Immunohistochemical Localization of Smooth Muscle Myosin in Human Spleen, Lymph Node, and Other Lymphoid Tissues

Unique Staining Patterns in Splenic White Pulp and Sinuses, Lymphoid Follicles, and Certain Vasculature, With Ultrastructural Correlations

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The anatomic distribution of smooth muscle myosin, a contractile protein, was determined in a variety of lymphoid tissues (spleen, lymph nodes, tonsils) with the use of highly specific rabbit antibodies to human uterine smooth muscle myosin and an indirect immunoperoxidase technique. In the spleen, in addition to the anticipated immunoreactivity in the walls of arteries, veins, splenic capsule, and trabeculas, other staining patterns were observed. Smooth muscle myosin-containing cells which comprised the adventitia of the trabecular arteries appeared continuous with myosin-containing reticular cells of the white pulp. The latter cells assumed a circumferential pattern within the periarteriolar lymphoid sheaths, then blended delicately with the red pulp at the marginal zone. Ultrastructurally, immunogold techniques demonstrated that smooth muscle myosin in these cells was localized to cytoplasmic filaments. Within the red pulp, a different and distinct staining pattern was observed for the splenic sinuses. Short, regular, orderly, and repetitive bands of immunoreactivity, aligned parallel to the long axis of the sinus, extended between contiguous ring fibers. By immunoelectron microscopy these structures corresponded to distinct bundles of filaments in the endothelial lining cells of the splenic sinuses. Factor VIII associated antigen was also identified in the splenic lining cells in cryostat and paraffin sections, and ultrastructurally. Within the red pulp of the spleen, the sheaths of sheathed capillaries also revealed strong immunoreactivity for smooth muscle myosin. Other sites of immunohistochemical localization of smooth muscle myosin included dendritic reticulum cells present in reactive follicles and in nodular non-Hodgkin's lymphomas. Certain vascular structures, specifically sinus lining cells and Schweigger-Seidel capillary sheaths of the spleen and postcapillary venules of lymph nodes and tonsils, coexpressed smooth muscle myosin and Factor VIII associated antigen. The patterns of localization of smooth muscle myosin are correlated with anatomic structures and possible tissue functions. (Am J Pathol 1986, 123: 440–453)

SMOOTH MUSCLE MYOSIN, a contractile protein, is a well-recognized component of smooth muscle cells of blood vessel walls and the muscular wall of the gastrointestinal tract. Myosin has been isolated from both smooth muscle and nonmuscle cells, even though myosin filaments are not apparent in the latter by conventional ultrastructural studies.[1] The different types of myosin are highly variable in their primary structures and enzymatic activities, permitting their distinction using immunohistochemical techniques.[2] The latter approach potentially represents a more sensitive method for

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identification of these contractile proteins and permits assessment of their structural organization in tissues. Using an immunoperoxidase method and rabbit antibodies specific for human smooth muscle myosin, we recently described the staining profile of smooth muscle myosin in most normal human tissues.[3] This technique permitted characterization of smooth muscle myosin in large and small bundles of fibers as well as in single cells or groups of cells, further delineated their architectural alignment, and verified the specificity of the antibodies to smooth muscle myosin. These studies were recently extended to lymphoid tissues and demonstrated staining patterns for smooth muscle myosin which were unanticipated, particularly its localization in the spleen, lymphoid follicles, and certain vasculature.

This report describes the immunohistochemical profile of smooth muscle myosin in lymphoid tissues including spleen (with particular attention to anatomic compartments), lymph nodes, and tonsils, and correlates the patterns of localization with known structural components of these tissues. Sites of smooth muscle myosin were further defined with immunoultrastructural techniques. The localization of Factor VIII associated antigen in vascular structures of these tissues was also determined using immunohistochemical methods and correlated with the distribution of smooth muscle myosin.

Materials and Methods

All tissues represented surgical specimens of the Brigham and Women's Hospital. Lymph nodes (4) exhibited nonspecific reactive changes. Ten nodes from patients with nodular non-Hodgkin's lymphoma were also evaluated. Tonsils (6) were obtained from patients with chronic tonsillitis. Normal small intestinal tissue (terminal ileum) was obtained from ilecocolectomy specimens (2). Spleens (13 total) were obtained from patients with no evidence of hematopoietic disorders (7), represented uninvolved spleens from negative staging laparotomies of patients with Hodgkin's disease (3), or were obtained from patients with idiopathic thrombocytopenia purpura (3). For cryostat section studies, tissues were embedded in OCT (Ames Co., Miles Laboratories Inc., Elkhart, Ind), were snap-frozen in isopentane-dry ice, then stored in a Revco freezer (−60 C). A few specimens were also fixed either in methacarn (methanol-chloroform-glacial acetic acid; 60:30:10, vol/vol/vol) overnight, dehydrated through absolute alcohol and xylene, and embedded in paraffin, or in 10% neutral buffered formalin. Prior studies[3] demonstrated that immunoreactivity for smooth muscle myosin was best preserved in cryostat sections, with limited antigenic preservation with Zenker's solution or Methacarn, and loss of nearly all reactivity with formalin fixation.

Rabbit antibodies to human uterine smooth muscle myosin were prepared as previously described.[2-4] By Ouchterlony double immunodiffusion, these antibodies formed a single precipitin line with extracts of human uterus and did not cross-react with extracts of skeletal, platelet, or cardiac myosin.[2] The specificity of this antiserum was further verified by immunoprecipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis.[5] Additional characterization on immunoblots of vascular smooth muscle extracts demonstrated the specificity of the antiserum for the heavy chain of smooth muscle myosin.[5] An extensive study of cryostat sections of normal human tissues further substantiated the specificity of the antiserum.[3]

Cryostat sections (4–5 μ) were placed on gelatin-coated slides, immersed for several seconds in acetone (room temperature), then placed in a cryostat to dry, generally overnight. Immediately prior to evaluation, sections were fixed for 5–7 minutes in cold acetone, then placed in 0.05 M Tris buffer, pH 7.6 (supplemented with 2% normal swine serum).

Immunoperoxidase studies of cryostat sections were performed as previously described.[3] Briefly, slides were sequentially incubated with rabbit anti-smooth muscle myosin antiserum (1:200, 1:500, 1:1000, 1:2000 dilutions) for 1 hour at room temperature, followed by swine anti-rabbit immmunoglobulin serum (1:30 dilution, Dako Corp., Santa Barbara, Calif), and horseradish peroxidase–rabbit anti-horseradish perodixase immune complexes (PAP, 1:100 to 1:300 dilution, Dako Corp.). The latter incubations were for 30 minutes. Antibody localization was determined with a peroxidase reaction with 3,3′-diaminobenzidine tetrahydrochloride (Aldrich Chemical Co., Milwaukee, Wis) as chromagen. Sections were counterstained with methyl green solution or hematoxylin.

For paraffin sections (methacarn-fixed tissues), immunoperoxidase studies were performed as described for cryostat sections, following deparaffinization and a 30-minute incubation in methanolic peroxide (5 parts methanol to one part 3% hydrogen peroxide, vol/vol). However, higher concentrations (1:25, 1:50, and 1:100 dilutions) of primary antibody were necessary for detection of smooth muscle myosin.

For all studies, sections of uterine smooth muscle wall were employed as positive controls. Negative controls included serial sections of a given tissue, substituting either Tris buffer, preimmune rabbit serum, or immune serum adsorbed with smooth muscle myosin, for the immune serum.

In some cases, additional studies with monoclonal

antibodies to Factor VIII associated antigen (1:25, 1:50 dilution; Dako Corp.) or with monoclonal antibodies to dendritic reticulum cells (1:25, 1:50 dilution; Dako Corp.) were performed, to verify cell types immunoreactive for smooth muscle myosin. In these studies, incubation with primary monoclonal antibody (1 hour) was followed by sequential incubations (45–60 minutes) with peroxidase-conjugated rabbit anti-mouse immunoglobulin antibody (1:40 dilution, adsorbed reagent, Dako Corp.) and peroxidase-conjugated swine antirabbit immunoglobulin antiserum (1:60 dilution, Dako Corp.). The latter reagents were diluted with 0.1 M Tris buffer supplemented with 4% human AB serum, to minimize cross-reactivity with human immunoglobulin. Antibody localization was determined by a peroxidase reagent, as noted for polyclonal antibody studies. For formalin-fixed specimens, either monoclonal or polyclonal antibodies to Factor VIII associated antigen (both from Dako) were evaluated. Formalin-fixed tissues were processed following preliminary trypsinization, as previously described,[6] to provide optimal immunoreactivity. For polyclonal antibodies to Factor VIII associated antigen (1:100, 1:200, 1:500 dilutions), a PAP technique similar to that employed for detection of smooth muscle myosin in paraffin sections was employed.

