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Heterotopic autologous splenic grafts in rat

Morphological studies

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Summary. Splenic grafts autotransplanted at splenectomy into the omentum of 23 Porton strain rats were compared with spleens from 10 sham-operated controls. Six months after transplantation, the grafts weighed between 81 to 545 mg (median 166 mg) compared to control spleens which weighed 775 to 1,250 mg (median 995 g). Histoquantitation of the grafts revealed marked reduction of the splenic white pulp when compared to control spleens. Morphological examination showed not only a reduction of lymphocytes but also a striking architectural abnormality in all grafts. In 2 of the transplants, no lymphoid aggregates were observed; small subcapsular collections were present in 7, while in 8, isolated perivascular aggregates of lymphocytes with poorly formed marginal zones were observed. Only 6 transplanted spleens showed linkage of adjacent lymphoid aggregates but the number and size of these aggregates were clearly less than normal. These findings indicate that autotransplanted splenic tissue in rats does not regain histological normality. The implications of these observations for autotransplantation in splenectomized patients are discussed.

Key words: Splenic grafts – Morphology – Histoquantitation – Lymphocytes – Splenectomy

Introduction

A major long-term consequence of splenectomy is the occurrence of overwhelming sepsis. Rapid downhill progression despite aggressive antibiotic therapy, disseminated intravascular coagulation and high mortality are hallmarks of these bloodborne infections. *Pneumococcus* alone accounts for the majority of cases but immunization has not been successful in covering

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for all possible causative organisms (Francke and Neu 1981). Restoration of splenic function by preservation of the spleen or by transplantation has therefore been considered as alternative prophylactic measures against post-splenectomy infections.

Patients who have had splenectomy following traumatic rupture, may subsequently show splenic tissue in multiple ectopic sites within the peritoneal cavity, even though splenic removal was thought to be complete at the time of surgery (Kiroff et al. 1983). This phenomenon of "splenosis" is thought to be the result of the growth of splenic cells dissociated from the organ during its rupture, or, at the time of surgical removal. The ability of splenic tissue to grow in ectopic sites has allowed heterotopic autologous splenic grafts to be surgically created and studied in experimental animals. Recently, such grafts have been performed in patients splenectomized following traumatic injury in an attempt to alleviate the problems of overwhelming postsplenectomy sepsis (Millikan et al. 1982; Moore et al. 1983). Both such surgically created autotransplants and spontaneous splenosis tissue have been shown to take up radio-labelled heat-damaged erythrocytes (Kiroff et al. 1985), and have the ability to remove vacuolar inclusions from circulating erythrocytes (Neilsen et al. 1982; Kiroff et al. 1985). However, it is not clear to what extent the immunological functions which are performed by the normal spleen are restored by autotransplanted tissue or splenosis. To date, experimental studies have reported conflicting results (Livingstone et al. 1983). Splenectomized patients have succumbed to septicaemia despite significant splenosis revealed at autopsy (Rice and James 1980). Immunological variables have not been measured in such patients and in instances of prophylactic autotransplantation, the splenic tissue has been found to contain reduced amounts of lymphoid cells, accounting for the inability to protect the splenectomized patient from overwhelming sepsis (Moore et al. 1983).

This report describes a model of splenic heterotopic autologous implantation in the rat in which detailed histological examination of the white pulp in six-month old grafts was made. Quantitative evaluations of the lymphoid tissues in the grafts were compared with those of normal rat spleen with the aim of assessing the extent of restitution of lymphoid cells in the transplanted tissue.

Materials and methods

Male Porton strain S.P.F. rats weighing 220–300 g were used. The rats were bred in a closed colony maintained at the Central Animal House, University of Adelaide, and were 7 weeks old at the time of operation.

Surgical procedures. The rats were lightly anaesthetized with sodium pentobarbitone (0.5 ml/kg body weight, i.p.) (Nembutal, Ceva Chemicals, Australia). The peritoneal cavity was opened by a small left para-median incision and the spleen mobilized outside the peritoneal cavity.

