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IMMUNOARCHITECTURE OF THE REGENERATING RAT SPLEEN: EFFECTS OF PARTIAL SPLENECTOMY AND HETEROTOPIC AUTOTRANSPLANTATION

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Abstract

To investigate the microstructure of in situ (eutopic) and autotransplanted (ectopic) splenic remnants, adult Sprague-Dawley rats were studied 60 days after 1) subtotal (~80%) splenectomy, 2) total splenectomy followed by single or multiple remnant intraperitoneal autotransplantation, or 3) sham operation. Total nucleated cell counts were determined in excised splenic remnants, and immunohistochemical staining using monoclonal antibodies to rat B- and T-cell antigens was performed in serial tissue sections.

Immunoarchitecture of eutopic remnants was indistinguishable from that of intact spleens and total nucleated cell counts remained proportional to weight. In contrast, ectopic remnants showed sparsity and abnormal mixing of B and T lymphocyte subpopulations with widespread loss of follicles and periarteriolar lymphoid sheaths in addition to lower density and marked reduction of total nucleated cells.

These findings provide immunohistologic evidence that preservation of intact vasculature is critical to splenic architecture, which may account in part for the demonstrable functional inferiority of ectopic remnants.

KEY WORDS: immunoarchitecture, monoclonal antibodies, lymphocyte antigens, spleen regeneration, spleen preservation, partial splenectomy, splenic autotransplants

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Introduction

Because of the danger of post-splenectomy sepsis, heterotopic autotransplantation to peritoneal or subcutaneous sites and partial splenectomy have been proposed as surgical alternatives to total splenectomy for the management of traumatic rupture and occasionally other splenopathies. Experimental support for autotransplantation, i.e. "free grafting" devoid of intrinsic vascular supply, has come from histologic confirmation of lymphoid repopulation after reticular proliferation of viable revascularized subcapsular tissue in necrotic splenic fragments [1-2, 14]. In addition, both restoration of "spleen-like" histology and evidence of trapping of damaged erythrocytes and 99mTc sulfacolloid have been documented in patients with spontaneously implanted intraabdominal splenic nodules (splenosis) arising after traumatic splenic rupture [6,9]. Nonetheless, serious reservations persist about the extent of the spleen's capacity to repopulate and regenerate in the absence of an abnormal workload as well as the protective value of ectopic implants to prevent overwhelming post-splenectomy sepsis [17].

To evaluate further the cell populations and architectural integrity of preserved splenic remnants, we examined the immunohistology of mature ectopic and eutopic remnants from rats using specific monoclonal antibodies directed against common lymphocyte antigens and also determined remnant nucleated cell counts as a gauge of total number of potentially immunocompetent cells.

Materials and Methods

Outbred adult male Sprague-Dawley rats (Hilltop, Chatsworth, CA) weighing approximately 230g were divided into 4 groups and under Innovar-vet anesthesia subjected to either sham-operation (splenic manipulation without incision or resection), subtotal splenic resection leaving behind a tailored eutopic remnant of ~200mg (~1/5 normal spleen), or total splenectomy followed by implantation of single (200mg ectopic) or multiple (25-50mg ectopic) autotransplants of the sliced whole spleen. Ectopic remnants were weighed, wrapped in omentum, and seeded in the peritoneal cavity.

At 60 days, a time interval after which splenic regeneration in the rat is essentially complete [3,18], rats were sacrificed by cervical dislocation. Splenic tissue was removed and weighed, and in one large stratified subset of rats from each group, spleen cells were teased into 2% acetic acid in normal saline and nuclei counted on a hemocytometer to determine total nucleated cell counts. In the remaining rats, splenic tissue was washed in normal saline, embedded in OCT medium (Ames Co., Division of Miles Laboratory, Elkhart, Indiana), and snap-frozen at -150°C in isopentane suspended in liquid nitrogen. Each specimen was wrapped in aluminum foil and air-tight plastic bags to prevent desiccation and stored at -70°C until sectioned. Frozen cryostat sections approximately 2 micra thick were prepared in serial order and mounted on numbered glass slides. Sections were preserved prior to staining by "quick-dipping" in 4°C acetone and stored at -20°C. Several serial tissue section samples from each rat were stained with hematoxylin and eosin for study of histologic features, and representative remnants from each group were selected for immunohistochemistry.

