BACTERIAL CELL WALL–INDUCED HEPATIC GRANULOMAS

An In Vivo Model of T Cell–Dependent Fibrosis

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Fibroblast proliferation and collagen synthesis are crucial in the repair of injured tissue associated with inflammatory lesions. However, the prolonged fibrotic response which may accompany chronic inflammatory lesions can also be detrimental to the host. Definition of the inflammatory-based mechanisms responsible for the regulation of enhanced connective tissue metabolism are central to an understanding of how these events may become pathologic and consequently, to potential avenues of therapeutic intervention. The ability of inflammatory mononuclear cells to regulate fibroblast growth and function has been well documented in vitro (1). Both T lymphocytes (2, 3) and monocyte/macrophages (4–7) generate mediators in vitro responsible for increased fibroblast proliferation. Therefore, it is reasonable to assume that the mononuclear cells are instrumental in regulating fibrosis during the inflammatory process in vivo. In this regard, recent studies have identified mononuclear cell-derived factors that can regulate fibroblast growth in the synovium of rheumatoid arthritis patients, which is characterized by hyperplasia of fibroblasts (8).

While these studies lend credence to T lymphocyte and monocyte- /macrophage-mediated regulation of connective tissue metabolism and pathology, there is a need for an experimental model to further explore these pathways and to determine mechanisms by which the process can be modulated. In this study, we have used an experimental animal model in which a chronic inflammatory lesion can be induced, and this inflammatory lesion is associated with the formation of fibrotic tissue. After the injection of group A streptococcal cell wall (SCW)[1] antigens into the peritoneum of genetically susceptible rats, the antigen is dispersed primarily to the spleen, liver, and peripheral joints (9, 10). The affected animals develop proliferative and erosive polyarthritis in the joints and granulomatous lesions in the liver (10). Both lesions require local deposition and persistence of the antigen and exhibit dramatic effects on the surrounding connective tissue. In this study, we focus on the fibrotic events that occur within

[1] *Abbreviations used in this paper:* CsA, cyclosporin A; FAF, fibroblast-activating factor; SCW, streptococcal cell wall.

the liver and their requirement for T lymphocytes, which includes the generation of a lymphokine that stimulates fibroblast proliferation. Furthermore, we show that functionally T cell–deficient animals not only fail to develop hepatic fibrosis after SCW administration but they also do not produce a cytokine, distinct from IL-1, that regulates fibroblast growth.

Materials and Methods

Animals. Specific pathogen-free inbred LEW/N female rats were obtained from the Small Animal Section, Veterinary Resources Branch, National Institutes of Health, Bethesda, MD (10). The animals were ~6 wk old at the initiation of each experiment and were housed in filter top cages (Lab Products Inc., Maywood, NJ) in an environment free of known microbial pathogens. For additional experiments, specific pathogen-free, congenitally athymic nude female rats (*rnu/rnu*) and their heterozygous, euthymic (NIH/*rnu*) littermates were also obtained from the Small Animal Section (NIH).

Induction of Granulomas. The preparation of peptidoglycan-polysaccharide fragments from group A SCW and the induction of the chronic inflammatory lesions have been described (10). Briefly, a sterile aqueous suspension of sonicated SCW in PBS, pH 7.4, was injected intraperitoneally into LEW/N, *rnu/rnu*, or NIH/*rnu* female rats at a dose equivalent to 20 μg of cell wall rhamnose per gram body weight. This dose of SCW has been shown previously to induce severe chronic polyarthritis and hepatic granuloma formation with nearly 100% incidence in LEW/N female rats (10).

Light and Electron Microscopic Analysis. Tissue specimens were cut into 1-mm^3 pieces and suspended in 5 ml of fixative solution containing 2% glutaraldehyde and 2% formaldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, with 0.05% $CaCl_2$. After fixation at room temperature for 2–3 h, the tissue pieces were washed and stored at 4°C in 0.1 M cacodylate buffer, pH 7.4, with 7% sucrose. After a 2-h postfixation with 1% OsO_4 in Veronal acetate buffer, pH 7.4, the liver pieces were stained for 2 h with 0.5% aqueous uranyl acetate, dehydrated in ethanol, and embedded in Spurr epoxy resin (11). Semithin (1 μm) sections were stained with toluidine blue and examined by light microscopy. Thin sections were stained with uranyl acetate and lead citrate and observed in a transmission electron microscope.

Immunofluorescent Detection of Collagen. Livers were snap-frozen in an embedding medium (Tissue-Tek II, O.C.T. Compound; Miles Laboratories, Inc., Naperville, IL) by immersion in a mixture of dry ice and acetone. Sections (8 μm) were cut on a cryostat at −20°C and placed on pretreated glass slides as described (12). The sections were fixed in acetone, washed, and incubated with affinity-purified rabbit anti–bovine type I or type III procollagens or with rabbit anti–bovine type IV or type V collagens. The washed slides were then incubated with an affinity-purified goat anti–rabbit serum conjugated to FITC (Cappell Laboratories, Cochranville, PA) as described (13) and analyzed for specific fluorescent staining.

Immunoperoxidase Stain for Group A SCW Antigens. Liver tissues were fixed in 10% buffered formalin, paraffin embedded, sectioned, and stained for SCW antigens using an immunoperoxidase technique (ABC VectaStain Kit; Vector Laboratories, Burlingame, CA) (12) with a rabbit antibody to the group A–specific SCWs. The localization of SCW antigens was indicated by the deposition of a black precipitate in the tissue sections; the specificity of the staining has been previously documented (12).

Granuloma Isolation and Culture. Livers of SCW-injected rats were aseptically excised at 3, 6, and 9 wk after injection for the isolation of granulomas. After washing, the livers were suspended in 50 ml DME (Quality Biologicals, Gaithersburg, MD) and homogenized intermittently for 30–60 s in a Waring blender (Waring Products, New Hartford, CT) (14). The granulomas that generally are 1–2 mm^3 are more dense than the homogenized hepatic tissue and can be separated by serial sedimentation. Granulomas were resuspended in PBS and isolated by repeated gravity sedimentation in PBS for 10 min at 20°C until the supernatants were free of debris. Isolated granulomas, of which up to several hundred can be obtained from a single liver, were counted and cultured at 5, 10, 20, or 40

granuloma/ml DME containing 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM Hepes, and 2 mM glutamine without serum in TC 24 well plates (Costar, Cambridge, MA). Supernatants were collected at varying time periods.

Mononuclear cells were isolated from 3–6-wk granulomas by enzymatic digestion. Isolated granulomas (5 ml) were incubated in 50 ml DME containing 35 U/ml collagenase (Cooper Biochemical Inc., Philadelphia, PA) for 30 min and the supernatant was removed. Fresh DME containing collagenase was added; this procedure was repeated four times. All the supernatants containing dispersed cells were pooled, centrifuged, washed three times, and the cells resuspended at 2×10^6/ml DME without serum. These granuloma-derived cells were >90% viable as determined by trypan blue dye exclusion and consisted primarily of esterase-positive adherent cells and a nonadherent population of lymphocytes. Some neutrophils, mast cells, and fibroblasts were also found in the enzymatically dispersed 3-wk granuloma cells. At later time points (>6 wk) increased numbers of fibroblasts and mast cells were identified.