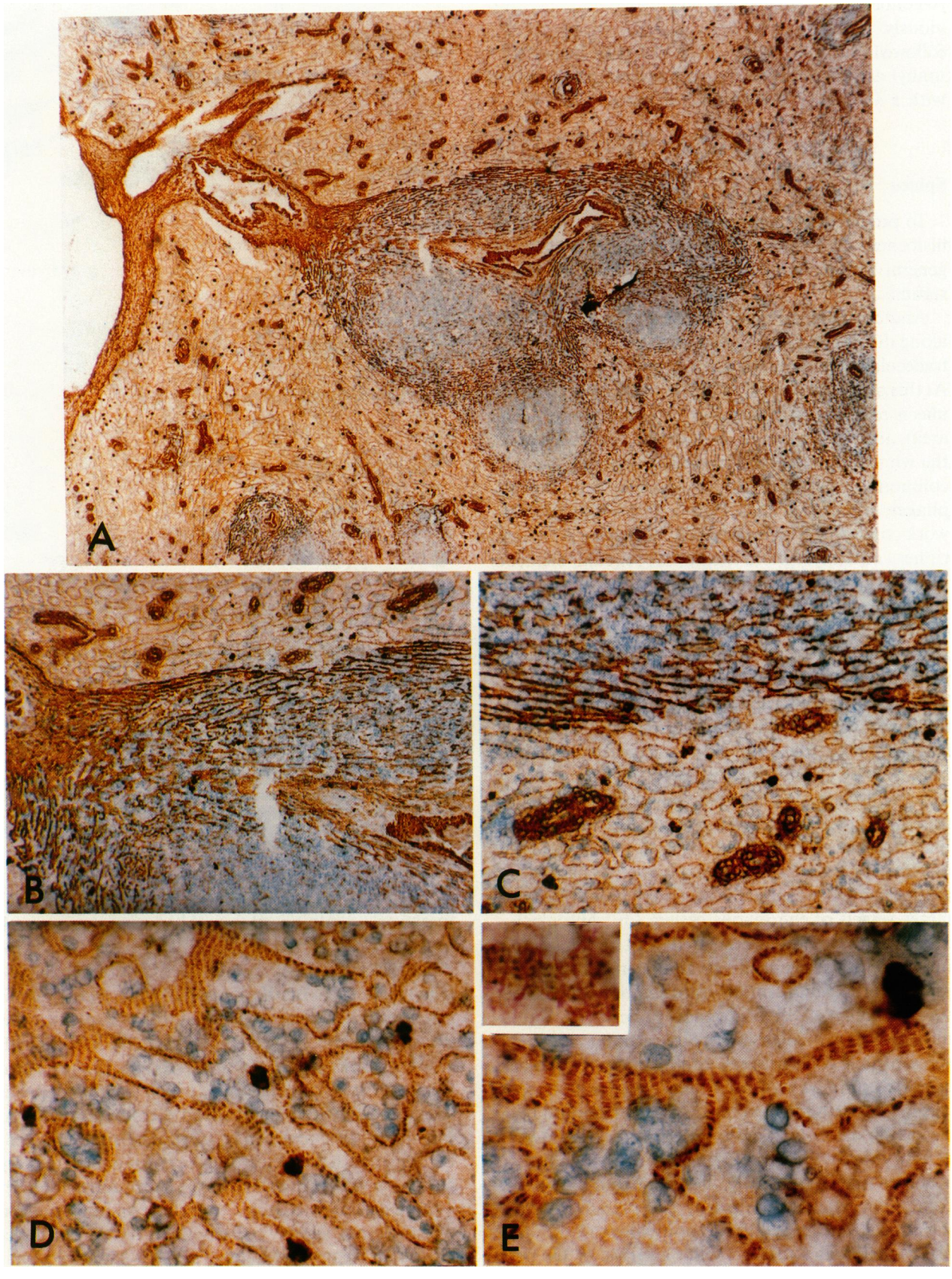
For routine electron microscopy, 1-mm blocks of spleen were obtained immediately after surgical removal. The tissue was fixed overnight in half-strength Karnovsky's fixative, postfixed for 2 hours in osmium tetroxide, dehydrated in alcohols, and embedded in Polybed (Polyscience, Warrington, Pa). Thick (1 μ) sections were cut and stained with toluidine blue for selection of areas for thin-sectioning. Thin, silver-gold, sections of representative areas of red and white pulp were cut and stained with uranyl acetate followed by lead citrate. The sections were examined on a JEOL-Co JEMS 100 electron microscope.

For immunoelectron microscopy, 1-mm cubes of spleen were fixed for 2 hours in a mixture of 3% paraformaldehyde, 0.1% glutaraldehyde. The sections were then washed three times in phosphate-buffered saline (PBS), pH 7.4. These washings were followed by a 10-minute incubation with 0.5 M NH_4Cl to quench unreacted aldehyde groups. The ammonium chloride was removed by several PBS washes, and the tissues were then embedded in Lowicryl K4M according to a protocol previously described.[7] Briefly, the tissues were first dehydrated in increasing concentrations of ethanol at −20 C. The tissues were then infiltrated with increasing concentrations of Lowicryl in absolute ethanol at −35 C. Final infiltration was accomplished by overnight incubation in pure Lowicryl. The tissue was polymerized initially at −35 C with ultraviolet light. Final polymerization took place at room temperature. Thick sections were then cut and stained with toluidine blue for selection of areas for thin-sectioning. Thin sections were then cut and picked up on formvar-coated nickel grids for immunoelectron microscopy.

Tissue was also processed for electron microscopy by immersion directly in 70% ethylene glycol at 4 C. The tissue was stored in the ethylene glycol until embedding. For embedding, the tissue was dehydrated with increasing concentrations of ethylene glycol at −35 C. The tissue was then infiltrated with increasing concentrations of Lowicryl K4M in ethylene glycol. Final infiltration was accomplished by an overnight incubation in pure Lowicryl. The tissue was then cut and sectioned as previously described.

For immunoelectron microscopy, the grids were first incubated for five minutes with 0.5% egg albumin to occupy nonspecific binding sites. This was followed by a 2-hour incubation with the anti-myosin antisera (dilutions 1:10, 1:20, and 1:40). The primary antisera was removed by two washes with PBS, and the tissues then reacted with protein A–colloidal gold for one hour. For studies employing monoclonal antibodies to Factor VIII associated antigen, incubation with the primary antibody was followed by sequential incubations with rabbit anti-mouse immunoglobulin antiserum (Dako Corp.), and protein A–colloidal gold reagent. All incubations were performed at room temperature. The protein

Figure 1—Spleen, cryostat section. **A**—At low power, immunoreactivity for smooth muscle myosin (*brown*) is apparent within the walls of large blood vessels within the trabeculas, including the adventitia of a trabecular artery (*upper left*) which splays, then appears in continuity with the circumferential "reticular" network of the white pulp. Smooth muscle walls of small arteries are also immunoreactive, as are sheathed capillaries within the red pulp. A delicate staining pattern outlines the sinuses, though a distinct pattern is not discernible at this magnification. The very small darkly staining cells scattered within the tissue represent myeloid cells with strong endogenous peroxidase activity. **B**—At higher magnification the adventitia of the trabecular artery appears continuous with the reticular network of the white pulp. Strong fibrillar/filamentous staining for smooth muscle myosin is noted. Smooth muscle wall of the central artery (*lower right*) and the sheathed capillaries (*upper*) are also reactive for smooth muscle myosin, with a delicate staining pattern noted for sinuses. **C**—At the marginal zone, the myosin positive "reticular" cells (*upper*) blend with the myosin containing splenic sinus cells (*lower*). Focally, delicate connections appear to join these areas. Sheathed capillaries (*lower*) in the red pulp are also strongly positive for smooth muscle myosin. Occasional peroxidase-positive (endogenous) myeloid cells are present, particularly in the red pulp. Lymphoid cells and splenic cords are nonreactive. **D**—Within the red pulp, the splenic sinuses exhibit a distinct pattern of immunoreactivity for smooth muscle myosin, characterized by short, repetitive orderly bands oriented parallel to the long axis of the sinus. These bands also occur parallel to each other and circumscribe the lumen of the sinus. The darkly staining foci represent myeloid cells (endogenous peroxidase). **E**—Higher magnification of the myosin-containing bands in the walls of the splenic sinuses. With a periodic acid–Schiff (PAS) counterstain, the bands of immunoreactivity extend between the PAS-positive ring fibers of the sinuses (*inset*). A neutrophil with strong endogenous peroxidase activity is present in the section (*upper right*). (Immunoperoxidase technique, methyl green counterstain; **A**, ×45; **B**, ×95; **C**, ×180; **D**, ×430; **E**, ×800; **Inset**, ×800)


A
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A-colloidal gold reagent was prepared as described previously.[8] The grids were again washed twice with PBS followed by distilled water. They were then stained with uranyl acetate followed by lead citrate and examined with a JEOL-Co JEMS 100 electron microscope.

