber with 28 µl of the primary antibody at 4°C for 1 hour. Following this the section was washed in PBS 3 times and then incubated with 28 µl of the secondary antibody, which was conjugated with horse radish peroxidase (HRP), for 1 hour at 4°C in a moist chamber. The section was then washed 3 times in PBS, and the peroxidase activity detected using 3'3'-diaminobenzidine (DAB) (Sigma Chemical, St Louis, USA).

Immediately prior to staining for the peroxidase activity, 200 µl of a 1% solution of H_2O_2 was mixed with 5 mg of DAB dissolved in 10 ml of Tris HCl (pH 7.6), and filtered through a 0.22 micron filter (Millex-GS). This solution was then added to the slide. Following the reaction, the section was dehydrated in absolute alcohol and counter stained for 10 seconds in haematoxylin. The section was then washed in tap water and dried in acid alcohol, saturated lithium carbonate and xylene. Negative controls were stained by substituting either the primary or secondary antibody with PBS.

3.19 Autoradiography

The histological zone in the spleen in which the phagocytosis of the radiolabelled colloid or RBC occurred was determined by autoradiography.

Autoradiography after clearance of stannous colloid was performed using a 100 µl sample of containing approximately 150 mBq of ^{99m}Tc, injected into rats as described in Section 3.4. After 30 minutes the spleen was removed and placed in buffered formalin for 30 minutes. After brief fixation in the formalin, the spleen was cut into thin slices (< 1 mm) and then sealed in a bag containing a small amount of formalin. This bag was then placed in a Harvey-Brenson contact printing frame (Model number 883696) so that the slices of spleen were exposed to a photographic film (Kodak XOMAT AR5 GBX-2). The length of exposure was varied from 6 - 24 hours to ensure an optimum exposure time.

Autoradiography after clearance of IgG coated red cells was performed using 260 µl of washed RBC labelled with ^{51}Cr containing 12 mBq of radioactivity and opsonised with the 25% dilution of rabbit anti-rat RBC IgG. The RBC were injected *i.v.* and allowed to circulate for 3 hours. The spleen was then excised, the radioactivity counted and then fixed in buffered formalin overnight. The tissue was embedded in paraffin and sections of 5 and 10 µ were cut. These sections were then exposed to a photographic film (Kodak XOMAT AR5 GBX-2) in a Harvey-Brenson contact printing frame for up to 4 weeks. Following exposure, all the sections were removed from the printing frame and stained with haematoxylin and eosin to permit identification of the histological regions in which radiolabel was detected.

3.20 Analysis of results

All results are reported as the median and range of the group. The uptake of radiolabelled colloid or labelled red cells were calculated as the percentage of counts in the relevant organ to the total counts injected (%). To correct for differences in the weight of the splenic tissue, the counts in each organ were divided by its weight and expressed as the percent counts per gram of organ (%/g), using the formula:

$$\frac{\text{cpm spleen}}{\text{weight of spleen}} \times \frac{100}{\text{total cpm injected}}$$

To assess the function of individual compartments of the spleen, the uptake per unit of white pulp and marginal zone was calculated, using the estimates described in Section 3.15.

Groups were compared using the Mann-Whitney U test. Correlations between groups were tested using the Spearman Rank test. Differences were considered statistically significant when $p < 0.05$.

CHAPTER 4

Studies on the phagocytosis of a colloid by normal and regenerated splenic tissue

4.1 Introduction

The normal spleen is an integral part of the reticulo-endothelial system and an important phagocytic organ. Many studies have illustrated the specific nature of splenic phagocytosis (Schreiber and Frank, 1972; Hosea *et al*, 1981; Peters *et al*, 1984; Yousaf *et al*, 1986). Colloidal substances and IgM coated particles have been shown to be taken up predominantly by the liver (Schreiber and Frank, 1972; Atkinson and Frank, 1974; Malangoni *et al*, 1985), whereas poorly opsonised, IgG coated particles are most efficiently taken up by the spleen (Yousaf *et al*, 1986).

Colloids phagocytosed by the fixed cells of the reticuloendothelial system, which includes macrophages of the liver, spleen, lungs and bone marrow. Colloidal carbon was used extensively in the 1970s to demonstrate phagocytosis by the normal spleen (Nossal *et al*, 1966; Burke and Simon, 1970; Mitchell and Abbot, 1971). More recently radiolabelled colloids have been used to demonstrate and quantitate phagocytic function by liver and spleen (Keramidas *et al*, 1980; Kiroff *et al*, 1985; Malangoni *et al*, 1985). The mechanism of colloidal phagocytosis is unclear, particularly the role of antibody.

The aims of this study were to determine the ability of regenerated splenic tissue to clear a colloid from the circulation and to investigate the site(s) within the spleen responsible for phagocytosis. This was performed by measuring the clearance of injected colloid by the liver, lungs and splenic tissue in control rats. This was compared to the clearance by regenerated splenic tissue in rats following either ligation of the splenic vasculature or splenectomy with splenic autotransplantation. The time course of the restoration of function by the regenerating spleen following either of these procedures was determined by measuring the clearance at varying time points after surgery. The histological regions within the spleen responsible for colloidal phagocytosis were identified. Correlations between phagocytic ability and histological appearance were sought.

4.2 Preliminary experiments with Tc-99m stannous fluoride colloid

Preliminary experiments were performed to identify specific factors that may influence the study results.

The effect of anaesthesia and operation on phagocytosis was studied by comparing uptake between 5 sham operated (SHAM) and 5 unoperated control (CON) rats. There was no

statistically significant difference in splenic uptake of radiolabelled colloid between the groups, 4.8% (3.2 - 10.4%) in the SHAM compared to 4.8% (1.9 - 9.0%) in CON rats. There was no significant difference in hepatic, pulmonary or renal uptake either. Hepatic uptake by SHAM rats was 92.6% (82.1 - 96.0%) compared to 92.7% (85.6 - 97.2%) in CON rats. Pulmonary uptake was 0.9% (0.4 - 7.5%) and 0.9% (0.3 - 6.8%) respectively. Renal uptake was also less than 1% in both groups.

To determine if saturation of the phagocytic system could occur with the concentration of colloid used, a large, unlabelled volume of colloid was injected prior to the radiolabelled colloid. Uptake by the spleen was then compared to that by control rats injected with radiolabelled colloid alone. Two mls of a concentrated solution of colloid was injected into 5 CON rats prior to the radiolabelled colloid, and another 5 CON rats were injected with radiolabelled colloid alone. The splenic uptake was 2.9% (2.0 - 3.5%) in those rats which received unlabelled colloid compared to 4.8% (3.3 - 11.5%) which did not. Hepatic uptake was 94.0% (91.5 - 95.0%) compared to 87.5% (84.1 - 93.8%) respectively. There was no significant difference in splenic, hepatic, pulmonary or renal uptake of radiolabelled colloid between the rats which received the unlabelled colloid prior to injection of labelled colloid, and those that did not, indicating that the amount of radiolabelled colloid used was non-saturating.

4.3 Weight of regenerated splenic tissue

Following splenic devascularisation or autotransplantation the splenic tissue regenerated, but never attained the weight of the normal spleen. There was no significant difference in the final weight of regenerated tissue in AT rats that were implanted with 150 mg (n = 14) compared to those implanted with 225 mg (n = 15). The final weights at sacrifice were significantly higher in the TIE rats than the AT rats (Figure 4.1). The median weight of CON spleens was 737 mg (681 - 1079 mg). At 3 months TIE spleens weighed 234 mg (193 - 292 mg) compared to 172 mg (108 - 395 mg) for AT spleens. At 6 months, TIE spleens weighed 262 mg (167 - 507 mg) and AT 116 mg (43 - 223 mg). At 15 months, TIE spleens weighed 194 mg (130 - 408 mg) compared to AT spleens 216 mg (30 - 355 mg) (Figure 4.1).

4.4 Clearance of Tc-99m stannous fluoride colloid

Splenic uptake was significantly less in both TIE and AT spleens compared to CON spleens. In the TIE rats, splenic uptake increased over time following devascularisation until approximately 4 months post-operatively with no significant improvement from 4 to 15 months after operation. The splenic uptake was 0.8% (0.5 - 1.8%) at 4 months after devascularisation and 0.5% (0.5 - 0.6%) at 15 months, both significantly less than in CON rats, 5.0% (1.9 - 10.4%) ($p < 0.001$) (Figure 4.2).

Autotransplanted splenic tissue phagocytosed significantly less colloid than CON spleens. At 3 months 0.05% (0.006 - 0.2%) was phagocytosed in the AT rats compared to 5.7% (1.9 - 8.2%) in CON rats ($p < 0.001$). At 6 months the transplants removed significantly more colloid than at 3 months, 0.2% (0.005 - 0.5%) ($p<0.02$), but still significantly less than controls with 4.8% (3.0 - 10.1%) ($p < 0.001$). At 15 months, the AT phagocytosed 0.2% (0.04 - 0.6%), compared to 5.5% (3.2 - 10.4%) by CON ($p < 0.001$) (Figure 4.3). There was no significant difference in splenic uptake at 15 months compared to 6 months post transplantation, nor between 6 and 15 month TIE or AT spleens.

There was no significant difference between the groups in the uptake by the liver, lung or kidney at any time point, and so the clearance by each organ for all time points have been pooled and treated as one group for reporting. The uptake by the liver was significantly higher in both the TIE and AT groups when compared to control rats. The median uptake by the liver of the TIE group was 97.3% (90.3 - 99.2%), AT 97.6% (87.5 - 99.3%) and CON 92.7% (82.1 - 97.2%). There was no significant difference in the hepatic clearance between the TIE or AT groups (Table 4.1).

Experimental Group	Mean uptake	Range
TIE	97.3%	(90.3-99.2%)
AT	97.6%	(87.5-99.3%)
CON	92.7%	(82.1-97.2%)

Table 4.1 The uptake of Tc-99m labelled stannous fluoride colloid by the liver

The uptake of the colloid by the lungs did not differ between groups. The lungs cleared 0.8% (0.3 - 7.4%) in TIE rats, compared to 1.4% (0.3 - 10.6%) in AT rats and 0.8% (0.3 - 7.5%) in CON rats.

No statistically significant difference in the renal clearance of colloid was found between the groups. The kidneys cleared 0.5% (0.04 - 1.6%) in TIE rats, 0.5% (0.2 - 1.1%) in AT rats and 0.6% (0.1 - 1.4%) in CON rats.

To allow for the differences in weight, the splenic uptake per gram of tissue was calculated, and this was significantly less in the TIE and AT rats than the CON spleens. Three months after surgery, TIE spleens phagocytosed 0.8 %/g (0.7 - 1.5 %/g), AT spleens 0.3 %/g (0.02 - 1.3 %/g), both less than CON spleens 6.2 %/g (2.5 - 9.9 %/g) ($p < 0.001$). Six months after surgery, the TIE spleens cleared 1.4 %/g (0.5 - 2.5 %/g), AT spleens 1.8 %/g (0.04 - 4.2 %/g) and CON spleens 5.8%/g (2.2 - 13.1%/g) ($p < 0.001$). Fifteen months after surgery, TIE spleens cleared 2.4 %/g (1.5 - 4.0 %/g) and AT spleens cleared 1.2%/g (0.2 - 2.9 %/g), compared to the CON splenic clearance per gram of 5.9%/g (2.1 - 13.1 %/g) ($p < 0.001$) (Figure 4.4 and 4.5).

To assess the contribution of the individual compartments of the spleen to colloidal uptake, the percentage uptake of colloid per gram of either WP or MZ was calculated. Those spleens in which these compartments comprised less than 1% of the tissue were excluded from the analysis because of the errors arising from working with radiation close to background and the difficulty in accurately measuring very small areas. The percentage splenic uptake per gram of white pulp was 83.9 %/g (22.2 - 189.4 %/g) for TIE, 68.0 %/g (7.2 - 187.3 %/g) for AT and 48.9 %/g (22.3 - 61.9 %/g) for CON. There was no significant difference between the percentage uptake per gram of splenic tissue between the AT ,TIE or CON rats. The percentage uptake per gram of marginal zone was 72.9 %/g (20.8 - 170.9 %/g) for TIE, 65.5 %/g (2.8 - 170.1 %/g) for AT and 27.5 %/g (22.1 - 40.6 %/g) for CON. The TIE spleens had significantly more uptake than CON ($p < 0.05$) but not AT tissue. There was no significant difference between AT and CON splenic tissue.

There was no significant statistical difference in uptake per gram of hepatic, pulmonary or renal tissue between any group at any time point (Table 4.2).

Experimental group	Mean uptake	Range
TIE	6.5 %/g	(4.5 - 9.1 %/g)
AT	6.3 %/g	(4.3 - 8.5 %/g)
CON	6.6 %/g	(3.0 - 9.6 %/g)

Table 4.2 The uptake per gram of Tc-99m labelled stannous fluoride colloid

4.5 Histological examination of normal spleens after injection of colloidal carbon

The phagocytosis of colloids has been used to determine phagocytic function in normal and abnormal spleens (Schroth *et al* , 1981) but the site of phagocytosis of colloids has not been investigated in regenerated splenic tissue. If the phagocytic function of regenerated splenic tissue is decreased, despite adequate amounts of tissue, it may be due the absence of specific compartments or cells responsible for phagocytosis. Therefore the histological site of phagocytosis of colloids within the normal spleen and regenerated splenic tissue was determined.

Colloidal carbon was prepared as in Section 3.5, injected and the spleens prepared for histology as in Section 3.6. Three groups of rats were investigated: TIE (n = 7) and AT (n = 14), both 6 months after operation and CON (n = 4). The photomicrograph of a section of normal spleen is shown in Figure 4.9. This shows an area of white pulp, made up of a periarteriolar sheath (PALS) and germinal centre/follicle, surrounded by the marginal zone, with red pulp enclosing this. Colloidal carbon was found to be localised to the cells within the inner layer of the MZ predominantly (Figures 4.9 & 4.10). There were also isolated accumulations of carbon in the red pulp. Colloidal carbon was also seen in the thin marginal zone of regenerated spleens predominantly.

4.6 Autoradiography of normal spleens after injection of radiolabelled colloid

Stannous fluoride colloid labelled with Tc-99m was injected into 5 CON rats to determine its site of phagocytosis in normal splenic tissue (Section 3.19). The autoradiographs

showed that the colloid was phagocytosed by cells in the marginal zone (Figure 4.12). Small accumulations of colloid were also found in the red pulp, with a similar distribution to that observed using colloidal carbon.

4.7 Histological examination

The macroscopic appearance of the regenerated splenic tissue was normal, but the clearance of colloid by it was diminished. The process by which the splenic tissue regenerates may provide a clue to the impairment in function. All TIE (n = 29) and AT (n = 28) splenic tissue were examined histologically.

The findings as shown in Figures 4.6 - 4.10 are consistent with other reports describing the changes seen following ischaemia and regeneration of the devascularised spleen (Wolf 1982). The necrotic central portion of the spleen is initially invaded by neutrophils, and only a peripheral rim of cells survive, presumably maintained by diffusion of nutrients through the cortex (Figure 4.6). The necrotic central region is replaced by fibrous tissue, with scattered lymphocytes apparent at 1 month following ischaemia (Figure 4.7). At 2 months, lymphocytes have congregated to form compartmentalised WP and MZ within the red pulp cords (Figure 4.8). The splenic tissue continues to increase in size with further increase in the size of the WP and MZ until approximately 4 months after ischaemia (Figure 4.8). There was no significant changes in weights or histology after this time seen in spleens at 5 or 6 months post-operatively.

The WP constituted 10.8% (8.8 - 20.1%) of the sections from the CON spleens, significantly more than the 1.1% (0 - 6.6%) from the TIE spleens and 1.4% (0 - 7.3%) from the AT spleens ($p < 0.001$). The MZ constituted 17.4% (12.4 - 21.9%) of the sections from the CON spleens, 1.0% (0 - 3.8%) from the TIE spleens and 1.1% (0 - 8.8%) from the AT spleens ($P < 0.001$). The RP constituted 89.2% (79.9 - 91.2%) of the sections from the CON, 97.4% (89.6 - 100%) from the TIE and 97.5% (83.9 - 100%) from the AT splenic tissue. ($P < 0.001$)

The MZ was shown in the previous 2 experiments to be the major site of phagocytosis of a colloid. To allow for differences in organ weight, the approximate amount of MZ present was calculated for each specimen by multiplying the weight of splenic tissue by the percentage of the section that comprised the MZ (volume index). The median volume index for MZ was

15.3 (13.1 - 26.6 g) in the CON, 0.20 (0 - 1.9 g) in the TIE and 0.18 (0 - 1.1 g) in the AT spleens ($p < 0.0001$).

The percentage of WP areas in which there was a central arteriole was 88% (87 - 95%) for CON, 27% (0 - 60%) for TIE and 30% (0 - 67%) for AT spleens ($p < 0.001$).

There was a direct correlation between the percentage of WP and MZ ($p < 0.0001$). There was also a direct correlation between the uptake of colloid and the percentage of WP or MZ (Spearman coefficient 0.49 and 0.42 respectively, $p < 0.05$). There was a direct correlation between the volume index of MZ and splenic uptake of colloid (Spearman coefficient 0.7, $p < 0.001$) (Figure 4.13). There was a direct correlation between the percentage of WP areas with a central arteriole and the uptake of colloid by the tissue (Spearman coefficient 0.6, $p<0.05$).

4.8 Discussion

Colloidal substances are phagocytosed by the reticulo-endothelial system, but the histological site is unknown. The results here show that the majority is of injected colloid is phagocytosed by the liver, as had been reported by Malangoni *et al* (1985) using ^{99m}Tc sulphur colloid.

The normal spleen contributes to colloidal clearance and phagocytoses approximately the same amount as the liver on a per gram basis. The results from the experiments reported here show that the liver and spleen are responsible for the phagocytosis of over 98% of colloid from the bloodstream. In the experimental groups, phagocytosis of colloid by the splenic tissue was markedly reduced. The regenerated spleens phagocytosed only 10% of the colloid that normal spleens cleared. When calculated on an per gram basis, which made allowance for the fact that the regenerated spleen never weighed as much as the normal spleen, regenerated spleens still had only 25% of the normal phagocytic function.

The region within the spleen in which colloidal uptake occurred was investigated. For both carbon colloid (as determined by light microscopy) and technetium-99m stannous fluoride colloid (as determined by autoradiography), the principal site of uptake was found to be the MZ. Very little phagocytosis of colloid occurred in the RP. The reduction in the relative area of the MZ previously reported (Moore *et al*, 1986) was confirmed in this study. It was found

that the MZ in regenerated spleens was reduced by 90% when compared to normal spleens. The area of WP was also reduced to the same extent.

In addition to an abnormally low amount of WP and MZ, the regenerated spleen often lacked a central arteriole within the WP. This may indicate that the spleen has an abnormal vascular supply, which may contribute to the impaired phagocytic function. This abnormality in the vasculature may also contribute to the reduction in the amount of lymphoid regeneration. A direct correlation between the percentage area of MZ (or WP) and the percentage of WP areas containing a central arteriole was found.

The MZ was demonstrated on histological and autoradiographic studies to be the site responsible for colloidal phagocytosis, there being a direct correlation between colloidal uptake and the volume index of the MZ.

In conclusion, the MZ is the predominant site of phagocytosis of colloids in the spleen. In regenerated splenic tissue this component is reduced and consequently phagocytic uptake was found to be reduced in direct proportion to the reduction in MZ.

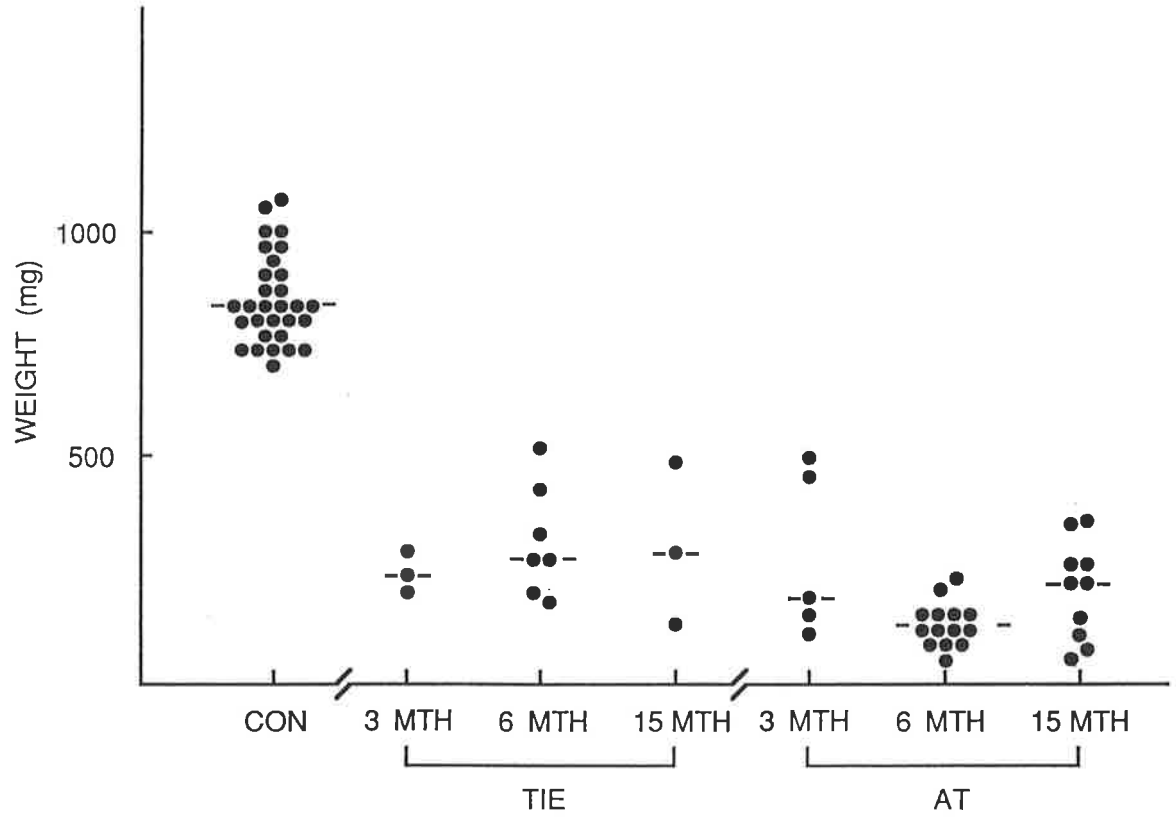

Figure 4.1. Splenic weight at varying time points after surgery.

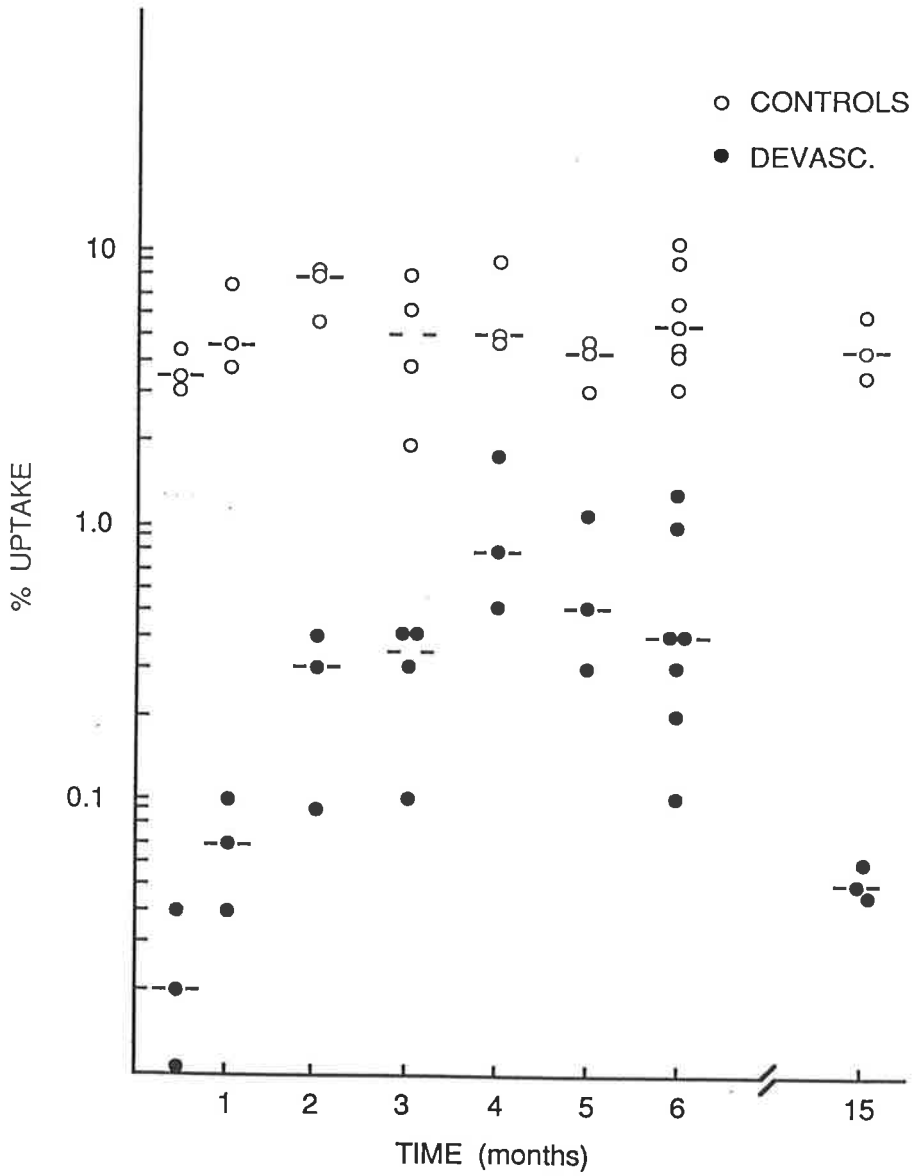

Figure 4.2. Splenic uptake of ^{99m}Tc Stannous colloid by TIE spleens at varying intervals after surgery.