Preparation and Characterization of Mononuclear Cell–derived Fibroblast Activating Factor (FAF). Single cell suspensions were prepared from the spleens of untreated LEW/N female rats under aseptic conditions. Spleens were minced, lysed with 0.14 M ammonium chloride (NIH Media Unit), and filtered through sterile gauze. The mononuclear cells were washed twice, and resuspended at 4×10^6/ml in DME without serum and 5 μg/ml Con A (Calbiochem-Behring, La Jolla, CA). Cell-free supernatants were harvested at 48 h. Intact granuloma (10/ml) and/or mononuclear cells (4×10^6) obtained after enzymatic dispersal of the granuloma were cultured under similar conditions, but received no exogenous stimulus. The 48-h cell-free supernatants (150–200 ml) were harvested, dialyzed, and lyophilized (2). Concentrated supernatants (2 ml) were applied to a 2.5 × 90-cm Sephacryl S-200 column (Pharmacia Fine Chemicals, Piscataway, NJ) precalibrated with blue dextran, OVA, and cytochrome *c* (Pharmacia Fine Chemicals) in 0.15 M NaCl, 0.02 M PBS, pH 7.2. The sample was eluted with PBS and the protein concentration of individual fractions determined by measuring the absorbance at 280 nm.

Fibroblast Proliferation Assay. Primary dermal fibroblast cultures established in DME containing 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, and 10% FCS (Gibco, Grand Island, NY) were maintained as described (2). For proliferation assays, trypsinized fibroblasts were plated at 5×10^4 cells/ml DME containing 10% FCS for 2–4 h in TC 24 plates, washed, and cultured 18 h without serum. This medium was removed and the supernatants to be tested were diluted in serum-free DME and added to replicate cultures. After a 48-h incubation, the cultures were pulsed for 4 h with 1 μCi/ml [^{3}H]-TdR (sp act 6 Ci/mM; Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, NY). Trypsinized cultures were harvested with an automated harvester and processed for determination of [^{3}H]TdR incorporation.

Results

Hepatic Granuloma Development and Fibrosis in SCW-injected Rats. As reported in previous studies, a single intraperitoneal injection of group A SCWs results in the formation of granulomas in the livers of the susceptible female LEW/N rats (10). The development of the granulomas involves a transient, diffuse infiltration of polymorphonuclear and mononuclear leukocytes, which is most apparent 3 d after SCW injection. This acute response subsides and is followed by a prominent chronic influx of mononuclear cells. During the first 1–2 wk after SCW injection, the mononuclear cells form aggregates consisting mainly of W3/13$^+$ (pan T) lymphocytes and Ia$^+$ monocyte/macrophages (15, 16). Variable numbers of plasma cells, multinucleated giant cells, and eosinophils are also apparent. As the granulomas develop within the portal areas and within the parenchyma of the liver, an influx of fibroblast-like cells becomes evident (Fig. 1*A*). The fibroblasts appear to localize primarily around the periphery of the granuloma adjacent to

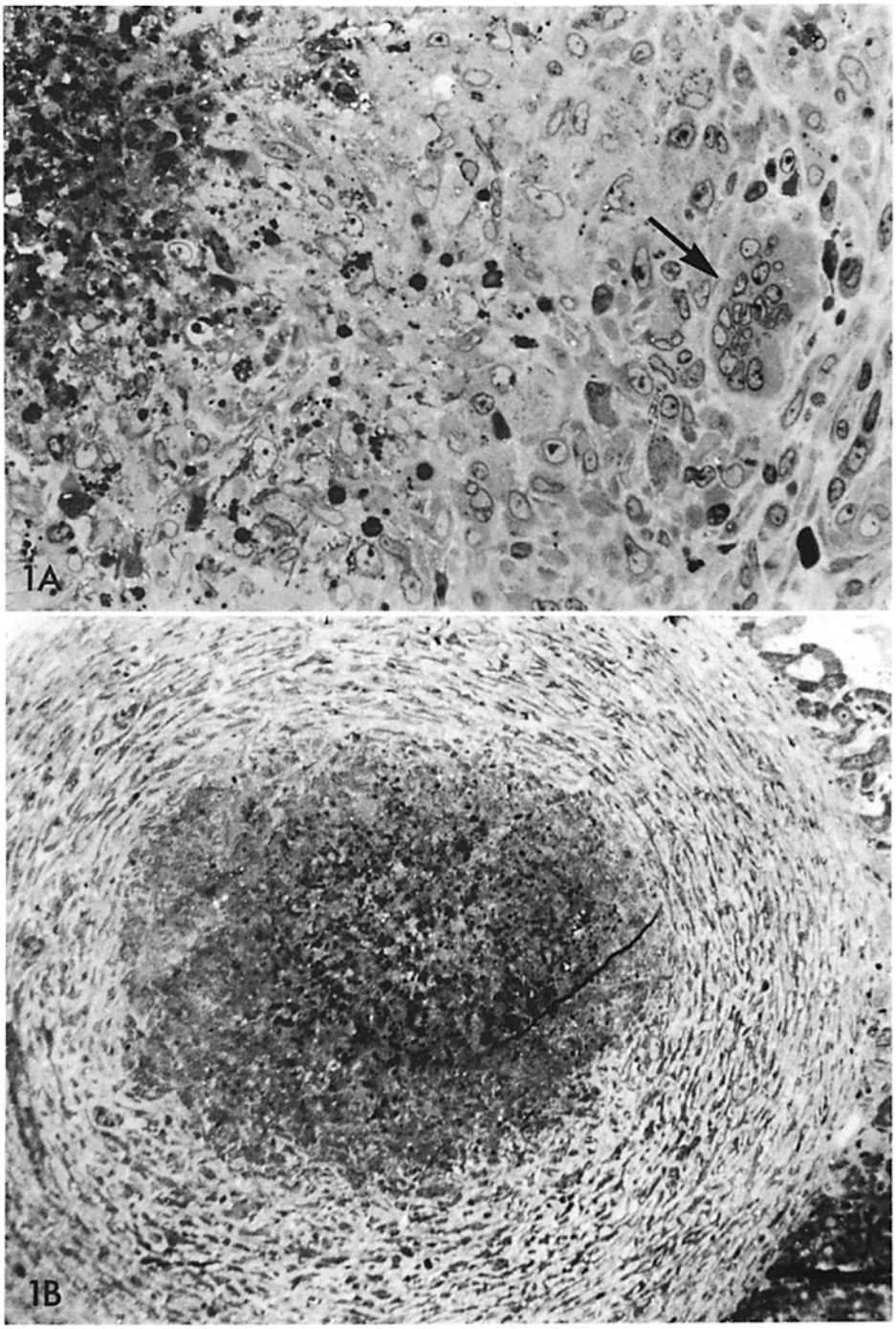
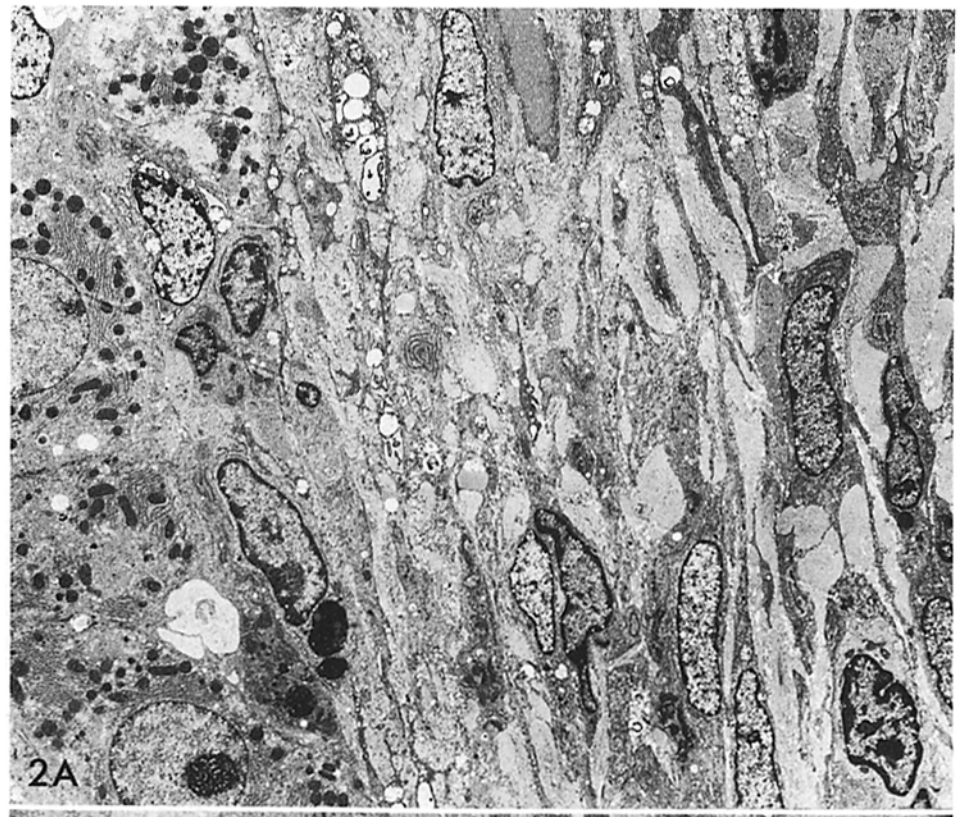