Results

Spleen

To provide a basis of comparison for the immunohistochemical studies, the known vascular network and general architecture of the human spleen are briefly summarized.[9-11]

Anatomically, the branches of the splenic artery pass along the trabeculas and repeatedly branch, leaving the trabeculas when reduced to a diameter of about 0.2 mm. At this point, the surrounding adventitia of the trabeculas is replaced by a sheath of lymphoid tissue (T-cell area), and the vessel is designated as a central artery of the white pulp. The periarteriolar lymphoid sheath is composed of lymphoid cells, plasma cells, macrophages, and other cells which lie in a reticular meshwork composed of reticular cells and fibers. At the periphery of the lymphoid sheath, the reticular elements tend to assume a circumferential pattern. The central artery continues to branch; and on reaching a diameter of 40–50 μ, the lymphoid sheath is markedly reduced and the vessel branches into two to six penicillar arteries or arteries of the red pulp. The penicillar arteries branch into several capillaries, some of which have a thickened sheath (Schweigger-Seidel sheath). At the marginal zone, the white pulp interfaces with the red pulp of the spleen, which is composed of cords and sinuses. Whether the circulation is open (from vessels through cords to sinuses) or closed (through endothelial lined channels only, ie, directly into the sinuses) is a matter of dispute, though a combination of both mechanisms has been suggested. Ultimately, blood enters the larger vessels of the venous system (in trabeculas).

Thirteen spleens were evaluated, with similar findings observed for all specimens. Immunoperoxidase studies of cryostat sections demonstrated smooth muscle myosin-containing cells in the splenic capsule, splenic trabeculas, and the muscular wall of large arterial vessels and veins. Muscle coats of small arteries and arterioles also exhibited staining for smooth muscle myosin. Beyond this point, the pattern of localization of smooth muscle myosin was unexpected and remarkable. As observed at low power in Figure 1A, which includes a trabecular area with arterial branching, the smooth muscle myosin-containing cells constituting part of the adventitia of the arterial wall appear continuous with the immunoreactive circumferential reticular network of the periarteriolar area (Figure 1A and B). Within this reticular meshwork, staining presumably represents smooth muscle myosin in reticular cells, which exhibit a cytoplasmic fibrillar staining pattern for this contractile protein (Figure 2). In the marginal zone, this complicated network appeared to blend with the splenic sinus walls and red pulp cords (Figures 1C and 3), with delicate connections noted in some sections (Figure 3). In the B-cell areas of white pulp, which generally appear eccentric to the lymphoid tissue surrounding the central artery, the circumferential reticular type cells containing smooth muscle myosin are noted only at the very periphery of this area. Within the B-cell zone, however, a more delicate stellate or branching staining pattern was observed, possibly corresponding to dendritic reticulum cells. Sequential cryostat sections employing pan-B or pan T-cell monoclonal antibodies demonstrated

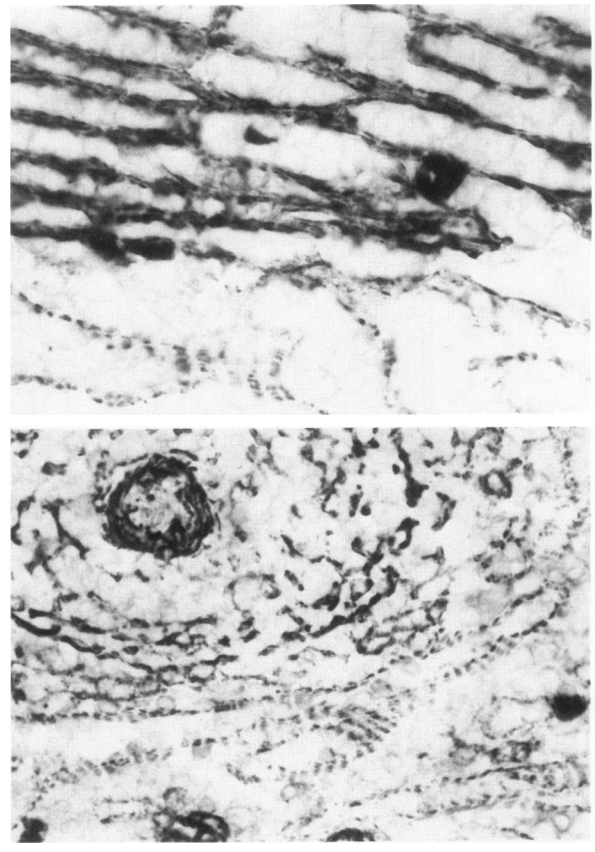

Figure 2—Spleen, marginal zone, cryostat section. Higher magnification of immunoreactivity patterns for smooth muscle myosin reveals fibrillar and/or filamentous staining (*black*) in cells of the white pulp (*upper*) and more delicate, short regular filament or bands in the contiguous red pulp. (Immunoperoxidase technique, methyl green counterstain, ×700) **Figure 3**—Spleen, marginal zone, cryostat section. This section illustrates focal delicate connections between the smooth muscle myosin-containing cells of the white pulp (*upper*) and those of the splenic red pulp. In the white pulp, staining for smooth muscle myosin is noted in the smooth muscle wall of the central artery and in "reticular" type cells of the periarteriolar sheath. In the red pulp, the staining pattern assumes the pattern of small regular bands in sinus lining cells. (Immunoperoxidase technique, methyl green counterstain, ×500)

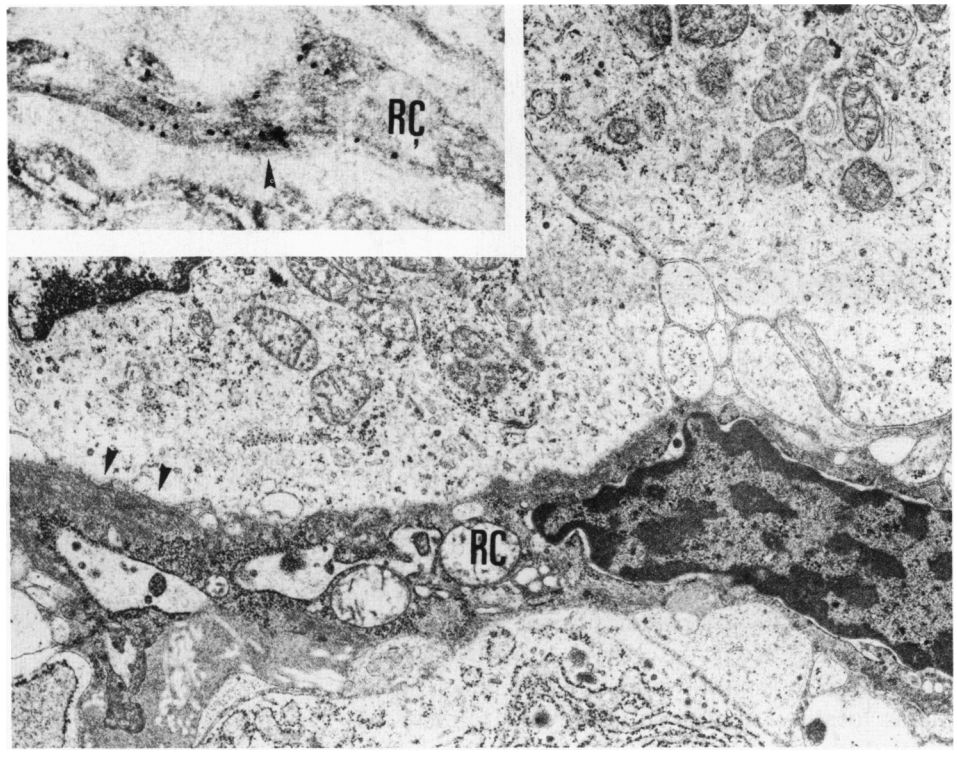

Figure 4—Splenic white pulp. In this electron micrograph, a reticular cell (RC) with a long cell process and bundles of cytoplasmic filaments (*arrows*) oriented parallel to the long axis of the cell interdigitates with lymphoid cells. With an immunogold technique, reticular cells (*RC*, **Inset**, *upper left*) reveal immunoreactivity for smooth muscle myosin, with localization to cytoplasmic filaments (*arrow*). (Ethylene glycol fixed tissue, uranyl acetate and lead citrate, ×6000; **Inset**, ×6000)

that the pattern of immunoreactivity for smooth muscle myosin in the white pulp was localized predominantly in the T-cell zones.