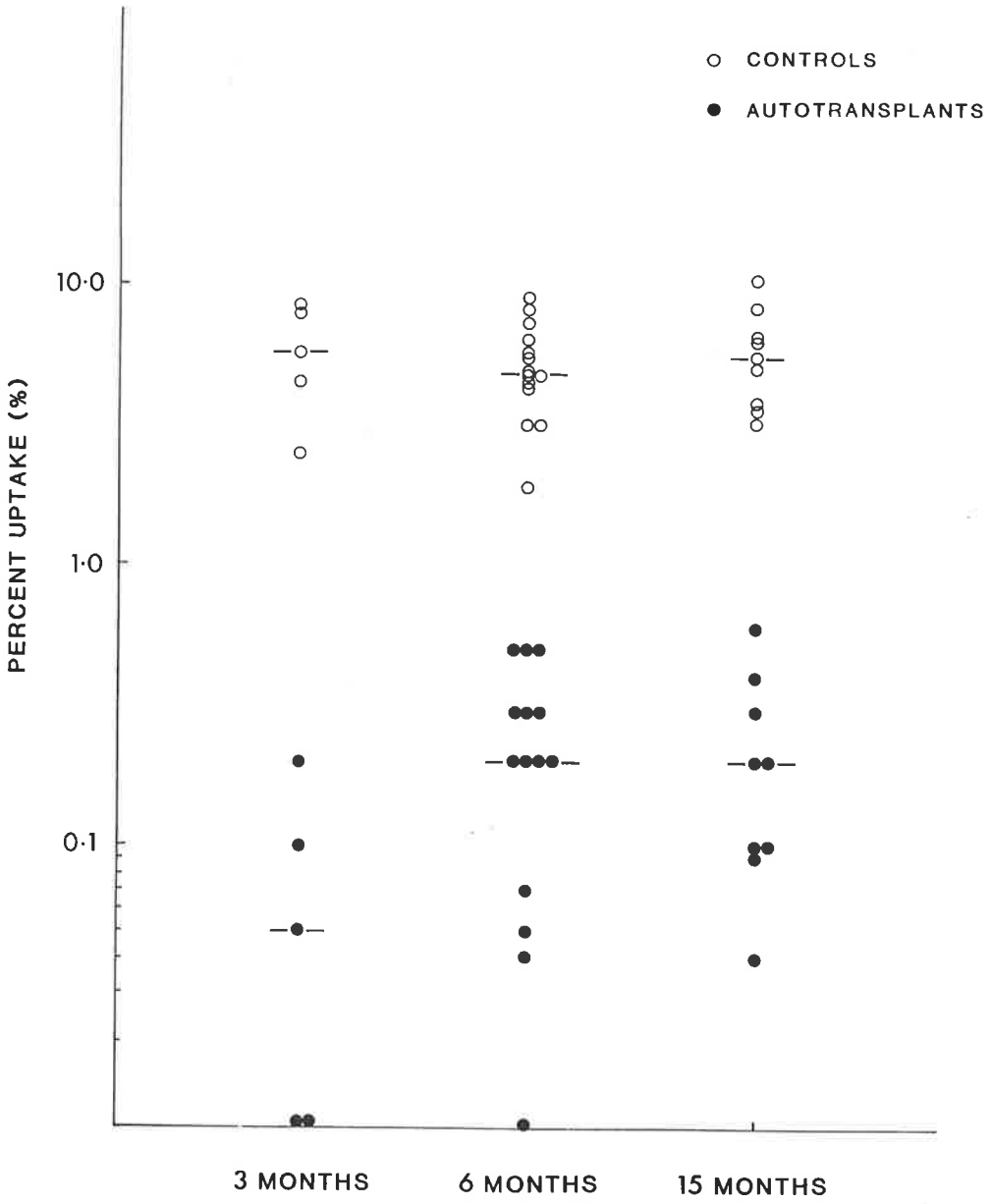

Figure 4.3. Splenic uptake of ^{99m}Tc stannous colloid by autotransplants at varying time points after surgery

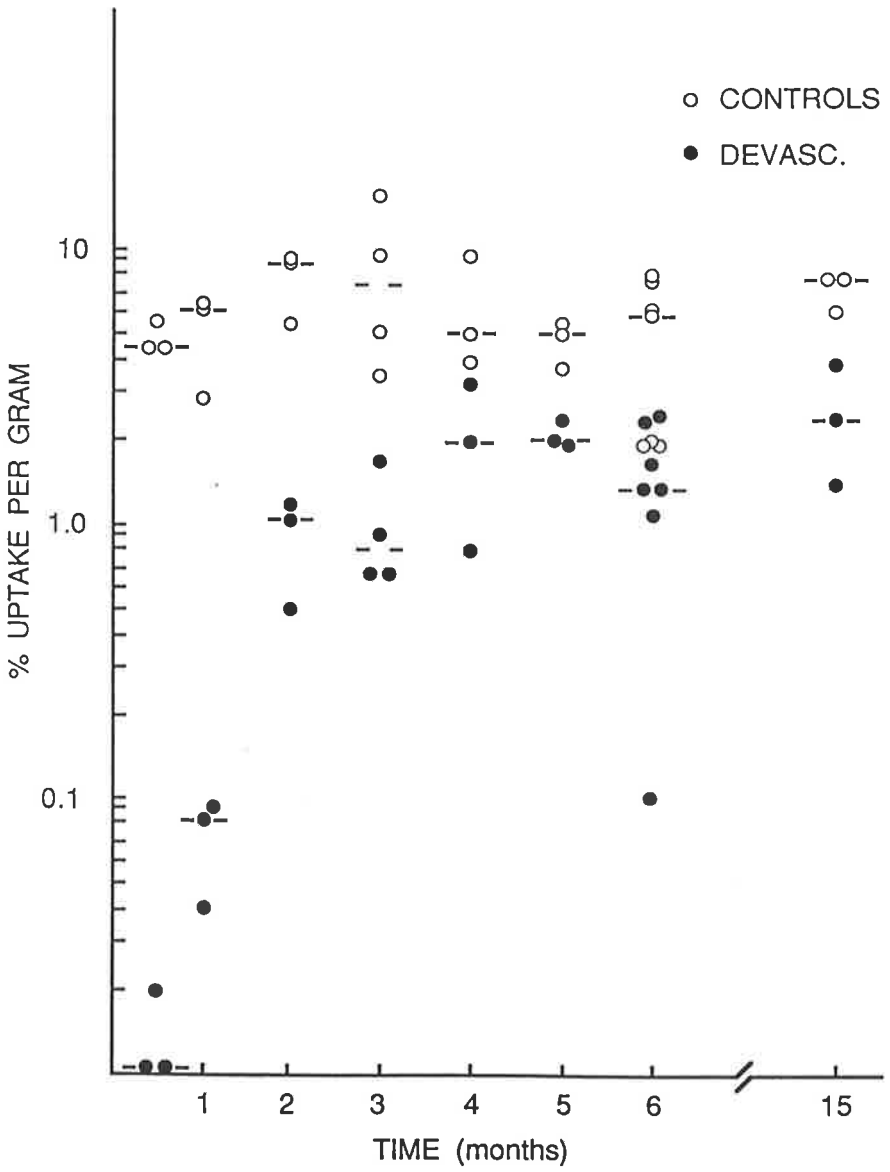

Figure 4.4. Splenic uptake per gram of splenic tissue by ligated spleens at varying time points after surgery.

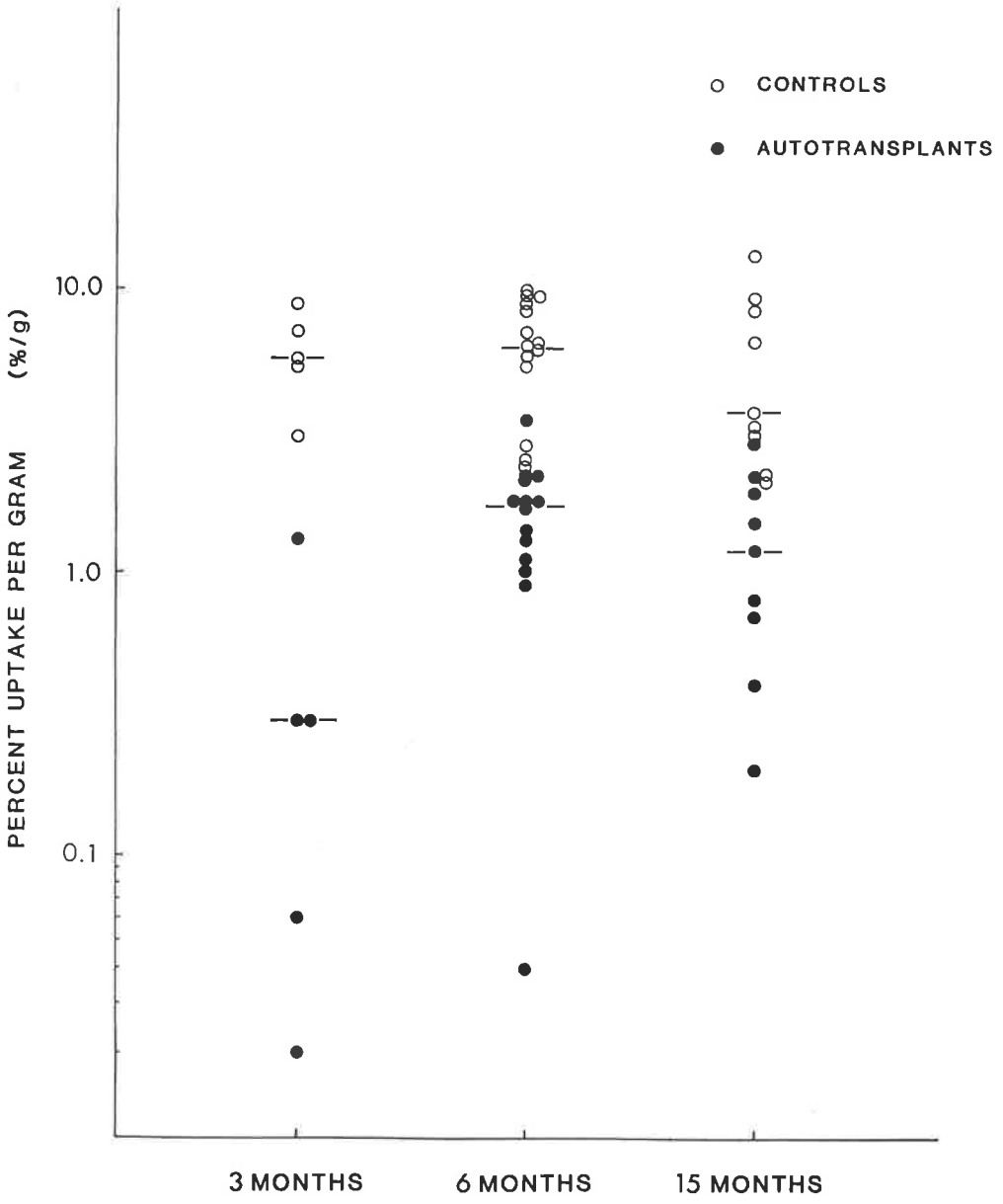

Figure 4.5. Splenic uptake per gram of splenic tissue by autotransplants at varying time points after surgery

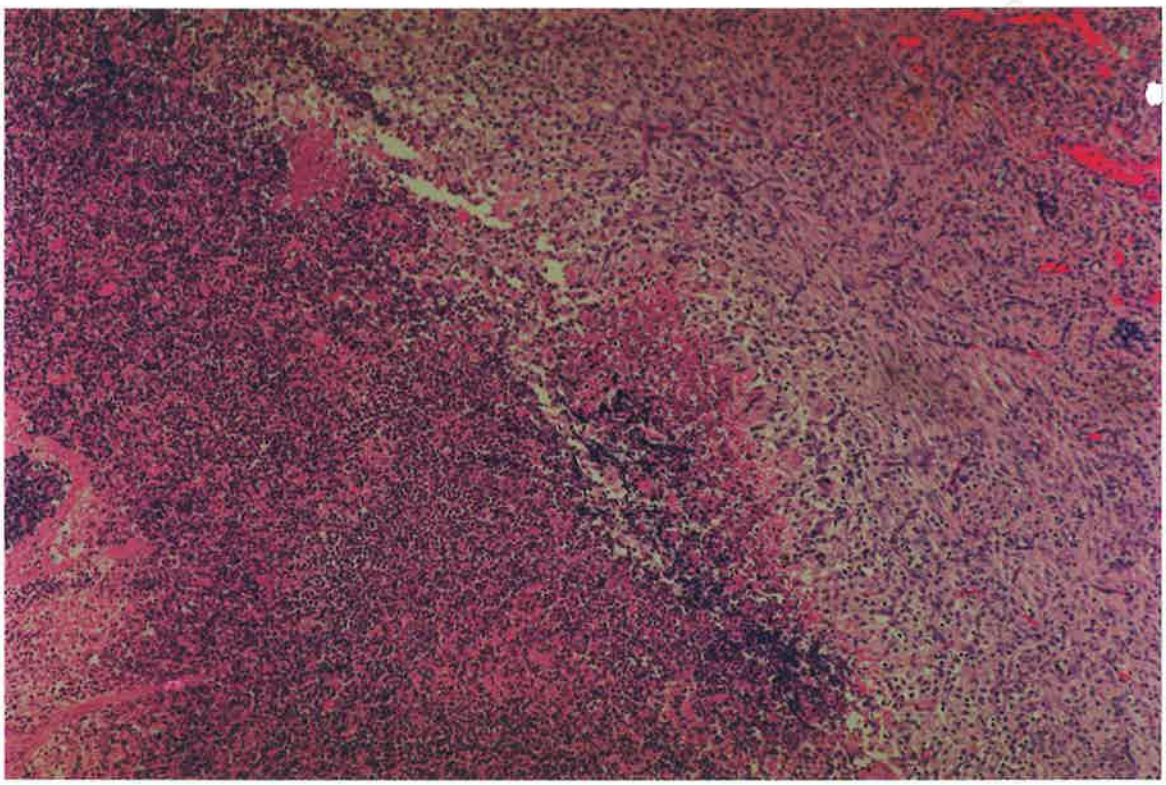

Figure 4.6. Low power view of a spleen 2 weeks after it has been ligated. Normal splenic tissue is seen against a wave of invading neutrophils in an area that is necrotic.
(Haematoxylin and Eosin, x100)

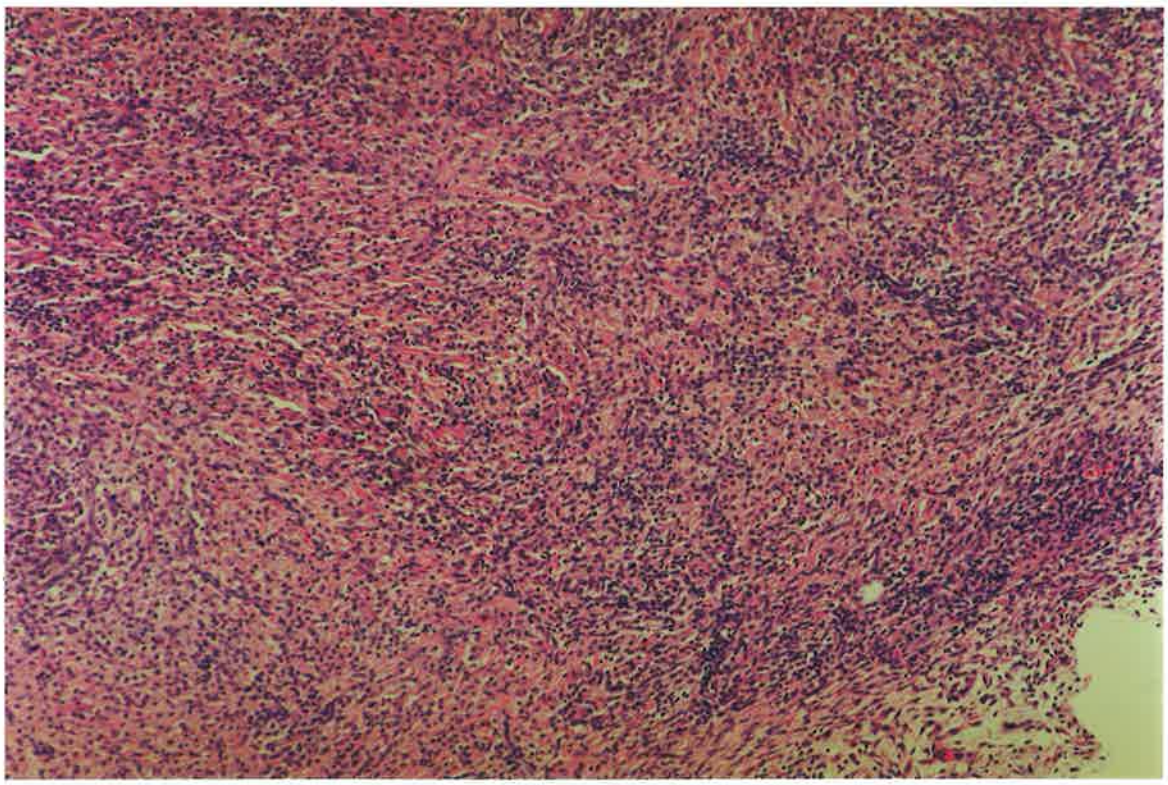

Figure 4.7. Low power view of a spleen 1 month after it has been ligated. The neutrophils have been replaced by fibrous tissue and re-establishing pulp cords. Lymphocytes are scattered throughout the field.

(Haematoxylin and Eosin, x100)

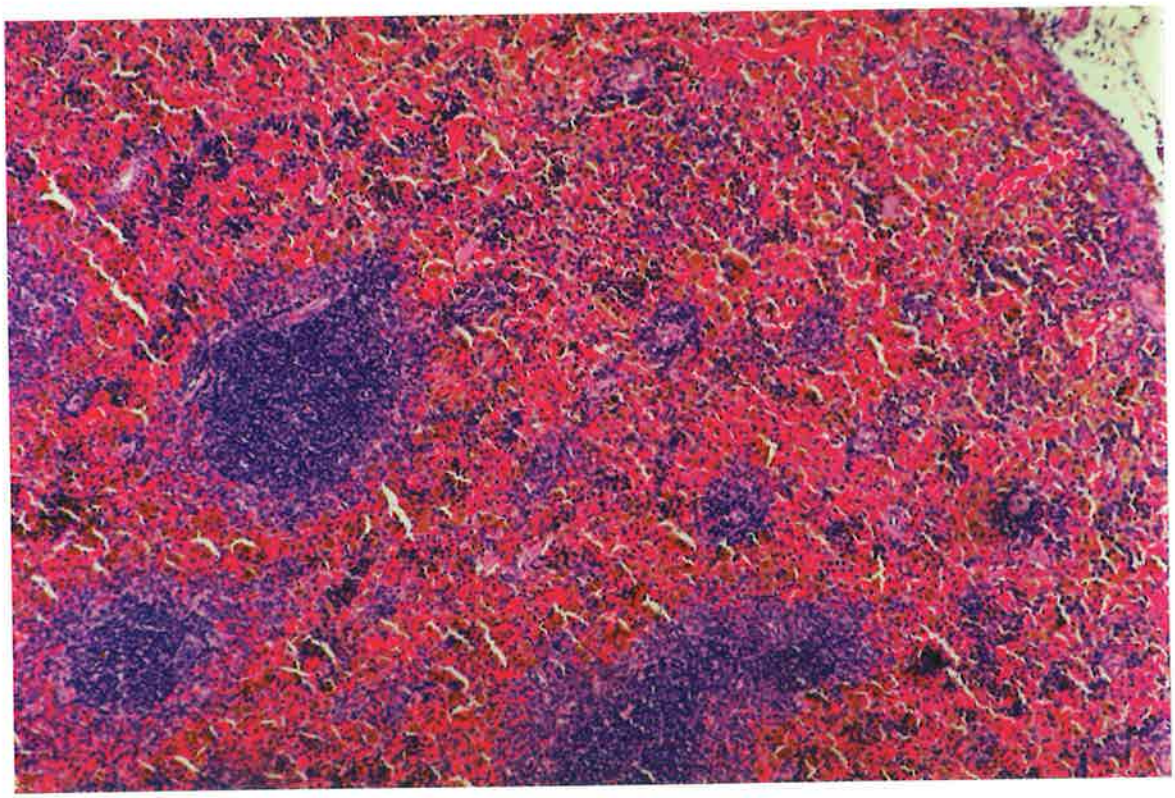

Figure 4.8. Low power view of a spleen 2 months after it has been ligated. The lymphocytes have aggregated to form WP and a surrounding MZ within a well developed RP cord system.

(Haematoxylin and Eosin, x100)

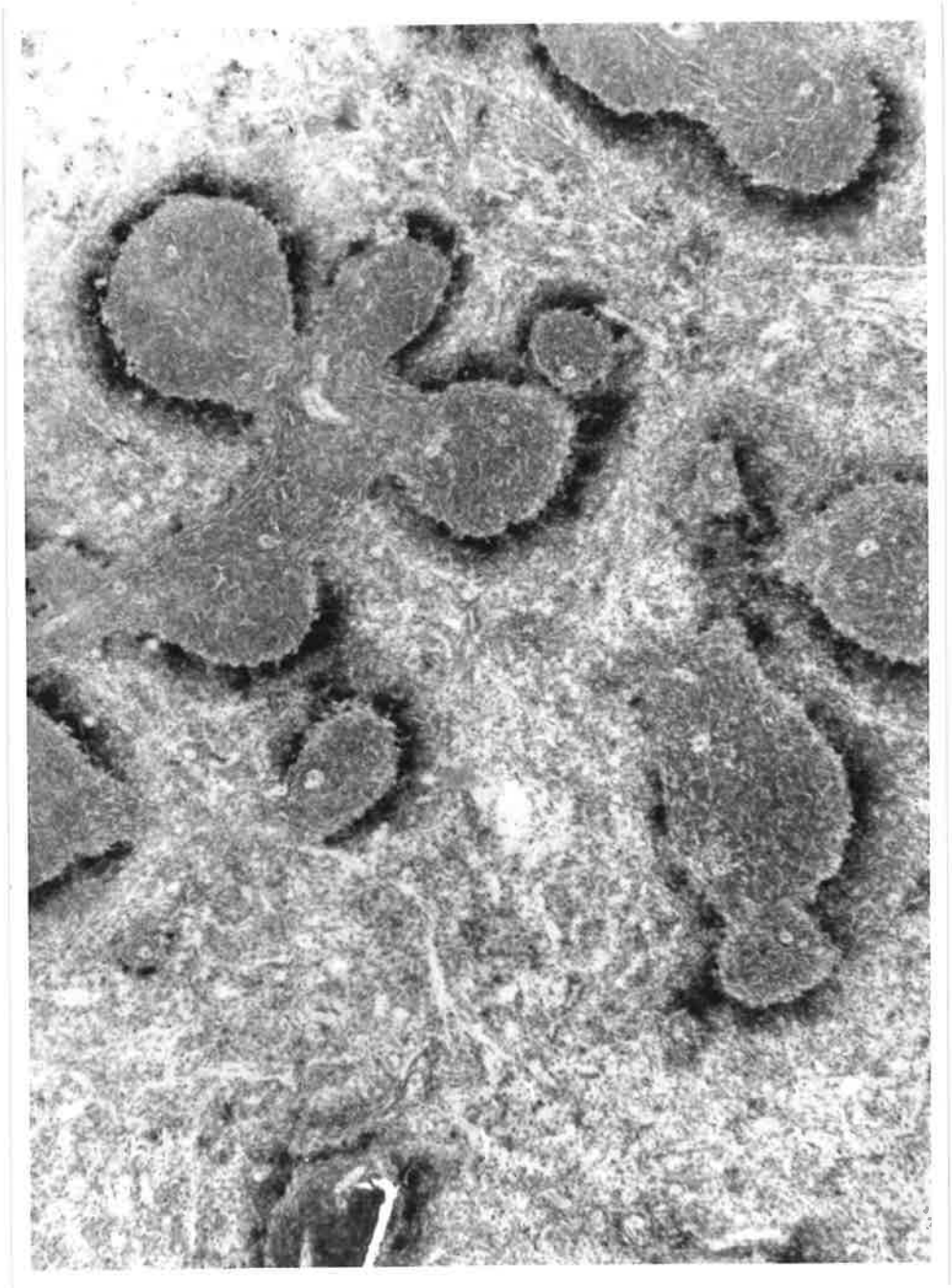

Figure 4.9. Low power view of normal splenic tissue following injection of colloidal carbon. Carbon is seen in the MZ surrounding the WP.