FIGURE 1. Histology of fibroblast infiltration and fibrosis in SCW-induced hepatic granulomas. Liver samples obtained 6 wk after SCW injection were fixed, sectioned, and stained with toluidine blue. (*A*) Cellular components of the granuloma are represented primarily by mononuclear cells and giant cells (*arrow*) in the center of the granuloma (*left*), and fibroblasts in the periphery (*right*). Densely stained particulate matter in center region represents material phagocytized by macrophages as well as glycogen and lipid droplets. × 560. (*B*) Lower power magnification of a granuloma showing fibroblasts surrounding the mononuclear cells to form a capsule-like structure. × 170.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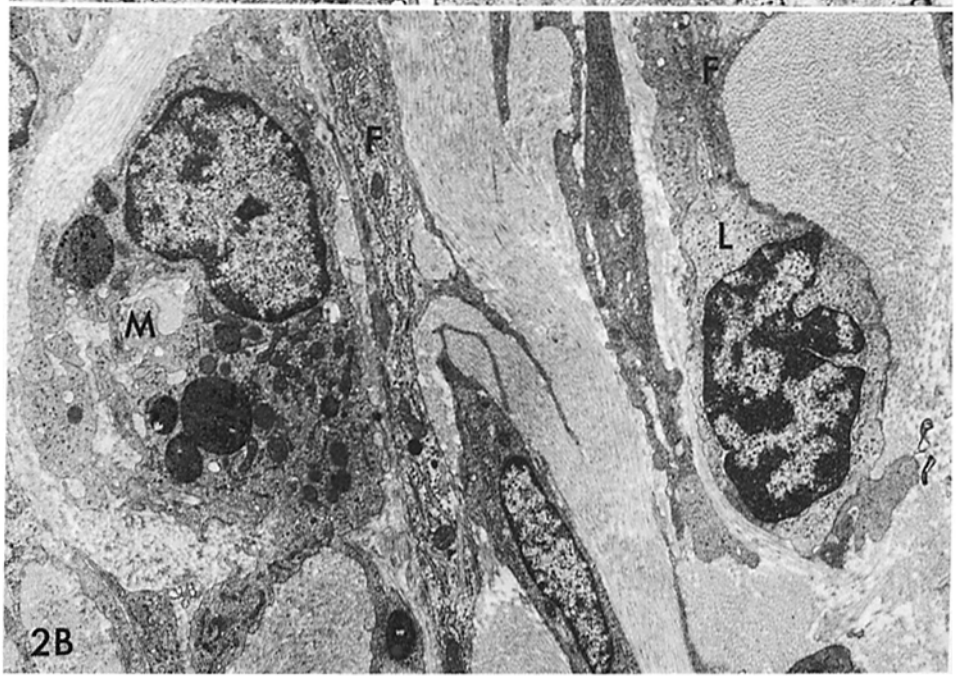
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the inflammatory cells that compose the central core of the granuloma. This increase in fibroblasts within the livers occurs only in association with the mononuclear cell aggregates. During the following 6 wk, there is a progressive fibroblast proliferative response, matrix generation, and the formation of fibrotic nodules throughout the liver. However, fibrosis does not occur at sites distinct from the granuloma.

Fibrotic Encapsulation of SCW-induced Hepatic Granulomas. With the infiltration of fibroblasts into the granulomatous lesion these cells assume an organized array in the peripheral region of the granuloma. The connective tissue cells, which appear to be actively generating matrix proteins, are frequently in direct apposition to the mononuclear cells of the granuloma. Furthermore, with the laying down of a connective tissue matrix, these cells create a capsular structure surrounding the mononuclear cell core of the granuloma (Fig. 1*B*). The proximity of the mononuclear cells and the fibroblasts encompassing them is also evident at the electron microscopic level (Fig. 2*A*). Mononuclear cells whose morphological features are consistent with those of lymphocytes and macrophages (Fig. 2*B*) were frequently found interspersed among cells of fibroblastic appearance, some of which were in mitosis (Fig. 3*A*). Furthermore, the fibroblasts exhibited a morphology typical of cells actively synthesizing protein, including a prominent Golgi apparatus and dilated rough endoplasmic reticulum (Fig. 3, *B* and *C*). The impressive quantity of interstitial collagen fibers (Fig. 2, *A* and *B*) surrounding the fibroblasts within the granuloma capsule underscores the synthetic activity of these cells. In addition, intracellular collagen fibrils (Fig. 3*C*) were frequently observed within vacuoles containing electron dense material similar to that described for secondary lysosomes (17), which suggests phagocytosis and degradation of collagen by these cells.

The formation of this connective tissue structure surrounding the granuloma appears to provide a barrier to the continual dispersal of the SCW antigen. As shown in Fig. 4, the SCW antigens identified histologically by positive staining with an antibody to the SCW coupled with the immunoperoxidase technique become segregated within the core of the granuloma. This walling off of the antigen may provide a mechanism for the host to sequester the poorly biodegradable and persistent SCW. During the course of the following weeks, the livers become studded with these fibrotic nodules.

Collagen Formation in Hepatic Granulomas. To determine what type(s) of interstitial collagen was being generated by the activated granuloma fibroblasts, granuloma sections were stained with antibodies to different types of collagen. As demonstrated in Fig. 5, the granulomas were found to contain significant amounts of both type I and III interstitial collagens. Type V collagen could also be shown within the granuloma sections, but no increase in type IV collagen was apparent (not shown).

