Localization of Smooth Muscle Protein in Myoepithelium by Immunofluorescence

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ARCHER AND KAO [1] HAVE SHOWN with the direct immunofluorescence (IF) technic that antisera to actomyosin extracted from human colon stained not only the smooth muscle of human blood vessels, but also the myoepithelial cells in human skin, breast and salivary gland. It was concluded that myoepithelial cells contain proteins that share common antigenic determinants with smooth muscle proteins.

In view of the importance of this observation and the known difficulty of ensuring specificity in IF staining by the direct method,[2] the present authors have extended these investigations, using antisera produced against actomyosin and myosin prepared from fresh human uterus, and employing the more sensitive indirect IF technic.

Materials and Methods

Preparation of Antigens

Actomyosin was extracted by a modification of the method of Knieriem *et al.*[3] The cervix, endometrium and peritoneum were dissected from human nonpregnant uteri within 1 hour after they were removed surgically, and 345 g of myometrium were passed through a tissue grinder. A total of four dilution-precipitations were performed and the final precipitate was taken up in an equal volume of buffered 1 M NaCl, yielding a viscous, opalescent solution, which was then centrifuged for 30 minutes at 35,000 g to remove subcellular particles. The resulting solution contained 440 mg% of protein, estimated by the method of Lowry.[4] All manipulations were carried out at 4 C.

The effect of adenosine triphosphate (ATP) on the viscosity of the solution was measured as described by Weber and Portzehl,[5] using an Ostwald-type viscosimeter with a capacity of 15 ml and a flow-time for the solvent (1 M NaCl) of 60 seconds at 20 C. The final concentration of $MgCl_2$ in the actomyosin solution was 10^{-3} M, and the concentration of ATP, 1.7×10^{-4} M. The abrupt fall in viscosity when ATP is added (Text-fig 1) is characteristic of actomyosin. A portion of the actomyosin solution was mixed 1:1 with glycerol and stored at −25 C, and the remainder used for preparation of myosin.

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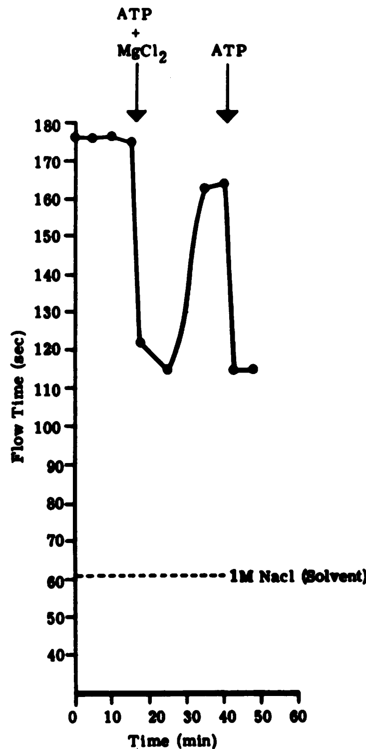

TEXT-FIG 1.—Effect of adenosine triphosphate on viscosity of human actomyosin (440 mg% in 1 M NaCl).

Myosin was prepared by ultracentrifuging the buffered actomyosin solution for 3 hours at 100,000 g, at 1 C, in the presence of adenosine triphosphate (3 mg/ml), and $MgCl_2$ (10^{-3} M), according to the method of Weber.[6] The upper one third of the supernatant containing myosin was collected. It was a water-clear, slightly viscous solution containing 174 mg% protein. Sedimentation studies in a Spinco Model E analytic ultracentrifuge yielded a curve with a single large peak, and two smaller peaks indicating the presence of a faster-moving component and a small slow-moving component (Fig 1). Sedimentation velocity of the material represented by the main peak was calculated as $S_{20,w} = 5.35$, a figure slightly lower than that recorded for skeletal muscle myosin by Weber ($S_{20,w} = 6.0$) under comparable conditions.[6] The myosin solution was mixed 1:1 with glycerol and stored at −25 C.

Preparation of Antisera

An aliquot of the actomyosin-glycerol solution was emulsified with complete Freund's adjuvant, and 0.5 ml containing 100 μg actomyosin was injected directly into a popliteal lymph node of each of 2 adult rabbits; 2 more adult rabbits were injected in identical manner with 50 μg of myosin. Booster injections containing 1 mg of antigen in incomplete Freund's adjuvant were given subcutaneously at 3 weeks and 5 weeks. Forty days after the primary immunization, single precipitation lines were present in an agar double-diffusion system at 1:4 dilution of serum in the animals injected with actomyosin, and at 1:2 dilution in the animals injected with myosin; sera were collected from all 4 animals and stored at −25 C.