(Haematoxylin and Eosin and Carbon, x40)

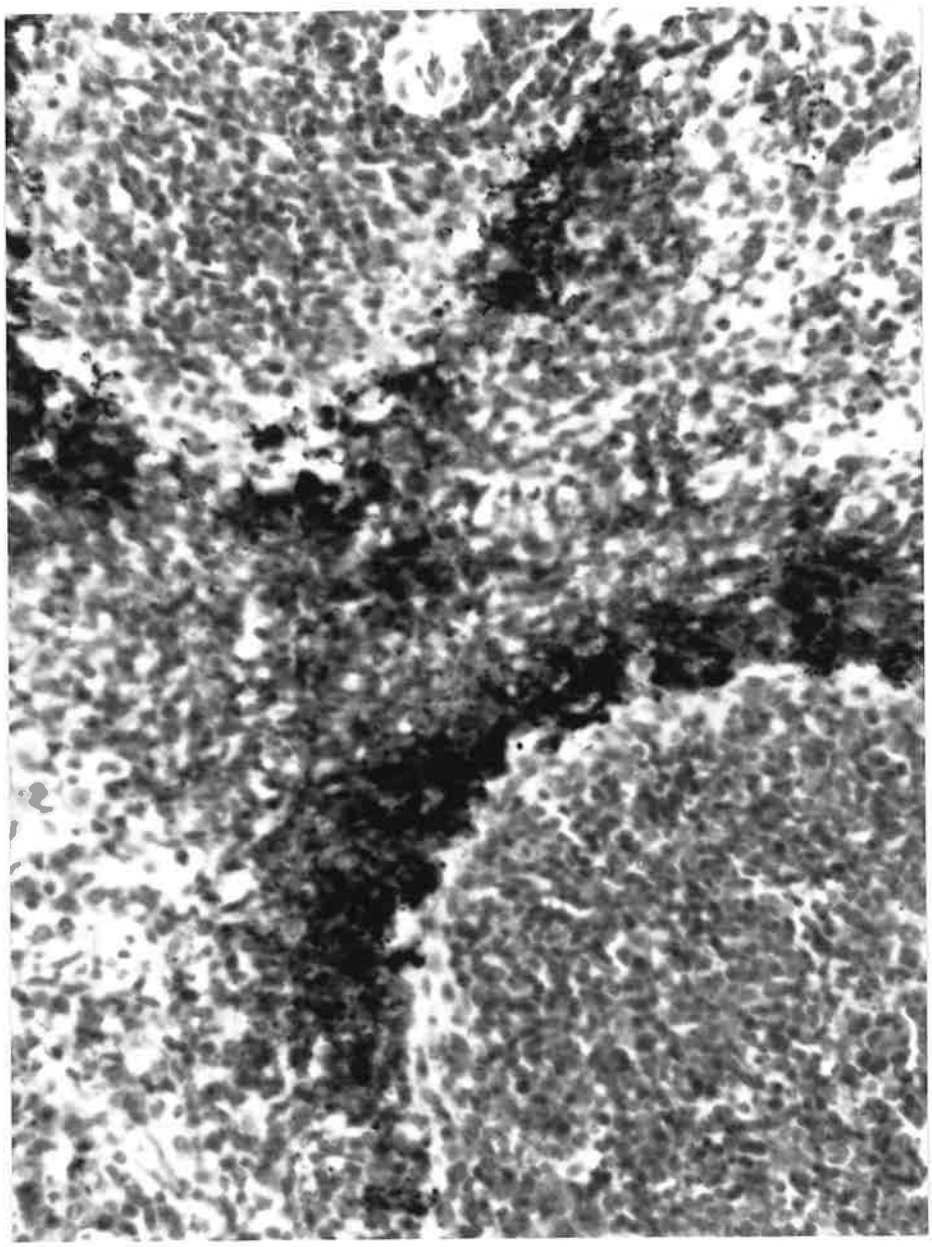

Figure 4.10. Higher power view of normal splenic tissue from figure 4.9., following injection of colloidal carbon. Carbon is seen in the MZ adjacent to the marginal sinus. (Haematoxylin and Eosin and Carbon, x200)

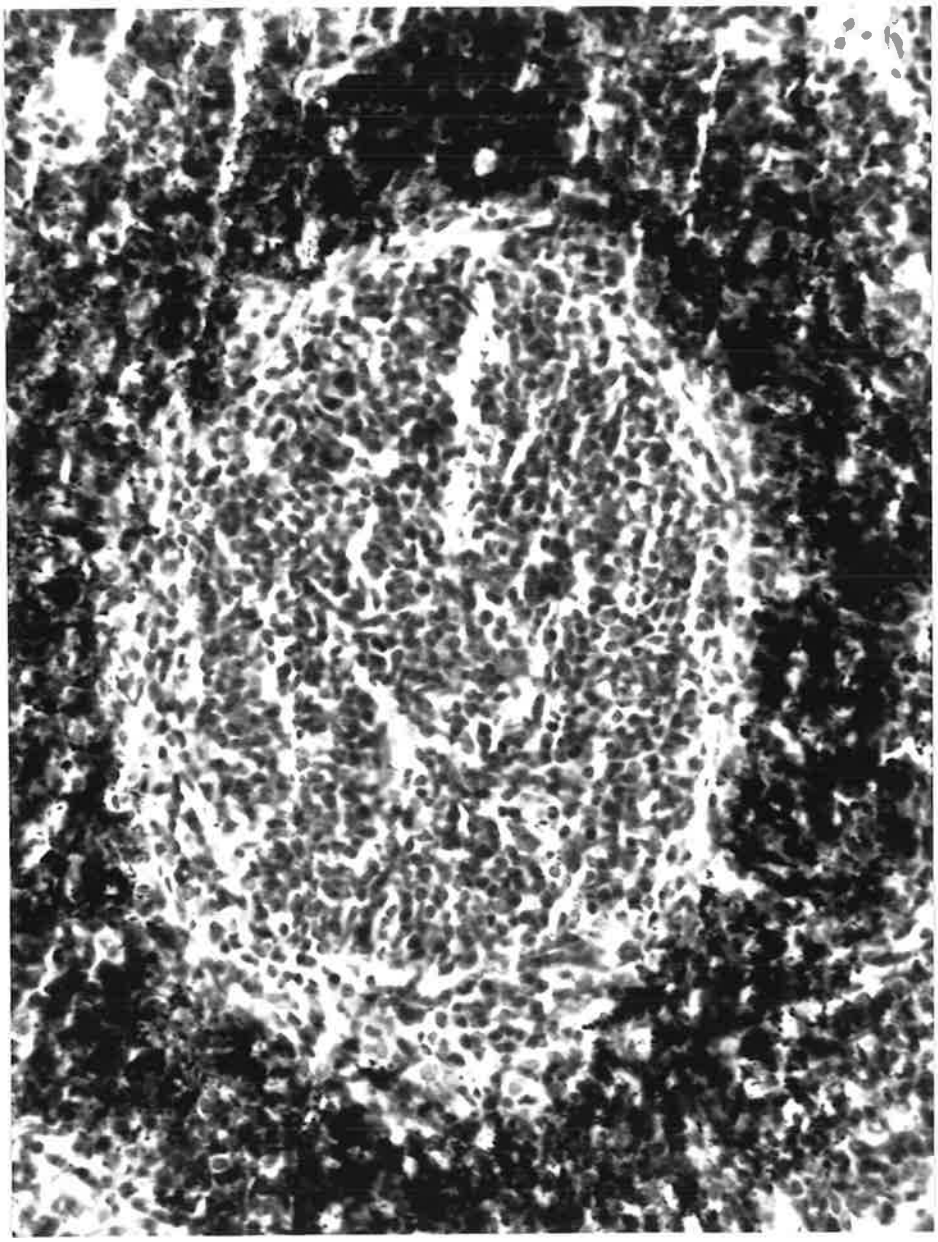

Figure 4.11. High power view of regenerated splenic tissue following injection of colloidal carbon. Carbon is seen in the MZ surrounding the WP once again adjacent to the marginal sinus.

(Haematoxylin and Eosin and Carbon, x200)

Figure 4.12. Low power view of autograph of normal splenic tissue following injection of stannous fluoride colloid. Areas of uptake are represented by dark rings surrounding "cold" central WP. RP in between the MZ also show minimal activity.

(x40)

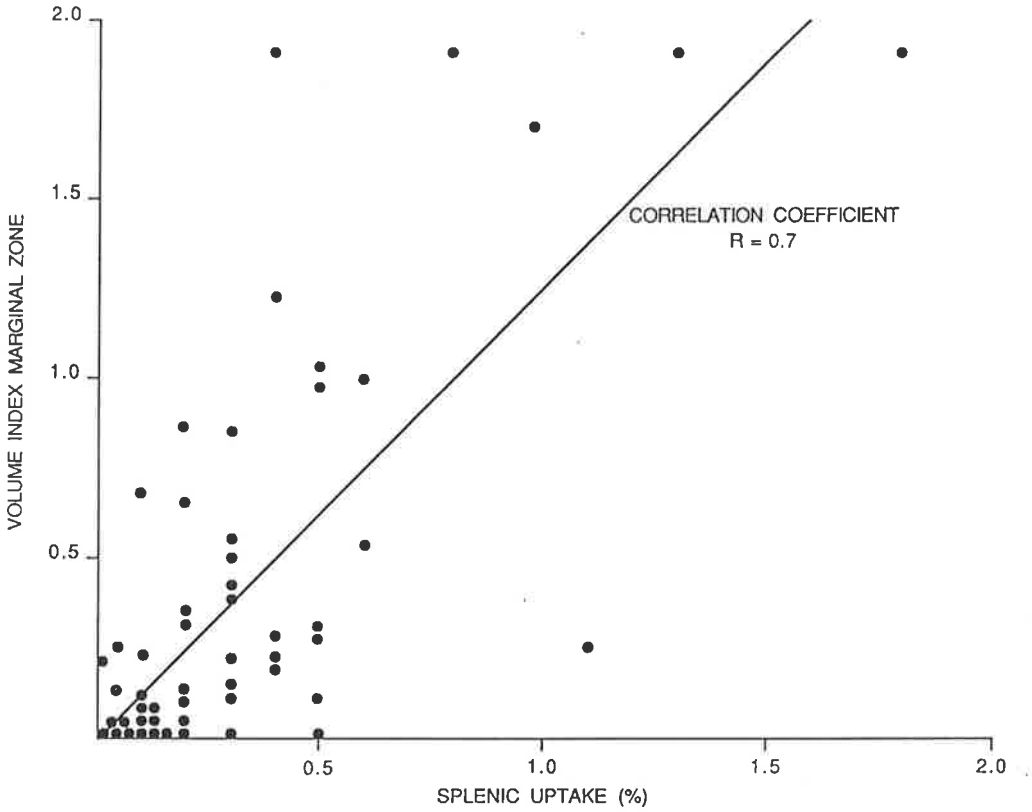

Figure 4.13. Volume index of MZ versus splenic uptake (%). Spearman correlation coefficient 0.7.

CHAPTER 5

Studies on IgG Fc-receptor mediated phagocytosis by normal and regenerated splenic tissue

5.1 Introduction

In Chapter 4 the phagocytosis of colloids was studied in both normal spleens and regenerated splenic tissue. These studies demonstrated that the spleen has only a minor role in colloidal clearance when compared to the liver (which clears 90% of an injected dose). This was reported by Atkinson and Frank (1974) and Malangoni *et al* (1985). In contrast, IgG coated particles are primarily removed by the spleen (Yousaf *et al*, 1986).

The IgG mediated clearance is dependent on the amount of IgG present on the cell surface. In low concentrations, complement is not bound and clearance is mediated by IgG Fc-receptors which are located in the highest concentration on splenic macrophages. When IgG is present at a high concentration, complement is bound to the cell surface and clearance is mediated via C3 receptors, which are present predominantly on macrophages in the liver. Schreiber and Frank (1972) demonstrated in the guinea pig that 2,000 IgG antibody molecules were needed on a surface to be certain that a single C1 (the first part of the complement cascade) binding site was formed, compared to only 1 IgM antibody molecule.

The macrophages in the spleen have the highest concentration of IgG Fc-receptors and therefore it is the most efficient organ for the removal of IgG coated particles. This has particular importance when the host is exposed to a bacteria for the first time. Only low concentrations of IgG will be present in the serum, resulting in poor opsonisation of the bacteria, which will in the main be cleared by the spleen. In the absence of the spleen, the bacteria are free to proliferate and the bacteraemia may progress to septic shock and overwhelming post-splenectomy infection (OPSI).

In this study the IgG Fc-receptor mediated phagocytosis of particles from the bloodstream of eusplenic and splenectomised rats was compared to that of rats which had undergone partial splenectomy, splenic arterial ligation or splenic auto-transplantation.

5.2 Effect of the amount of antibody on splenic clearance

The dilution of the IgG used for opsonization which mediated relatively greater splenic clearance compared with the liver was determined in control rats. Labelled erythrocytes were opsonized with one of four dilutions of IgG: 100%, 50%, 25% or 10% of an HA dose. Unopsonized erythrocytes were prepared by incubating the labelled cells with saline alone.

Using a 100% haemagglutinating dose of IgG resulted in over 80% of the red cells

being cleared, of which the liver was responsible for over 90%. Using the 50% haemagglutinating dose resulted in similar hepatic uptake and increasing splenic phagocytosis. When the 25% haemagglutinating dose was used, the spleen cleared 25% of injected red cells which compared to 4.5% using the 100% dose and 15% using the 50% dose. The 10% dose resulted in 21% cleared by the spleen and 6% by the liver. Saline incubated red cells were poorly cleared with less than 10% phagocytosed. The lungs cleared less than 2% in all groups.

The 25% haemagglutinating dose of IgG produced the best splenic uptake with minimal hepatic uptake (Figure 5.1), which was therefore used for all subsequent experiments. The majority of the clearance of IgG labelled red cells occurred within 1 hour of injection. This was irrespective of the amount of IgG used to coat the red cell.

5.3 Clearance of IgG coated red cells and organ uptake

Rats were allocated into one of 5 experimental groups, with 15 rats in each. The groups consisted of rats which were unoperated controls (CON), hemisplenectomised (HS), splenic artery ligated (TIE), splenectomised with autotransplantation (AT) or splenectomised (SPX). The rats were allowed 6 months to recover from the operation prior to investigation. To maximise splenic uptake the 25% haemagglutinating dose of antibody to the RBC was used. Using this dilution of antibody, 71% (48 - 90%) of labelled red cells were removed from the circulation by CON rats which compared to 71% (38 - 98%) by HS rats ($p > 0.05$). The TIE rats cleared 50% (33 - 68%), AT rats 47% (35 - 68%), SPX rats 47% (17 - 63%) and rats which were injected with saline incubated red cells cleared 8% (7 - 14%) (Figure 5.2). Both CON and HS rats cleared significantly more red cells from the circulation than all other groups ($p < 0.02$). There was no significant difference between TIE, AT or SPX rats ($p > 0.05$).

The spleen in CON rats phagocytosed 25% (17 - 49%) of the injected red cells which compared to 16% (4 - 50%) by HS, 3% (0.5 - 11%) by TIE, 2.5% (0.05 - 15%) by AT and 2% (1 - 4%) by saline treated rats (Figure 5.3). The CON spleens phagocytosed significantly more RBC than all other groups ($p < 0.05$). The HS spleens phagocytosed significantly more RBC than either TIE, AT or the saline treated group ($p < 0.05$). There was no significant difference between TIE, AT or saline treated spleens.

The liver in CON rats phagocytosed 38% (27 - 65%) of the RBC injected, in HS it cleared 49% (33 - 61%), 47% (31 - 75%) in TIE, 50% (20 - 67%) in AT, 47% (25 - 63%) in

SPX and 6% (5 - 8%) in saline treated rats (Figure 5.3). There was no significant difference between any group with the exception that all groups had significantly better clearance than the saline treated group ($p < 0.001$).

The lungs cleared 0.9% (0.3 - 1.3%) in CON rats, 0.9% (0.2 - 1.5%) in HS, 1.1% (0.6 - 1.6%) in TIE, 1.1% (0.5 - 2.8%) in AT, 1.2% (0.9 - 4.3%) in SPX rats and 1.7% (1.0 - 1.9%) in the saline treated group. The AT and SPX rats had significantly better pulmonary uptake but this is probably not clinically significant due to the small amounts cleared.

To compare the splenic and hepatic phagocytic function irrespective of organ weight, the uptake was calculated per gram of organ tissue as described in Chapter 3. The splenic uptake per gram for CON spleens was 22.3 %/g (14 - 49 %/g), 19.2 %/g (7.7 - 40.5 %/g) for HS, 20.7 %/g (2.7 - 35.5 %/g) for TIE, 18.2 %/g (0.9 - 55.2 %/g) for AT and 1.5 %/g (0.8 - 5.1 %/g) for saline treated rats (Figure 5.4). There was no significant difference between any group with the exception of that the saline treated group had significantly less splenic uptake than all other groups. There was no significant difference between any group in the hepatic uptake per gram, with the exception of the saline treated group which had significantly less hepatic uptake than all other groups.

No rat had serum antibody directed against the donor rat RBC as demonstrated by passive haemagglutination assay. At completion of the experiment 97% (88-98%) of the radioactivity present in the blood was bound to the blood cells.

5.4 Autoradiographic localisation of RBC

Clearance of the RBC was reduced following AT or TIE when compared to CON and HS rats. In particular, the spleen was again identified as the abnormal site of clearance. The site of uptake of the IgG coated RBC was investigated in 5 CON rats.

Exposure of the sections for 4 weeks resulted in the best autoradiographs. It was apparent from these that the opsonised RBC were cleared by cells in the RP of the splenic tissue. In contrast to colloidal uptake, very little uptake occurred in the WP or MZ (Figure 5.5).

5.5 Histological examination

All spleens were examined histologically (n = 60) and the relative compartments measured by planimetry as described in Chapter 3. Histological examination of the normal spleen demonstrated similar results found in Chapter 4. There was a marked reduction in the amount of WP and MZ in the TIE and AT spleens. The WP present was almost always confined to the subcapsular region of the section with the central region consisting of red pulp and fibrous tissue. The WP comprised 14.3% (11.7 - 21.6%) of the CON spleen. This compared to 15.6% (9.1 - 21.2%) of HS, 1.6% (0.2 - 3.7%) in TIE and 1.9% (0.1 - 4.5%) in AT spleens. The MZ was reduced to the same extent. Consequently the percentage of red pulp was higher in the TIE and AT spleens. It was 70.4% (60.3-77.6%) in CON, 68.6% (54.8-79.4%) in HS, 96.9% (91.1-99.8%) in TIE and 96.0% (87.8-99.9%) in AT. The percentage of the compartments in each section was used to calculate a relative amount of each compartment as described in Chapter 3. The results are shown in Table 5.1.

Region	CON	HS	TIE	AT
WP	15.5	11.3	0.36	0.21
	(11.4 - 27.4)	(6.2 - 33.2)	(0.03 - 1.4)	(0.01 - 1.21)
MZ	18.0	11.5	0.42	0.3
	(9.2 - 29.9)	(7.6 - 42.7)	(0 - 1.96)	(0 - 1.56)
RP	76.5	48.5	21.3	11.1
	(53.7 - 106.8)	(32.9 - 288.7)	(8.3 - 37.5)	(4.8 - 30.4)

Table 5.1 The relative amounts for each compartment within the spleen

There was a direct correlation between the relative amount of red pulp and the splenic uptake (Spearman co-eff 0.80, $p < 0.001$) (Figure 5.5).

A central arteriole was found in 89% (84 - 100%) of WP areas in CON spleens. This compared to 87% (79 - 96%) in HS spleens ($p > 0.05$), 36% (5 - 64%) in TIE ($p < 0.001$) and 46% (11 - 64%) in AT ($p < 0.001$). There was no significant difference between the AT and TIE spleens. The percentage of WP with a central arteriole was directly correlated with the percentage and relative amount of WP. The histological examination demonstrated an increase percentage of red pulp in both AT and TIE spleens. As previously shown, the uptake of RBC

was directly correlated to the amount of RP. On a per gram basis, AT and TIE spleens have equivalent RBC uptake as normal splenic tissue. To determine if this is due to the increased amount of red pulp, the uptake per gram of RP was calculated by multiplying the total weight of the splenic tissue by the percentage area of the RP. The splenic uptake per gram of RP for CON spleens was 31.5%/g (21 - 47%/g), 27%/g (12 - 66%/g) for HS, 22.5%/g (3 - 36%/g) for TIE and 19%/g (1 - 58%/g) for AT. There was no significant difference between CON and HS splenic uptake per gram of RP. There was a significant difference between CON and TIE ($p < 0.01$) and AT spleens ($p < 0.005$). There was no significant difference between TIE or AT spleens.

5.6 Discussion

This study has demonstrated that following splenic artery ligation or splenic autotransplantation in the rat the regenerated splenic tissue is neither histologically nor functionally normal. The regeneration process resulted in impaired regrowth of WP and MZ with an increased proportion of the spleen consisting of RP. Phagocytosis of RBC opsonised with a low concentration of IgG on their cell surface was significantly reduced. This reduction correlated with an overall reduction in the amount of splenic tissue following splenic artery ligation or autotransplantation. The uptake per gram of splenic tissue was equivalent in each group but this was due to unequal regeneration of the different compartments of the spleen. The lymphoid compartments of the spleen (WP and MZ) are reduced in preference to the RP. Despite this, the RP formed in transplants or following splenic artery ligation had significantly reduced phagocytic uptake compared to an equivalent amount of normal splenic tissue. As this phagocytic mechanism is the most important for removal of bacteria which are poorly opsonised, splenic autotransplantation would seem to be of value provided enough red pulp splenic tissue is present.

This study has demonstrated that, in the rat, splenic autotransplantation results in the regrowth of splenic tissue that has abnormal IgG Fc-receptor mediated phagocytic function which is only partially compensated for by a relative increase in the amount of RP. The reduction in total splenic mass, however, results in significantly reduced phagocytosis (to levels no better than in splenectomised rats). This study shows that WP is abnormal in both content

and amount. It also suggests that the blood supply may be abnormal as demonstrated by the significant reduction in the number of central arterioles.

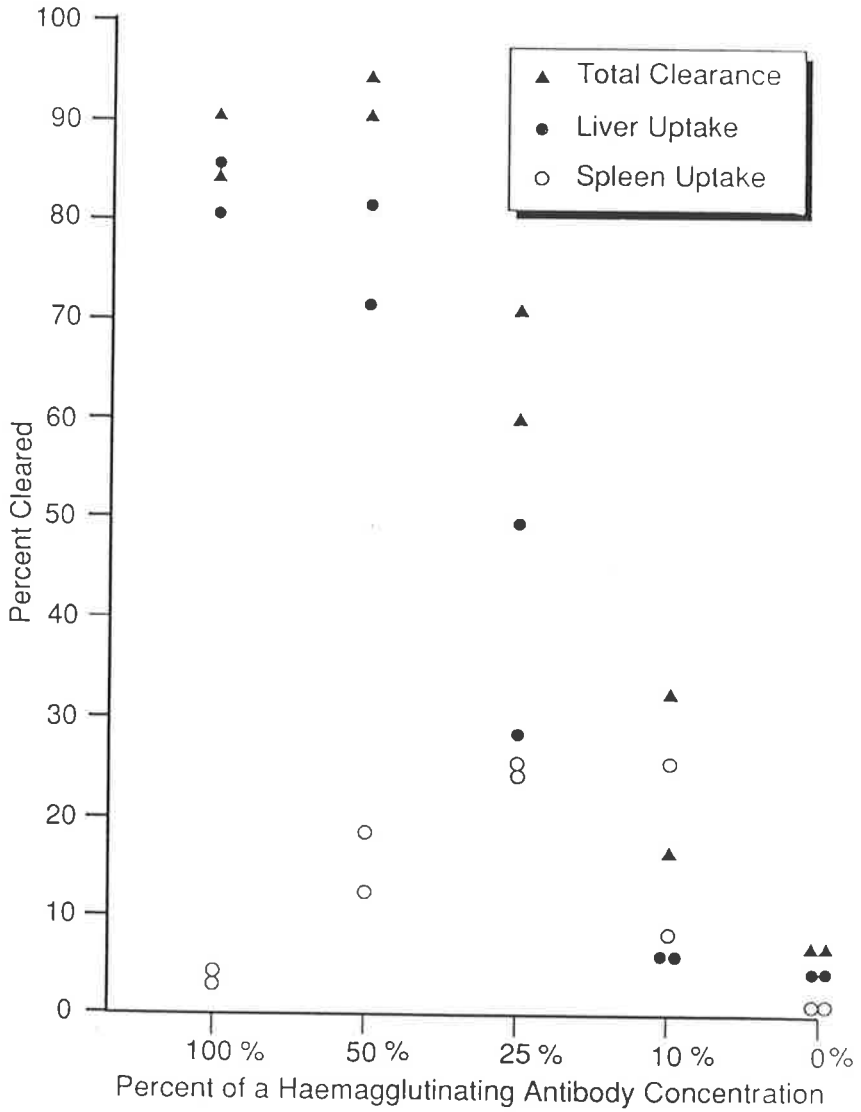

Figure 5.1. The influence of increasing antibody concentration on phagocytosis.