In the auto-transplanted group ($n=23$), the spleen was removed following ligation of the splenic vessels with silk. Each spleen was weighed immediately upon removal. A weighed longitudinal slice of spleen (about 100 mg) was then stitched into a surgically-created pouch in the mesentery of the small bowel, before closure of the abdomen, in two layers, by suture.

The same procedure was performed on the sham-splenectomised rats ($n=10$) except that the ligatures were not tied and the spleen was not removed from its normal position.

Following operation, the animals were housed in individual cages and immediately allowed free access to standard rat pellets and fresh drinking water. Terramycin antibiotic powder (Pfizer Laboratories, Australia) at a final concentration of 200 mg/litre was added to the animals' drinking water for one week postoperatively. The animals showed no evidence of infection. Six months postsurgery, the spleens from the sham-operated rats and the splenic auto-transplants were removed from the experimental animals. Adherent tissue was dissected off before the specimens were weighed.

Histological assessment. The specimens were divided at the waist and one half fixed in 10% buffered formalin and embedded in paraffin (the other half was deep frozen for immunological studies, the subject of another report). Five micron sections of each specimen were then stained with haematoxylin and eosin and Gomori's reticulin stain. Assessment of splenic white pulp was based on both the quantity of lymphocytes as well as their architectural arrangement. A score of 0 to 4 was assigned according to the following criteria:

0 = no lymphoid aggregates present
1 = less than 5 lymphoid aggregates in a subcapsular location without evidence of periarteriolar localization or marginal zonification.
2 = occasional aggregates of lymphocytes in periarteriolar location, mainly in the periphery of the spleen section, with or without evidence of early marginal zone formation. No confluence of lymphoid aggregates.
3 = frequent, circumscribed aggregates of periarteriolar lymphocytes diffusely distributed throughout the section but less than normal in number. Well-established marginal zones and confluence of lymphoid aggregates, i.e. linkage of adjacent marginal zones occasionally present.
4 = normal amount and distribution of lymphoid aggregates with normal architecture.

The scoring of the coded sections was made independently by two of the authors (AS-YL, MTFM) and in two instances of disagreement, a common score was assigned after discussion.

Quantification of splenic white pulp. Images of the whole tissue sections were projected at a fixed magnification and outlines of the sections and white pulp were traced. Limits of the marginal zones were clearly discernable, however, the periarteriolar lymphoid sheath could not be readily separated from the primary follicles and germinal centres, so that these structures were traced together. The tracings were then analysed with the Quantimet Image Analyser. The total areas of the section, the lymphoid aggregates, the periarteriolar lymphoid sheath including lymphoid follicles, and the marginal zone were separately measured. These various variables from each section were measured three times and the mean of the values obtained were expressed as a percentage of the total area of the section. Variation of each measurement did not exceed 5%. The results for each group were analysed using the Wilcoxon Rank Sum Test for unmatched pairs, and differences were considered significant when $P<0.05$.

Results

Histological findings

The histological scores assigned are shown in Table 1. All 10 spleens from sham-operated rats had scores of 4 i.e. they showed normal amounts of lymphoid cells and normal architecture. The white pulp of these spleens displayed distinct zonification of lymphocytes around small arterioles. Periarteriolar lymphoid sheaths, primary follicles, some with germinal centres and a less dense marginal zone were evident (Fig. 1). The white pulp was seen diffusely throughout the sections and confluence of adjacent lymphoid