Tissue section immunohistochemistry

B and T cell populations within frozen rat spleen section specimens were identified using a modification (vide infra) of a previously described immunohistochemical method [5,15], which employed biotinylated antibodies and an avidin D-horseradish peroxidase (Avidin D-HRP) conjugate to form irreversibly bound biotin-avidin-horseradish peroxidase complexes on the non-variable region of labeling antibodies. The antibody binding site was then detected with the addition of diaminobenzidine tetrahydrochloride (DAB), which imparted a brown color to the antibody complex sites.

Briefly, cryostat sections were fixed for 10 minutes in 4°C acetone, air-dried at room temperature, and washed in 2% phosphate-buffered saline (PBS) prior to application of the primary antibody. T-lymphocyte subsets were identified using monoclonal antibodies directed against rat T-helper cell antigens (MAS 1131 or W3/25, Pel-freeze Biologicals, Rogers, Arkansas, a mouse IgG anti-rat T-helper, 1:10 in 2% bovine serum albumin) and T non-helper (cytotoxic/suppressor) cell antigens (MAS 041 or OX8, Pel-freeze, mouse IgG anti-rat T non-helper, 1:100 in 2% bovine serum albumin) followed by a secondary biotinylated antibody (biotinylated goat F(ab')2 anti-mouse IgG, Tago, Inc., Burlingame, California, 1:10 in 2% rat serum), B-lymphocytes and plasma cells were identified using a biotinylated primary antibody directed against the mu heavy chain of surface immunoglobin (IgM) (biotinylated goat anti-rat IgM, American Qualex, Inc., La Mirada, California, 1:50 in 2% normal human serum). Mouse ascitic fluid (BRL-Life Technologies, Inc., Gaithersburg, Maryland, 1:200 in PBS) was substituted for the primary antibody as a negative control for non-specific background staining in each run. Incubation of each antibody application was performed at room temperature in a moist chamber for 20 minutes. Following the respective biotinylated antibody steps, slides were washed in 2% PBS and incubated with avidin D-HRP (Vector Labs, Sunnyvale, California, 1:80 in 2% rat serum), then washed again and incubated in 50ml of 2% DAB (Sigma Chemical Co., St. Louis, Missouri) in PBS with 0.5ml of 30% hydrogen peroxide for 5 minutes. After a serially repeated PBS wash and distilled water rinse the slides were incubated in a 5% copper sulfate solution for 5 minutes, serially washed and rinsed again, air-dried, and cover-slipped with Permount.

Modification of technique

Both biotinylated primary antibodies and biotinylated secondary antibodies in combination with an unmarked primary antibody were used. The main advantage conferred by the latter was cost saving and reagent standardization, but with the potential drawbacks of a slightly lengthened experimental procedure and non-specific binding of the secondary antibody causing background staining. Non-specific binding was minimized using a blocking antiserum (2% goat serum in PBS), but highly satisfactory results were more easily obtained using the biotinylated primary antibody. In addition, non-specific background staining caused by endogenous pseudoperoxidase activity was minimized by adding 0.5ml of hydrogen peroxide to the DAB bath.

Results

Eutopic remnants weighed more than ectopic remnants of equal initial weight and contained disproportionately more nucleated cells (much higher cell density), albeit proportionately fewer than the much larger sham-operated whole spleens (Table 1).

Table 1. FINAL SPLENIC MASS, TOTAL NUCLEATED CELL COUNTS (TNCC) AND CELL DENSITY (mean±SE)

Group	n	Mass (mg)	TNCC ($\times 10^6$)	Cell Density (TNCC/100mg)
200mg remnant				
Eutopic	14	330±28	470±48	144±9.0
Ectopic	14	92±17	49±16	37±8.7
p value		<0.001	<0.001	<0.001
Whole spleen				
Eutopic	18	1016±48	1725±168	166±12
Ectopic	12	410±52	134±37	33±7.9
p value		<0.001	<0.001	<0.001

On histologic examination, eutopic remnants were indistinguishable from sham-operated whole spleens (Fig. 1), both of which exhibited features characteristic of normal rat as well as human spleens. The distinctive white pulp morphology consisted of closely packed lymphocytes in the periarteriolar lymphoid sheath (PALS) and mantle zone (MT) as well as slightly larger and lighter-staining cells of the reactive-germinal center (GC). Outside of a parafollicular fibrous reticulum lay a thinner band of lymphocytes, the marginal zone (MZ), and beyond that, the red pulp. Characteristic cell shapes and sizes and mitotic activity common to clonal B-cell expansion were clearly seen (Fig. 1B) within the reactive germinal centers.