Ultrastructural studies corroborated and further elucidated the light-microscopic observations. The splenic white pulp consisted predominantly of lymphoid cells in various stages of transformation with occasional plasma cells, admixed with "reticular" cells. The latter cells resembled myofibroblasts with spindle-shaped nuclei and abundant anastomosing rough endoplasmic

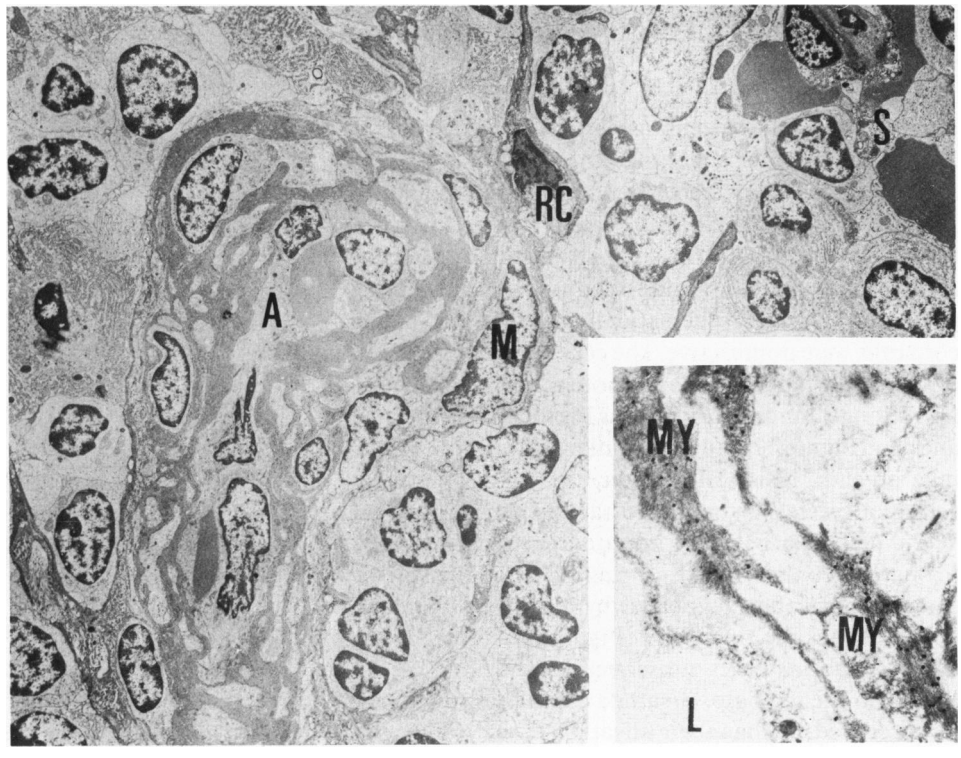

Figure 5—Electron micrograph of splenic arteriole at the junction of red and white pulp. Smooth muscle cells (*M*) are present within the arteriolar wall, and reticular type cells (*RC*) are seen within the adventitia. A sinus (*S*) is seen in the *upper right*. The **Inset** demonstrates another site of immunoreactivity for smooth muscle myosin (*MY*), with localization to filaments of cells comprising the Schweigger-Seidel sheaths of a sheathed capillary (*L*), as defined by an immunogold technique. (Ethylene glycol fixed tissue, uranyl acetate and lead citrate, ×1000; **Inset**, ×5000)

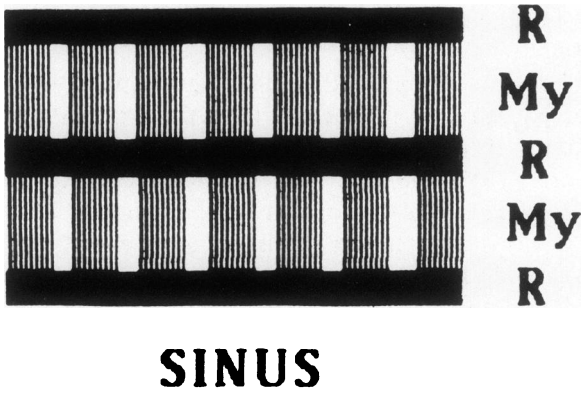

Figure 6—Schematic diagram of the wall of a splenic sinus. Short bands (filaments) aligned in the long axis of the sinus are immunoreactive for smooth muscle myosin (*MY*) and occur in a regular pattern between contiguous ring fibers (*R*). Compare with Figure 1E.

reticulum (Figure 4). In addition, these cells have aggregated bundles of cytoplasmic filaments arranged parallel to the long axis of the cells (10–12 nm in diameter; 300–500 nm in length). Immunoelectron-microscopic studies localized smooth muscle myosin to these filaments (Figure 4). These cells also have extemely long cell processes which interdigitate with the lymphoid cells. In addition, the "reticular" cells of the white pulp resembled cells seen within the adventitia of small arterioles (Figure 5). There appeared to be continuity between these adventitial cells and cells within the white pulp and the cords of the splenic red pulp. Smooth muscle myosin was also localized in the cytoplasm of these adventital cells with the use of an immunogold technique.

The staining pattern for smooth muscle myosin in the red pulp of the spleen was particularly noteworthy. A distinct, regular, repetitive linear staining pattern (stepladder effect), characterized by short bands aligned parallel to the long axis of the splenic sinuses, was observed consistently in the splenic sinus lining cells of all spleens evaluated (Figure 1D and E). This staining pattern is presented schematically in Figure 6. Splenic cord cells were nonreactive. The cells containing the distinct linear arrangements of smooth muscle myosin were also strongly immunoreactive for Factor VIII associated antigen (Figures 7 and 8; granular cytoplasmic staining), an endothelial cell marker,[12] as were endothelial cells of all vessels, in both cryostat and paraffin studies. The short linear bands of immunoreactivity appeared to span or connect adjacent ring fibers of the splenic sinuses, as further delineated by a PAS counterstain which defines the ring fibers (Figure 1E, inset) and by ultrastructural studies subsequently described.

Within the red pulp, sheathed capillaries were readily discerned in that their sheaths were strongly im-

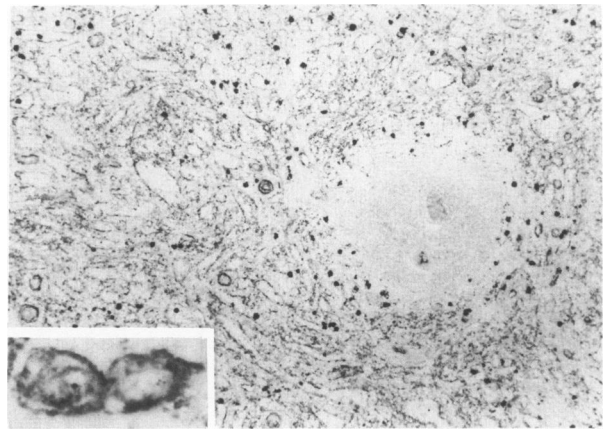

Figure 7—Spleen, cryostat section. Using monoclonal antibodies to Factor VIII associated antigen, at low power, immunoreactivity is predominantly observed in the red pulp, particularly in splenic sinus cells and Schweigger-Seidel sheaths. The staining pattern for cells comprising capillary sheaths is not readily discerned at low power and is therefore illustrated at high magnification in the inset. The limited staining in the white pulp is present in arterial endothelium. Small darkly staining foci scattered throughout the tissue represent myeloid cells with strong endogenous peroxidase activity. (Immunoperoxidase technique, methyl green counterstain, ×65; **inset**, ×400)