Antiserum against rabbit gamma globulin was produced in a goat, the globulin

fraction of this serum (GARG) was conjugated with fluorescein isothiocyanate, and unreacted fluorescent material was removed by gel filtration in a Sephadex column (G-25).[7] Nonspecific staining was removed by absorption with acetone-dried rat liver powder. The specificity of this antiserum was established by methods described previously.[8]

Procedures for Immunofluorescent Staining

With the exception of rat liver powder used in absorption of GARG, all tissues stained and all tissues used in preparation of homogenates for absorption procedures were obtained from adult human subjects. Samples of female breast, stomach and uterus were obtained from surgical specimens within 30 minutes of excision; all other tissues were removed postmortem up to 16 hours after death. All tissue samples were quick-frozen at −76 C.

Unfixed cryostat sections cut a 7 μ were stained by the indirect method, mounted in buffered glycerol, and examined in blue-violet/ultraviolet illumination, using a darkfield condenser. The details of the staining procedure and microscopy have been described previously.[8] Photographs were taken with Kodak high-speed Ektachrome film, and black-and-white prints were made from the color transparencies.

In preliminary experiments, it was found that 10-minute fixation of tissue sections in acetone or 95% alcohol at room temperature increased the intensity of specific staining, while similar treatment with 4% neutral buffered formaldehyde abolished staining.

All absorptions of antisera were done for 1 hour at room temperature with saline homogenates of human postmortem tissues.

Results

All antisera were tested against the following human tissues: uterus, prostate gland, submaxillary salivary gland, parotid gland, pancreas, testis, skin, female breast, skeletal muscle, cardiac muscle, stomach, collagen (fascia), kidney and small intestine. All structures known to contain smooth muscle were stained positively: specifically, all blood vessels from large arteries and veins (Fig 2) down to arterioles and venules (Fig 3), muscularis mucosae of the stomach (Fig 4) and small intestine, smooth muscle of the uterine myometrium, the muscular stroma of the prostrate (Fig 5), and arrectores pilorum of the dermis. The outlines of the smooth muscle cells, both individually and in small bundles, were well defined in both longitudinal and cross section.

In addition to smooth muscle, cells with the characteristic distribution of myoepithelium were stained in the breast, salivary glands and dermal sweat glands. In the breast, the stained cells were distributed with equal frequency in the intralobular acini and the interlobular ducts (Fig 6 and 7), while in both parotid and submaxillary glands, they were confined to acini and intercalated ducts, and were absent from large interlobular ducts (Fig 8 and 9). In the dermal sweat glands, positively stained cells were confined to the coiled, secretory

portion of the gland, and were not seen in the straight portions of the duct (Fig 10). In all of these locations, the cells in question had numerous attenuated processes extending between secretory epithelial cells, as well as forming a discontinuous layer in the region of the basement membrane. The intervening basement membranes were unstained. All other cells of the tissues examined remained unstained, as well as all intercellular material, including basement membranes of all glandular structures, renal glomeruli and tubules. Cells in the basal region of prostatic glands were not stained. In all of the tissues, positively stained blood vessels were present as controls.

All of the seminiferous tubules of two specimens of testis studied were surrounded by a band several layers thick of positively stained cells (Fig 11) that were elongated and similar in shape to smooth muscle cells, and were interspersed with strands of weakly autofluorescent collagen.

Cardiac or skeletal muscle were not stained (Fig 12), although blood vessels in these tissues showed brightly positive specific fluorescence.

All four of the antisera gave the same staining results, with no difference between the anti-myosin and anti-actomyosin sera. Gamma globulin prepared from two of the sera—one anti-myosin and one anti-actomyosin—gave the same results as the whole serum.

Absorption of the antisera with homogenates of human liver, thyroid, brain, kidney, skeletal muscle, cardiac muscle and fascia had no effect on their specific staining properties, while absorption with homogenates of prostate or uterus completely abolished specific staining.