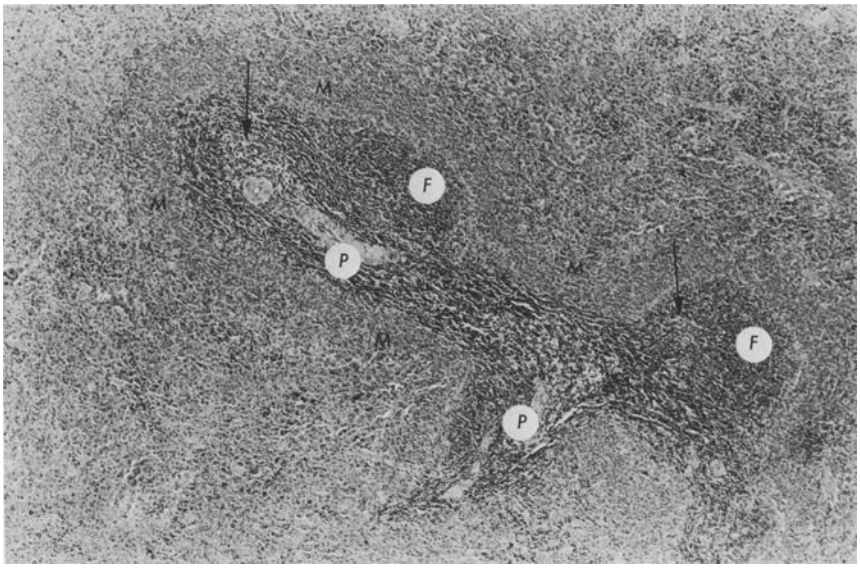

Fig. 1. Sham operated control spleen showing the normal zonification of white pulp. The dense peri-arteriolar sheath (P) is surrounded by an imperceptible rim of lymphocytes which includes discernible lymphoid follicles (F) some with germinal centres (→). A distinct, less dense marginal zone (M) is evident. Note the confluence of the lymphoid aggregates to produce the branching pattern (H&E, ×200)

Table 1. Histologic scoring of white pulp in auto-transplanted and control spleens

Histologic score[a]	Number of specimens
0	2
1	7
2	8
3	6
4	10
Total	33

[a] For definition of scores refer to text

aggregates was common. Control spleens weighed between 775.4 mg to 1,250 mg (median 995 mg).

The transplanted spleens weighed between 81 mg to 545 mg (median 166 mg). They showed a wide range of morphological appearances and were characterised by a marked depletion of the splenic white pulp which showed abnormal architectural arrangement of the lymphocytes. There was a diffuse distribution of prominent haemosiderin-laden macrophages and scattered foci of extramedullary haematopoiesis were present. These features made the transplanted specimens readily separated from the controls even on casual microscopic observation.

Two grafts were scored "0" as no lymphoid aggregates were present

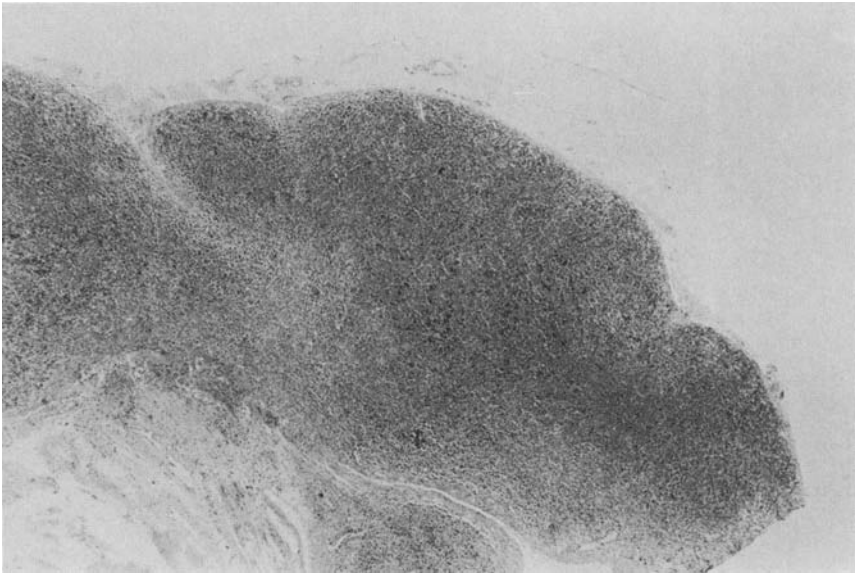

Fig. 2. Transplanted splenic tissue devoid of lymphoid aggregates, Score 0. Scattered foci of haematopoiesis are seen (H&E, ×100)

(Fig. 2). Seven grafts were scored "1" and they displayed scattered loose aggregates of lymphocytes, numbering less than 5 in total and arranged in a subcapsular location with no evidence of periarteriolar localization or marginal zonification (Fig. 3). The 8 specimens scored as 2 showed a dense aggregation of lymphoid cells in a periarteriolar pattern. An ill-defined, thin marginal zone was sometimes visible but lymphoid follicles were not observed and the aggregates remained isolated (Fig. 4). A score of 3 was assigned to 6 transplants in which the frequent, circumscribed, periarteriolar aggregates showed occasional follicle formation and a well-defined marginal zone. Occasional linkage or confluence of adjacent marginal zones was seen (Fig. 5).