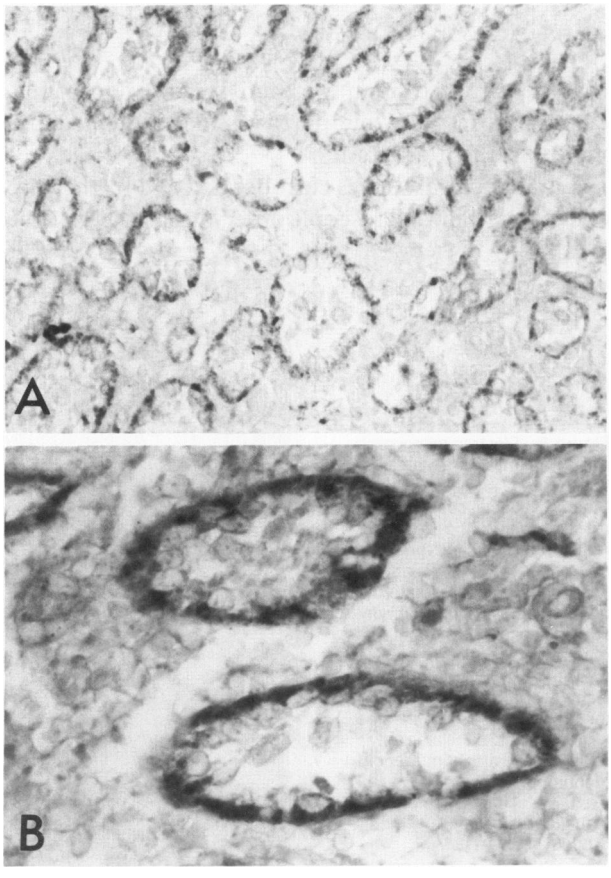

Figure 8—Spleen, red pulp, paraffin section, formalin fixation. **A**—Using polyclonal antibodies, Factor VIII associated antigen is localized to splenic sinus lining cells, with a granular staining pattern. Cords are nonreactive. **B**—Higher magnification of the staining pattern observed for Factor VIII associated antigen. (Immunoperoxidase technique, methyl green counterstain; **A**, ×400; **B**, ×850)

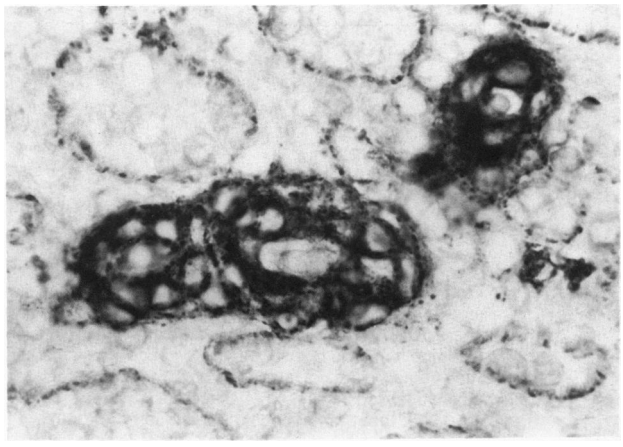

Figure 9—Spleen, red pulp, cryostat section. Strong cytoplasmic staining for smooth muscle myosin is noted for the sheaths of the sheathed capillaries. A more delicate pattern is observed for sinus lining cells. Cords are nonreactive. (Immunoreactive technique, methyl green-counterstain, ×550)

munoreactive for smooth muscle myosin (Figures 1 and 9). These capillaries are difficult to identify in routine sections of human spleens. Cells of the sheaths also exhibited immunoreactivity for Factor VIII associated antigen (Figure 7, inset) as did the endothelial cells of these sheathed capillaries.

Routine electron microscopy confirmed previous observations regarding splenic architecture within the red pulp.[9-11,13] As illustrated in Figure 10, ring fibers provided a scaffolding from which sinusoidal lining cells are suspended. A striking feature of the sinusoidal lining cells is the presence of bundles of aggregated thin (5–6 nm in diameter) and thick (12 nm in diameter) filaments, resembling dense bodies of smooth muscle cells, which extend between and insert into ring fibers (Figure 11). These cells also have numerous pinocytotic vesicles, microtubules, and some cells have rodlike structures with parallel laminations which resemble Weibel-Palade bodies (Figure 12). Thus the sinus lining cell appears to be a "hybrid," exhibiting features of endothelial and smooth muscle cells. Using an immunogold technique, smooth muscle myosin was localized to the bundles of filaments in sinusoidal lining cells (Figure 11). These structures would correspond to the bandlike pattern of immunoreactivity for smooth muscle myosin observed in cryostat sections (Figure 1). Immunoelectron microscopy also localized smooth

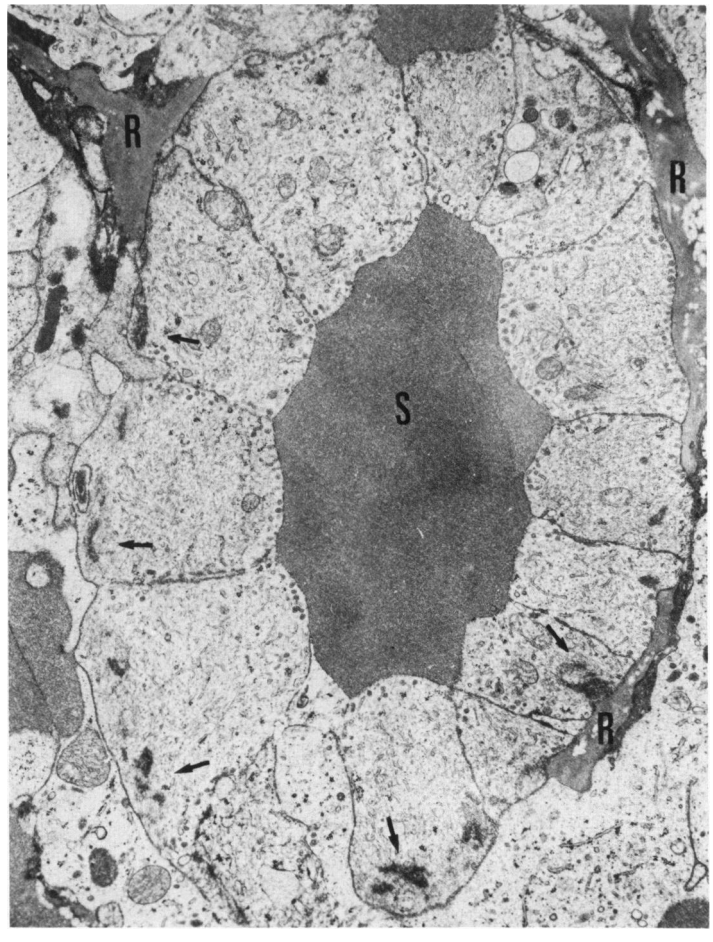

Figure 10—This electron micrograph of spleen illustrates a cross-section of a normal sinus (*S*). The ring fibers (*R*) forms a discontinuous scaffolding for the splenic lining cells. Bundles of thick and thin filaments (*arrows*) run circumferentially around the sinus, oriented at the basal aspect of the cells. (Uranyl acetate and lead citrate, ×6000)

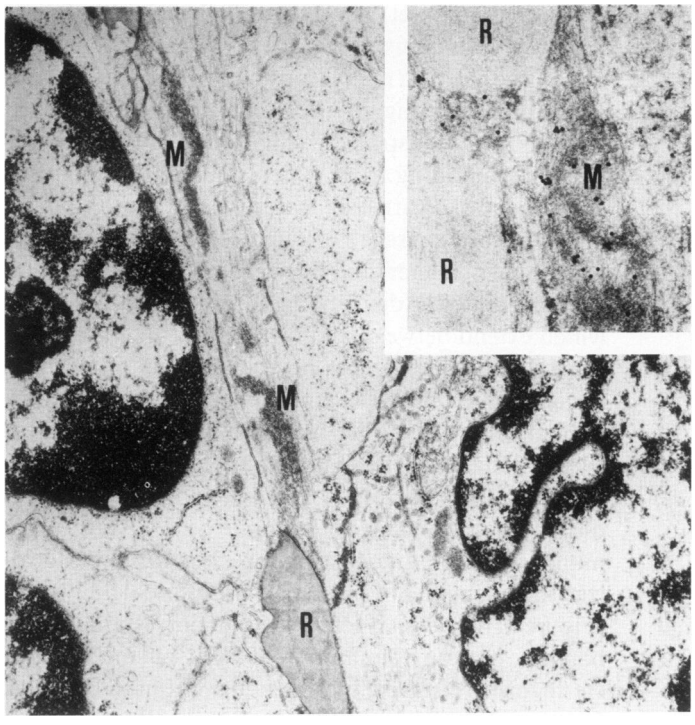

Figure 11—Splenic red pulp. Bundles of thick and thin filaments (*M*) in a sinus lining cell appear to insert into the ring fibers (*R*). The *inset* illustrates labeling of the filaments (*M*) for smooth muscle myosin with colloidal gold as a marker. Ring fibers (*R*) are not labeled. (Paraformaldehyde–glutaraldehyde fixed tissue, uranyl acetate and lead citrate, ×10,000; **inset**, immunogold technique, ×15,000)

muscle myosin to filaments within the cytoplasm of cells comprising sheaths of some arterial capillaries (Figure 5, inset). With monoclonal antibodies to Factor VIII related antigen, immunoreactivity was observed in Weibel-Palade bodies and cytoplasmic vesicles of the same cells (Figure 12). Within the splenic cords is a melange of cell types, including histiocytes, mast cells, plasma cells, occasional neutrophils, and cells with elongate cell processes resembling "reticular cells." These cells were not labeled for either smooth muscle myosin or Factor VIII related antigen with the use of an immunogold technique.