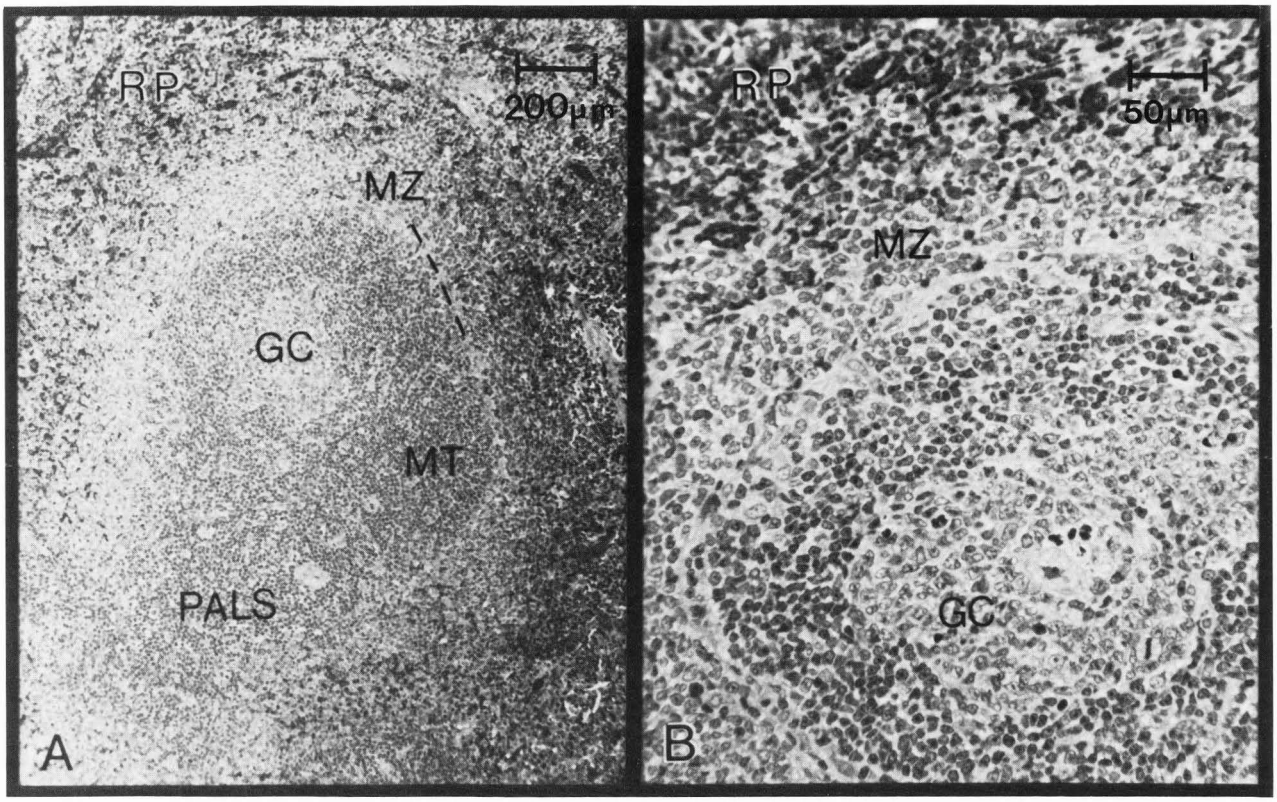

Fig. 1. Light microscopy (H & E) of whole rat spleen 60 days after sham operation. In eutopic remnants histology was similar. A) Intact white pulp architecture with prominent germinal center (GC) and adjacent mantle zone (MT). The marginal zone (MZ) is separated from the mantle zone by a strand of fibrous tissue (----). The periarteriolar lymphoid sheath (PALS) lies adjacent to a central arteriole. Red pulp (RP) surrounds white pulp (WP). B) Higher magnification of germinal center area in A. Note mitoses in GC indicating clonal expansion.