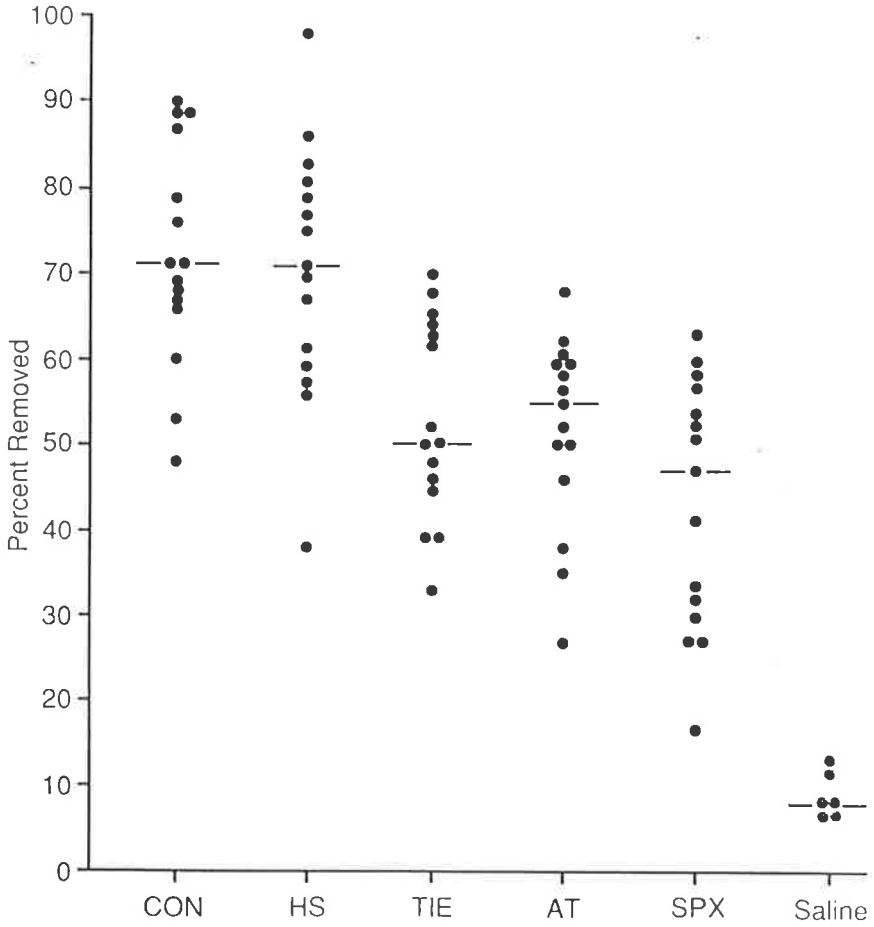

Figure 5.2. The percentage of labelled cells removed from the circulation.

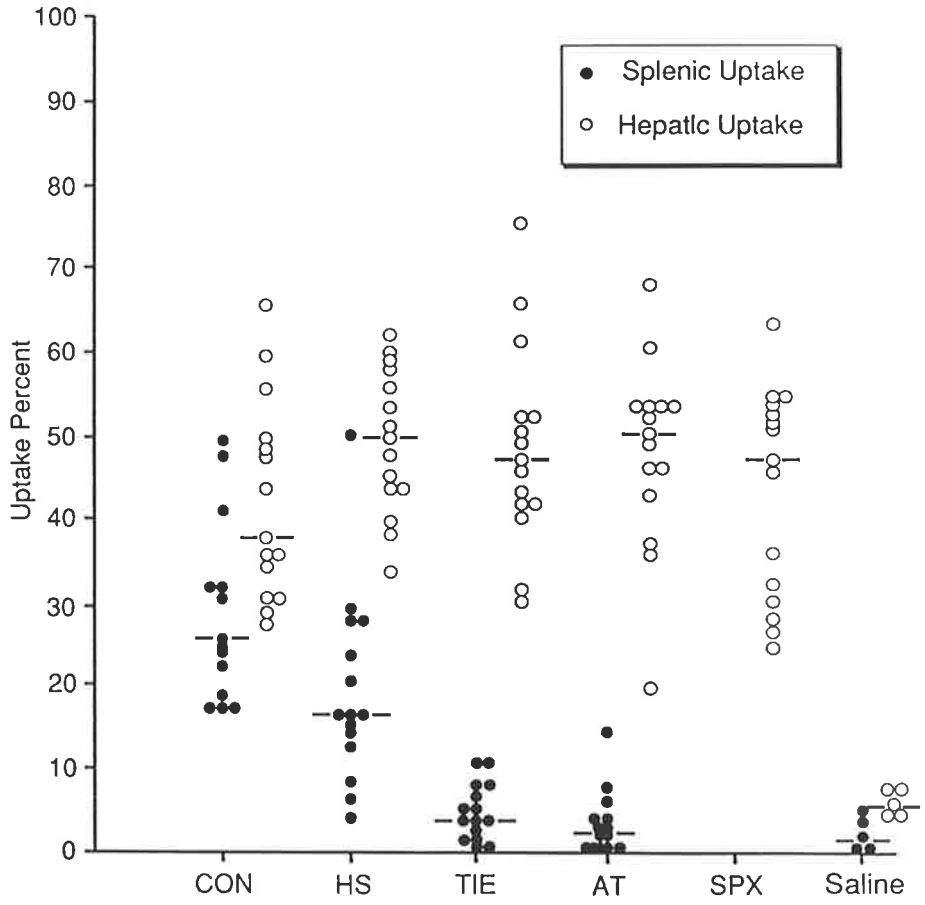

Figure 5.3. The hepatic and splenic clearance of red cells.

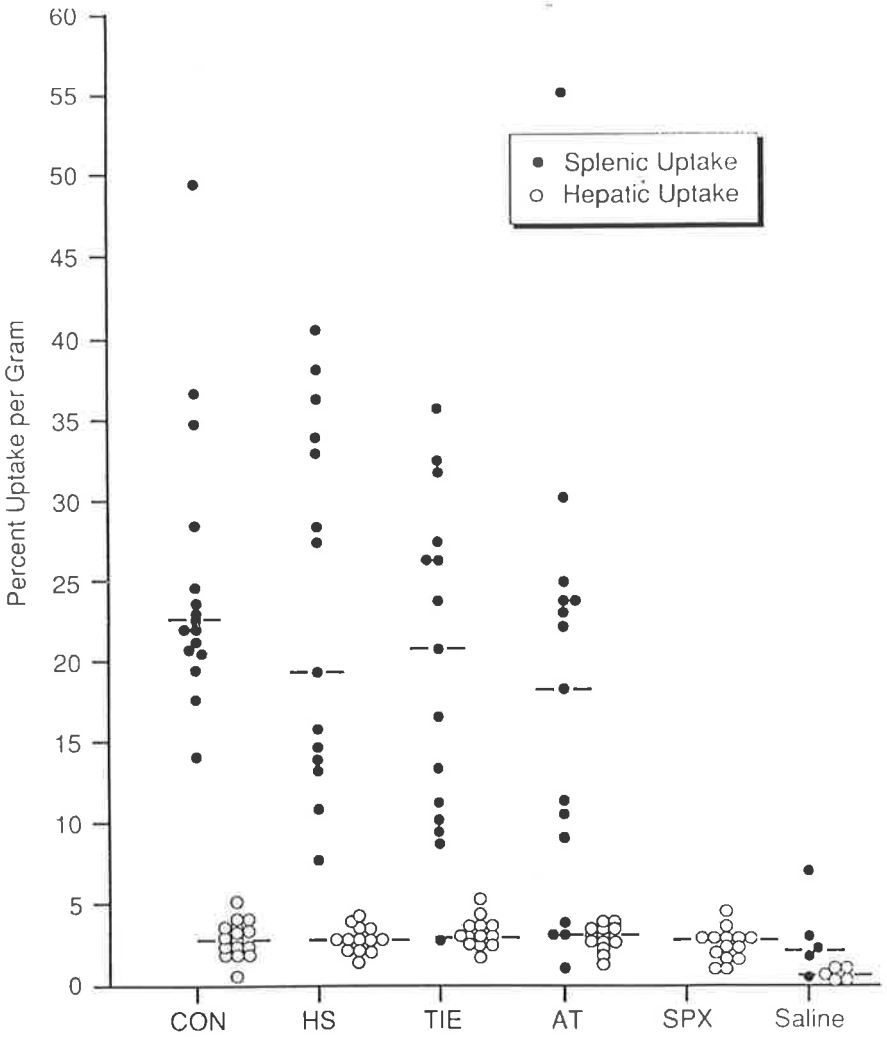

Figure 5.4. The splenic and hepatic uptake per gram of organ.

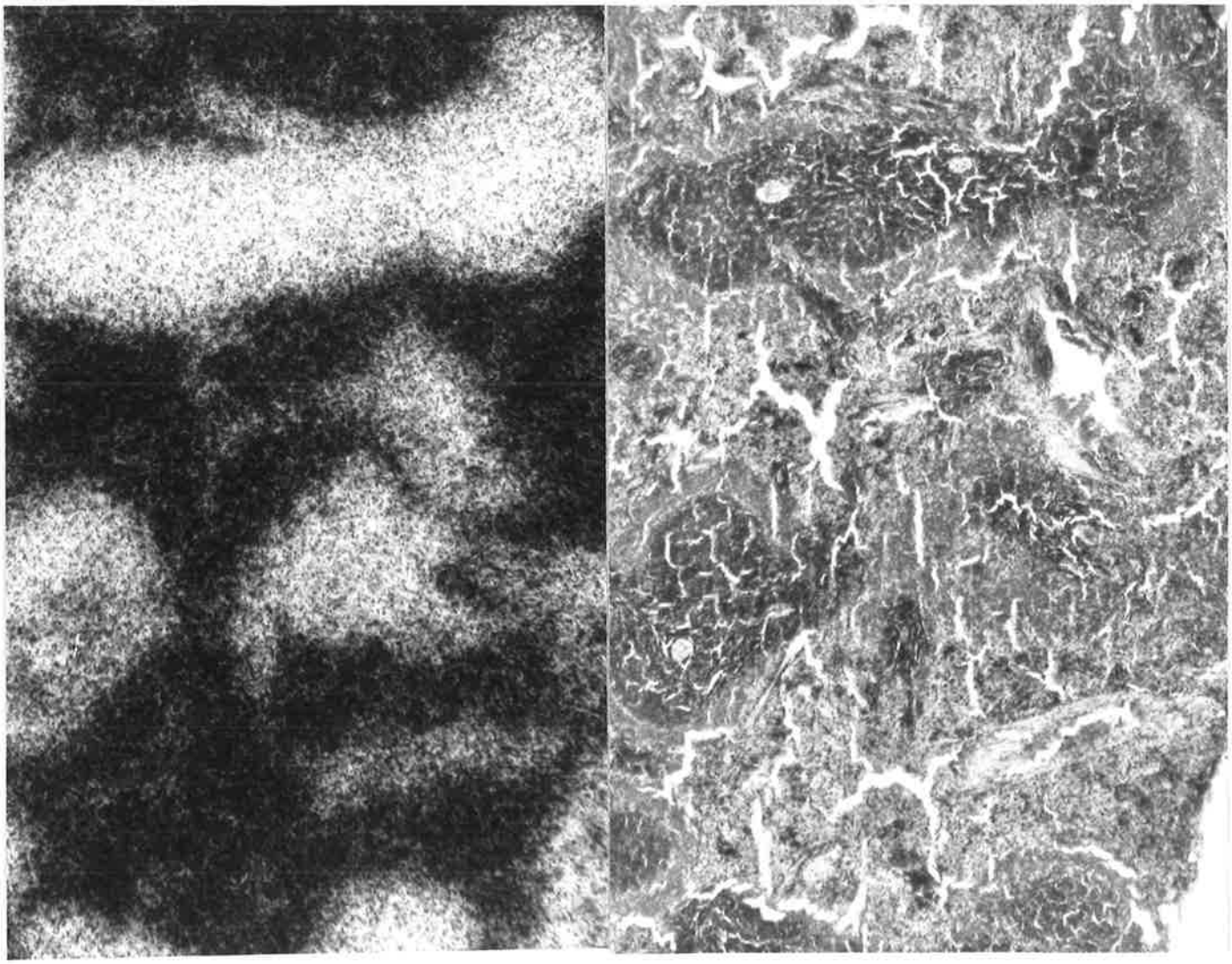

Figure 5.5. The autoradiographic localisation of phagocytosed radiolabelled red cells in normal splenic tissue. Autoradiograph on the left with the corresponding light microscopy of the section on the right (H & E x25).

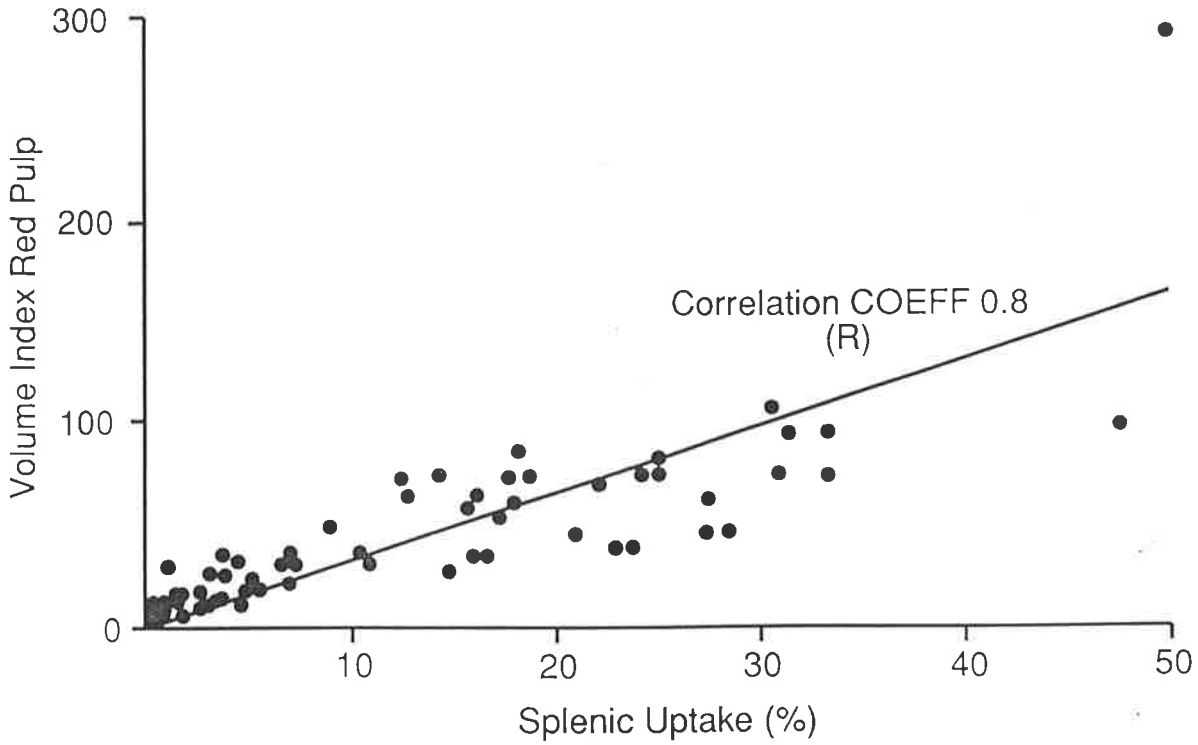

Figure 5.6. The volume index of RP versus splenic uptake (%). Spearman correlation coefficient 0.8.

CHAPTER 6

The vascular supply of normal and regenerated splenic tissue

6.1 Introduction

The spleen has a unique vascular system which allows exposure of immunocompetent cells to the blood circulation. The sluggish flow through the RP cords allows prolonged exposure of antigen to phagocytic and antigen presenting cells. In addition these antigen presenting cells are in direct communication with both B and T lymphocytes which allows early exposure of circulating antigen to these cells thus enabling a prompt antibody response. This unique and efficient system of antigen trapping of blood borne antigen and processing is lost when the spleen is removed or becomes non-functional.

Splenic autotransplantation has been suggested as a technique to retain splenic tissue following splenectomy for traumatic rupture. Autotransplants undergo almost complete necrosis before regenerating (Dijkstra *et al*, 1983; Tavassoli *et al*, 1973). Histological studies of the regenerated transplant have produced contradictory results. It has been reported that regeneration produces normal splenic tissue by some authors (Dickerman *et al*, 1979; Fasching and Cooney, 1980) and abnormal splenic tissue by others (Moore *et al*, 1983; Moore *et al*, 1986). Functional studies have demonstrated that transplants are capable of removing colloidal substances, heat-damaged erythrocytes, corpuscular inclusions and pitted RBC (Lanng Nielsen *et al*, 1984). In contrast, antigen clearance and antibody production may be reduced (Kiroff *et al*, 1985). It has been suggested that this may be due to an abnormal blood supply resulting in the loss of prolonged exposure of antigen to immunocompetent cells. Few studies have investigated the blood flow of these organs.

Reilmann *et al* (1983) used ^{99m}Tc labelled albumin microspheres and Westermann *et al* (1988) used rubidium chloride to determine the perfusion, and both found a reduction in the blood supply, however, this method does not allow direct visualisation of the blood vessels.

The making of corrosion casts of the microvasculature is a well established technique to visualise the size, shape and number of vessels, as well as giving an impression of the appearance of the luminal surface. Casts have been used to study the normal spleen (Schmidt *et al*, 1985), but there is no study of the vasculature of splenic autotransplants using this technique.

The aim of this study was to compare the microvascular anatomy of regenerated splenic tissue with that of normal splenic tissue, particularly the capillary networks of the MZ and RP

which my previous studies have shown are the regions in which phagocytosis and antigen trapping occur.

6.2 Vascular anatomy of normal and regenerated splenic tissue

Corrosion casts were made of the microvasculature of splenic tissue from CON (n = 5) and AT (n = 8) rats.

The microvasculature of the normal spleen has been previously reported and is described in Chapter 2 (Barnhart and Baechler, 1974; Fujita *et al*, 1985; Schmidt *et al*, 1985; Sasou *et al*, 1986). Briefly, the trabecular arteries give off smaller arteries that form the central arterioles of the WP. The central arteriole is surrounded by the WP, which is composed of densely packed lymphocytes and appears as an open area in corrosion casts (Figures 6.1 & 6.2). Surrounding the WP is the marginal sinus. This is formed by a capillary network that originates from the central arteriole via follicular capillaries that radiate through the WP, with frequent anastomoses. The marginal sinus consists of a network of anastomosing flattened capillaries that surround the entire WP. Outside the marginal sinus lies the marginal zone which has the appearance of small grape-like clusters due to the filling of small irregularly shaped vascular spaces among the reticular meshwork (Figures 6.1 & 6.2).

The central arteriole also gives off the penicillar arterioles which pass directly through the WP where they branch to form the penicilli. These may then terminate into the red pulp cords (of Billroth) or the outer aspect of the marginal sinus. The red pulp cords also have a flattened appearance and form a network around the MZ (Figures 6.2 & 6.3). The red pulp cords drain into venous sinuses that eventually drain to the trabecular veins (Figures 6.3 & 6.4). The larger sinuses produce indents on the corrosion casts due to the protuberance of the nucleus of the endothelial cells (Figure 6.4).

The most obvious feature of the splenic autotransplants is the paucity of areas of microvasculature typical of WP. This is consistent with my light microscopic findings (see also Moore *et al*, 1986). In the casts, the central arterioles found in splenic autotransplants give off follicular and penicillar arterioles. These radiating vessels appear to have larger diameters than the controls and, in addition, they appear distorted in shape. The fine vascular network produced by anastomosing arterioles appears to be lost (Figure 6.5). In places the follicular capillary can be seen terminating into the MZ with dilation of the saccules up to 200 μm in

diameter (Figure 6.6). The marginal sinus can also be seen and this too is dilated, and has a sheet-like quality (Figure 6.7).

The red pulp is also dilated and the branching pattern seen in normal splenic tissue is replaced by a globular pattern (Figure 6.8). In some areas this globular pattern has been further exaggerated and large vascular lakes have formed (Figure 6.9).

6.3 Discussion

The ability of the transplant to phagocytose material from the blood is dependent on its vascular supply. It determines the blood flow through the organ and the microvasculature determines what happens to material within the organ. The direct exposure of circulating antigen to (phagocytic and APC) immunocompetent cells without the interposition of endothelial cells is unique to the spleen. To achieve this the normal splenic vasculature has 2 major components, vessels that supply the RP and those that supply the MZ and WP. Both these circulatory systems originate from the central arteriole which derives from the trabecular arteries. These in turn originate from the tributaries of the splenic artery. Following autotransplantation, the transplant becomes ischaemic and the parenchyma necroses back to a peripheral rim that remains viable by diffusion of oxygen and nutrients from surrounding structures. The capillaries grow in and revascularise the necrotic central core. With revascularisation, the peripheral rim of viable cells proliferate and form new areas of MZ, RP and WP. From my and previous experimental animal studies, the central areas of transplants often heal with fibrous tissue and the area of WP and MZ is dramatically reduced compared to normal splenic tissue. Very few histological studies have been performed in humans but those reported have indicated similar fibrotic changes and lymphocyte depletion (Moore *et al*, 1983).

Histological studies reported in earlier chapters indicate that WP in regenerated splenic tissue is often lacking a central arteriole. Results reported in this chapter demonstrate that the microvasculature of transplants is abnormal. The revascularisation process occurs via capillary ingrowth and not via the original single vessel. The structure is disordered and the fine network of small capillaries that engulf the phagocytic cells of the MZ is replaced by dilated capillaries. The red pulp cords are replaced by dilated vessels which form "vascular lakes" in places. The spleen is the site for phagocytosis of poorly opsonised antigens. The direct contact of blood with phagocytic cells in the red pulp combined with sluggish flow (thereby increasing

time of exposure of antigen) may be the mechanism for the greater efficiency of the spleen over other phagocytic organs. The autotransplants have lost this close approximation of antigen to phagocytic cells due to dilation of the RP and MZ vessels.

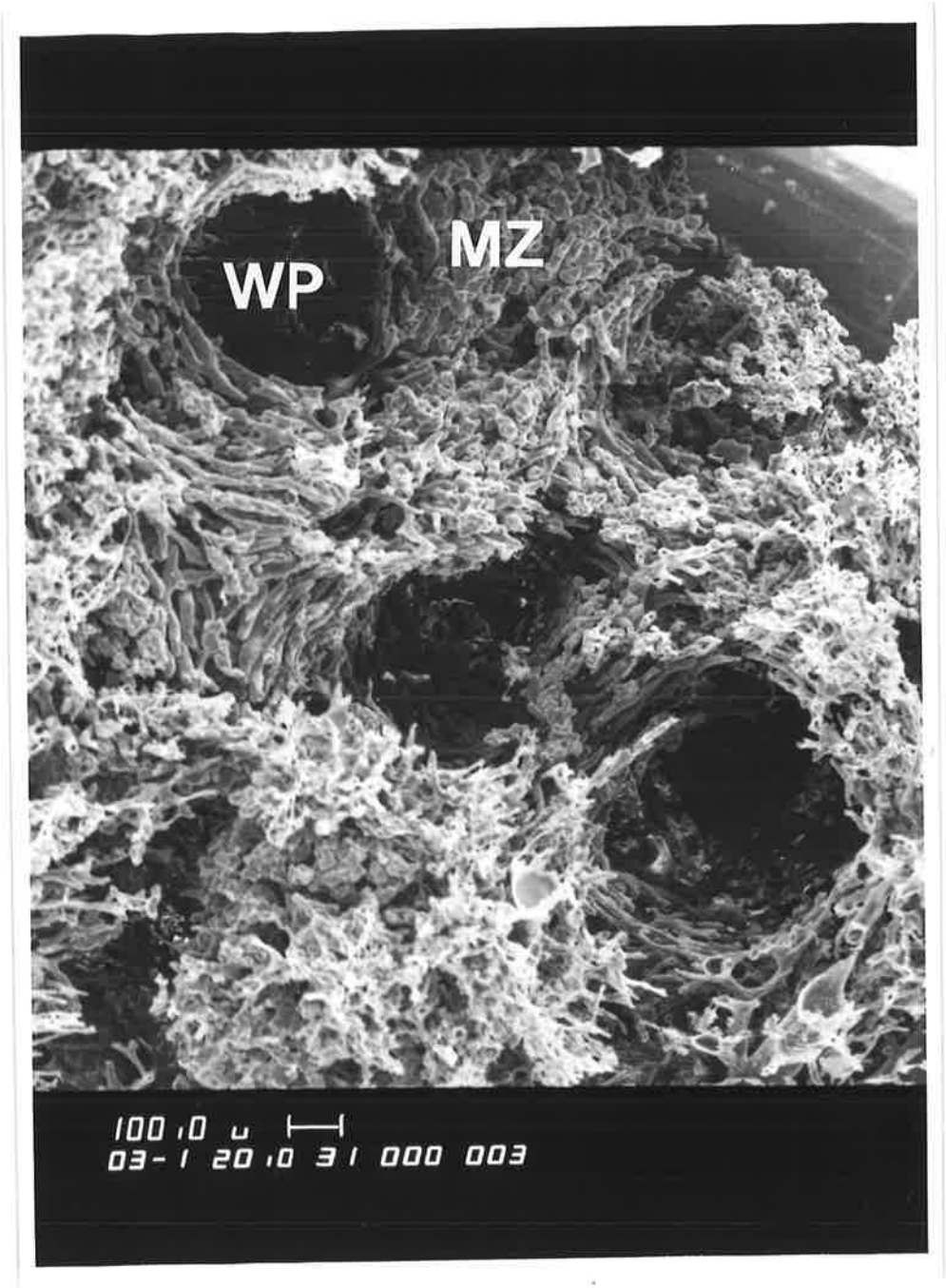

Figure 6.1. Normal splenic vasculature. WP consists of densely packed lymphocytes which appear hollow following digestion (**WP**). Surrounding the WP are flattened blood vessels that form the marginal sinus. These drain further to the MZ which consists of small vascular spaces interspersed with B lymphocytes and macrophages (**MZ**)

(x 300).