Immunoreactivity for smooth muscle myosin proved to be extremely sensitive to fixation with aldehydes. In

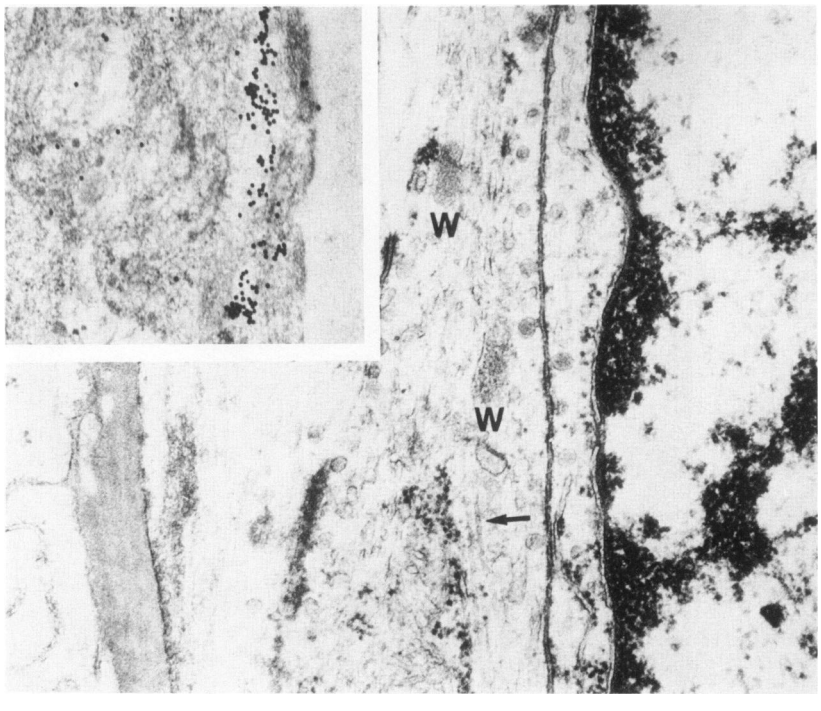

Figure 12—Ultrastructure of a sinusoidal lining cell with Weibel-Palade bodies (*W*) and microtubules (*arrow*). By an immunogold technique, the *inset* demonstrates localization of Factor VIII related antigen with labeling of the rod-like Weibel-Palade bodies and also cytoplasmic vesicles. (Paraformaldehyde–glutaraldehyde fixed tissues, uranyl acetate and lead citrate, ×5000; **inset**, ×5000)

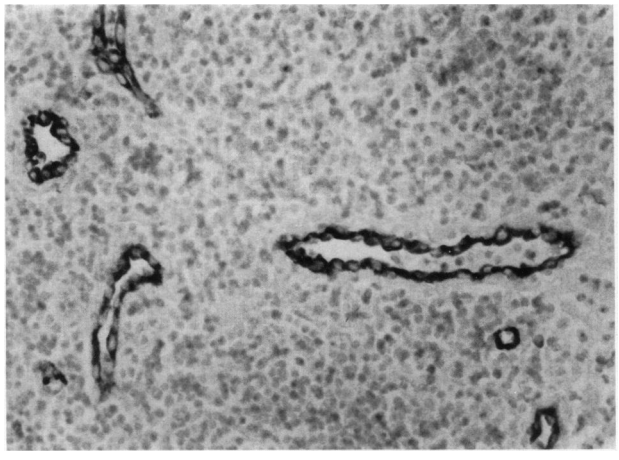

Figure 13—Tonsil, paraffin section, Methacarn fixation. Luminal lining cells of postcapillary venules, the predominant type of blood vessel present in the illustration, reveal strong cytoplasmic immunoreactivity for smooth muscle myosin. Lymphoid cells are nonreactive. (Immunoperoxidase technique, methyl green counterstain, × 250)

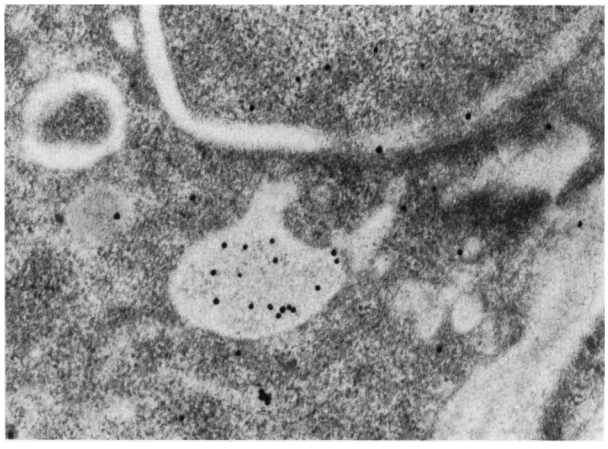

Figure 15—Postcapillary venule. The immunogold technique demonstrates localization of Factor VIII related antigen to cytoplasmic vesicles of endothelial lining cells. (Paraformaldehyde–glutaraldehyde fixation, uranyl acetate and lead citrate, × 10,000)

order to demonstrate myosin in "reticular" type cells and smooth muscle of arteriole walls, tissue was processed directly in ethylene glycol for immunoelectron microscopy.

Lymph Nodes/Tonsils

Four nonneoplastic lymph nodes and 6 tonsils were evaluated, with relatively similar findings observed in both tissues. Occasional cells in the capsule of the node were immunoreactive for smooth muscle myosin. Arterial and venous blood vessels revealed strong staining of smooth muscle walls and an absence of endothelial cell staining. However, a unique staining pattern was noted for postcapillary venules. Cells lining the lumens of these vessels, typically a single layer of tall cuboidal type cells, exhibited strong diffuse cytoplasmic staining (granular/filamentous) for smooth muscle myosin (Figure 13). These cells were also immunoreactive for Factor VIII associated antigen (diffuse, granular cytoplasmic staining; Figure 14), as were all other endothelial cells in the tissue. Except for staining of the vascular elements, interfollicular (T cell) zones were nonreactive. Ultrastructurally, endothelial cells of the postcapillary venules contained microtubules, filaments, pinocytotic vesicles, and elaborated a basal lamina. Weibel–Palade bodies were extremely rare. Immunoelectron microscopy demonstrated localization of Factor VIII related antigen primarily to cytoplasmic vesicles filled with faintly electron-dense material (Figure 15).