FIGURE 2. Association of mononuclear cells and fibroblasts in hepatic granulomas. Liver samples obtained 6 wk after SCW injection were fixed, thin-sectioned, and stained with uranyl acetate and lead citrate for electron microscopic analysis. (*A*) Section of the peripheral region of the granuloma adjacent to liver parenchyma (*left*), showing a few mononuclear cells and numerous fibroblasts embedded in a collagenous matrix. × 2,900. (*B*) A macrophage (*M*) and a lymphocyte (*L*) in direct contact with granuloma fibroblasts (*F*) and prominent collagen bundles. × 7,400.

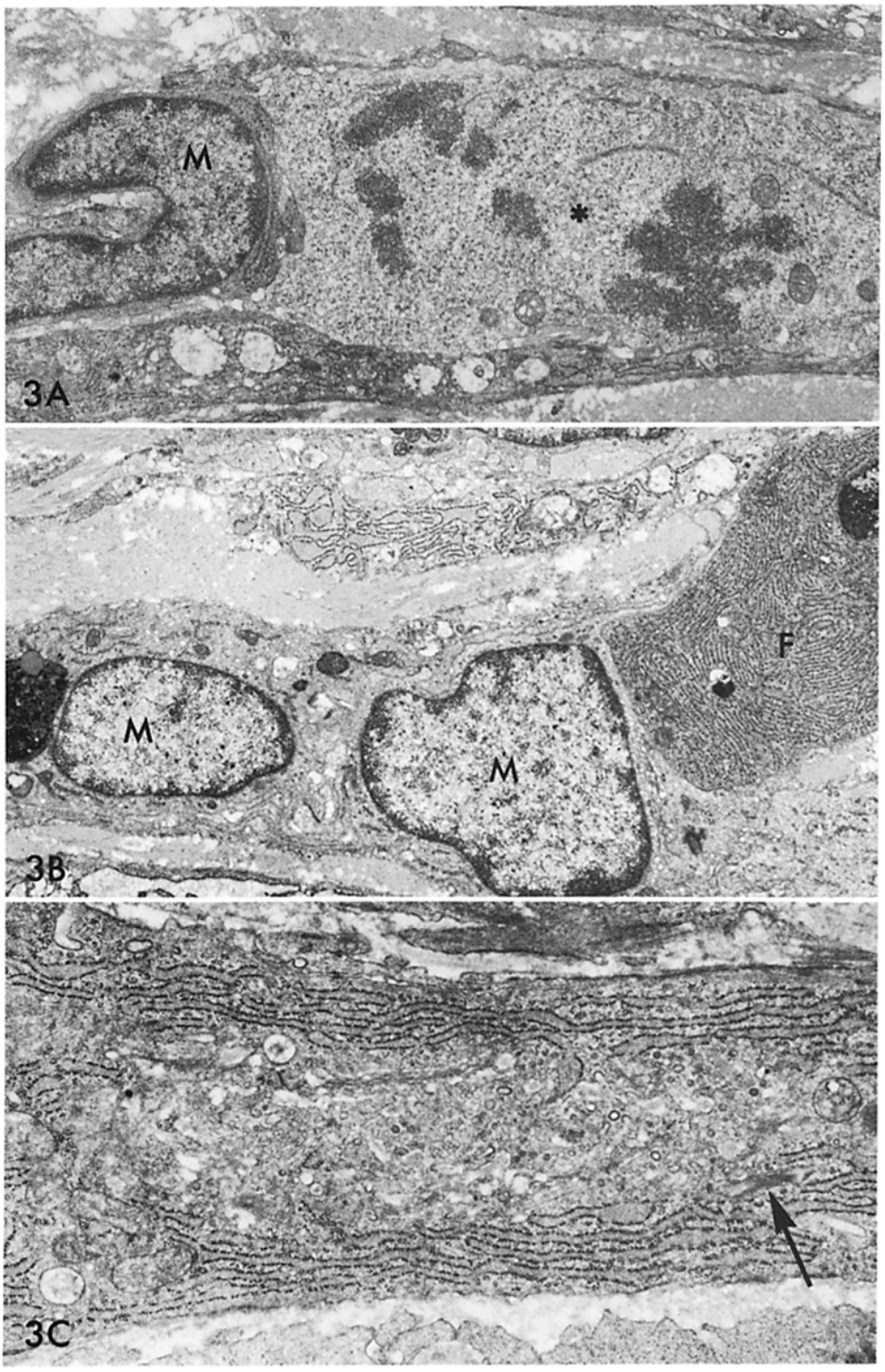

FIGURE 3. (*A*) A mitotic cell (*), probably a fibroblast, adjacent to a mononuclear cell (*M*). × 7,300. (*B*) Mononuclear cells (*M*) next to a fibroblast (*F*) with extensive rough endoplasmic reticulum. × 7,100. (*C*) Golgi region of a fibroblast that exhibits morphology consistent with active protein production. Fibroblast also contains intracellular collagen fibrils associated with electron dense material resembling the contents of secondary lysosomes (*arrow*). × 14,600. See Fig. 2 for fixation technique.

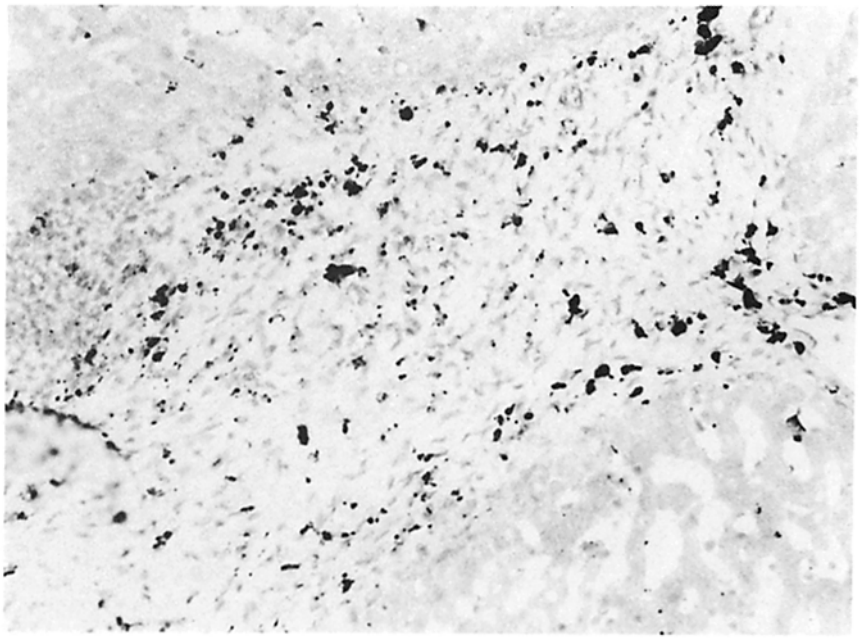

FIGURE 4. Localization of SCW antigens within the granuloma. Formalin-fixed sections of liver were stained using an antibody to group A SCW antigens with the immunoperoxidase technique. SCW antigens are indicated by the deposition of a black precipitate and are sequestered within this 6-wk granuloma. × 130.