Immunoarchitecture

Serial section immunoperoxidase staining of sham-operated spleens and eutopic remnants (Figs. 2A, B, and C) demonstrated immunoarchitectural features of normal spleen, in particular, the characteristic distribution of B and T cell neighborhoods within the white pulp [5]. Biotinylated goat anti-IgM antibody delineated an interconnected ring-like pattern, characteristic of surface membrane staining, in the reactive germinal center (GC) as well as the adjacent marginal zone (MZ) of a reactive follicle. Darkly globular, cytoplasm-stained mature plasma cells were seen along the margins of normal periarteriolar lymphoid sheaths (PALS) (white or non-staining in Fig. 2A). A few scattered plasma cells were found within the mantle zone (MT) and in the peripheral red pulp (RP). The perifollicular reticulum lying between the germinal center and marginal zone and the central arterioles (seen adjacent to the letter S in PALS in Fig. 2A) failed to stain with anti-IgM or anti-T cell antibodies and thus appeared white in all sections.

In marked contrast, a double-staged biotinylated goat F(ab')2 anti-mouse IgG and mouse IgG anti-rat T-helper antibody complex demonstrated dense clusters of T-helper cells in the PALS, where IgM+ B cells were virtually absent. T-helper cells were also concentrated in the mantle zone (MT) where IgM+ B cells were rare. Throughout the rest of the reactive follicle (including GC) and peripheral red pulp, T-helper cells were present but in markedly reduced numbers.

Similarly, a double-staged antibody complex against rat T-nonhelper antigens (cytotoxic/suppressor cells) demonstrated abundant non-helper T cells in the PALS. The ratio of T-helper to T-cytotoxic/suppressor cells was approximately 2 to 1. T cytotoxic/suppressor cells were also prevalent in the mantle zone and red pulp but rare in the reactive germinal center and adjacent marginal zone.

In contrast, and consistent with the predominance of red pulp and paucity of centrally located (c.f. subcapsular) reactive follicles and periarteriolar lymphoid sheaths on H & E stained sections, single and multiple splenic autotransplants showed corresponding defects in immunoarchitecture. B and T cells were scattered in a disorganized distribution over wide areas, including regions adjacent to peripheral arterioles (Fig. 3A,B) where distinctive B and T cell zones were found in normal spleen (vide supra). In ad-

Fig. 2. Immunologic features of adjacent tissue sections of eutopic remnant 60 days after partial resection (immunoperoxidase stain). A) Anti-IgM antibody delineates abundant IG+ B cells in the white pulp, particularly in the germinal center (GC) and mantle zone (MT). B) Anti-T-helper antibody delineates abundant T-helper cells particularly in the periarteriolar lymphoid sheath (PALS). C) Anti-T-nonhelper antibody delineates abundant T suppressor/cytotoxic cells, albeit fewer than T-helpers, in the PALS. A, B, and C serial sections taken together define the B and T cell neighborhoods of "normal" spleen.

dition, follicles were reduced in number and largely confined to subcapsular areas.

Discussion

The spleen's immunoprotective function depends on initial trapping and filtering of poorly opsonized blood-borne particles such as encapsulated bacteria in the sinuses of the red pulp and thereafter both humoral and cellular responses in the B and T cell regions of the white pulp. Serial section immunohistochemical techniques delineate more specific features of the white pulp architecture: the localization, prevalence, and mutual near exclusivity of B and T cell neighborhoods. When these integrated functioning units comprising the splenic mass are reduced quantitatively or qualitatively below a critical level by operative removal, rupture, ischemia, or replacement (e.g. by reticuloendothelioses), the orderly sequence of particulate antigen processing is disrupted, rendering the host susceptible to fulminant septicemia from a variety of microorganisms.

This study found substantial architectural alterations in mature ectopic splenic remnants ("free" grafts with impaired vascularity) compared to eutopic remnants ("pedicle" grafts with intact blood supply), providing a structural basis for functional disparities [17,18]. Eutopic remnants retained their share of nucleated (largely lymphoid) cells in proportion to weight and displayed the mutually exclusive distribution of lymphocyte subpopulations characteristic of normal spleen. Ectopic remnants, in contrast, demonstrated less mass and even more sharply decreased total number of nucleated cells (accounting for a much lower cell density, only $\sim$ 20-25% of eutopic remnants), a deficiency of follicles, and widespread B and T cell disorganization.