Discussion

These results confirm the previous demonstration [1] of a close relationship between the specific protein of smooth muscle and that of myoepithelium. Particular care was taken in the present study to test the reproducibility and specificity of the immunohistochemical reactions. Beck *et al* [2,8] have discussed the criteria for reliability of such reactions, pointing out that, besides characterizing the antigens, it is necessary to demonstrate that for each of the antisera used in the indirect immunofluorescence method the reaction is immunologic in nature, reproducible and specific.

The Antigens

Although the abrupt fall in viscostiy of the actomyosin solution when ATP and $MgCl_2$ are added is sufficiently unique [9] to establish

the presence of actomyosin as the principal constituent, it is likely that impurities were present in the solution. Needham *et al*[10,11] have shown that the high-dilution precipitate of salt extracts of the uterus contains significant amounts of tonoactomyosin and salt-soluble collagen. Antibodies to such contaminants were probably responsible for some of the nonspecific staining, which required several absorptions for removal. These same substances, and possibly small amounts of depolymerized G-actin, may have been responsible for the slow-moving component appearing in the sedimentation curve of the myosin. The faster-moving component probably consisted of high-molecular-weight aggregates of myosin.[12,13] No precipitation lines were obtained when the rabbit antisera were tested in the agar diffusion system against normal human serum, and it is unlikely that human serum proteins were present in the solutions of antigens in significant amounts. In any case, results of the subsequent staining procedures indicated that the antibodies formed were specific for an antigen or antigens peculiar to smooth muscle, and there was no evidence of significant interference with the specific staining reaction by contaminating substances.

Specificity of the Staining Reactions

The specificity of the goat anti-rabbit conjugate was established by methods analogous to those used by Beck *et al*[8] to confirm the immunologic specificity of rabbit anti-goat conjugate. The results of these tests fulfilled the criteria for immunologic specificity of immunofluorescent staining laid down by Beck and Currie.[2] The consistent localization of positive staining and the effect of absorptions are evidence for the specific nature of the rabbit antisera. All of the cells and tissue components known to contain smooth muscle and myoepithelium always stained positively, and no other tissue components were stained. The absence of staining of basement membranes is especially notable, since these are invariably closely apposed to myoepithelium and are difficult to distinguish histologically. The complete ablation of specific staining by absorption with tissues with a high content of smooth muscle, together with the failure of absorption with a number of other tissue homogenates to affect positive staining further indicate the specific nature of the reaction. The ability to reproduce the same localization of staining and the specificity with the gamma-globulin fraction of the antisera is evidence for the immunologic nature of the reaction.

Evidence thus far indicates that, although myosin and actomyosin of skeletal muscle are identical in many of their physicochemical prop-

erties with the same proteins extracted from smooth muscle, significant differences exist.[14] The finding that antisera against uterine smooth muscle proteins failed to cross-react with skeletal muscle in the immunofluorescence system is in agreement with the observations of Becker and Murphy,[15] and constitutes further evidence for differences in the physical or chemical properties of these proteins, or in the manner of their linkage in the two types of muscle. On the other hand, Knieriem *et al*[3] obtained positive staining of smooth muscle cells with labeled antibody prepared against actomyosin and myosin from cross-striated muscle. Further work is needed to clarify the immunologic relationships of the contractile proteins from these sources.

Myoepithelium

The appearance and distribution of myoepithelium in the present study is identical with that demonstrated in the previous study,[1] and conforms to descriptions in the literature based on histologic and electron microscopic observations.[16-18] In their immunofluorescence studies of contractile cells in cardiovascular lesions, Becker and Murphy,[15] using labeled antibody against uterine smooth muscle, also noted staining of myoepithelial cells in human breast, salivary gland and skin.

Rowlett and Franks,[19] using electron microscopy, found myoepithelium in the mouse prostrate gland, but other investigators have not described such cells in human prostate,[20] and the immunofluorescence studies reported here show no evidence of contractile cells in the basal region of human prostatic glands.

The presence of contractile cells surrounding the seminiferous tubules of the testis is confirmed by these immunofluorescence studies. The positively stained cells lie outside of the tubular basement membrane and have the elongated shape of smooth muscle cells. Their location and appearance correspond closely to the description by Ross,[21] based on electron microscopic studies. Contractile cells in this location that stain positively with fluorescein-labeled anti-actomyosin antibody have also been described by Straus and Kao.[22]

Summary

Antisera prepared against human uterine myosin and actomyosin, labeled with fluorescein isothiocyanate and tested against a spectrum of human tissues, react specifically with smooth muscle and with myoepithelium, but not with skeletal muscle or cardiac muscle. The reaction is immunologic in nature, and specific.