In 4 specimens (2 each with scores of 2 and 3), a distinct rim of lymphocytes was visible, clearly located external to the splenic capsule (Fig. 6). These rims of lymphocytes were often linked to periarteriolar aggregates deep to the capsule (Fig. 7). All transplants, even those scored "0", revealed a delicate reticulin background very similar to that of normal spleen (Fig. 7).

Quantimet image analysis

Results of Quantimet analysis are shown in Fig. 8. The percentage area of the splenic section occupied by white pulp in the control spleens ($n=10$) ranged from 20.04 to 37.40% (median 24.82%). In the autotransplanted spleens ($n=23$), the values were 0.07 to 23.19% (median 5.53%) ($P<0.0001$). The marginal zones in controls occupied 11.40 to 24.80% (median

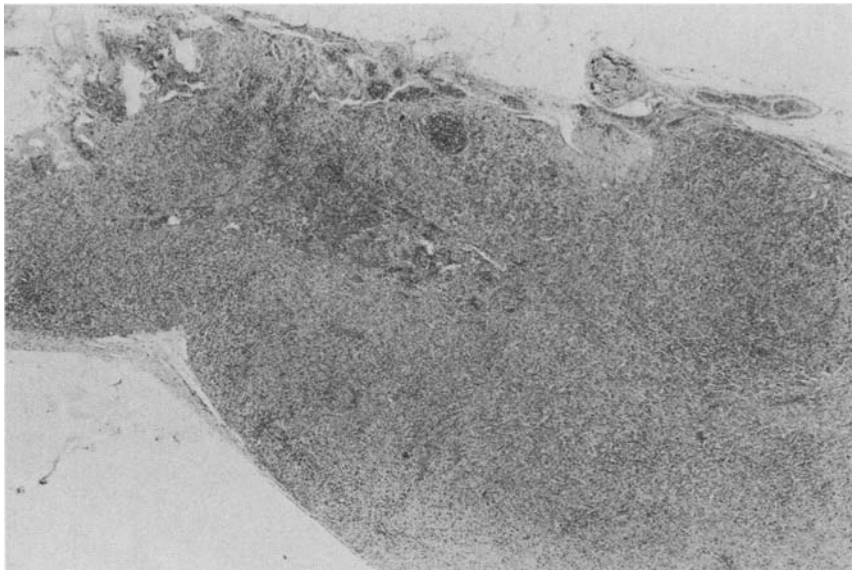

Fig. 3. Transplanted splenic tissue, Score 1. The specimen is largely devoid of lymphoid aggregates except for a few small subcapsular collections which are not associated with arterioles (H&E), × 100)