Fibroblast Proliferation Mediated by Granuloma Products. Because of the apparent association between the mononuclear cells and the expansion of the fibroblast population within the liver granulomas, it was of interest to determine whether products of granuloma cells participated in the regulation of this fibroplasia. Initially, intact granulomas were isolated from the livers of SCW injected rats, quantitated, and cultured at various concentrations. No enzymatic digestion of livers or granulomas was required for the isolation of the granulomas. Consequently, the presence or absence of biological activity within supernatants derived from the granulomas is independent of any enzymatic activity or of any enzyme preparation contaminants as has been suggested in other systems (18). Supernatants from these granulomas were then assayed for their ability to regulate fibroblast growth. Intact granulomas, cultured in the absence of serum, generated a soluble factor(s) which could stimulate quiescent fibroblasts to proliferate as measured by the incorporation of [^{3}H]TdR into DNA (Fig. 6*A*). Because of the high metabolic activity of the cultured granulomas, supernatants from cultures containing more than 10 granulomas/ml were frequently found to contain inhibitory materials.

In further studies, mononuclear cells were isolated from the granulomas and were cultured at $1-2 \times 10^6$ cells/ml DME without any exogenous stimulation in vitro. These mononuclear cells, which were primarily lymphocytes and adherent macrophages, spontaneously released activity into the culture supernatants, which stimulated fibroblast [^{3}H]TdR incorporation (Fig. 6*B*) in contrast to

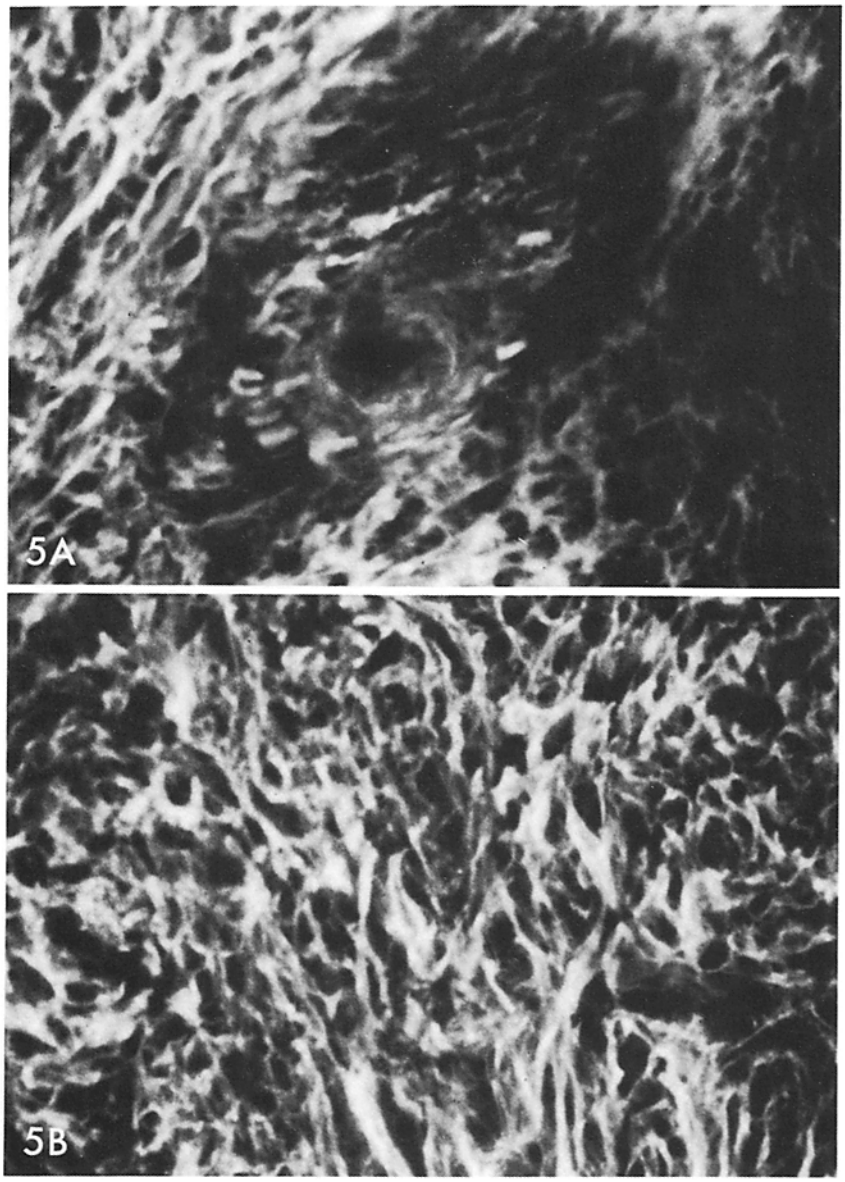

FIGURE 5. Immunofluorescent staining of granuloma collagen. Liver samples obtained 6 wk after SCW injection were frozen, sectioned, and incubated with affinity-purified rabbit anti-bovine antibodies to type I (*A*) and type III (*B*) procollagen. The sections were then incubated with affinity-purified FITC-conjugated goat anti-rabbit serum for fluorescent analysis. Fluorescent collagen bundles are seen throughout the granuloma.

unstimulated spleen cells cultured under identical conditions which do not spontaneously elaborate FAF activity. Spleen cell suspensions not exposed to exogenous stimuli were used as a control population representing a mixture of lymphocytes and macrophages. In the absence of stimulation, negligible levels of fibroblast growth activity were generated, but when optimally stimulated these

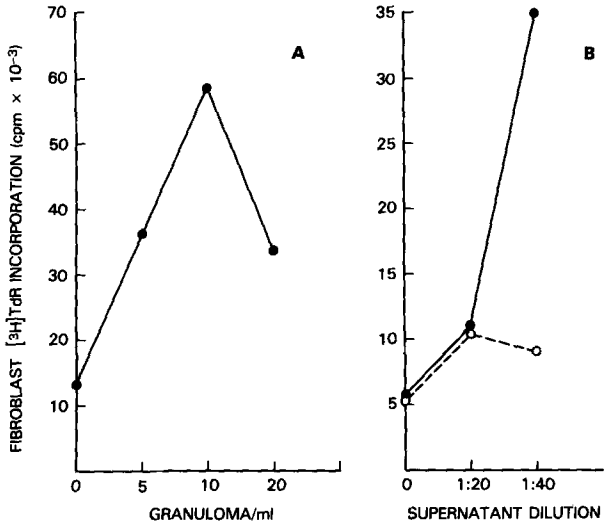

FIGURE 6. FAF produced by hepatic granulomas. (*A*) Individual granulomas were isolated from the livers 3–6 wk after SCW injection and cultured at 5, 10, or 20 granulomas/ml DME without serum. The 24-h supernatants were then tested for their ability to stimulate fibroblast [^{3}H]TdR incorporation as described in Materials and Methods. (*B*) Mononuclear cells obtained from the granulomas or from the spleens of control animals were cultured at 2 × 10^6/ml in the absence of an exogenous stimulus. 48 h supernatants were tested for their ability to stimulate fibroblast [^{3}H]TdR incorporation. ●, granuloma; O, spleen.

spleen mononuclear cell populations release levels of fibroblast growth activity comparable to that elaborated constitutively by the granuloma-derived mononuclear cells. The constitutive release of a FAF(s) by these granuloma mononuclear cells indicates that the cells had been previously activated in vivo and continued the generation of this activity in vitro.