The distribution of myoepithelium in normal human breast, salivary

gland and skin, as demonstrated by this technic, conforms to descriptions in the literature based on electron microscopic evidence, but no myoepithelium could be demonstrated in the human prostate gland. The presence of contractile cells resembling smooth muscle in the lamina propria of the testes is confirmed in this study.

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Legends for All Figures

Fig 1.—Ultracentrifugal pattern of myosin derived from human uterine smooth muscle (protein concentration, 1.7 mg/ml in 1 M phosphate-buffered NaCl; temperature, 514 C; speed, 59,780 rpm; diaphragm angle, 50°).

Fig 2.—Small arteries in submucosa of stomach, showing binding of specific antiserum by smooth muscle of arterial wall. Brightly stained band at inner edge of larger artery is autofluorescent elastic membrane (anti-actomyosin, × 340).

Fig 3.—Localization of labeled immune globulin in smooth muscle of small vessels between gastric glands (anti-actomyosin, × 340).

Fig 4.—Specific fluorescence in muscularis mucosae of stomach. In adjacent glandular and submucosal regions, staining is virtually absent except for occasional small vessel (anti-actomyosin, × 340).

Fig 5.—Bands of smooth muscle cells adjacent to gland in prostate. There is no indication of band of positively stained contractile cells in region of basement membrane. White spots that appear in glandular epithelium are autofluorescent ceroid pigment (anti-myosin, × 340).

Fig 6.—Specific immunofluorescence localized in myoepithelial cells in terminal ducts and acini of normal breast lobule (anti-myosin, × 340).

Fig 7.—Pattern of distribution of myoepithelium in large duct of normal breast is revealed by localization of labeled antibody (anti-myosin, × 340).

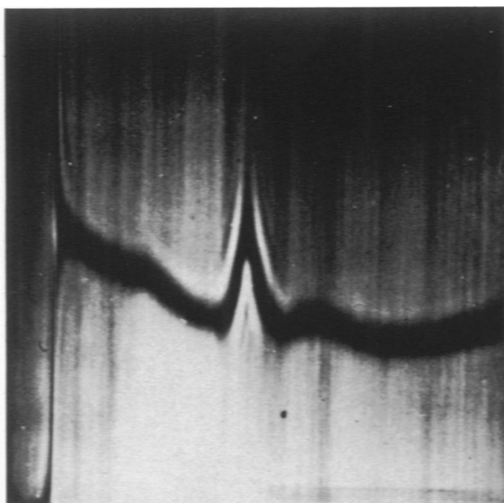
Fig 8.—Cytoplasmic processes of myoepithelial cells and their characteristic basket-like arrangement around acini are illustrated in this section of submaxillary salivary gland stained with anti-actomyosin antiserum. Note absence of myoepithelium in interlobular duct *(D)*. Droplets in duct epithelium are autofluorescent ceroid pigment (× 340).

Fig 9.—Myoepithelium in parotid gland has similar appearance and distribution to that in submaxillary gland. Duct contains ceroid pigment and no myoepithelium (anti-myosin, × 340).

Fig 10.—In eccrine sweat glands of skin, immunofluorescent staining reveals myoepithelium in terminal ducts; it is absent in straight ducts and in those parts of duct system that contain no ceroid pigment (anti-myosin, × 340).

Fig 11.—Immunofluorescent staining of section of normal adult testis, showing localization of immune globulin in band of cells surrounding each seminiferous tubule. Positively stained cells lie among strands of dimly autofluorescent collagen fibers. Small artery is at center (anti-myosin, × 340).