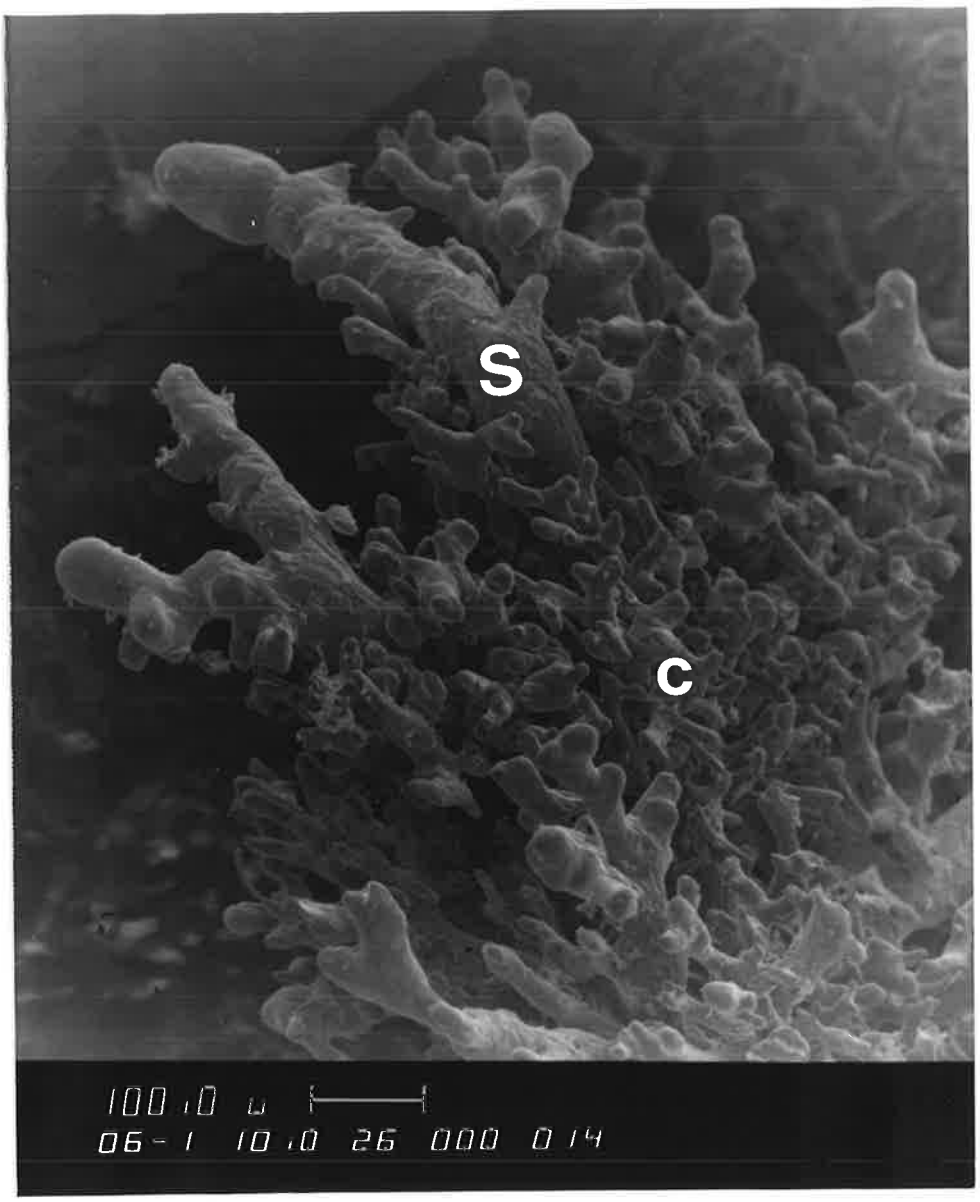

Figure 6.2. Normal splenic vasculature. RP cords (**c**) ending blindly and abbutting the much larger early venous sinuses (**S**). The RP cords are lined by macrophages which phagocytose antigenic substances including senescent red blood cells that are unable to pass through pores connecting the pulp cords to the sinuses. These sinuses drain to larger sinuses. (x 600).

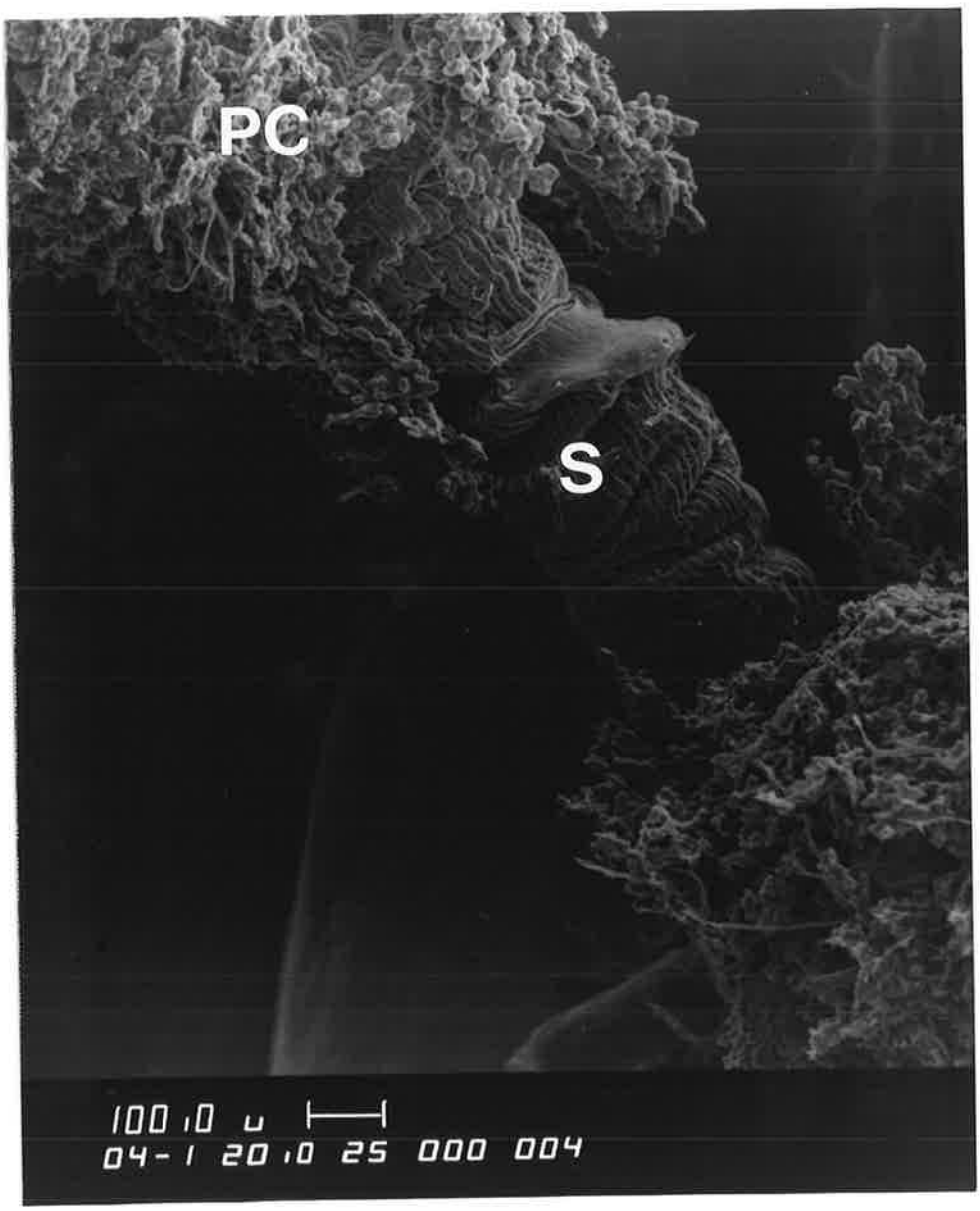

Figure 6.3. Normal splenic vasculature. Large venous sinus (**S**) demonstrating a ridged appearance due to the impression of the nuclei of the endothelial cells imprinting the cast. Small penicilli can be seen traversing the pulp cords (**PC**) (x 400).

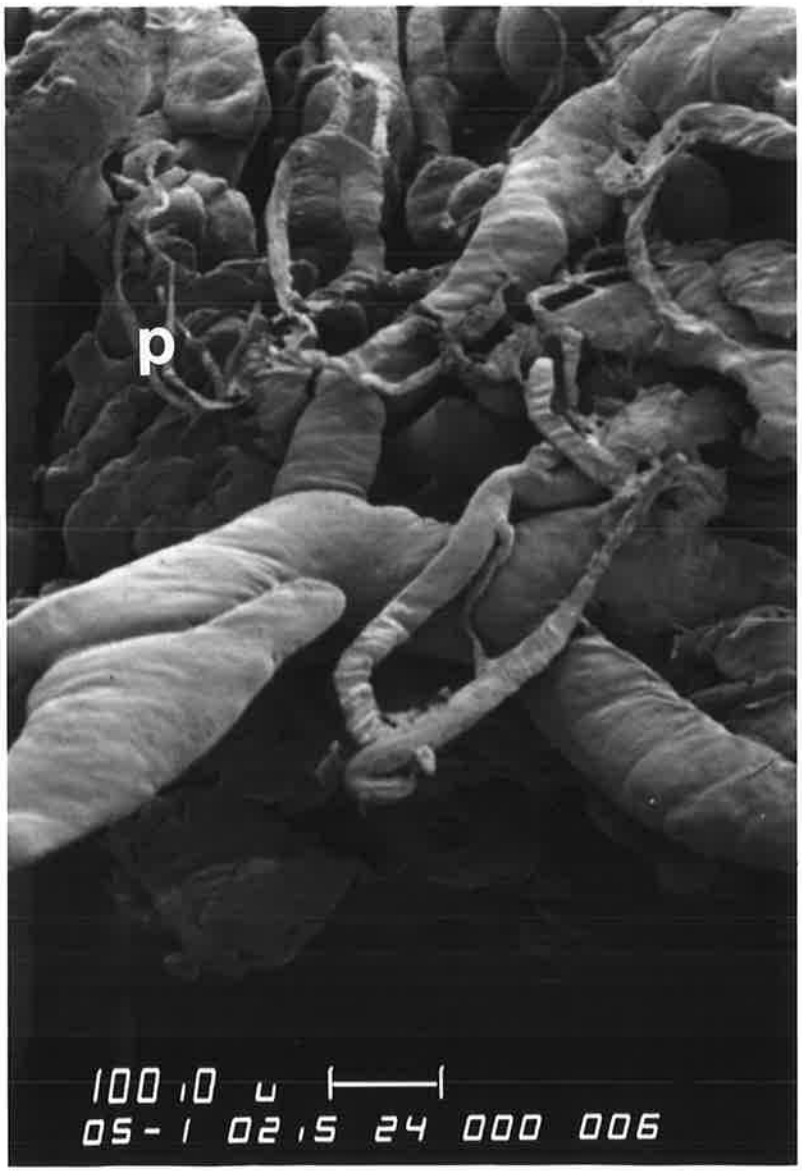

Figure 6.4. Splenic autotransplant vasculature. Abnormally dilated blood vessels which in places form flattened vascular "lakes". No clear central arteriole can be seen in this preparation. Small penicilli (**p**) can be seen arising from the larger arterioles and in 1 area appear to terminate into an area of the dilated and flattened vascular "lakes" (x 500).

Figure 6.5. Splenic autotransplant vasculature. An area of WP is shown with the normal flattened vessels of the marginal sinus and the "grape-like" appearance of the marginal zone replaced by grossly dilated saccules (**MZ**). Penicillar capillaries can be seen to drain into these saccules. This exposure has equivalent magnification as figure 1 (x 300).

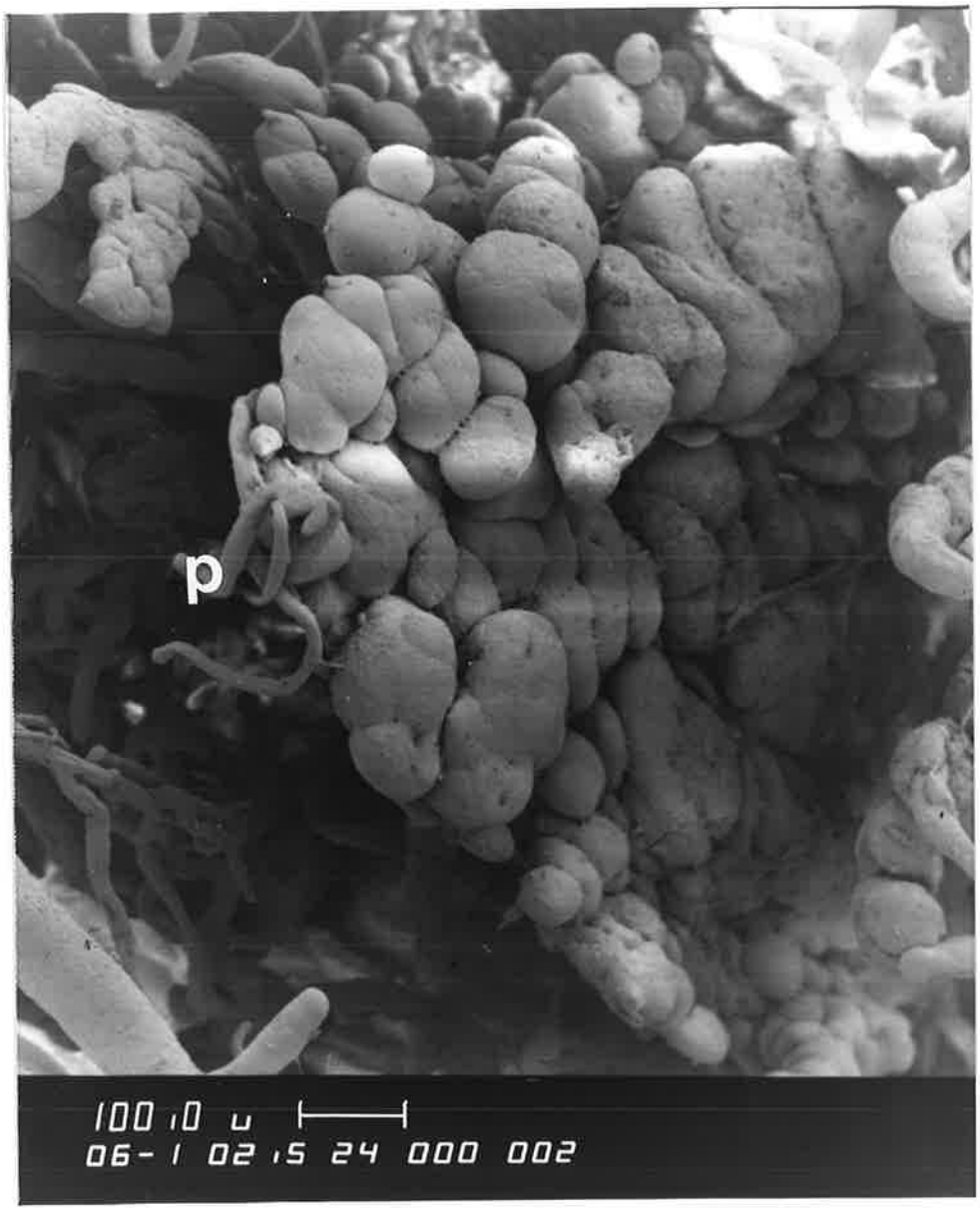

Figure 6.6. Splenic autotransplant vasculature. A higher magnification of figure 4.5 demonstrating penicillar capillaries (**p**) connecting directly with the probable marginal sinus surrounding the WP (x 600).

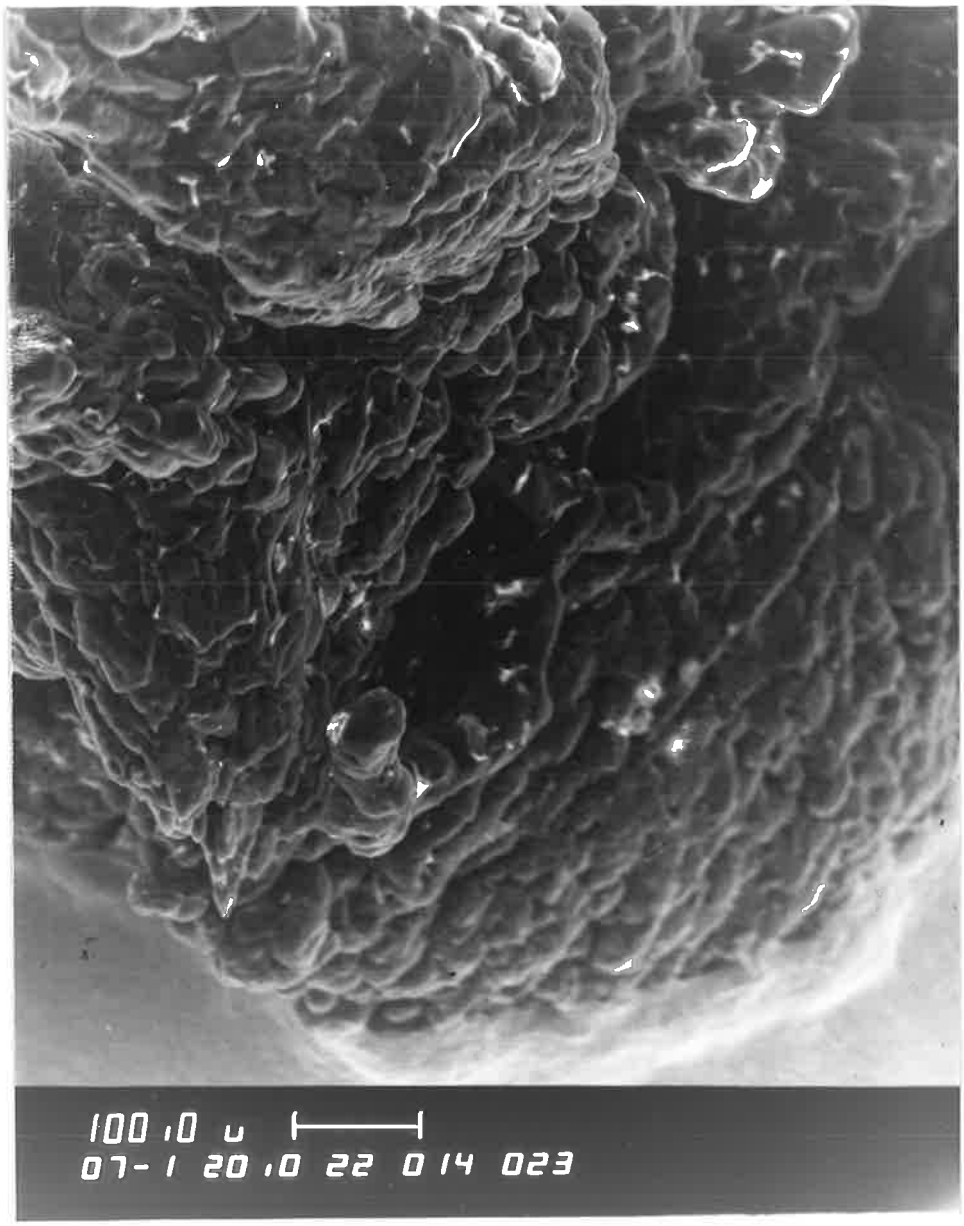

Figure 6.7. Splenic autotransplant vasculature. An area of splenic autotransplant adjacent to the subcapsular region. The fine network of blind ended RP cords intermingled with venous sinuses is replaced by dilated, interconnected cords which form a globular pattern. (x 700).

Figure 6.8. Splenic autotransplant vasculature. An area of dilated RP cords which have formed large "vascular lakes" with very little intervening cellular matrix. (x700).

CHAPTER 7

Studies on the cellular components of normal and regenerated splenic tissue

7.1 Introduction

In the previous chapter the microvasculature of normal and regenerated splenic tissue was investigated. In the normal spleen, arterial blood reaches the veins by 1 of 3 pathways, namely through the red pulp cords, the white pulp capillary network to the marginal sinus and marginal zone network or directly into venous sinuses. I have shown that the first 2 pathways have roles in phagocytosis of antigen. I have also shown that these regions have a specificity for the type of antigen that is cleared. It was shown in the previous chapter that the vascular network was abnormal in splenic autotransplants, particularly in the marginal zone.

The normal spleen has 3 main cellular regions and within these regions are some specialised cells (Buckley *et al,* 1987). The periarteriolar sheath contains T cells and interdigitating reticulum cells (Wood *et al,* 1985; Matsumo *et al,* 1989). Germinal follicles contain B cells, follicular dendritic cells and Tingible body macrophages (Timens and Poppema, 1985; Buckley *et al,* 1987). Surrounding these is a layer of macrophages known as marginal metallophils (named because of their appearance when stained by Marshall's silver-impregnation method) (Snook, 1964). The marginal sinus then separates this macrophage layer from the marginal zone. This is divided into 2 zones, an inner layer (mantle zone) that is composed of small B lymphocytes and an outer layer that contains medium sized B lymphocytes and macrophages (marginal zone) (Hsu, 1985; Van den Oord et al, 1986). The mantle zone is seen in other lymphoid organs (lymph node, Peyer's patches) whereas the marginal zone is unique to the spleen (Hsu, 1985).

The red pulp is formed by T and B cells, macrophages, reticular fibres and sinusoidal lining cells (Faller *et al,* 1985; Buckley *et al,* 1985; Buckley *et al,* 1987).

The immunohistochemical staining of the normal spleen has attracted interest due to its importance in the immune system (Buckley *et al,* 1987; Matsumo *et al,* 1989), but the staining of splenic autotransplants has not been reported in detail. The aim of this study was to type the cells that constitute the regenerated spleen, in particular the cells of the white pulp and marginal zone.

7.2 Cellular components of the white pulp and marginal zone

All autotransplanted and ligated spleens from the functional studies reported in earlier chapters were available for histological examination. The normal spleen was histologically

similar to the general structure as presented in Chapter 3. Briefly, white pulp, distributed throughout the sections of normal splenic tissue, was composed of approximately equal amounts of PALS and follicles and had a wide surrounding MZ. The RP intertwined between the WP areas. Fibrous tissue was only evident in the trabeculae and the surrounding capsule (Figure 7.1).

Specific monoclonal antibodies (Table 3.1) were used to type the cellular constituents within the compartments. The antibody W313 showed the distribution of T lymphocytes (Figures 7.2 and 7.3). In the normal spleen they were seen within the white pulp, surrounding the central arteriole, to form the periarteriolar sheath. Adjacent to these collections of T cells were usually seen non-staining areas corresponding to the follicle. Occasional T cells were also seen in the red pulp although they did not form any discrete accumulations. The anti-immunoglobulin antibody is specific for B cells. These were found predominantly in the follicles and MZ, with some scattered in throughout the red pulp (Figures 7.4 - 7.6). Macrophages, B cells and interdigitating dendritic cells (IDC) were identified by their staining with the antibody OX6 and their characteristic morphology (Figure 7.7). Macrophages were found in the MZ, follicles and scattered through the RP. The IDC were seen in association with T cells in the PALS.

The autotransplanted splenic tissue and ligated spleens differed obviously from normal splenic tissue in a reduction in WP and an increase in fibrous tissue which was predominantly in the central portions of the tissue sections (Figure 7.8). Because there were no obvious differences between ligated or autotransplanted splenic tissue, subsequent descriptions will refer only to regenerated splenic tissue.