Characterization of the Granuloma-derived FAF. Earlier studies have shown that T lymphocytes and macrophages produce fibroblast growth factors both in vitro and in vivo (1–7). To determine whether the characteristics of the granuloma-derived fibroblast growth factor(s) were consistent with a mononuclear cell derivation, preliminary physicochemical analysis of this activity was initiated. Since FAF has not previously been defined in the rat system, we first prepared supernatants from Con A–activated rat spleen cells and characterized this lymphoid cell–derived FAF. As clearly shown in Fig. 7, the primary peak of fibroblast stimulating activity produced by Con A–activated rat spleen cells elutes with ar apparent M_r near 40,000, similar to that described for a T cell product in guinea pig (19) and human (2). This activation of fibroblast proliferation could not be reproduced using purified rat or human IL-2 or human IFN-γ (not shown). A smaller peak of activity frequently eluted near the cytochrome *c* marker, which may represent IL-1 (14). However, the serum-free fibroblast proliferation assay used in these studies is not optimal for quantitation of the fibroblast growth factor activity of IL-1, which is serum dependent (20). Nonstimulated spleen cells did not generate soluble factors that stimulate fibroblast growth.

Based on this profile of rat spleen cell FAF, supernatants obtained from cultures of isolated granulomas (10/ml) were similarly analyzed by Sephacryl

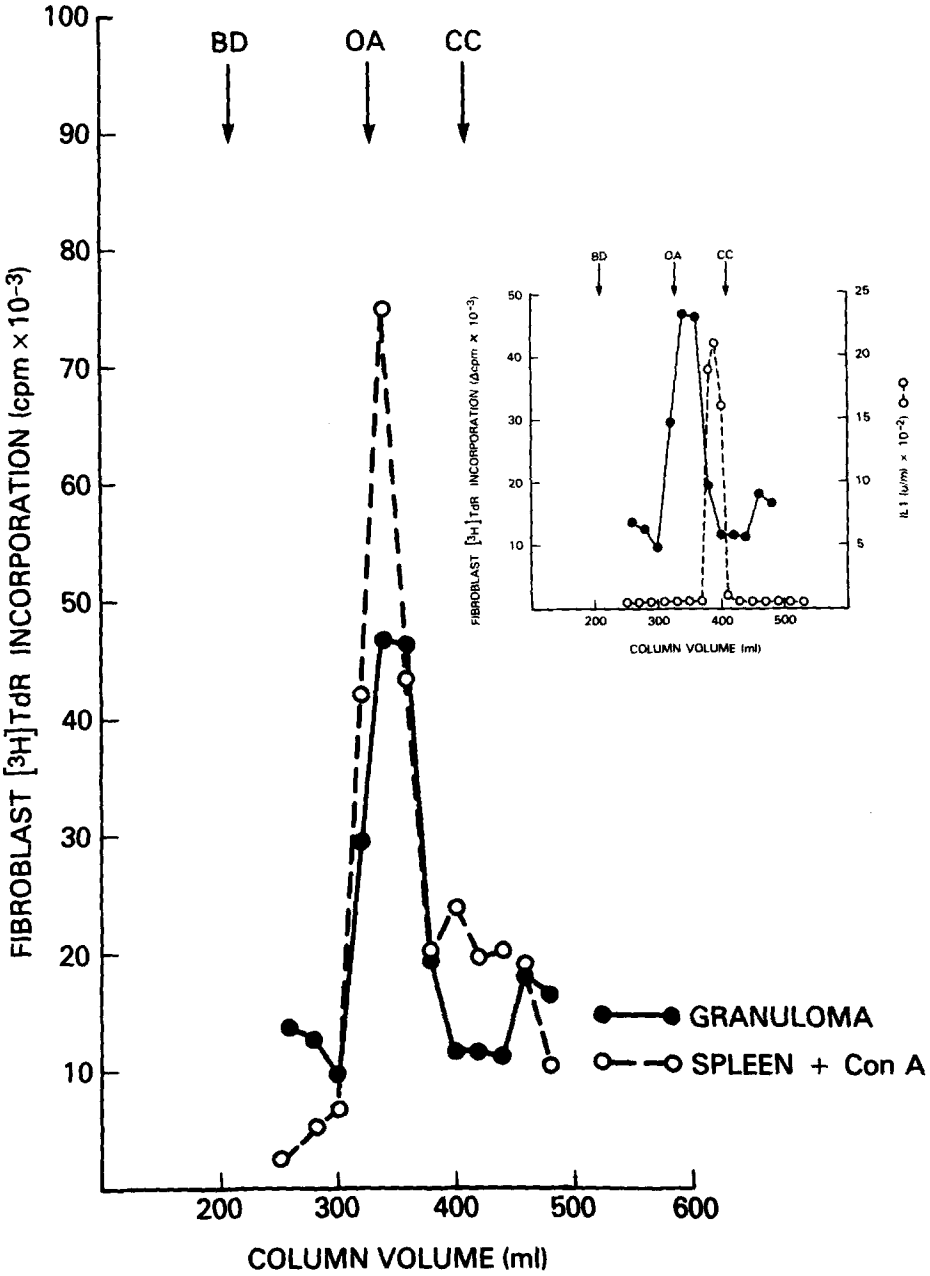

FIGURE 7. Characterization of Con A–stimulated spleen cell–derived FAF and granuloma-derived FAF. 200 ml of supernatants from Con A–stimulated (5 μg/ml) spleen cells (4×10^6/ml) or unstimulated granulomas cultured at 10 granuloma/ml for 48 h were dialyzed and concentrated by lyophilization. The media products were resuspended in 2 ml PBS and applied to a precalibrated Sephacryl S200 column. Fractions were eluted in PBS and the individual fractions were assayed for their ability to stimulate fibroblast proliferation ([^{3}H]TdR incorporation) in replicate cultures. In parallel experiments (*inset*), the same fractions were analyzed for IL-1 activity. Twofold dilutions of the fractions were added to murine thymocytes (1.5×10^6/well) in the presence of 1.5 μg/ml PHA. IL-1 activity is represented as units per milliliter based on comparison of [^{3}H]TdR incorporation to an IL-1 standard containing 100 U/ml.

S200 column chromatography. The granuloma-derived fibroblast growth activity was found to elute in the same apparent molecular weight range (~40,000) as that shown for Con A–stimulated spleen cells. Furthermore, although the granuloma mononuclear cells elaborated high levels of IL-1, the primary peak of FAF activity did not coelute with IL-1 (Fig. 7, inset). These data suggest that mononuclear cells within the granuloma are involved in the production of a factor(s) that stimulates fibroplasia, and that this primary cytokine activity is similar to that produced by Con A–activated spleen cells.

T Lymphocyte Dependence of Fibroplasia. Further support for the T cell–dependent fibroblast proliferative and synthetic response associated with the SCW antigen-induced granuloma formation comes from additional studies. In two T cell–deficient systems, we have shown that granuloma development and liver fibrosis after the injection of SCW are dramatically inhibited. Athymic (nude) rats that exhibit defective T cell proliferative responses and lymphokine production do not develop granulomas nor fibrotic nodules when injected with SCW (12, 15). To determine whether the production of FAF was deficient in these animals and might contribute to the lack of SCW-antigen–induced fibrosis in the livers, mononuclear cells were obtained from the spleens of athymic animals and their heterozygous littermates and stimulated with Con A in vitro. The supernatants were then compared for their ability to stimulate fibroblast proliferation. As shown for the LEW/N rats, the euthymic (NIH/*rnu*) rat spleen cells also produced lymphokine activity which could stimulate fibroblast proliferation (Fig. 8*A*). Again, the major fibroblast growth factor eluted in the 40,000 M_r range. When supernatants prepared under identical conditions from the athymic nude (*rnu/rnu*) rats were similarly analyzed, it was clear that little or no FAF activity eluted from the column (Fig. 8*A*). Clearly, the 40,000 M_r peak of FAF-like activity was absent in the athymic rat supernatants. Consequently, it appears that in the absence of a fully functional T cell response, the ability of these athymic animals to generate a T cell–specific fibroblast growth factor is curtailed. Furthermore, these data suggest that the apparent absence of FAF and not an inhibitor of this activity, unless it coelutes with FAF, is likely associated with the lack of granuloma fibrosis in the livers of athymic rats as compared with their euthymic littermates.