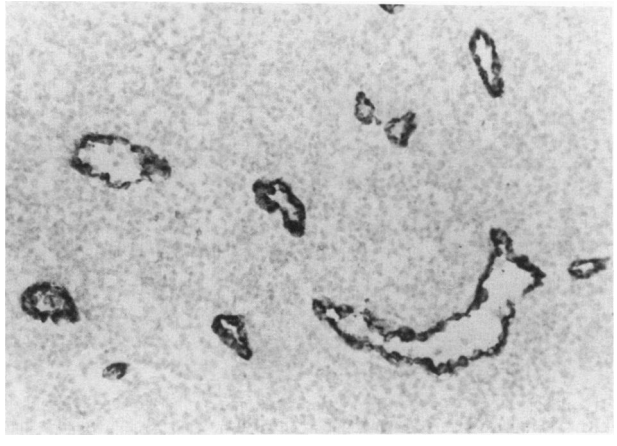

Figure 14—Lymph node, cryostat section. Luminal lining cells of postcapillary venules also exhibit cytoplasmic immunoreactivity for Factor VIII associated antigen, by the use of monoclonal antibodies. Endothelial cells of all vessels present in the tissue were also immunoreactive for this antigen. Lymphoid cells are negative. (Immunoperoxidase technique, methyl green counterstain, × 150)

The pattern of immunoreactivity for smooth muscle myosin which was observed in follicles, particularly follicular centers, was totally unanticipated. Immunohistochemical localization in these B-cell areas of either tonsils or nodes was consistently characterized by a reticular pattern, reminiscent of the distribution of dendritic reticulum cells. Immunoreactive cells revealed fibrillar, filamentous, and/or poorly localized cytoplasmic staining (Figures 16 and 17). Studies of sequential cryostat sections with monoclonal antibodies to dendritic reticulum cells revealed a relatively similar, though not identical, staining pattern. However, absolutely identical staining patterns would not be anticipated, because myosin antibodies define a cytoplasmic protein, whereas the monoclonal antibodies to dendritic reticulum cells predominantly identify a surface membrane epitope. Control studies with Tris buffer, preimmune rabbit serum, and immune serum adsorbed with anti-

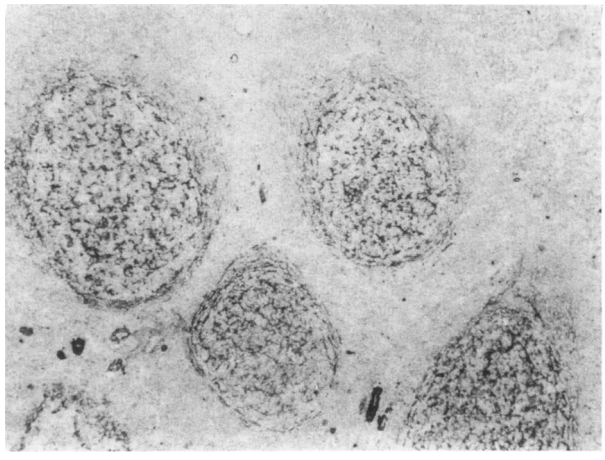

Figure 16—Tonsil, cryostat section. At low power, immunoreactivity for smooth muscle myosin assumes a reticular staining pattern within follicles. Occasional vessels present in the section also exhibit immunoreactivity for smooth muscle myosin in their walls. Lymphoid cells are nonreactive. (Immunoperoxidase technique, methyl green counterstain, ×60)

gen were all negative, except for endogenous peroxidase activity in myeloid cells, verifying the specificity of the staining pattern for smooth muscle myosin. Additional studies with irrelevant antibodies, eg, to keratin proteins (polyclonal and monoclonal) failed to reveal staining of lymphoid tissue, vascular structures, or follicles.

Staining patterns for smooth muscle myosin were similar in cryostat sections and methacarn-fixed paraffin sections of these tissues. However, higher concentrations of primary antibody were required for the latter. Factor VIII associated antigen was detected in cryostat and paraffin sections, including formalin-fixed tissues. For the latter, preliminary trypsinization was required for optimal immunoreactivity.

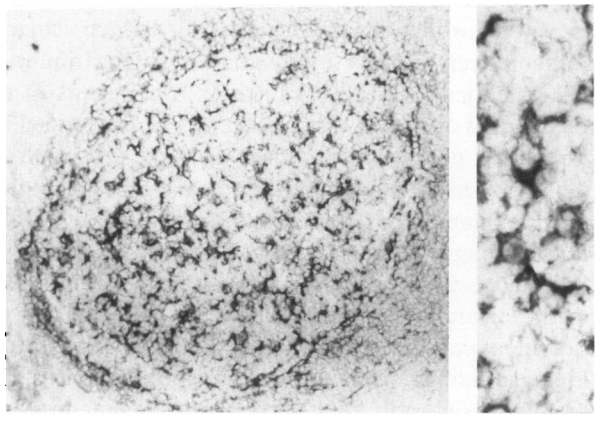

Figure 17—Tonsil, cryostat section. Higher magnification of a follicle (*left*) demonstrates the reticular staining pattern for smooth muscle myosin which appears to correspond to cytoplasmic staining of dendritic reticulum cells. This pattern of localization is even better discerned with further magnification (*right*). (Immunoperoxidase technique, methyl green counterstain, ×130, *left*; ×410, *right*)

To examine further the possibility that the immunoreactive cells in follicles represented mainly dendritic reticulum cells, we evaluated other tissues containing follicles, eg, Peyer's patches of small intestine. Staining patterns within follicles were similar to those noted in lymph nodes. Ten nodes involved by nodular non-Hodgkin's lymphomas (6 small cleaved, 2 mixed large and small, 2 large cell types), ie, neoplasms of follicular center cell derivation, were also assessed, since dendritic reticulum cells have been identified in the neoplastic nodules of these lymphomas[14,15] (personal observations), though in smaller numbers, as compared with reactive follicles. Immunoreactivity patterns for smooth muscle myosin in the neoplastic nodules of these proliferations (Figure 18A) resembled those apparent in follicles of reactive lymphoid tissues and were similar to the localization pattern observed for dendritic reticulum cells in these tissues (Figure 18B).

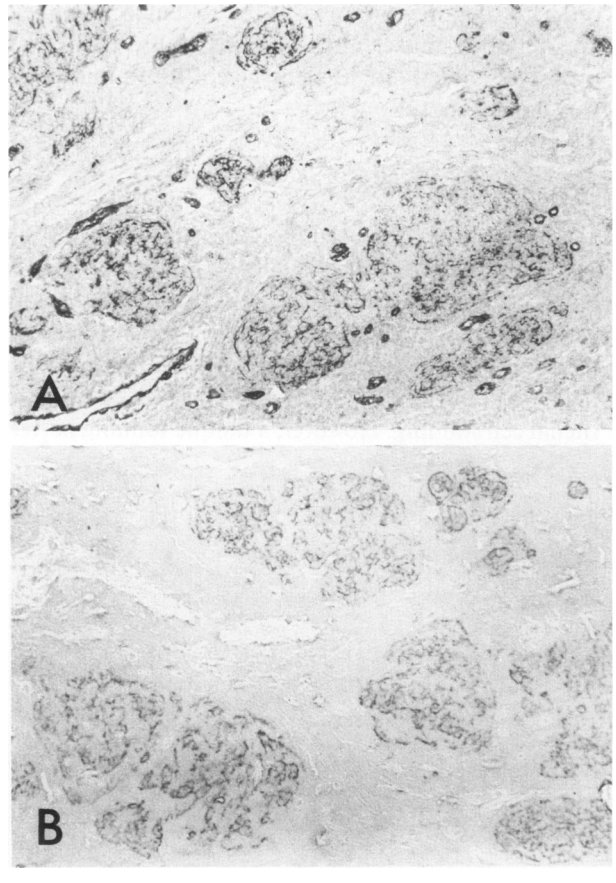

Figure 18—Non-Hodgkin's lymphoma, nodular; mixed follicular center cell type; lymph node, cryostat section. **A**—Smooth muscle myosin is localized to nodular areas of the infiltrate in an irregular reticular pattern. Immunoreactivity is also observed within the walls of various blood vessels. **B**—With monoclonal antibodies to dendritic reticulum cells, nodules of this infiltrate exhibit a staining pattern relatively similar to that observed for myosin-containing cells. Blood vessels and lymphoid cells are nonreactive. (Immunoperoxidase technique, methyl green counterstain; **A**, ×65; **B**, ×65)

Other Observations

For light-microscopic studies, immunohistochemical localization of smooth muscle myosin was best achieved with the use of cryostat sections and was detected at all dilutions employed. In methacarn-fixed paraffin sections, smooth muscle myosin could be detected in vascular structures (eg, Figure 13), with suboptimal preservation of antigenicity within apparent dendritic reticulum cells of follicular centers. However, the degree of immunoreactivity was considerably diminshed as compared with cryostat sections, requiring much higher concentrations of antibody. In paraffin sections of spleen, little immunoreactivity was apparent, noted only for larger vascular structures, with a total absence of staining in splenic sinus lining cells. Factor VIII associated antigen was more easily detected in paraffin sections, though immunoreactivity appeared better preserved in cryostat sections. However, for formalin-fixed tissues, preliminary proteolytic digestion, as previously described,[6] was necessary for optimal immunoreactivity. In all smooth muscle myosin studies, control sections employing Tris buffer, preimmune serum, or immune serum adsorbed with antigen were used and yielded negative results, except for endogenous peroxidase activity in cryostat sections.