WP was found almost exclusively in the subcapsular regions of the section. The organisation of the WP varied from section to section. In some sections WP was formed by B cells only, in other areas, T cells could occasionally be seen scattered amongst the B cells, but not forming a PALS (Figure 7.9). In some, the structure resembled normal splenic tissue with regions staining positively for T cells around a central arteriole (Figure 7.10). Follicles and a MZ were found occasionally, but, even in the best examples of this organised WP, there was a disproportionate representation of B cells (Figures 7.11 & 7.12). In no sections was a PALS found which did not have an associated follicular area and MZ. In addition, the MZ, when present, was significantly narrower than normal.

Lymphoid aggregates of B cells could also be found in areas throughout the RP, but without an associated PALS or MZ. Using the antibody OX6, the IDC were seen in the PALS area only, in association with T cells. When a PALS had been formed in the transplant, IDCs were seen. They were not present in the absence of T cells.

The B cells were stained with anti-IgM and anti-IgD. In normal spleens, the inner layer of the MZ contained B cells that stained for both IgM and IgD. The outer layer contained B cells that stained for only IgM. In the sections of regenerated spleens, these 2 layers were rarely seen (Figure 7.13). The double layer was seen only in areas where a PALS had developed in conjunction with a follicle and MZ (Figures 7.14 - 7.16).

7.3 Discussion

The transplanted splenic tissue undergoes necrosis back to cortical rim of viable cells prior to regeneration (Tavassoli *et al*, 1973). New lymphoid tissue replaces fibrous tissue along the subcapsular region but rarely is found in the central portions. RP also regenerates and is found in almost all regions of the transplant, however, the central regions always remain replaced by fibrous tissue. These findings have been supported by earlier studies (Pabst and Reilmann, 1980; Malangoni *et al*, 1985; Moore *et al*, 1986).

The amount and organisation of the WP in the transplant varied markedly between sections. This varied from splenic tissue consisting entirely of RP and fibrous tissue through to tissues which contained WP with normal compartmentalisation (although reduced in amount). In the sections with a paucity of WP, the WP lacked compartments and consisted entirely of B cells, sometimes with a very thin surrounding MZ. With increasing amounts of WP, an increase in the size of the MZ was often noted. In sections with more WP again, the presence of T cells in conjunction with follicles were observed. Where there were T cell aggregates of any size they were invariably surrounding a central arteriole and IDC were present. On rare occasions, a section was found in which there was a well developed T and B cell region with differentiation of the MZ into an inner and outer layer.

It therefore appears that during regeneration the splenic transplant progressed through phases of maturation. The first phase consisted of the reconstitution of the B cells. These were disorganised and formed occasional accumulations, sometimes with a rudimentary MZ. The next phase consisted of the appearance of T cells in association with the B cells with no defined

T or B cell compartments. The next phase consisted of compartmentalisation of the T and B cells into a PALS and follicles respectively, in which a central arteriole was invariably present. This suggests that the blood supply is an important factor in the regeneration process. The final phase of maturation consisted of maturation of the MZ into 2 layers.

Westerman *et al* (1988) demonstrated that the age of the transplant significantly altered the outcome of the regeneration of the spleen. Transplants in adults regenerated with significantly less PALS and MZ than foetal or neonatal splenic transplants. These latter regenerated with a more normal mix of B and T cell compartments and a well developed MZ (although whether differentiation into inner $\mu^+ \delta^+$ and outer $\mu^+ \delta^-$ zones occurred was not examined).

This lack of WP organisation and the development of an "immature" MZ in the regenerated splenic tissue may be relevant to the continued susceptibility of autotransplanted or splenotic individuals to OPSI.

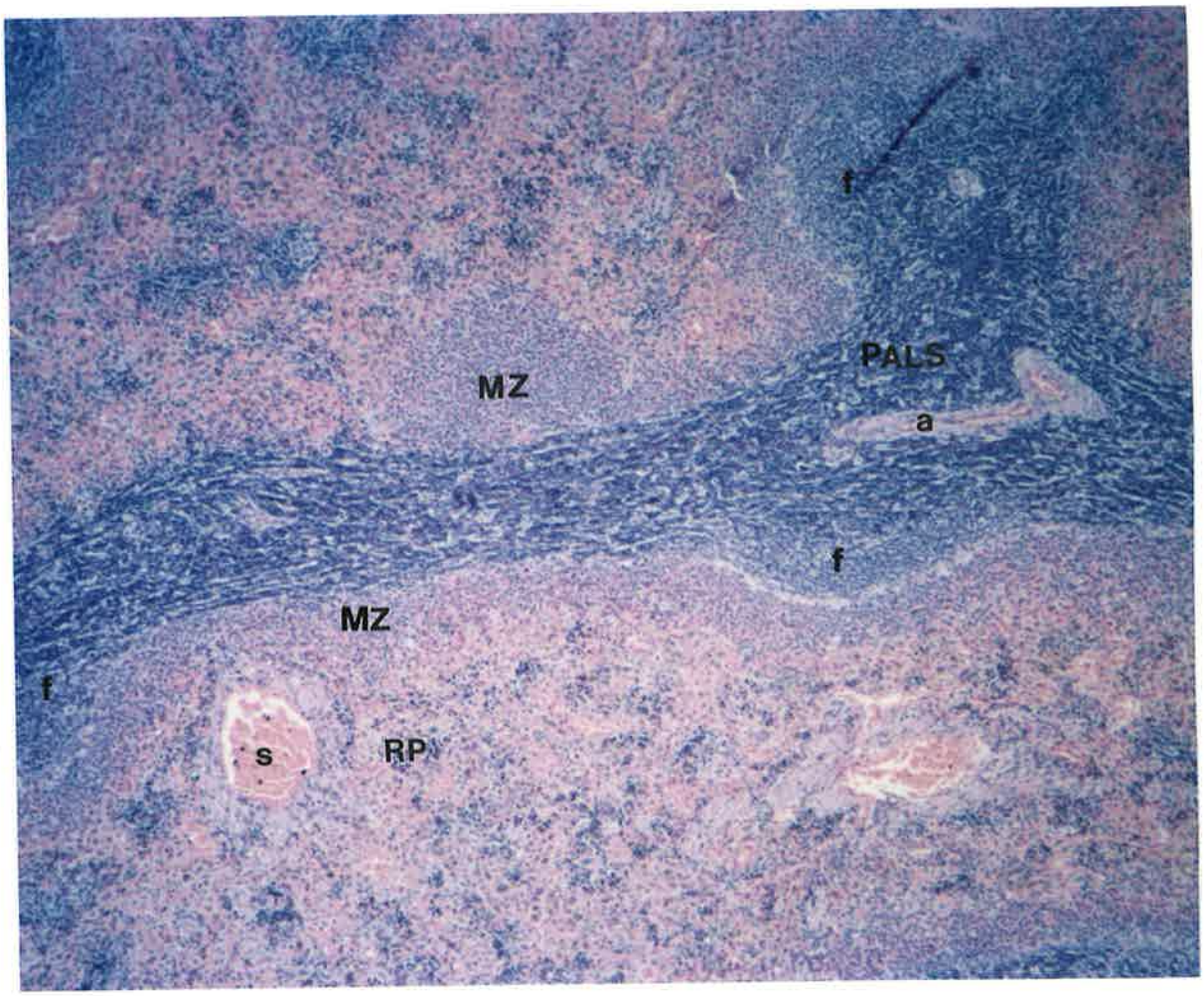

Figure 7.1. Normal splenic tissue. Overview of white and RP and MZ. A large muscular central arteriole (**a**) is surrounded by the periarteriolar sheath (**PALS**). 3 germinal follicles (**f**) are seen associated with the PALS with a surrounding marginal zone (**MZ**). A venous sinus (**s**) is seen in the RP (**RP**).

(Haematoxylin and Eosin, x40)

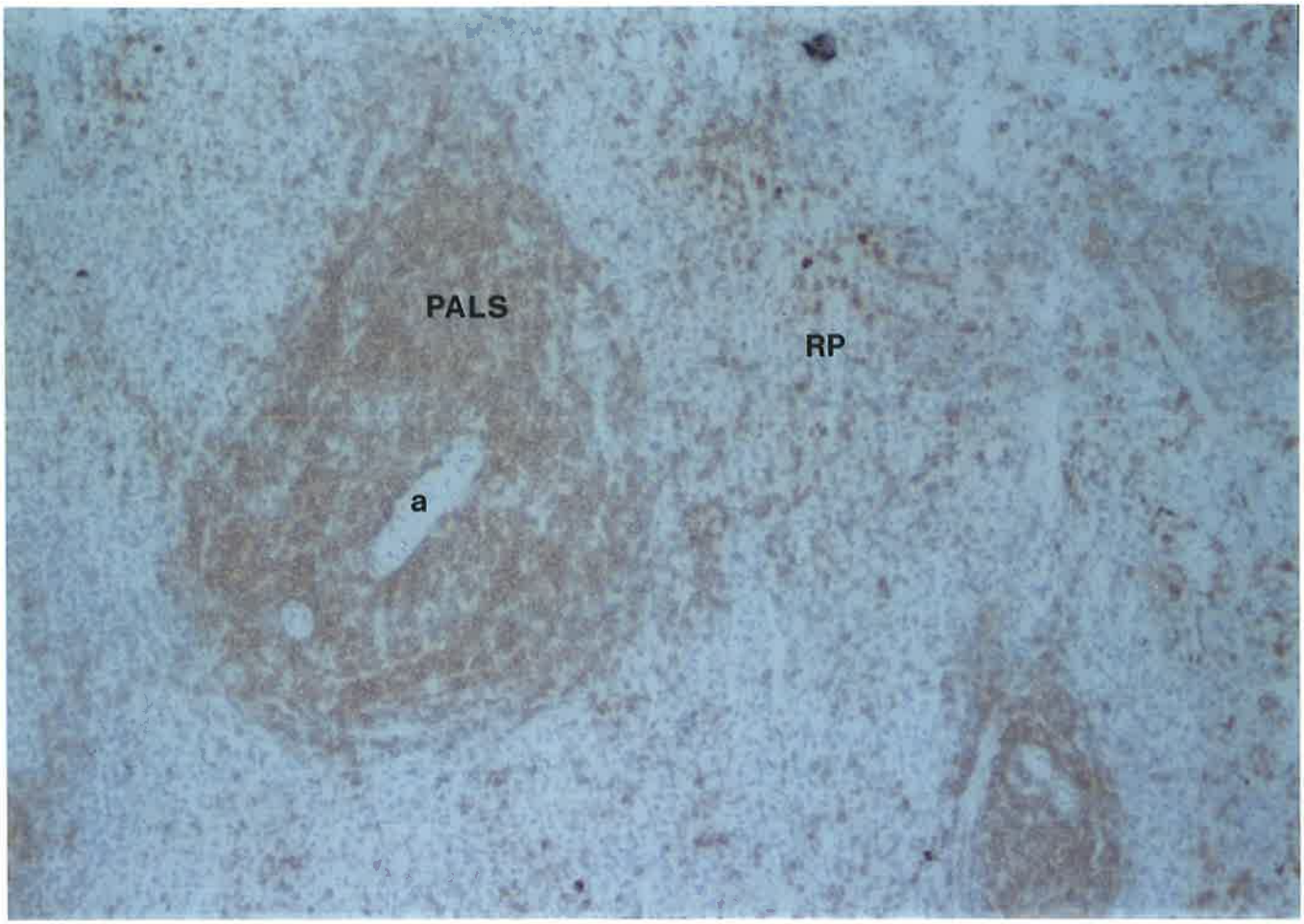

Figure 7.2. Normal splenic tissue. A central arteriole (**a**) surrounded by the periarteriolar sheath (**PALS**) which has been stained with W313. All cells in PALS demonstrate W313 membrane staining. A few W313 positive cells are seen scattered throughout the red pulp (**RP**).

(W313 and haematoxylin, x100)

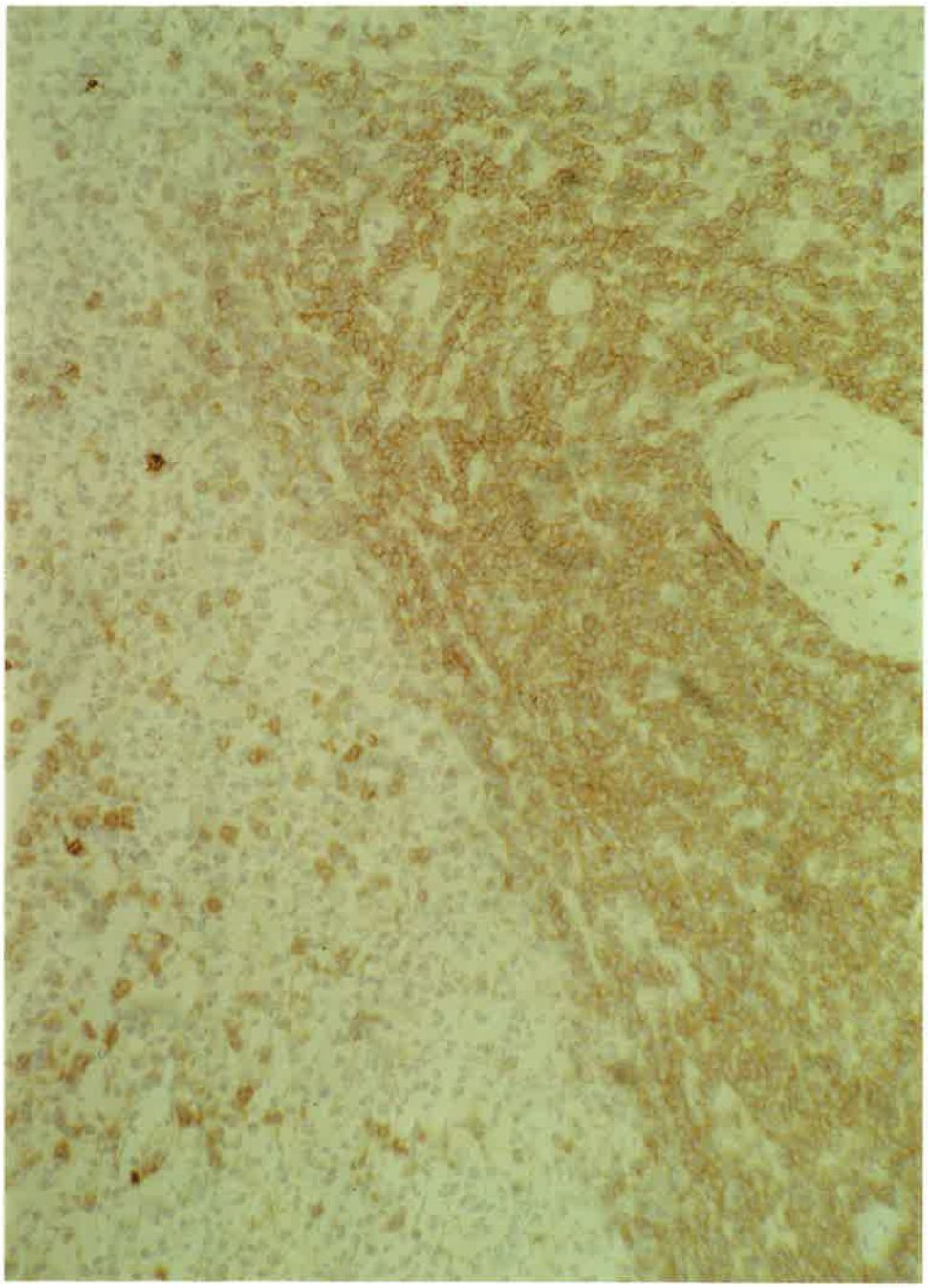

Figure 7.3. Normal splenic tissue. Higher definition of PALS stained with W313 demonstrating clear membrane staining of T cells.

(W313 and haematoxylin, x200)

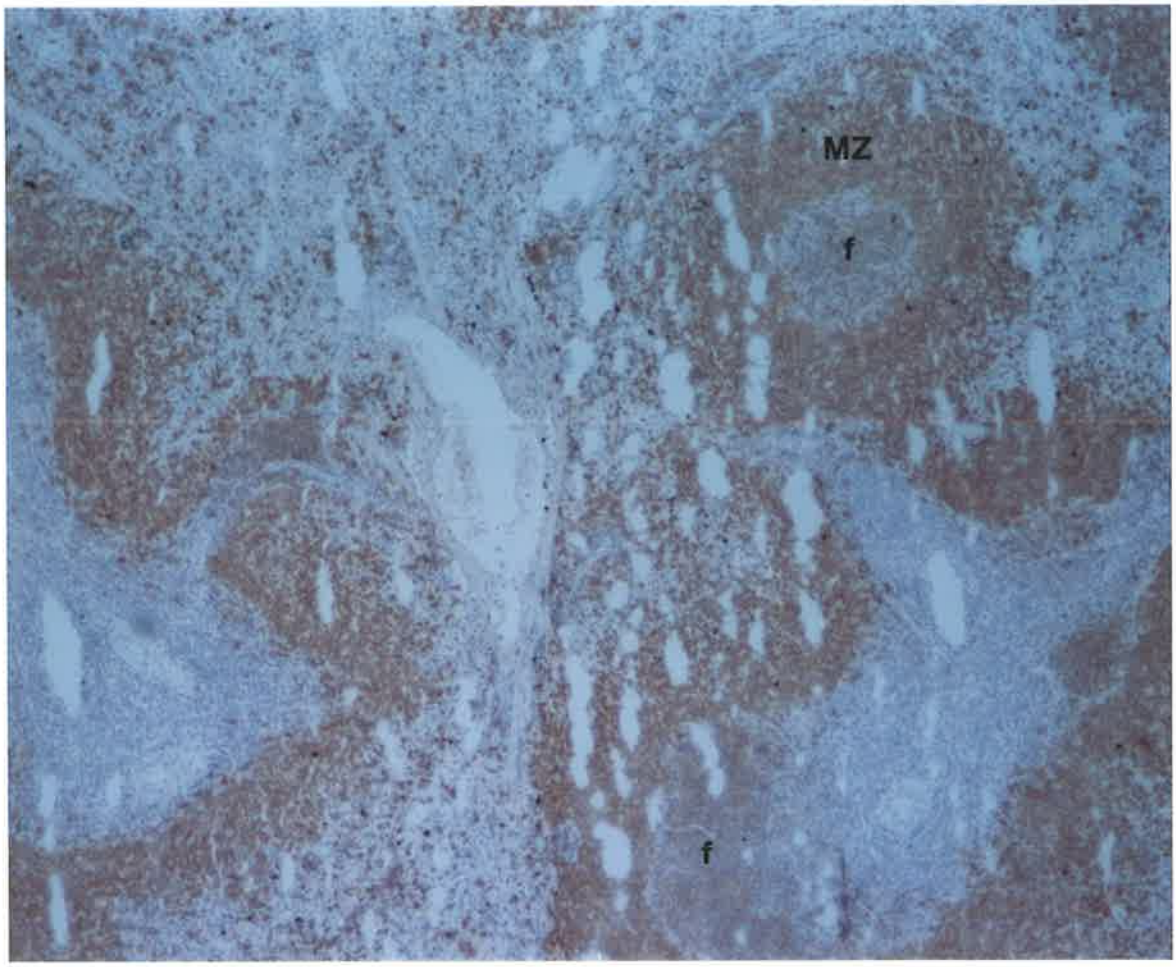

Figure 7.4. Normal splenic tissue. Overview of white and RP and MZ stained with anti-IgM. The PALS does not stain in contrast to the heavily stained germinal follicle (**f**) and marginal zone (**MZ**).

(Anti-IgM and haematoxylin, x40)

Figure 7.5. Normal splenic tissue. Overview of white and RP and MZ stained with anti-IgD. The inner layer of the MZ and the lymphocyte corona (**lc**) of the germinal follicles (**f**) are the only areas with IgD^+ B cells.

(Anti-IgD and haematoxylin, x40)

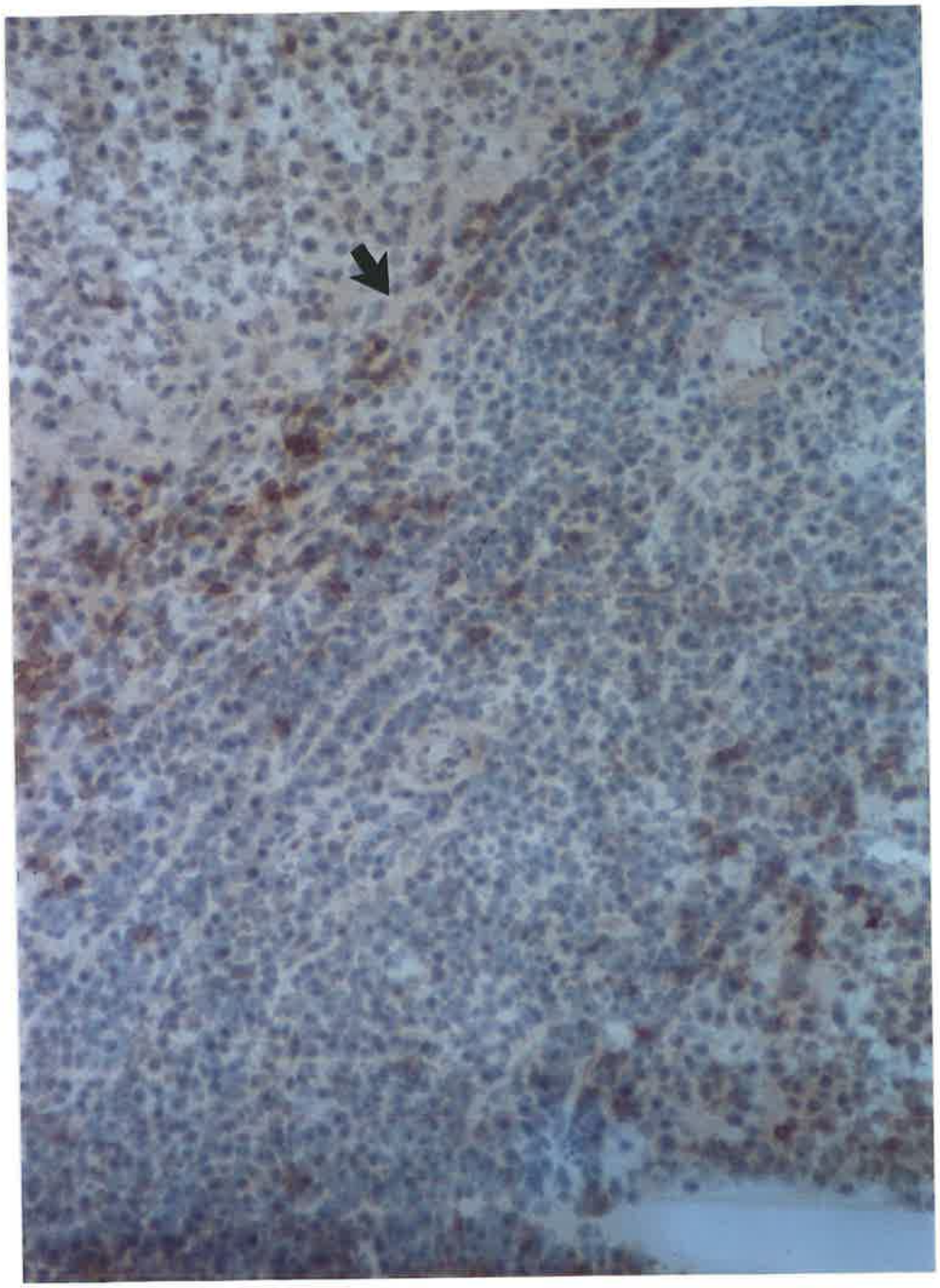

Figure 7.6. Normal splenic tissue. Higher power view of the inner layer of the MZ demonstrating a thin layer of IgD^+ B cells (arrow).