In related studies, treatment of LEW/N rats with the T cell inhibitor cyclosporin A (CsA) has also been shown to inhibit the development of hepatic granulomas and fibrosis in SCW-treated rats (16, 21, 22). In animals treated with CsA (25 mg/kg/d, kindly provided by Sandoz Pharmaceuticals, E. Hanover, NJ) from the time of SCW injection, no pathologic lesions develop within the liver. To evaluate whether CsA could influence the production of FAF by lymphoid cells of the rat, spleens were obtained from LEW/N rats treated intramuscularly with CsA (25 mg/kg/d). When Con A–stimulated supernatants from spleens of rats treated with CsA for 3 d were analyzed for the presence of FAF, a significant decrease in the 40,000 M_r peak of FAF activity was apparent as compared with placebo-treated control rats (Fig. 8*B*). These data suggested that the in vivo administration of CsA was sufficient to inhibit the production of this lymphokine by lymphocytes in situ, and that the inhibitory effect was sustained without further exposure to CsA during culture and Con A stimulation in vitro. Because

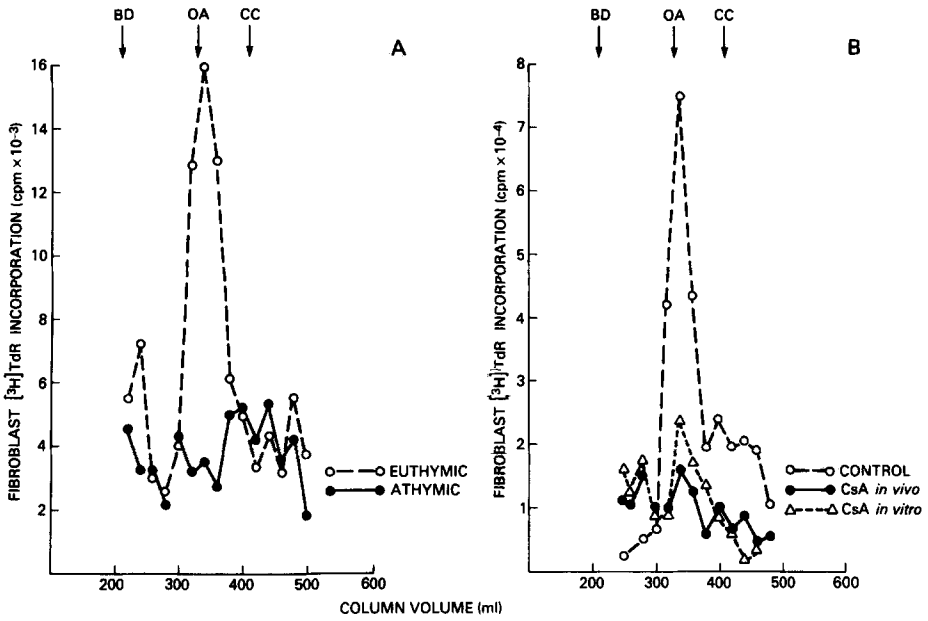

FIGURE 8. T cell–dependent FAF production. (*A*) Con A–stimulated spleen cell supernatants from athymic (*rnu/rnu*) and euthymic (NIH/*rnu*) rats were concentrated and eluted from a Sephacryl S200 column. Individual fractions were assayed for their ability to stimulate fibroblast proliferation ([^{3}H]TdR incorporation). (*B*) Con A–stimulated spleen cell supernatants from LEW/N control rats (—○—), or LEW/N rats treated with CsA (25 mg/kg) for 3 d (----) were concentrated and eluted from a Sephacryl S200 column. In additional experiments, LEW/N control rat spleen cells were stimulated with Con A in the presence of CsA (1 μg/ml) for 48 h and the concentrated supernatants eluted from a Sephacryl S200 column (—△—). Individual fractions were assayed for their ability to stimulate fibroblast proliferation ([^{3}H]-TdR incorporation).

the effect of CsA on FAF production has not previously been described, we further analyzed the effect of CsA on FAF production by adding CsA directly to the rat spleen cells in culture (Fig. 8*B*). Spleen cells obtained from untreated rats were simultaneously incubated with CsA (0.1–1 μg/ml) and Con A, and the supernatants compared with supernatants from spleen cells treated with Con A only. CsA directly suppressed the production of FAF by the Con A–activated spleen cells, as is evident by the decrease in the peak of fibroblast growth activity after gel filtration of the supernatants. Con A–activated supernatants to which CsA was added at the time of harvest were not inhibited, nor did CsA directly alter the fibroblast proliferative response to FAF, in agreement with previous studies (22). Consequently, it appears that CsA directly inhibits lymphocyte production of the lymphokine(s), which stimulates fibroblast proliferation.

Discussion

SCW-induced liver granuloma formation provides a useful model for exploring the mechanisms of pathogenesis of hepatic fibrosis. In this model, the antigen is defined, the time of initiation of the antigenic response is known, the requirement for T lymphocytes has become evident (12, 15, 21), and the chronic lesions are associated with the development of fibrosis. Furthermore, the ability to phar-

macologically regulate these events and to inhibit fibrosis may provide insight into avenues for controlling hepatic fibrosis initiated by various other agents including *Schistosoma mansoni*, viruses, toxins, or alcohol (23) which adversely affect liver function. In SCW-induced liver fibrosis, a single injection of the bacterial cell walls initiates a prescribed course of events which leads to fibroplasia and fibrogenesis. The SCW antigen is captured by hepatic Kupffer cells within hours after SCW injection. After an acute reaction characterized by edema and diffuse polymorphonuclear and mononuclear leukocyte exudation, which is maximal 3 d after SCW injection, a primarily mononuclear cell infiltration occurs. Initially, mononuclear cells diffusely infiltrate the liver in the periportal areas and in the parenchymal tissue. As the mononuclear cells continue to be recruited, presumably under the influence of T cell–derived chemotactic factors (15, 16), loose aggregates of T cells and macrophages form which become organized into mature granulomas within 3 wk.

