Fibroblasts of Granulation Tissue: Immunofluorescent Staining with Antismooth Muscle Serum (35920)

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It is an established fact that granulation tissue is capable of contracting. Since the work of Abercrombie *et al.* (1) showing that the mechanism of this contraction cannot be sought in the collagen fibers, attention has focused on the fibroblasts (2). Recent work from this laboratory (3, 4), on experimental models of contracting granulation tissue, has shown that fibroblasts can indeed come to resemble smooth muscle cells: (a) By electron microscopy, they were found to contain a system of intracellular fibrils (40–80 Å in diam) with "attachment sites," as well as distorted nuclei typical of contracted cells. (b) Strips of granulation tissue from wounds and Selye pouches (5) could be made to contract *in vitro* with bradykinin and other smooth muscle stimulants. (c) Actomyosin was extracted from Selye pouches, giving a yield of 4 mg/g of wet weight, and an ATPase activity of 10 nmoles of ATP split/mg of protein/min—virtually identical values were obtained with similarly prepared extracts from pregnant rat uterus. We have proposed the term *myo-fibroblast* to designate the modified fibroblasts in contracting granulation tissue (4).

The present study aimed to provide further evidence of the similarity between smooth muscle and myo-fibroblasts, *i.e.*, the presence of common antigens in the two types of cells. As a source of antibody we used the human antismooth muscle serum that is produced by many patients with "autoimmune" hepatitis (6).

Materials and Methods. Male Wistar rats weighing 100–150 g were used. Granuloma pouches were produced according to Selye (5); and 2 × 2 cm open, full-thickness wounds were made on the skin of the back, as described previously (3). The rats were killed by cervical dislocation 7 to 60 days later.

Human antismooth muscle (HASM) serum was obtained from a 16-year-old girl with chronic biliary cirrhosis (courtesy Drs. Cruchaud and Nicod, Clinical Immunology Laboratory, Geneva). Interaction with smooth muscle of rat stomach occurred with dilutions up to 1:320; tests for antimitochondrial and anti-DNA antibodies, and for antinuclear factor, were negative.

To demonstrate smooth muscle antigens, the double layer technique was used (7). Sections of all tissues were treated with HASM serum at a dilution of 1:6 and stained with sheep antihuman IgG fluorescent serum (Miles-Seravac, Maidenhead, England). Fluorescence was compared with that of sections treated with normal human serum instead of HASM serum.

Photographs were taken on a Zeiss UV photomicroscope with UG I or II excitor filter and a Zeiss No. 50/65 barrier filter, using Ektachrome HS B or Ilford HP 4 film. The same fields were photographed with visible light after staining the sections with hematoxylin and eosin (Figs. 1 and 2).

Results. Specificity of HASM serum. To establish the specificity of HASM serum, we prepared fluorescent slides of rat stomach, cecum, kidney, urethra, bladder, prostate, testicles, liver, tendons, skeletal muscle, and skin. In all these tissues, the distribution of fluorescence coincided with the distribution of smooth muscle as seen in paraffin-embedded hematoxylin–eosin sections (Fig. 1). In the kidney, we found slight staining of glomeruli, as already described (8). Skeletal muscle also fixed HASM serum, but this result is sometimes obtained with normal human serum (9). Normal fibroblasts were never stained.

These results proved that the HASM

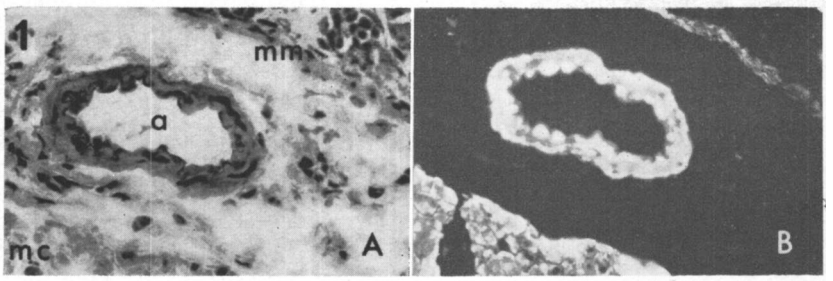


FIG. 1. An arteriole (a) in rat cecum, also showing muscularis mucosae (mm) and muscle coat (mc): (A) stained with hematoxylin–eosin; (B) treated with HASM serum and stained with fluorescent antihuman IgG; ×250.

serum was specifically directed against *smooth muscle cells*. It was not possible to pinpoint the responsible antigen—it was probably not actomyosin itself because: (a) pretreatment of HASM serum with purified rat uterus actomyosin (10) did not abolish fluorescence [although pretreatment (7) with rat uterus homogenate did]; (b) in agar gel immunodiffusion (11), no precipitation lines formed when HASM serum was allowed to diffuse against actomyosin; and (c) HASM serum failed to specifically label the deposit left by a dried, acetone-fixed drop of a suspension of actomyosin precipitate.

Immunofluorescent staining of granulation tissue. In the *granuloma pouches,* labeling was at first recognizable in a few cells on day 7. It reached a maximum between 20 and 30 days (Figs. 2 and 3), when fluorescent cells were regularly distributed through the whole wall, with the exception of the innermost layer that contained essentially polymorphs and macrophages. Later, the outer layer of the wall lost its fluorescence (Fig. 4): by 50 days, less than half of the wall thickness was labeled.