(Anti-IgD and haematoxylin, x200)

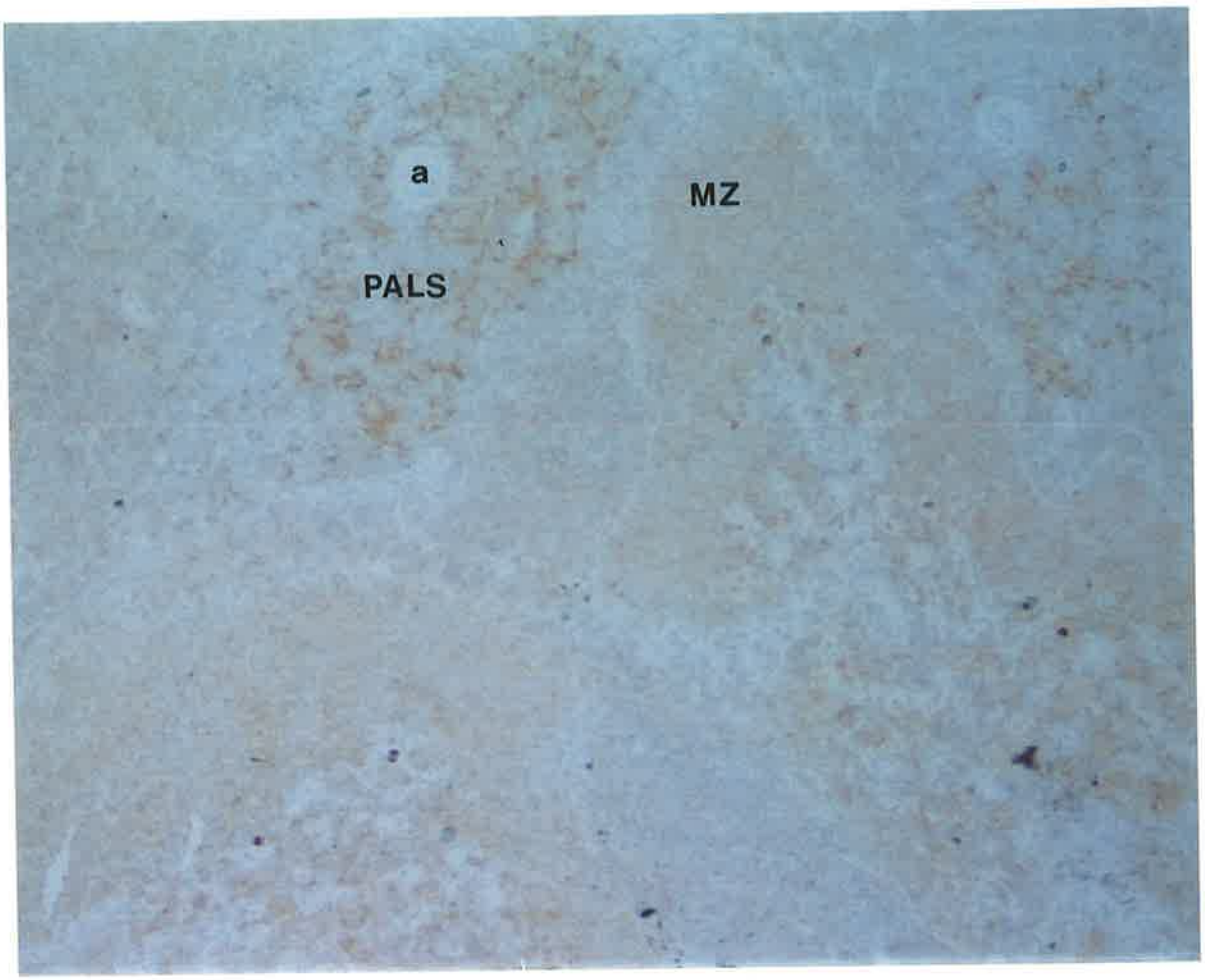

Figure 7.7. Normal splenic tissue. Low power view of WP lightly stained with OX6 to demonstrate the IDCs in the PALS surrounding a central arteriole (**a**). B cells and macrophages are lightly stained in the marginal zone (**MZ**).

(OX6 and haematoxylin, x100)

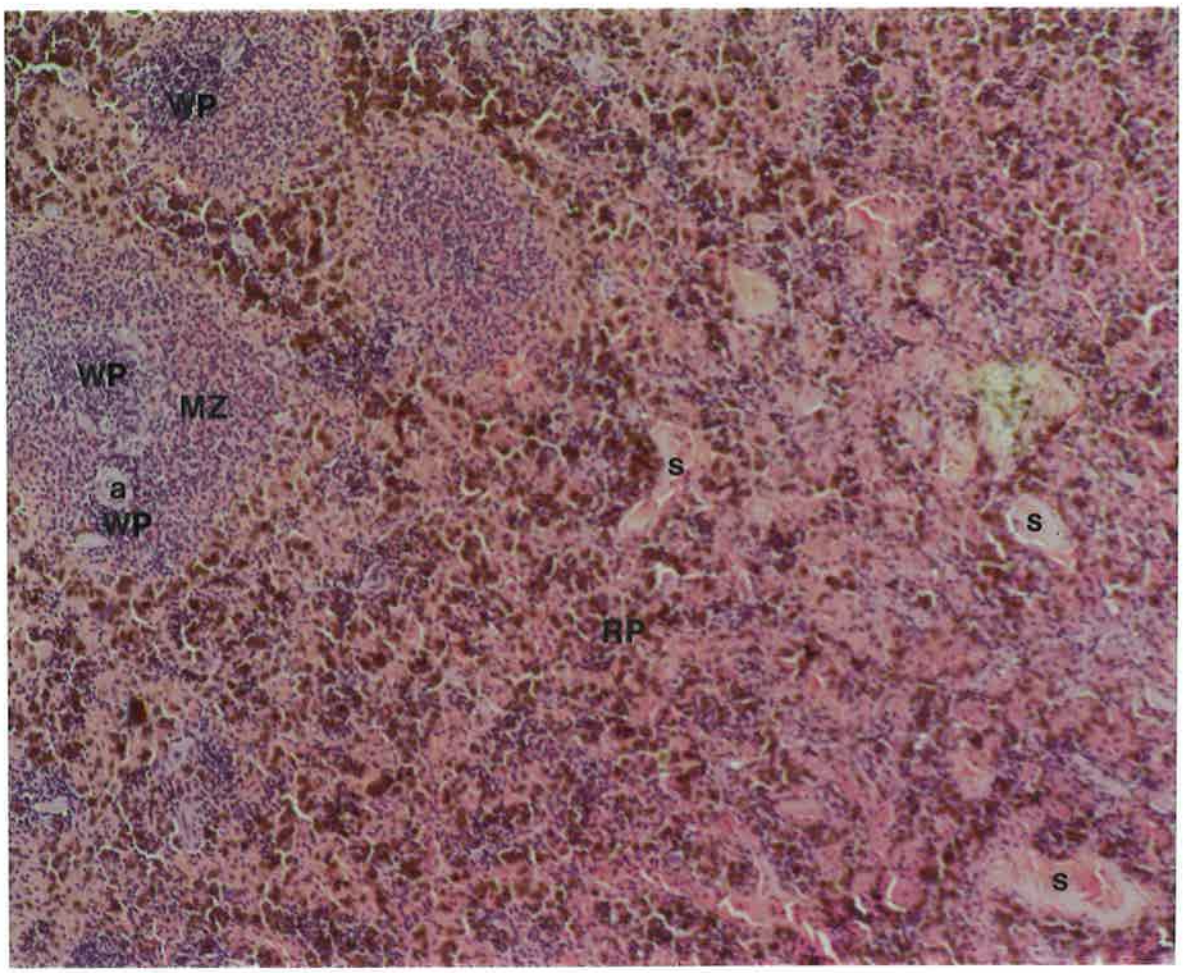

Figure 7.8. Regenerated splenic tissue. Overview of white and RP and MZ. A central arteriole (**a**) is seen in 1 of 3 areas of white pulp (**WP**). The WP is very small and has a much larger marginal zone (**MZ**). The section is predominantly red pulp (**RP**) and aggregations of lymphocytes can be seen scattered through the pulp cords. A venous sinus (**s**) is seen in the RP.

(Haematoxylin and Eosin, x40)

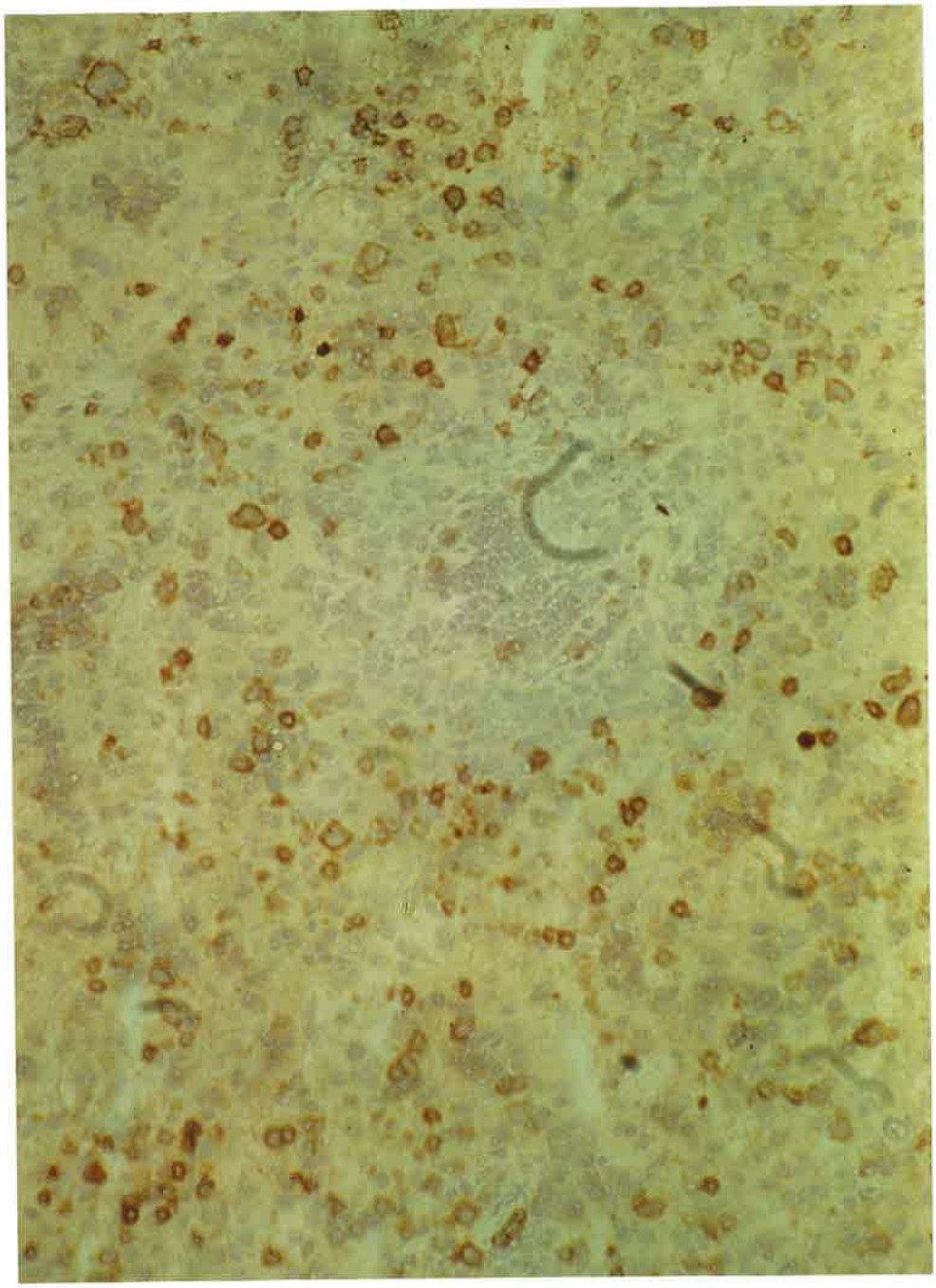

Figure 7.9. Regenerated splenic tissue. An area of WP that has been stained with W313. There is no obvious central arteriole and the lymphocytes that form the WP do not stain positively. W313^{+} cells are seen scattered throughout the section but never form a PALS. (W313 and haematoxylin, x200)

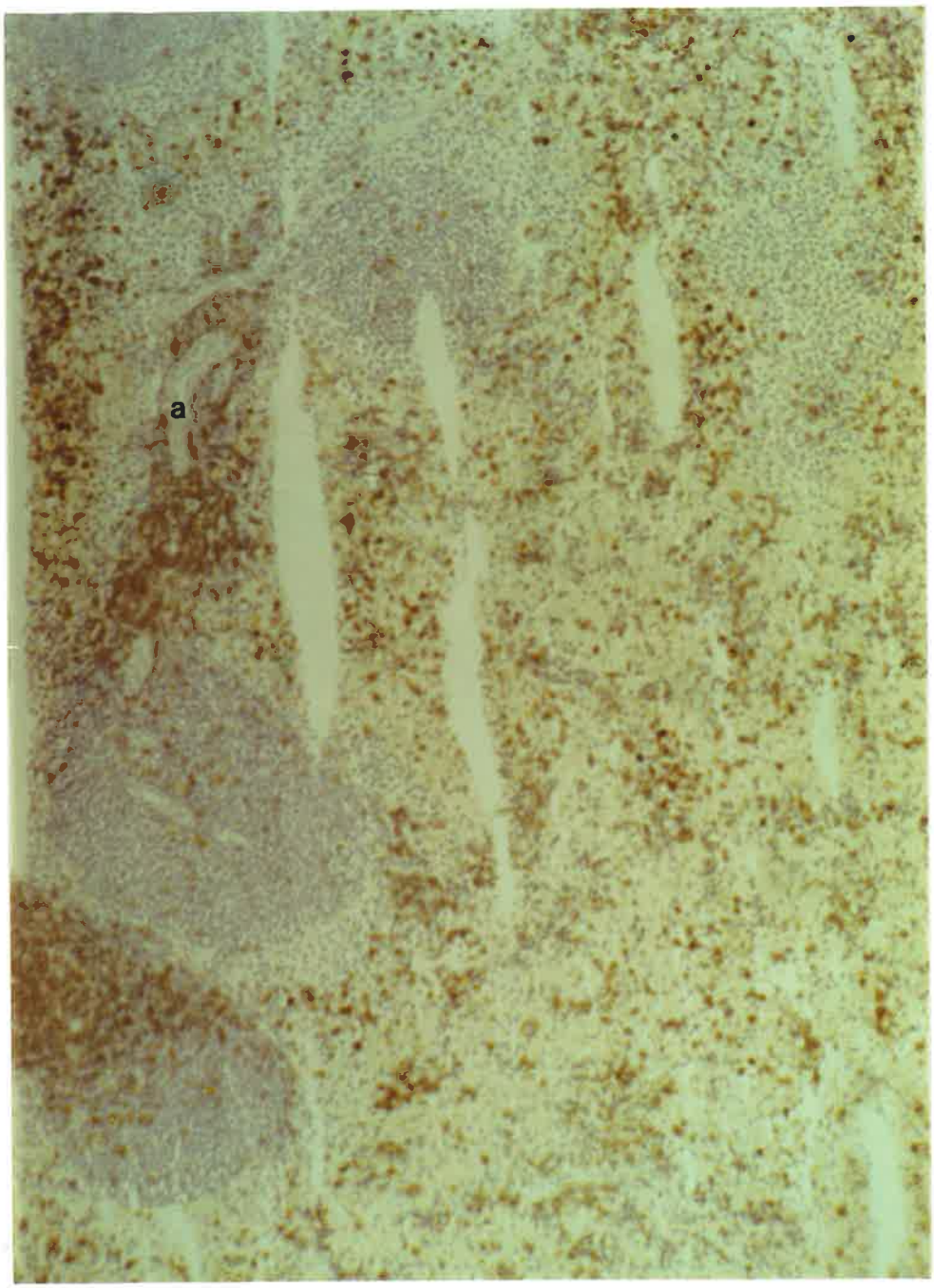

Figure 7.10. Regenerated splenic tissue. A low power view of WP that does have a central arteriole (**a**) with an attempt to form a PALS although the predominant cell type forming the WP remains unstained (likely B cells).

(W313 and haematoxylin, x40).

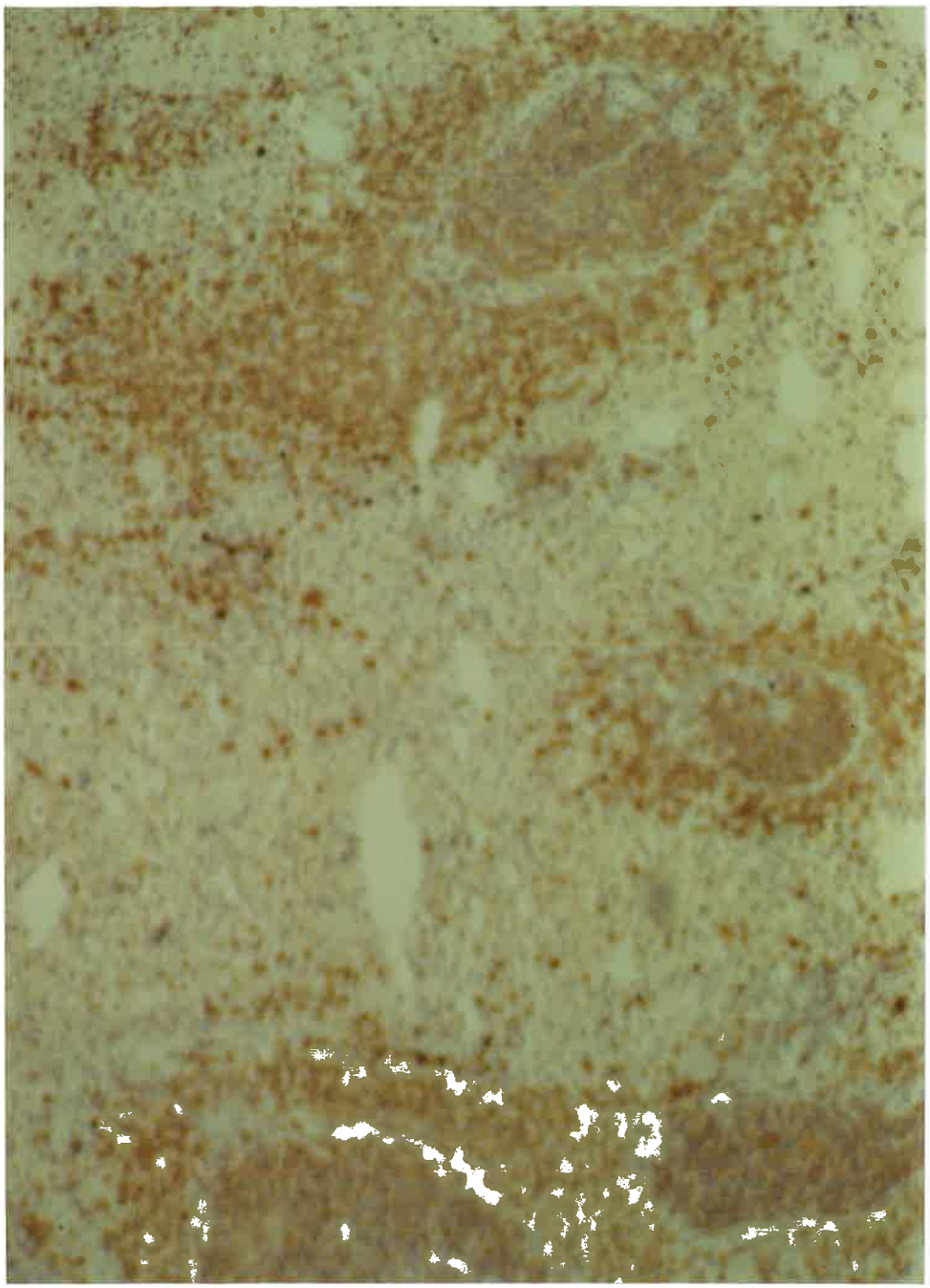

Figure 7.11. Regenerated splenic tissue. A low power view of WP that has been stained with anti-immunoglobulin to demonstrate B cells. All areas of WP are formed by B cells alone. The small MZ is also strongly stained for B cells.

(anti-IgM and haematoxylin, x40).

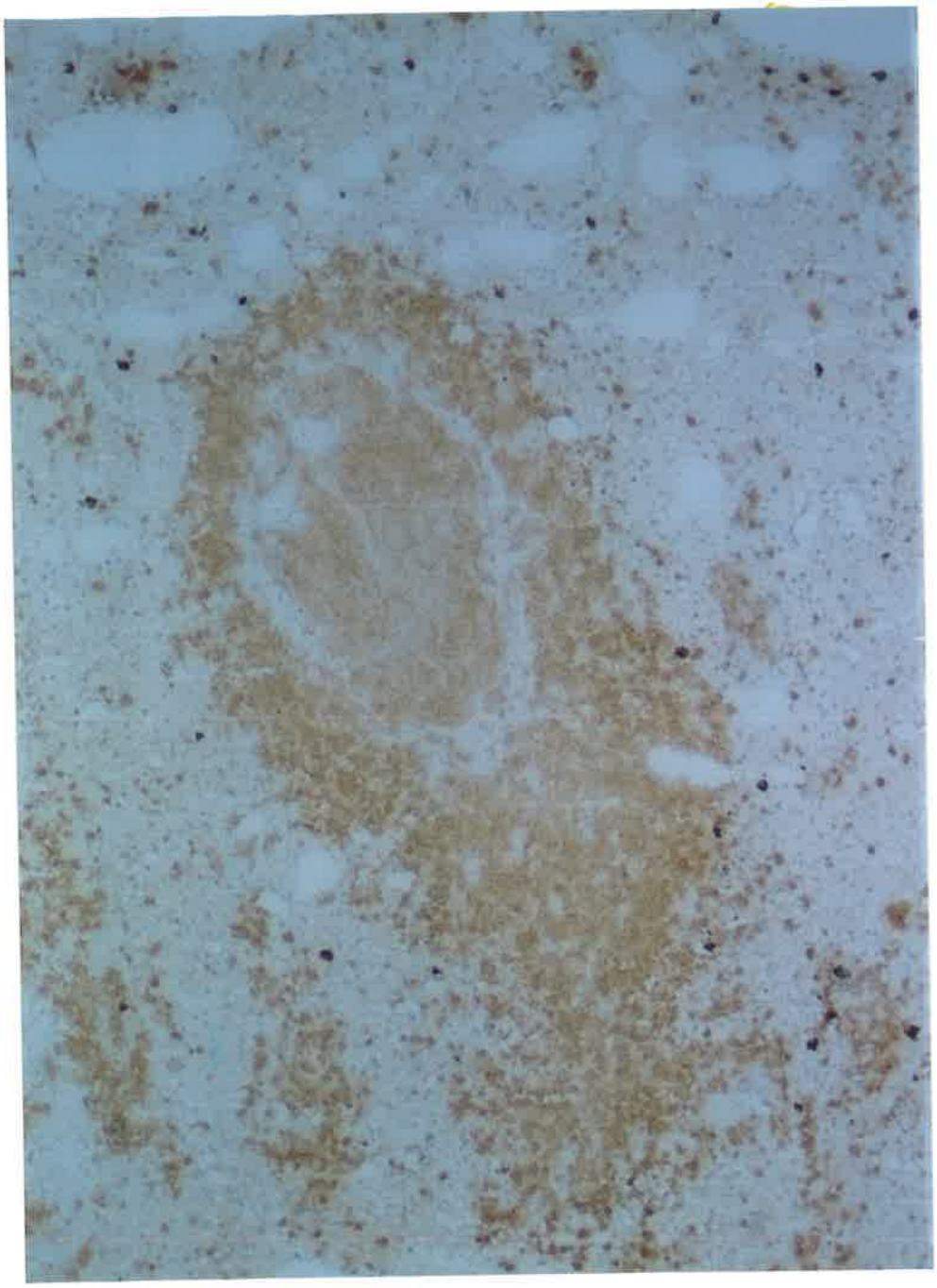

Figure 7.12. Regenerated splenic tissue. Higher power view of an area of WP shown in figure 5.11. Despite 2 small central arterioles, all cells have stained for IgM indicating a failure to form a PALS.

(anti-IgM and haematoxylin, x100).