During the course of the mononuclear cell recruitment and organization, fibroblasts also infiltrate the area, likely as a consequence of T lymphocyte– (24) and macrophage-derived (25) fibroblast chemotactic factors. These connective tissue cells localize primarily in the peripheral region of the inflammatory lesion, and these cells proliferate, presumably under the influence of the lymphocytes and macrophages, resulting in a markedly expanded fibroblast population. Ultrastructurally, the lymphocytes and macrophages frequently appear to be in direct contact with the activated fibroblasts. Products of lymphocytes and macrophages have been shown also to enhance fibroblast collagen synthesis (5, 19, 26, 27), and these granuloma fibroblasts are actively synthesizing matrix proteins, which is apparent at both the light and electron microscopic levels. Also apparent was evidence suggesting an active role for the fibroblasts in the degradation of collagen. The intracellular vacuoles containing collagen fibrils within the granuloma fibroblasts are similar to those frequently identified in tissues such as the periodontal ligament (17, 28) and in carageenin-induced granulomas (29) where rapid and continuous connective tissue remodeling occurs. Thus, fibroblasts appear to be involved in the simultaneous synthesis and breakdown of collagen with synthesis predominating. The net generation of collagen around the granuloma is substantial. Collagen types I and III, with some increase in type V, primarily account for this accumulation of matrix proteins, consistent with what has been described in the murine model of schistosome-induced hepatic granulomas (30). Unlike the schistosomiasis model, however, the SCW-induced granuloma fibrosis pattern is restricted to the peripheral region of the granulomas. During the development of schistosome-induced granulomas in the murine livers, the portal vessels become occluded by fibrosis both upstream and distinct from the actual granulomatous lesions (31). The localized fibrosis in the SCW model suggests that the mechanisms involved in the regulation of hepatic fibrosis in these two models may differ. In addition to the mononuclear cells, the maturing granulomas also characteristically exhibit increasing numbers of mast cells. Since recent studies implicate mast cells in the regulation of fibrosis (32), this model may provide a useful approach to the exploration of mast cell, T lymphocyte, and fibroblast interactions.

In generating the matrix proteins, the fibroblasts produce a capsular structure

around the chronic inflammatory lesion, providing a barrier to the influx and efflux of the SCW antigens. While this host response provides a very effective means for sequestering this poorly biodegradable and persistent antigen, it also results in pathologic displacement of the liver tissue with collagen. As has been previously shown, the livers of these SCW-treated rats often become densely studded with fibrotic nodules (16). During the course of the next several months, many of these fibrotic nodules shrink in size with a diminution of the inflammatory response but may persist indefinitely.

The documentation for the T lymphocyte role in this series of events that leads to fibrosis is several-fold. First, the morphological and immunohistological evidence at the light microscopic level show that T lymphocytes as well as macrophages are the primary cells comprising the granulomatous inflammatory lesion during the period when fibroblasts are being recruited, proliferating, and generating collagenous proteins. Additionally, the mononuclear cells are found closely interspersed among the activated fibroblasts; this is especially apparent at the electron microscopic level. Secondly, granulomas isolated from the livers of SCW-injected animals spontaneously generate a factor(s) that stimulates fibroblast proliferation. Although not previously characterized in the rat, we have shown that rat spleen cell–derived FAF appears to have an apparent molecular weight similar to that observed for FAF produced by T cells in other species (1–3, 19), and is distinguishable from IL-1, IL-2, and IFN-γ. However, since macrophage-derived growth factors for fibroblasts have also been described with the same molecular size characteristics (5, 14, 33), the contribution of macrophages to this primary peak of Con A–induced spleen cell FAF cannot be excluded. While production of FAF requires activation of the spleen cells in vitro, the granulomas spontaneously generate this activity, consistent with their activation in situ. The granuloma-derived FAF coelutes by gel filtration with the Con A–activated spleen cell FAF. Lymphoid cells derived from the isolated granulomas also generate FAF without in vitro activation. Thus, the evidence suggests that the lymphoid cells of the granuloma have been activated in vivo and continue to generate mediators, including FAF, without further activation in vitro.

Finally, compelling evidence that this granulomatous response and its fibrotic sequelae are indeed T lymphocyte–dependent is provided by studies in two functionally T lymphocyte–deficient systems. Using the NIH/*rnu* euthymic rats and their homozygous (*rnu/rnu*) athymic littermates as an experimental model, it is possible to dissect the contribution of the T cell to these events. In the athymic rat that lacks functional T lymphocytes (12, 34), the injection of SCW, while initiating acute, low intensity leukocyte infiltration and small focal areas of loosely organized mononuclear cells in the periportal regions, does not progress to organized granuloma formation. Furthermore, fibrosis associated with mononuclear cells is not apparent at any point in the livers of these animals. Thus, while the athymic animals can muster a minimal response to the SCW antigens, the response cannot be sustained because of the lack of T cell participation, whereas the euthymic littermates develop the pathologic sequelae leading to fibrosis. Essential T cell–derived mediators including FAF are not generated by the mononuclear cells of these animals. Although the macrophage is also a source

of fibroblast growth factors including IL-1 (4–7), the generation of these factors requires sustained activation of the macrophages (5), and in the absence of the cascade of lymphokines produced by SCW-activated T lymphocytes, the macrophages apparently are not able to independently mediate SCW-induced granuloma development and fibrosis. While IL-1 production by macrophages from the athymic animals is not impaired after exposure to macrophage-activating agents in vitro (12, 15), stimulation may not adequately occur in the athymic animals in vivo. Thus, the lack of a functional T cell component and the failure to generate granuloma and fibrotic lesions emphasize the key role of the T cell in this process.

In additional studies, CsA, an inhibitor of T cell function, was also found to block the SCW-induced pathogenesis of hepatic fibrosis when administered prophylactically to the animals (21, 22). While CsA has been documented to inhibit IL-2 production at the transcriptional level (35, 36), and to inhibit the generation of several other lymphokines (37), its effect on the production of FAF has not previously been reported. Since CsA blocks FAF production by rat spleen cells in vitro and also blocks fibrosis in vivo, these data indicate that T lymphocyte–derived cytokines are instrumental in the fibrotic events associated with this model system. These data, as well as other data that show that CsA does not inhibit monokine synthesis (16), do not rule out the contribution of macrophages to these events, but only emphasize that the T cell is responsible for driving the response. Additionally, while CsA effectively blocks antigen-induced hepatic fibrosis, its potential role in toxic or nonantigen-mediated liver fibrosis remains unclear. However, these studies provide direct evidence for the T cell–dependent pathway of fibrosis in this model and suggest additional avenues for pursuing the role of T lymphocytes and their products in fibrosis, and for regulation of the potentially pathologic fibrotic events associated with chronic inflammatory lesions.

Summary

In vitro studies implicate a molecular link between inflammatory mononuclear cells and alterations in fibroblast growth and function. We have extended these observations in an experimental animal model in which we document the T cell–dependence of fibrosis that occurs after activation of the cell-mediated immune system by specific antigen. Chronic granulomatous lesions were induced in the livers of susceptible rats by the intraperitoneal injection of group A streptococcal cell walls (SCW). The development of granulomas that are composed primarily of lymphocytes and macrophages was associated with the recruitment and proliferation of connective tissue cells. Furthermore, this expanded population of fibroblasts generated a collagenous structure consisting primarily of types I and III collagen around the granuloma. The progression of these chronic inflammatory lesions leads to the formation of fibrotic nodules throughout the livers of the treated animals.

Intact granulomas, as well as mononuclear cells derived from the granulomas, spontaneously elaborated a soluble factor(s) that stimulates fibroblast proliferation. Physicochemical analysis revealed that the primary granuloma-derived peak of fibroblast growth activity corresponded to an apparent M_r of 40,000, which

is consistent with a previously described T lymphocyte–derived fibroblast-activating factor (FAF) in guinea pig and human. Furthermore, the fibrosis that occurs in the granuloma is apparently T cell–dependent, since no fibrotic lesions developed in SCW-injected athymic nude rats nor in SCW-injected animals treated with the T cell inhibitor, cyclosporin A (CsA). Mononuclear cells from neither of these functionally T cell–deficient animals could generate FAF activity. These data show a role for T lymphocyte–derived cytokines in the development of hepatic fibrosis in SCW-injected rats.

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