Fig 12.—Cross-striated skeletal muscle, showing negative reaction after exposure to anti-actomyosin antiserum. Small artery at center is brightly stained (× 340).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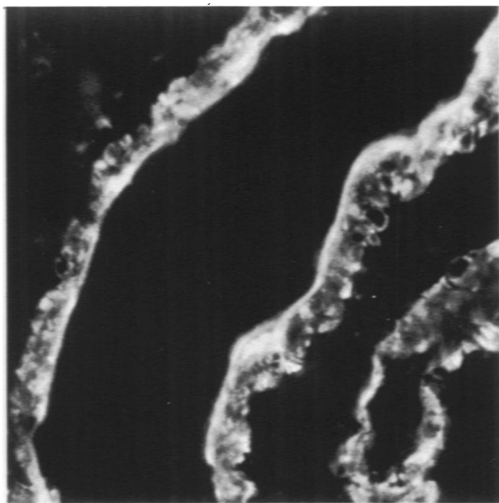
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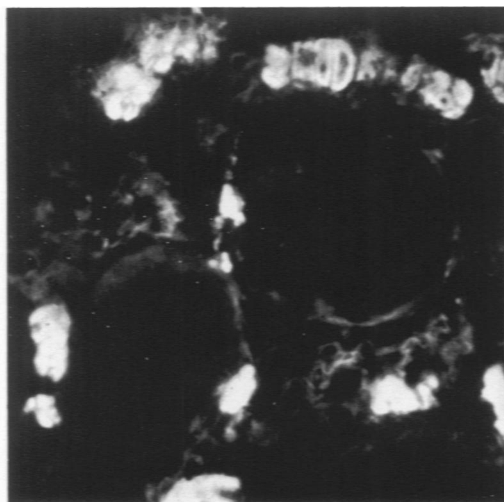
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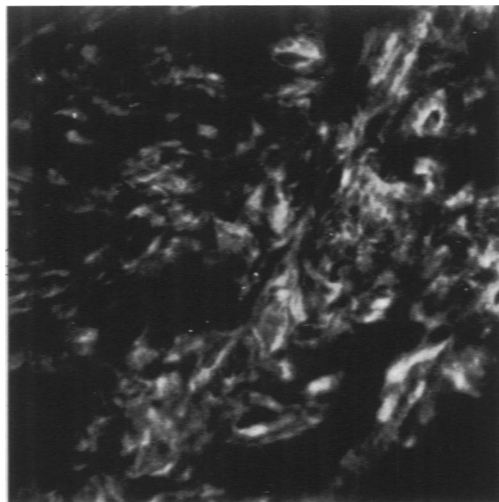
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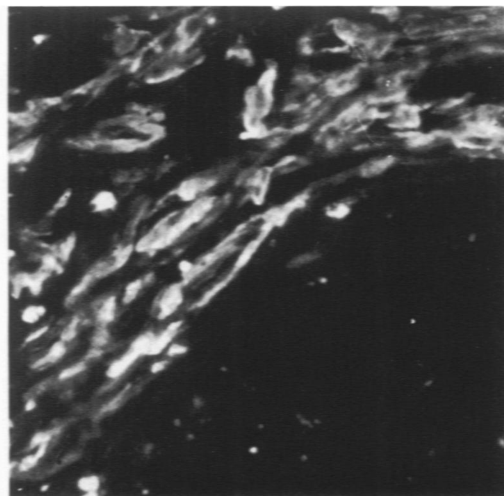
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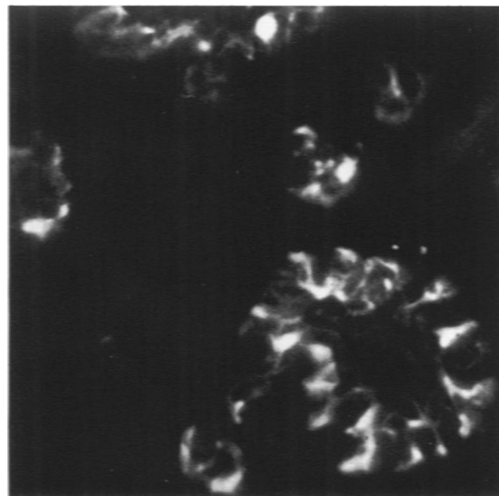
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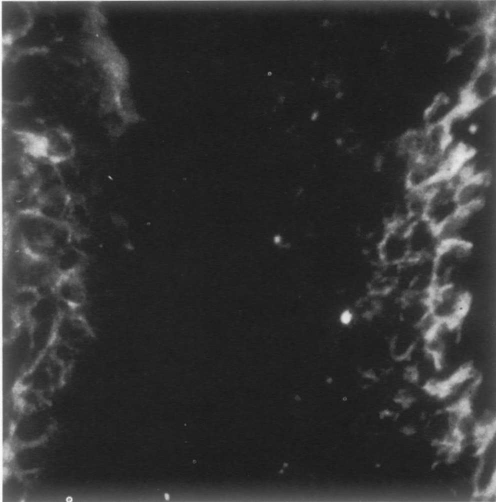