For immunogold techniques, smooth muscle myosin was best detected in tissues processed directly into ethylene glycol. As noted previously, immunoreactivity for this protein was readiy destroyed by fixation in aldehydes. However, immunoelectron-microscopic localization of Factor VIII associated antigen was readily achieved following paraformaldehyde-glutaraldehyde fixation.

Discussion

Immunohistochemical localization of smooth muscle myosin in normal human lymphoid tissues, as described in this study, provides a unique comprehensive architectural assessment of lymphoid tissue constituents containing this contractile protein. This technique offers certain advantages in detection of smooth muscle myosin, even compared with electron microscopy. Ultrastructurally, the smooth muscle cell may contain three sets of myofilaments, namely, thick (15-nm), intermediate (10-nm), and thin (average 5 nm).[16] However, some filaments, particularly thick ones, are difficult to preserve in fixed tissues. Based on biochemical analysis and x-ray diffraction studies, the latter filaments probably contain myosin. It has also become apparent that myosin and/or actin, which is not assembled into filaments, may also occur in cells of nonmuscle type.[17] Presumably these proteins have a function in cytoplasmic motility and possibly also in maintaining cytoskeletal integrity.[2]

In the spleen, immunoreactivity patterns for smooth muscle myosin strikingly demonstrated abundant quantities of this contractile protein, with an interesting overall distribution. Smooth muscle myosin-containing cells of the trabeculas, which comprised the adventitia of large vessels, appeared in continuity with the central artery and the concentric reticular network of the white pulp, delicately blended with the red pulp in the marginal zone, and assumed a distinct pattern in the splenic sinus lining cells. Cells immunoreactive for smooth muscle myosin were also observed in the muscle walls of arteries and veins as anticipated. Splenic reticular cells in the white pulp are known to contain distinctive cytoplasmic structures, including dense material comprised of closely packed microfilaments.[11,18] As suggested by our light-microscopic studies and further corroborated by our immunoultrastructural studies (Figure 4), the microfilaments apparently represent the cytoplasmic correlate for smooth muscle myosin staining in these cells. Using rabbit antibodies to chicken gizzard myosin, Muller-Hermelink and co-workers[18] also demonstrated immunoreactivity for that contractile protein in smooth muscle cells and myofibroblast type cells of the splenic capsule and trabeculas and the marginal zone of the white pulp. However, based on the descriptions and illustrations in that report, the white pulp staining appeared more limited than that observed with our anti-human uterine smooth muscle myosin antibodies, and red pulp staining apparently was not observed. The central artery, arterioles, and the arterial capillaries, most of which terminate in the marginal zone, are also invested with varying quantities of smooth muscle in their walls. This overall distribution of smooth muscle myosin may play a role in altering blood flow through the white pulp and its exit to the red pulp through the marginal zone. Within the red pulp, cells comprising the sheaths of terminal branches of the arterial vessels also contained abundant smooth muscle myosin (Figures 1 and 9; Schweigger–Seidel sheath), and potentially may function to alter blood flow in these vessels.

The pattern of smooth muscle myosin staining in the splenic sinuses of the red pulp was unique. Short, regular small bands immunoreactive for smooth muscle myosin extended between adjacent ring fibers. These bands corresponded ultrastructurally to the dense bands of finely filamentous material which is observed to lie parallel to the long axis of the sinus and to extend from one rib of basal lamina to the next.[9,19] These structures in the sinus lining cells of the red pulp were referred to as "basal plates" by early investigators.[20,21] Though the precise nature of the basal plates was initially unclear, the schematic diagram of the wall of the splenic

sinus provided by Mangubi-Kudrjavtzewa in 1909[20] beautifully portrays these structures. Histochemical studies for α-helical proteins of the myosin group have also demonstrated bands, apparently comprised of myosin, which were located between the ring fibers[22] and corresponded to the basal plates or filamentous bundles described in the ultrastructural studies.[20,21] These bands were thought to resemble the A bands of striated muscle. Meloan et al[22] provide an excellent historical discussion of observations pertinent to the structure of splenic sinuses. Subsequent ultrastructural studies of rat spleens[23] suggested that filaments, about 5 nm in diameter, were assembled into distinct bundles to form contractile units about 4 μ in length and 0.25 μ in diameter. Such filaments were noted to be oriented parallel to the long axis of the sinus with a repeating pattern, contained "dense bodies," and were attached to the plasmalemma at the margins of the indentations of the endothelial cells, at the site of the annular components of the basement membrane ("ring fibers"). Based on these ultrastructural features, DeBruyn and Cho[23] proposed that the filaments were contractile smooth muscle elements. Our immunohistochemical studies corroborate the smooth muscle derivation for these distinct structures of the splenic sinus wall and represent the first specific characterization of these filaments using antibodies to uterine smooth muscle myosin. The presence of myosin in splenic sinus lining cells has previously been demonstrated with antibodies to calf thymus myosin.[19] However, thymus myosin, a vertebrate nonmuscle myosin, is immunologically distinct from smooth muscle myosin; and antibodies raised to this antigen have different specificities from those of our uterine smooth muscle myosin antibodies. For example, anti-thymus myosin antibodies are immunoreactive with all endothelia,[19,24] as well as lymphocytes, neutrophils, hepatic cells, and other tissues, and do not exhibit the selective staining patterns described in this study and in our prior report[3] using antibodies raised against human uterine smooth muscle myosin. The myosin-containing contractile elements in the splenic sinus walls potentially may function in producing cyclic volume changes, as observed in the transillumination studies of Knisely[25,26] and those of Peck and Hoerr.[27] Also, the presence of contractile proteins in a highly fenestrated lining cell may also serve to alter the permeability of the sinus wall.

The presence of smooth muscle myosin within follicles, presumably in dendritic reticulum cells, represented another unanticipated finding. Ultrastructurally, dendritic reticulum cells of follicles are known to contain fine cytoplasmic filaments[14,28] which represent the most likely anatomic correlate for smooth muscle myosin staining. These cells are reportedly associated with antigen processing; thus, the presence of this contractile protein may provide cytoplasmic motility germane to optimal cell function. Interestingly, immunoreactivity for smooth muscle has been described in another type of reticulum cell, ie, fibroblastic reticulum cells of tonsil and the paracortex of node, with the use of antibodies to chicken gizzard myosin.[18] Ultrastructurally, the latter cells are similar to dendritic reticulum cells and may be derived from a common precursor cell.[18]

In our previous study of human tissues (exclusive of brain and lymphoid tissue), endothelia were nonreactive for smooth muscle myosin with the use of antibodies to uterine myosin.[3] In our current study, splenic sinus lining cells and luminal lining cells of postcapillary venules in lymph nodes and tonsils were unusual, exhibiting immunoreactivity for both smooth muscle myosin and Factor VIII associated antigen (Figures 13 and 14). The endothelium of other vessels (arteries, veins, capillaries) in these tissues was nonreactive for smooth muscle myosin. In contrast to our observations, Drenckhahn[19] observed endothelial cell staining only in cerebral vessels with antibodies to human uterine myosin. Similarly, antibodies to chicken gizzard myosin were also reportedly nonreactive with human endothelia, though all endothelial cells, including postcapillary venules of lymphoid tissues, were immunoreactive with the use of antibodies to nonmuscle myosin of calf thymus.[24] Differences in antibody specificities may account for these varied patterns of immunoreactivity. The unique nature of postcapillary venules in certain lymphoid tissues has been appreciated by various observers.[28,29] These structures lack a muscular coat and are lined by a single layer of tall endothelial cells which are tightly bound by close interdigitations and contain abundant cytoplasm with many lysosomes, phagolysosomes, and filamentous structures. The latter probably represent the site of smooth muscle myosin localization, although definitive evidence is unavailable. Apparently, many lymphoid cells from the peripheral blood traverse the walls of these vessels.[29] Thus, the presence of abundant smooth muscle myosin in these cells may be pertinent for lymphocyte migration by altering the contours of the endothelial cells.

In summary, immunohistochemical detection of smooth muscle myosin using specific antibodies and cryostat sections permits unique topographic visualization of this contractile protein. The overall distribution of smooth muscle myosin in lymphoid tissues, even in unanticipated sites, could be correlated with subcellular structures of immunoreactive cells, particularly cytoplasmic filaments. This study is the first to provide specific immunohistochemical characterization of these

filaments in lymphoid tissues by the use of antibodies to human uterine smooth muscle myosin. Although this report does not resolve the issue of open and/or closed circulation in the spleen, it does further establish the presence of a contractile protein as an integral component of both red and white pulp, providing a basis for regulation of blood flow in these anatomic sites.

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