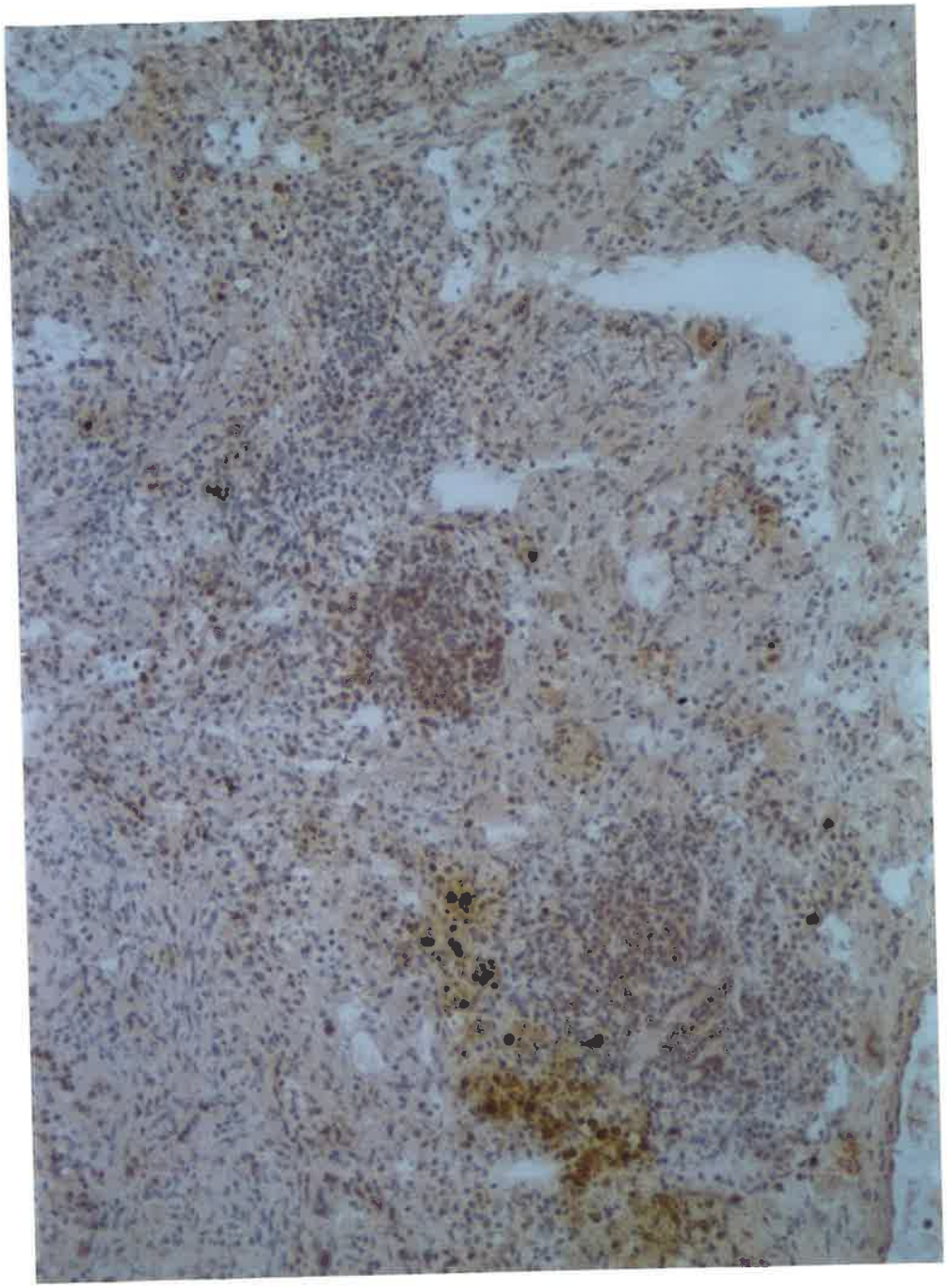

Figure 7.13. Regenerated splenic tissue. 2 areas of WP stained with anti-IgD to demonstrate the presence of IgD^- cells. There is no well defined, double layer MZ or lymphocyte corona.
(anti-IgD and haematoxylin, x100).

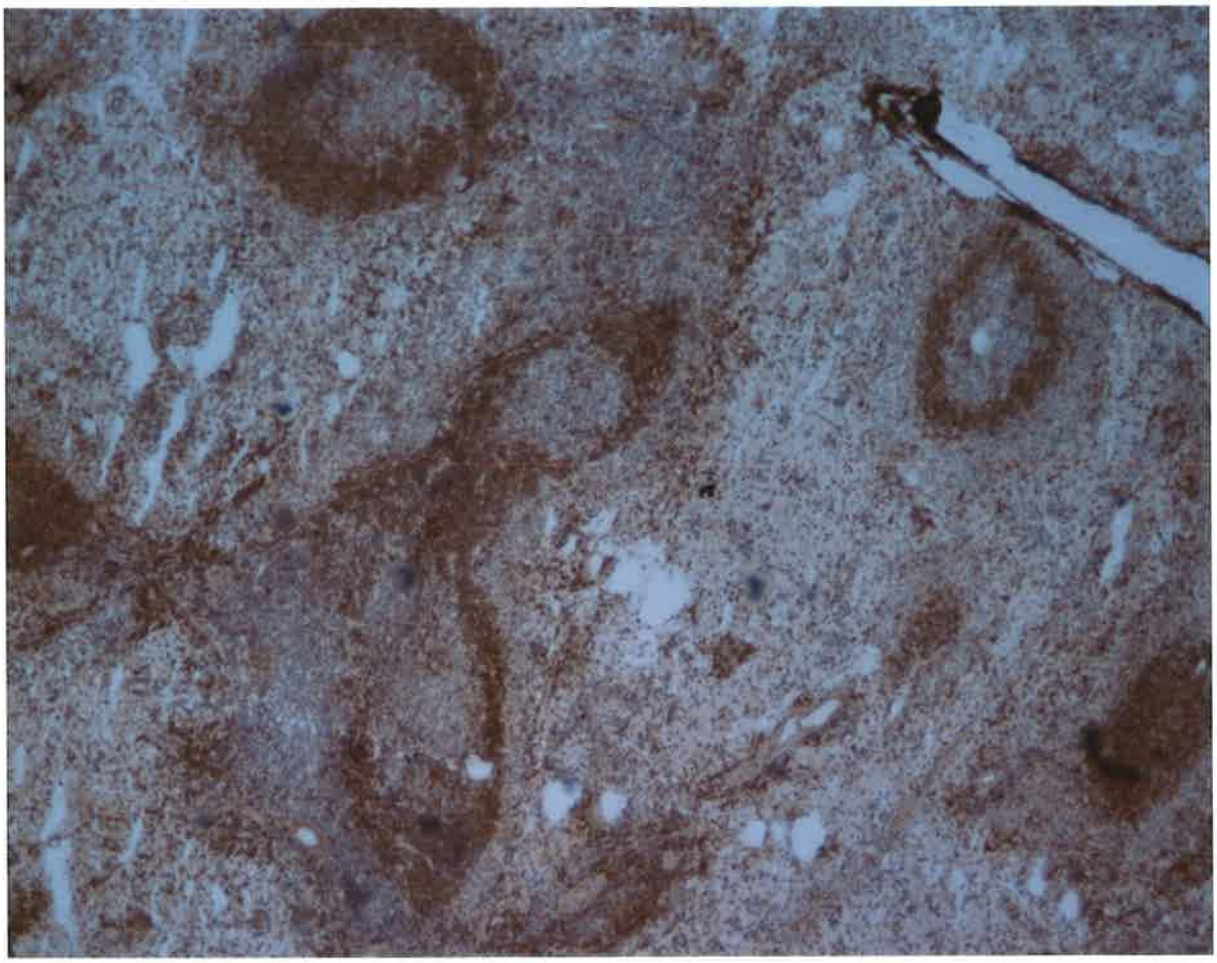

Figure 7.14. Regenerated splenic tissue. A low power view of well developed WP that has been stained with anti-IgD. The WP has been clearly formed into follicular areas and the MZ has 2 layers, an inner IgD^+ and outer IgD^- layer of B cells.
(anti-IgD and haematoxylin, x40).

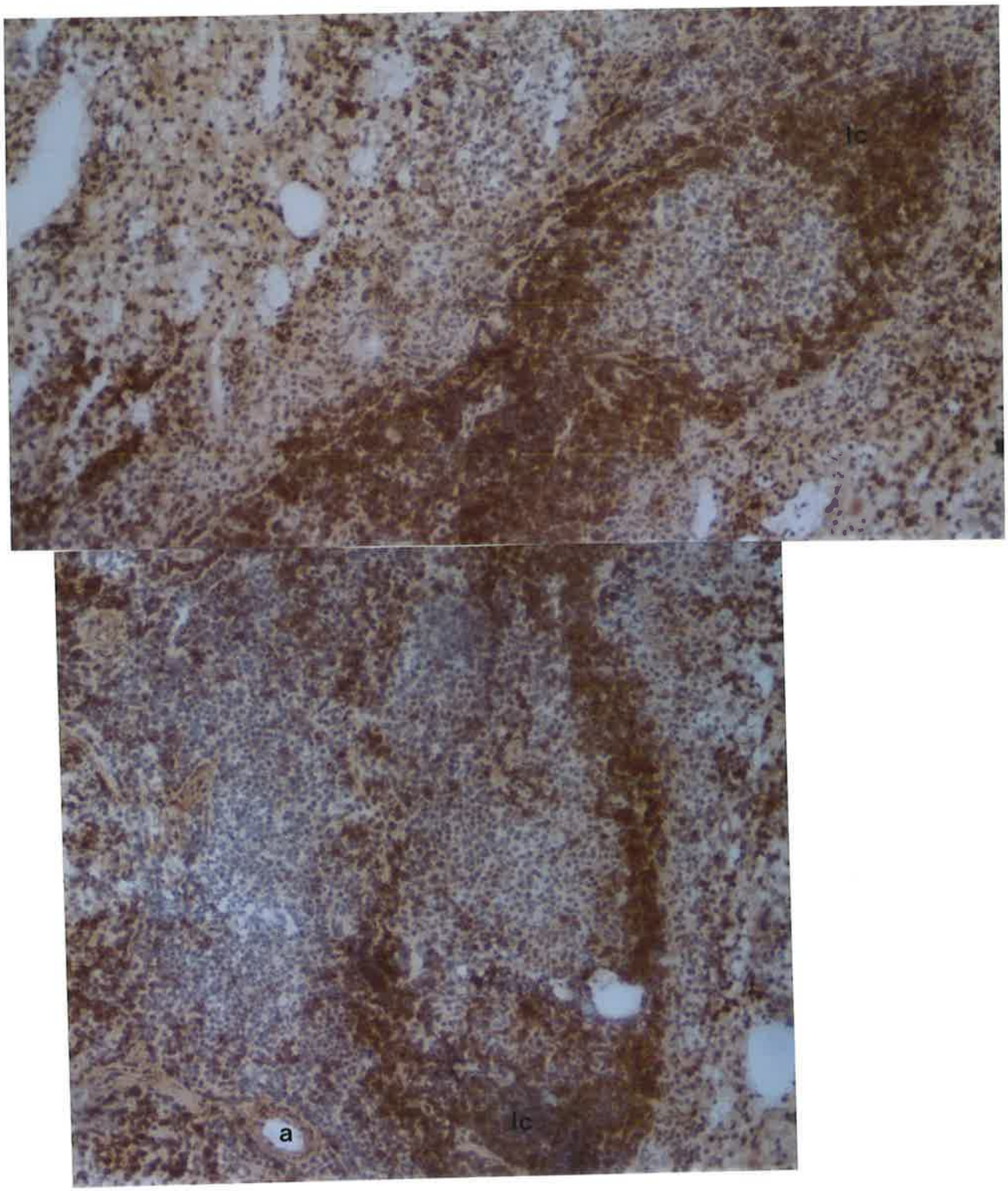

Figure 7.15. Regenerated splenic tissue. A higher power view of figure 5.14 which demonstrates a central arteriole (**a**) that has IgD^-cells surrounding it and lymphocyte coronas (**lc**) capping 2 follicles.

(anti-IgD and haematoxylin, x100).

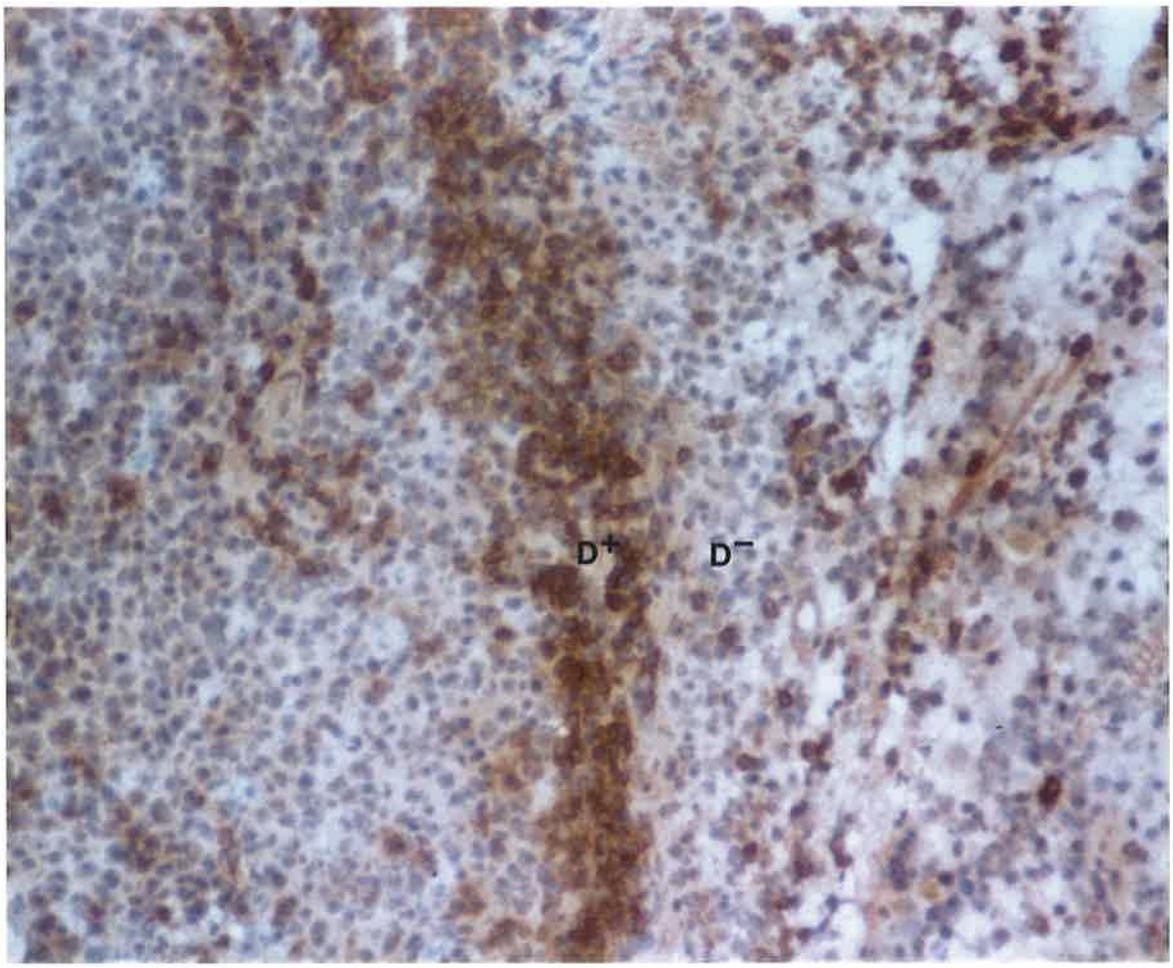

Figure 7.16. Regenerated splenic tissue. A higher power view of figure 5.14 which demonstrates the double layer of the MZ, inner IgD$^+$ (**D$^+$**) and outer IgD$^-$ (**D$^-$**). (anti-IgD and haematoxylin, x200).

CHAPTER 8

Discussion

Galen described the spleen as an organ which was full of mystery, involved in the purification of the blood. The function of the spleen has until recent times remained a mystery, permitting the view that the organ is expendable. Kocher (1911) reported that "Injuries of the spleen demand excision of the gland. No evil effects follow its removal...". It was not until Morris and Bullock (1919) first published the observation that in rats there was an increased risk of sepsis following splenectomy that the interest of the scientific community was stimulated in the role of the spleen in protection against infection. King and Shumacker (1952) reported that splenectomised children were at an increased risk of sepsis, but the mechanisms by which the spleen conferred protection against such infections was not the subject of investigation until Najjar (1970) identified tuftsin and linked its synthesis to the spleen.

The realisation of the importance of preserving splenic tissue to prevent septic complications stimulated the development of operations designed to repair or replace the traumatised spleen. The splenosis tissue that was frequently found after splenectomy indicated that splenic tissue implanted in the peritoneum retained the potential to revascularise and regrow. This provided surgeons with the incentive to induce splenosis deliberately at the time of splenectomy, by autotransplantation of splenic tissue to the omentum. Benjamin *et al* (1978) reported the planned autotransplantation of splenic tissue, but the value of this operation in the prevention of post-splenectomy sepsis was uncertain. Cooper and Williamson (1984) stated that "a combination of splenosis and regular immunisation may well be protective" and emphasised the need for further study before the value of autotransplantation could be determined. Peters (1984) reported that "Overall, the trauma patient with splenosis appears better protected against overwhelming sepsis than the truly asplenic individual". The filtering functions of the spleen, the clearing of senescent blood cells and the removal of vestigial cytoplasmic inclusions, were shown to be restored in patients with splenosis (Kiroff *et al,* 1983). Whether the other important functions of the spleen, phagocytosis, antigen transport and presentation, and antibody formation , are adequately restored remains unclear despite much experimental endeavour. In the absence of conclusive experimental findings the value of regenerated splenic tissue in the prevention of OPSI has continued to be strongly debated.

The bacteria commonly associated with OPSI generally have a short generation time. With low concentrations of specific antibody in the serum, the spleen will be the organ responsible for the clearance of the bacteria from the circulation. In the absence of the spleen,

bacteria will only be cleared from the bloodstream if a sufficiently high concentration of antibody is present to opsonise for clearance by the liver.

The aim of the studies reported in this thesis was to compare the phagocytic ability of splenosis tissue to that of normal spleen. Clearance of bacteria by splenic phagocytosis (which is most important when the serum antibody concentration is low) may be sufficient to reduce the bacterial load on the host. If this clearance function is adequately preserved, it may allow the slower responding immunocompetent organs valuable time to mount an effective antibody response. The phagocytic function of regenerated splenic tissue was investigated in rats in which splenic tissue had regenerated following either splenic autotransplantation or splenic devascularisation by ligation of the splenic vasculature.

The ability of the regenerated splenic tissue to phagocytose and remove radiolabelled colloid from the circulation was investigated 3, 6 and 15 months after the operation. The autotransplanted and devascularised spleens regenerated and macroscopically looked like normal tissue, the new organ never regrew to the full weight of the normal spleen. The regenerated tissue reached its maximum weight 3 months after surgery. Radioactivity was measured in the splenic tissue in each rat in each of the surgical groups following the *i.v.* injection of colloid. The amount of the circulating, ^{99m}Tc stannous fluoride colloid phagocytosed by the regenerated splenic tissue increased with the length of time after the operation. The maximum clearance was reached at 6 months, when, the regenerated splenic tissue phagocytosed one tenth of that cleared by the control spleens. The uptake of radiolabel per gram of splenic tissue was calculated, which corrected for the differences in weight between the different specimens. When calculated in this way, the phagocytosis by the regenerated spleens was 20% of the normal splenic clearance.

The histological zone in which the phagocytosis of colloid within the spleen occurred was studied. Autoradiographic studies following injection of radiolabelled colloid, and light microscopy studies following *i.v.* injection of colloidal carbon proved that the splenic uptake of colloid occurred in the inner layer of the MZ. Measurement of the percentage of the area of the spleen sections occupied by the MZ demonstrated a significant reduction in this compartment in the regenerated spleens, and a direct correlation between the amount of MZ and the phagocytosis of radiolabelled colloid by the total splenic tissue. On a per gram basis, the WP

and MZ in the regenerated tissue had equivalent ability to clear colloid as normal splenic tissue. A direct correlation between the amount of MZ and WP in the regenerated tissue was noted.

Yousaf *et al* (1985) investigated the clearance from the circulation of N-ethylmaleimide-treated and IgG coated red cells in normal and splenectomised rats. The time taken to clear the opsonised cells in the splenectomised rats was significantly prolonged. The ability of splenic tissue to phagocytose IgG opsonised syngeneic RBC was measured in rats 6 months following splenectomy and splenic autotransplantation, splenic artery ligation, total or partial splenectomy and compared with eusplenic controls. In eusplenic and partially splenectomised rats 71% of the label was cleared at 3 hours, compared with approximately 50% in rats following total splenectomy, splenectomy and splenic autotransplantation or splenic artery ligation. The autotransplanted and the ligated splenic tissue cleared less than 10% compared with normal spleen, but there was no difference between them when clearance was expressed as uptake per gram of tissue. The site of phagocytosis within the spleen was determined by autoradiographic studies. These identified the RP as the region for uptake of the RBC. Digitised measurement of this compartment demonstrated a significant increase in regenerated splenic tissue and a direct correlation between the amount of RP and the phagocytosis of radiolabelled RBC was identified. Despite the increase in RP, on a per gram basis the regenerated RP was a less efficient in the phagocytosis of red cells.

These results showed that regenerated splenic tissue had reduced phagocytic function for colloids and IgG opsonised RBC. It also permitted the demonstration of the specific regions within the spleen in which phagocytosis occurs. The histological studies confirmed that the regenerated tissue was abnormal in the amount, distribution and cellularity of the WP and MZ. These findings extend earlier reports (Pabst and Reilmann, 1980; Malangoni *et al*, 1985; Moore *et al*, 1986; Westerman *et al*, 1988).

In the WP areas observed in the regenerated tissue there frequently was no central arteriole. This prompted an investigation of the microvascular anatomy within the regenerated splenic tissue. Reilmann *et al* (1983) and Westerman *et al* (1988) used indirect methods to determine the blood supply of autotransplants by measurement of radioactivity after injection of radiolabelled albumin or rubidium chloride microspheres, and found a reduction in the total blood flow to the tissue on the basis of reduced radioactivity found in the spleen. In this study the microvascular anatomy within the autotransplanted spleens was studied using methyl

methacrylate corrosion casting and compared to that in normal spleens. These experiments enabled the study of the detail of the intrasplenic vessels.

Scanning electron microscopy of the vascular casts of the autotransplanted spleens demonstrated a number of abnormalities. The normal rich capillary network of the WP was lost. The normal discontinuous flattened vascular spaces of the marginal sinus were replaced by rounded spaces. The MZ network of anastomosing capillaries were also dilated into large capillary saccules. The "reticular meshwork" of the RP as described by Schmidt *et al* (1985) was flattened but abnormally dilated in places to form large vascular "lakes". The close approximation of matter in the blood to phagocytic cells which occurs in the normal spleen by virtue of the rich capillary circulation in the WP, flattened spaces abutting the MZ and flattened reticular spaces, was diminished in the autotransplant.

As previously reported, regenerated splenic tissue has a reduction in the percentage of WP and MZ. These regions are important in the development of many if not all antibody responses in the spleen. Matsumo *et al* (1989) reported that a thymus independent type 2 antigen was selectively phagocytosed by macrophages in the MZ, MZ bridging channels and the outer PALS, and the development of antibody forming cells directed against the antigen was confined to the outer PALS. The follicular region did not contain antibody cells nor did cell proliferation occur there, as determined by cell uptake of 5-bromo-2'-deoxyuridine. This dependence on the MZ for developing an antibody response to certain thymus independent antigens has been reported in both rats and man (Amlot *et al* , 1985; Amlot and Hayes, 1985). Within the MZ and WP in the normal spleen specialised cell types are distributed to specific regions within the compartments (Buckley *et al*, 1987; Westerman *et al*, 1988). To determine if the fine cytoarchitecture is maintained in the lymphoid aggregates of the regenerated splenic tissue, the WP and MZ were studied using specific immunohistochemical stains. The T cells were seen scattered through the WP, but were rarely organised around a central arteriole to form a periarteriolar sheath. The ratio of T cells to B cells was abnormal, with most areas of WP comprising B cells exclusively. Primary follicles were apparent in the WP, but secondary follicles were rarely seen. The MZ was abnormal with the normal thin inner layer of $\mu^+\delta^+$ B lymphocytes and outer layer of $\mu^+\delta^-$ B lymphocytes usually replaced by a narrow, single layer of $\mu^+\delta^-$ B cells.

The data presented in this thesis show that the regenerated spleen has markedly altered phagocytic function. This reduction in total clearance from the circulation is primarily due to the reduction in splenic mass. The clearance of a colloid and of IgG coated red cells was measured, and the underlying mechanisms were found to differ. The clearance by the regenerated tissue was different for the two particles, because of the specific nature of uptake within the spleen. The phagocytosis of the colloid was reduced due to the disorganisation of the lymphoid compartments , altered microvasculature and abnormal cellular regeneration, principally affecting the WP and MZ. In contrast, the clearance of the IgG coated red cells was carried out in the RP, and the regenerated spleen was as effective on a weight for weight basis as the normal spleen.

The extent to which the regenerated spleen had the features of normal splenic tissue, the degree of WP regeneration and the maturation of the lymphoid cells, was highly variable between specimens. Occasionally splenic transplants were seen with near normal cytoarchitecture.

Asplenia results in reduced immunity. Splenic autotransplantation has been suggested to reverse this immunodeficiency. Previous studies have stated that antibody production by splenic autotransplants is not restored (Rowley, 1950; Kiroff *et al,* 1985). This thesis has investigated the other important splenic function of phagocytosis. The experimental work has demonstrated that splenic autotransplants do not have normal phagocytic function, that the areas of the spleen that are involved in phagocytosis are not regenerated to that of normal spleens and the blood supply is abnormal. In conclusion, splenic autotransplants are not normal "regenerated" spleens and can not be considered to be protective against OPSI.

The mechanisms and regulatory controls involved in the "regeneration" of the spleen and its repopulation with its various constituent cells is not known. One possible factor may be the nature and adequacy of the restored blood supply, which would be an area for future research. Another factor in regeneration may be the preservation of specific splenic homing receptors that may attract circulating cells to restock the growing splenosis tissue. The advent of new cell markers will also further differentiate the cells that repopulate the splenic transplants. The identification of specific cells may be of value. The spleen contains 3 subsets of macrophages which are known to participate in antigen phagocytosis and presentation and

they have not been specifically identified in the transplants. A specific line of MZ B cells are unique to the spleen but poorly restored in the transplants.

If the splenic "regeneration" can be stimulated to repopulate to the normal histology, this may improve the phagocytic function. Enhanced splenic phagocytosis may provide protection against OPSI.

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