Although these findings differ from several other studies [1-4,6,14], part of the difference may stem from interpretation of the predominant histology in ectopic remnants. For example, Dijkstra et al focus on follicular development in the peripheral subcapsular areas and interpret the presence of follicles as representing restored normal architecture. Yet they note: "follicles were not found in every section and per section only in 1 or 2 white pulp regions" [4]. We and others [7,8] focus on the dominant and widespread dearth of follicles, general disorganization of B and T cell populations and poor cellularity of these autotransplants. In addition, superimposition of intentional or inadvertent splenotropic "workloads" (such as foreign erythrocytes, carbon particles, or Bartonellosis), complicating other experiments but avoided by us, may have factitiously stimulated cellular enlargement and cellular proliferation (apparent regeneration) in ectopic remnants.

Further support for our conclusions is found in other clinical and experimental studies. Defective restoration of architecture and cellularity has been described in spontaneously engrafted splenic nodules in patients many years after traumatic splenic disruption [16]. Experimentally, whereas eutopic remnants produce antibody, trap damaged red blood cells, and protect against blood-borne pneumococcal challenge in proportion to remnant weight, autotransplants, even of substantial weight, function poorly and fail to protect in a highly spleen-sensitive model of postsplenectomy pneumococcemia [17,18]. Indeed, deaths from fulminant pneumococcemia occur in patients despite the presence of numerous and large ectopic splenotic nodules [10].

While a scar-free interstitial environment and intact lymphocyte homing and cell-cell interactions are no doubt essential to splenic architectural restitution, these features themselves likely depend on restoration of the normal pattern of blood flow to the devascularized ectopic splenic remnant. Regeneration of implanted tissues depends on the degree of angiogenesis induced [11-13]. While ectopic, freely engrafted liver induces minimal angiogenesis and undergoes little or no regeneration, bone marrow stimulates intense angiogenesis and undergoes rapid regeneration [12,13]. The spleen demonstrates some angiogenetic capacity although far less than marrow, perhaps accounting for suboptimal splenic regeneration [11]. The present study provides further immunohistochemical evidence that an intact splenic blood supply is necessary for maintenance of the complex integrated architecture that underlies the unique immunoprotective function of the spleen.

Acknowledgement

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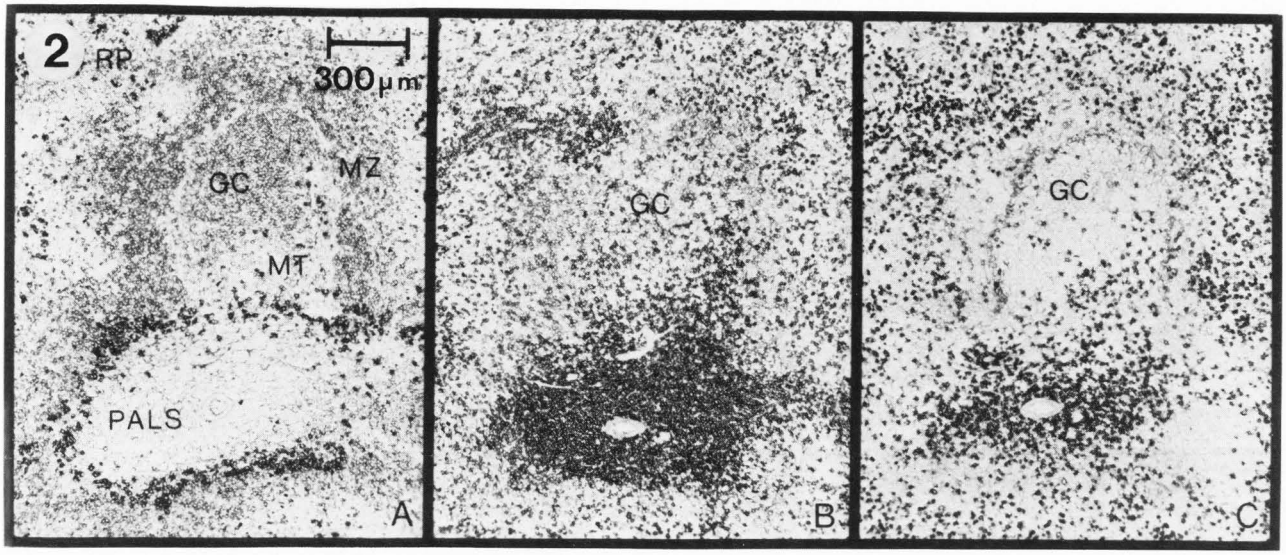