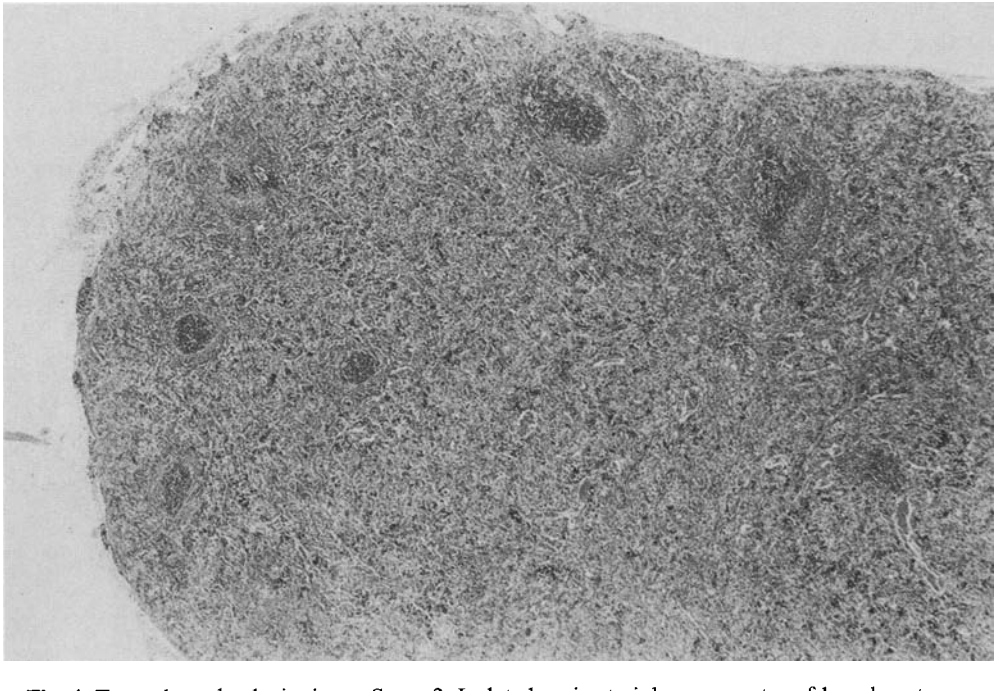

Fig. 4. Transplanted splenic tissue, Score 2. Isolated peri-arteriolar aggregates of lymphocytes are seen in the periphery of the specimen. The marginal zone is visible around some of the aggregates but no lymphoid follicle formation is evident (H&E, × 100)

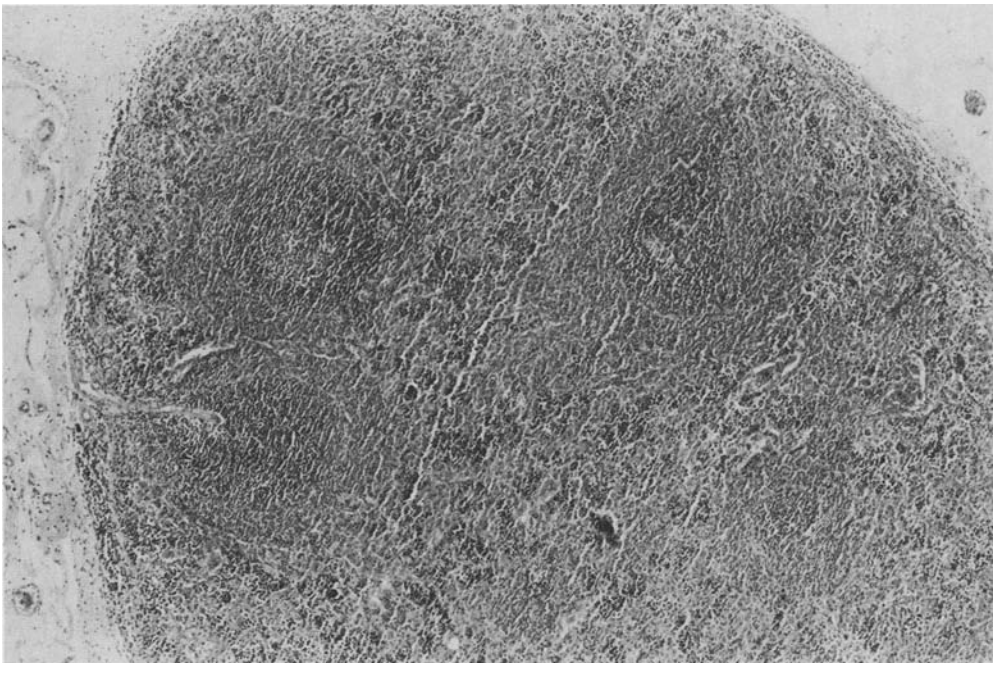

Fig. 5. Transplanted splenic tissue, Score 3. Three large peri-arteriolar aggregates are present, two of which show abutting marginal zones. Although lymphoid follicles are not distinct, the presence of clusters of blast cells indicate germinal centre formation (H&E, ×470)