The *open wounds* took about 15 days to close; the slides examined were cross sections of the granulation tissue constituting the floor of these wounds. Labeled cells were first detected in 9-day wounds; they were prominent at 11 days (Fig. 5), and still present, although less numerous, at 13 and 15 days. The total number of fluorescent cells was always less in wounds than in Selye pouches.

Discussion. Our data show that human antibody against smooth muscle binds to fibroblasts of rat granulation tissue.

Antisera for the study of muscle have been used extensively (11); muscle proteins being weak antigens, one must resort to long periods of immunization (12) or to immunization across a wide phylogenetic gap (11). We did attempt to immunize rabbits against purified rat uterus actomyosin, but without success. The antismooth muscle antibody, discovered accidentally (6) in patients suffering from hepatitis, provided an easy way out; the sera are available in all large hospitals and maintain their activity for years if stored

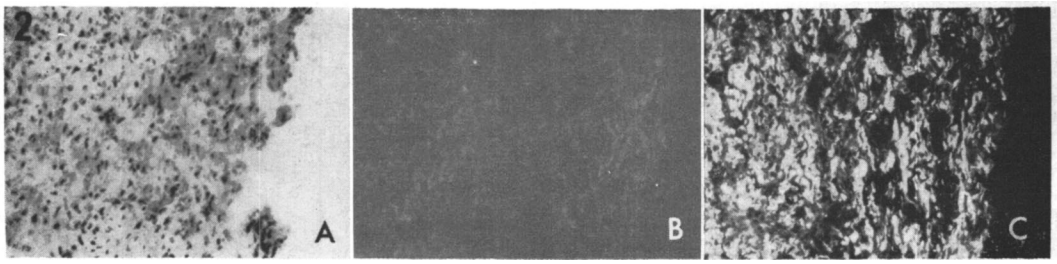
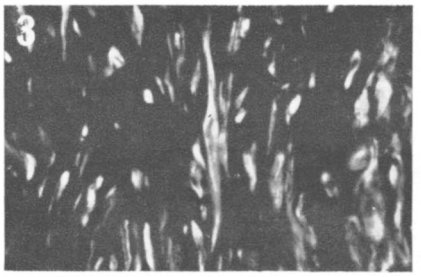


FIG. 2. Twenty-eight-day-old granuloma pouch: (A) stained with hematoxylin–eosin; (B) treated with normal human serum, followed by fluorescent antihuman IgG; (C) treated with HASM serum, followed by fluorescent antihuman IgG; ×96.



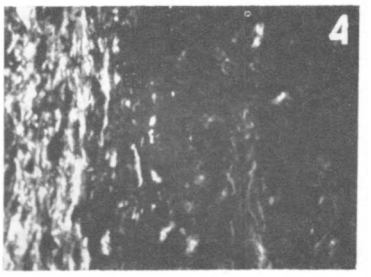


FIG. 3. Twenty-two-day-old granuloma pouch treated as in 2C: Cytoplasmic contents fluoresce; whereas extracellular material is unstained; ×380.

FIG. 4. Forty-two-day-old granuloma pouch: Fluorescence has disappeared from the outer part of the wall; ×100.

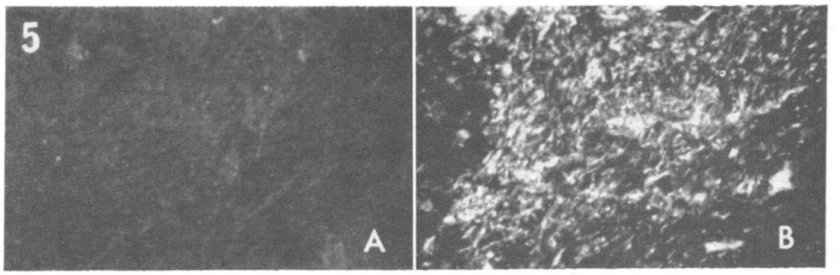


FIG. 5. Eleven-day-old skin wound: (A) treated as in 2B. (B) treated as in 2C; ×100.

at −65°.

The use of human antiserum, rather than artificially produced antibody, represents of course a compromise, since the corresponding antigen is not known; however, the compromise is acceptable because the antibody shows, at the histological level, a high degree of specificity for smooth muscle cells (apart from some reaction with skeletal muscle and glomeruli).

The findings reported here correlate well with our earlier data (3, 4). By electron microscopy, normal fibroblasts contain few fibrils; significant bundles are visible in both Selye pouches and wounds by the time specific fluorescence is detectable. Strips of Selye pouches contracted *in vitro* more strongly than strips of wound granulation tissue—and correspondingly more fluorescent cells were found in pouch walls than in wounds.

Further work will be needed to elucidate the nature and location of the common antigen(s) within granulation tissue fibroblasts and smooth muscle; in any event, our results provide a further link between these two types of cells.

Summary. Human antibody against smooth muscle such as found in patients with autoimmune hepatitis binds to rat fibroblasts in contracting granulation tissue, but not to normal rat fibroblasts. This provides another link between granulation tissue fibroblasts and smooth muscle cells.

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