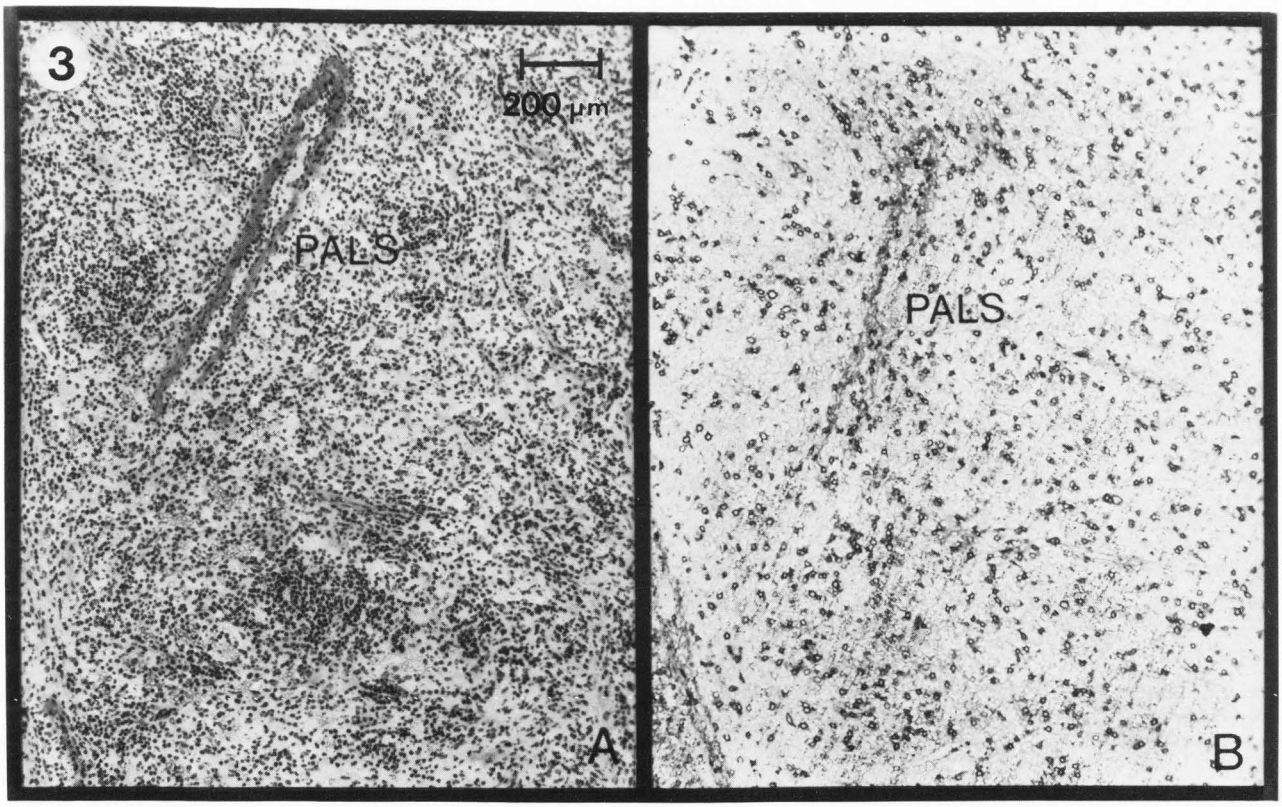

Fig. 3. Histologic and immunologic features of adjacent sections of splenic autotransplants 60 days after ectopic implantation (free grafts). A) Histologic features (H & E): loss of white pulp morphology (GC, MT, MZ); including distinction between white and red pulp. Further, the central arteriole is diagonally cut and adjacent PALS, normally well seen (Fig. 1), is absent. B) Immunologic features (immunoperoxidase stain): anti-T-suppressor cytotoxic antibody demonstrates diffuse scattering of T suppressor/cytotoxic cells in contrast to their abundance in normal PALS (Fig. 2C). Anti-mu and anti-T-helper antibody (not shown) show similar wide scattering of B and T helper cells in contrast to the mutual exclusivity of B and T cell zones in normal spleen.