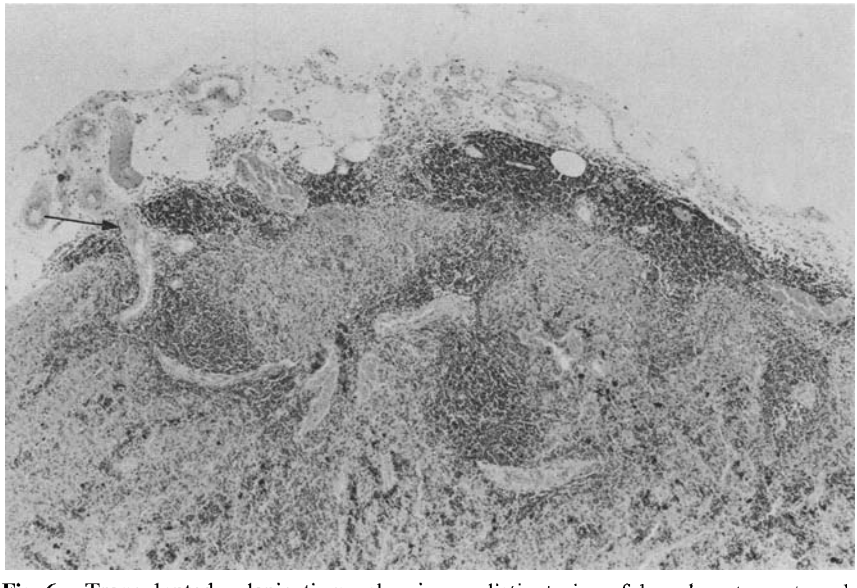

Fig. 6. Transplanted splenic tissue showing a distinct rim of lymphocytes external to the thin fibrous capsule. There is continuity between this rim and the peri-arteriolar lymphoid aggregates within the spleen. Note the prominent arterioles which enter the spleen from the adjacent connective tissue (*arrow*) (H&E, ×470)

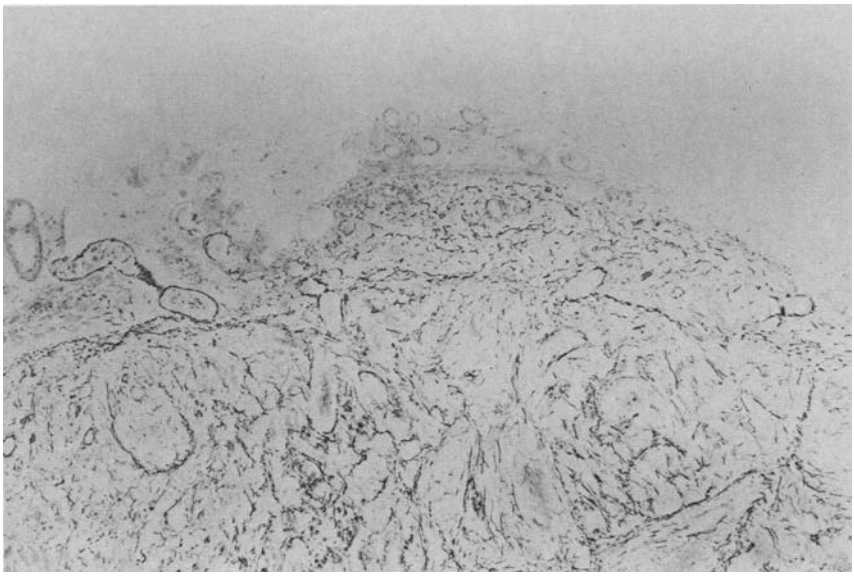

Fig. 7. Reticulin pattern of the same area as shown in Fig. 6. The rim of lymphocytes are confirmed to be external to the splenic capsule and the reticulin network replicates that of normal spleen with the formation of sinusoids and condensation around lymphoid aggregates (Gomori's reticulin, ×470)