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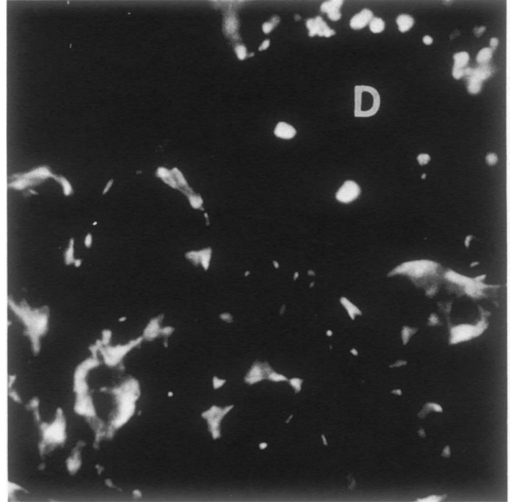

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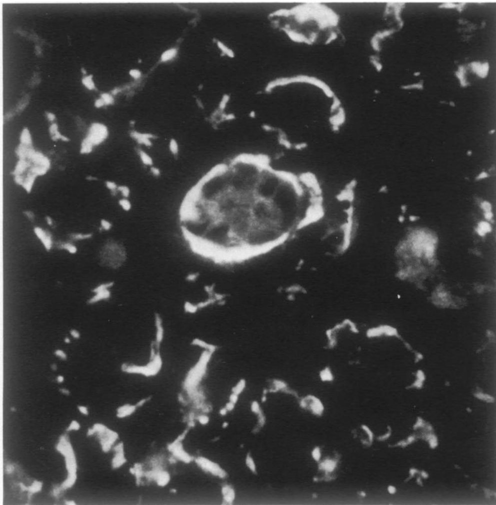

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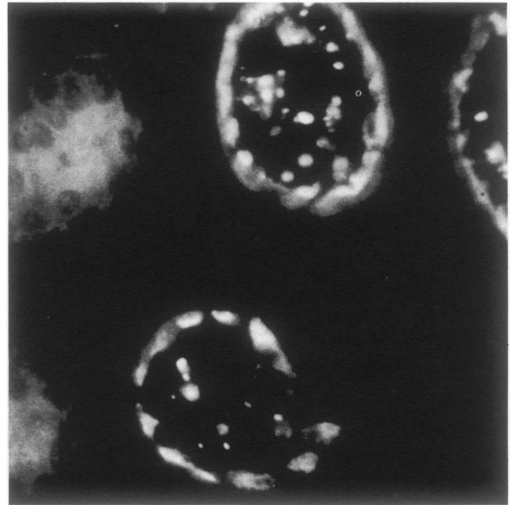

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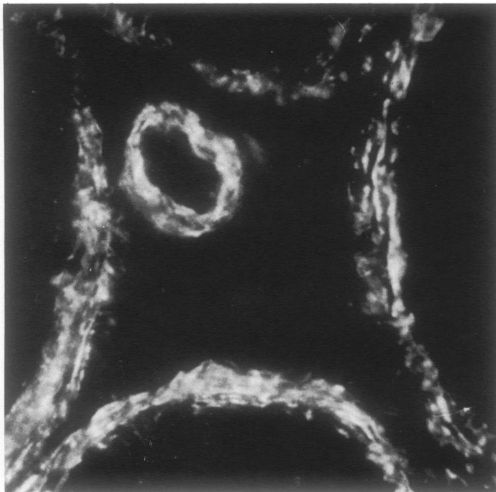

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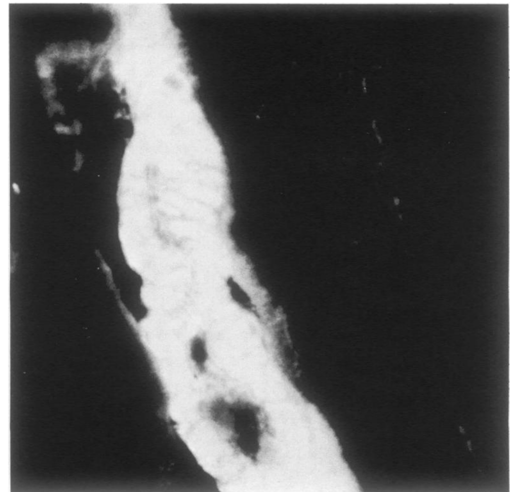

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