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Discussion with Reviewers

M. Tavassoli: The authors have focused on the white pulp. Do they have observations on the organization of the red pulp?
Authors: Mutual exclusivity of B and T cell populations characterized the white pulp, forming the focal point of comparisons between eutopic and ectopic remnants. As revealed in the figures, scattering of B and T cells is normally found in red pulp and persists in both eutopic and ectopic spleens.

H. Gamliel: Why do the authors not provide higher magnification to illustrate their results and particularly to define background labeling and cross reactivity of anti-T conjugates?
Authors: Lower magnification was preferred to emphasize the "immunoarchitecture" pivotal to differentiating eutopic and ectopic remnants. Cross reactivity was obviated by monoclonal antibodies and effectively illustrated in Fig. 2, which demonstrates clearly separable B and T cell neighborhoods. Control studies excluding, in turn, primary and secondary antibody, biotin-avidin, and DAB revealed no significant background. The monoclonal antibodies are antigen-specific for T-suppressor and T-helper cells [3,5,15].

H. Gamliel: Except for higher density of labeling/staining, what were the criteria for classifying lymphocytes as plasma cells? How were monocytes identified and distinguished?
Authors: Eccentric nucleus and abundant cytoplasm were additional morphologic criteria used to identify plasma cells. Butyrate esterase was used to detect monocytes and histiocytes scattered through the spleen. To avoid cross reactivity with monocyte Fc receptors, we specifically used F(ab')2 reagents.

T.H. Ermak: Is the technique for eliminating nonspecific background staining correct? The term endogenous peroxidase should also be substituted for "pseudoperoxidase".
Authors: Initially hydrogen peroxidase neutralizes endogenous peroxidase obviating background staining. Peroxidase oxidizes the hydrogen peroxide, which subsequently reduces the DAB producing a colored precipitate. Pseudoperoxidase is a bona fide term referring to the peroxidase-like activity found in red cells in the red pulp of the spleen.

T.H. Ermak: Why have the figures not been designed to compare in the same figure eutopic and ectopic remnants and the magnifications made uniform for comparison?
Authors: The difference in magnification is intentional. Lower power Fig. 2 reveals the complexity of normal architecture while higher power Fig. 3 emphasizes the striking absence of architecture in ectopic remnants.

T.H. Ermak: Figs. 2B,C and 3B contain unaccounted for light-labeling patterns. Does this represent labeling by the secondary reagent, i.e., cross-labeling of cell-surface rat Ig on B cells by biotinylated goat anti-mouse Ig?
Authors: Our biotinylated goat anti-mouse Ig is an F(ab')2 reagent designed to obviate nonspecific binding due to Fc receptors. Cross reactivity of T antigens is highly unlikely since highly monospecific monoclonal antibodies were used as the primary antibody and F(ab')2 reagents as the secondary antibody. Perhaps the reviewer perceives the slight photographic shadow cast by the negative cells.

M. Tavassoli: Is it possible that 60 days was not adequate time for implant regeneration to be completed? Is it possible that the abdominal site is not an optimal supporting bed and implants made in other sites regenerate better?
Authors: Previous experience with subcutaneous implants substantiates that these ectopic grafts are no better than and probably inferior to intra-abdominal implantation, which is used clinically probably reflecting the better vascular supply of the omentum and peritoneum.

M. Tavassoli: A major question in this work is the absence of functional parameters. Is it possible that these implants, despite the poor organization, can still carry out some normal splenic functions? It is at least likely that they can carry out normal splenic functions if these functions are not challenged by extraordinary workload, i.e. the difference may be a quantitative one.
Authors: The functional parameters questioned here are cited in the bibliography. Indeed, trapping function is detectable in the remnants. However, the bulk of evidence suggests that these remnants function poorly in the integrated activity of protection against postsplenectomy sepsis, the key danger of the asplenic state. It is possible that ectopic remnants could be "boosted" by a variety of immunomodulating agents to optimal function.