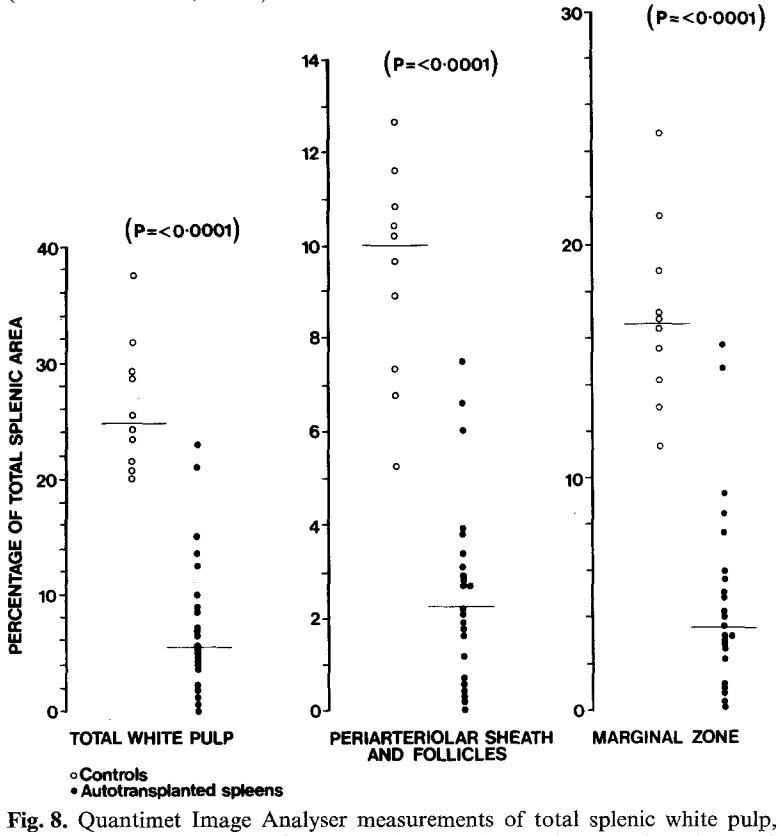

Fig. 8. Quantimet Image Analyser measurements of total splenic white pulp, peri-arteriolar sheaths including lymphoid follicles and marginal zones in controls and transplanted spleens

16.60%), while the marginal zone values for transplanted spleens were 0 to 15.67% (median 3.52%) ($P<0.0001$). The periarteriolar lymphoid sheath including primary follicles and germinal centres in controls occupied 5.26 to 12.63% (median 9.93%) of the sections while the transplanted group showed values of 0.08 to 7.50% (median 2.28%) ($P<0.0001$).

Discussion

While the importance of the spleen in the clearance of bacteremia in both experimental animals and humans is well recognized, the potential role of auto-transplantation in the protection of patients from overwhelming post-splenectomy infection is yet to be established. Studies of the immune competence of autologous splenic grafts in experimental animals have reported conflicting results (Grosfeld and Malagoni 1980; Cooney et al. 1979). It has been suggested that the depression of opsonin and leukophilic gamma globulin activity observed in the sera of chronically splenectomized animals can be avoided after ectopic transplantation of splenic tissue (Likhite 1975). Scintigraphy studies have indicated a normal function of the white pulp after antigenic stimulation (Cooney et al. 1979) and reimplanted splenic tissue has resulted in normal immunological response to intravenous challenge with pneumococcus and sheep erythrocytes (Rice and James 1980; Patel et al. 1982).

Several studies have indicated a protective effect of splenic autotransplantation in rats exposed to pneumococcal septicaemia (Patel et al. 1982; Livingstone et al. 1983; Neilsen et al. 1983). However, other workers have failed to find conclusive evidence of protection against sepsis in postsplenectomized animals (Schwartz et al. 1977, 1978; Moxon and Schwartz 1980). Our findings clearly show that although auto-transplanted splenic tissue may grow and increase in weight in the root of the mesentery, it remains markedly deficient in lymphoid content even 6 months after implantation. Not only was the lymphoid tissue quantitatively reduced but organization of the splenic white pulp remained abnormal. By histoquantitation, significant differences were found between the transplant and the controls ($P<0.0001$). Only two transplant spleens appeared to have as much lymphoid tissue as the controls. However, when examined by histological criteria, these specimens were scored only as 3 as they showed abnormal architecture. Transplanted spleens and spleens from sham-operated animals were clearly different when analysed by the set morphological criteria which were based not only on the quantity of lymphoid elements but also the organisation of the tissues. The histological differences between both groups were so distinct that separation could be made by casual microscopic observation.

Detailed chronological studies of the regeneration of subcutaneously implanted pieces of spleen (Tavassoli et al. 1973) and ligated mouse spleen (Wolf 1982) have shown that whole organ necrosis is followed by a centripetal pattern of reconstruction which derives from a thin layer of surviving cells including and beneath the splenic capsule. Cellular regeneration appeared to proceed inward along remaining noncellular reticular fibre tracts

throughout the spleen, resulting in architectural restoration (Wolf 1982). Knake (1952) showed that autotransplants of rat spleen placed intraperitoneally survived without evidence of partial necrosis and revealed normal splenic architecture as early as 14 days after transplantation. A similar histology was observed in the autotransplants 13 months later. It has been claimed that the histological appearances of implants and regenerated spleens resembled those of their normal counterparts (Tavassoli et al. 1973; Warner and Krueger 1975; Wolf 1982; Reilman et al. 1983). These conclusions resulted from studies on autotransplanted spleens in rats (Knake 1952; Tavassoli et al. 1973), mice (Warner and Krueger 1975); pigs (Reilman et al. 1983) and in regenerated spleens in mice following predicle ligation (Wolf 1982). Histological assessment in these studies were based on casual microscospic examination and no grading or histoquantitation was performed; furthermore, the specimens were not compared to normal spleens. While Metcalf (1963) did quantify the amount of lymphoid tissue using camera lucida drawings of subcutaneous isologous spleen grafts in splenectomized and nonsplenectomized mice, he failed to compare them with normal spleens. Histoquantitation as well as histological grading in the present study indicate definite deficiencies in the white pulp per unit area of autotransplanted spleens.

The total amount of splenic white pulp would even have been less taking into consideration the much lower weights of the autotransplanted tissues (median 166 mg) compared with that of control spleens (median 995 mg). A recent study by Melangoni et al. (1985) revealed marked reduction in the phagocytic index after splenic autotransplantation in rats, and the splenic grafts showed a relative decrease in white pulp with increased fibrous tissue within the red pulp.

In humans, the demonstration of isotype uptake attests to the phagocytic activity of autotransplanted splenic tissue and the reduction of circulating vacuolated erythrocytes reflects some restoration of the pitting function of the implants (Neilson et al. 1982; Kiroff et al. 1983). There is, however, no evidence that splenosis tissue is protective and the presence of large numbers of ectopic splenic tissue has failed to protect against fatal pneumococcal septicaemia (Rice and James 1980; Moore et al. 1983). The many immunological functions which are altered in splenectomized patients remain abnormal in the presence of splenosis. We have shown that splenosis tissue does not correct the abnormalities of serum immunoglobulin concentrations nor the abnormal *in vitro* immunoglobulin synthesis by peripheral blood mononuclear cells in splenectomized patients (Drew et al. 1984; Kiroff et al. 1985). Antibody responses to subcutaneous immunization with pneumococcal polysaccharide vaccine (Kiroff et al. 1985), natural killer cell activity and lymphocyte responses to mitogen (Ferrante et al. 1985) continue to remain abnormal.

Moore et al. (1983) described a patient who died of overwhelming postsplenectomy infection despite splenic autotransplantation. At autopsy 5 months after surgery, all five implants showed microscopic lymphocyte depletion although technesium scan had revealed active uptake by the im-

plants. The clinical evidence that phagocytic activity occurs is supported by our findings or numerous haemosiderin laden macrophages in the grafts. While the pitting function of the spleen may also be performed to some extent by the grafts (Kiroff et al. 1983) they represent only two of the many immunological activities of the normal spleen.

The present experimental findings indicate that autotransplanted splenic tissues contain decreased amounts of lymphocytes which also show abnormal architectural organization. These findings provide a histological basis for the failure to restore normal immunological functions by autotransplantation in splenectomized patients as well as experimental animals.

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