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IMMUNOFLUORESCENT TRACING OF SMOOTH MUSCLE CONTRACTILE PROTEIN ANTIGENS IN TISSUES OTHER THAN SMOOTH MUSCLE

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SUMMARY

Rabbit antisera were prepared against six contractile proteins of smooth muscle—actomyosin, actin, myosin, heavy meromyosin, light meromyosin and tropomyosin—extracted from human uterus by standard procedures. Antibodies were sought by double-diffusion in gel and by immunofluorescence on cryostat sections of rat tissue. All the antisera except anti-actin gave precipitin lines with their respective antigens, and all gave specific fluorescence with smooth muscle fibres and produced staining of liver cells in the region of their cell membranes (polygonal pattern). Anti-actin sera stained in addition renal glomeruli, and also a microfilamentous network in HeLa cells and chick embryo fibroblasts. Anti-heavy meromyosin (and less brightly anti-actin) stained the cytoplasm of bile duct epithelial cells, and the apical and basal regions of intestinal muscosal cells in a linear fashion. These patterns of staining between them reproduce the range of staining patterns seen with human sera positive for smooth muscle antibody by immunofluorescence.

INTRODUCTION

Human smooth muscle autoantibodies were first discovered in the sera of patients with chronic active hepatitis (Johnson, Holborow & Glynn, 1965) and have since been found in several other conditions (Doniach & Walker, 1969; Farrow *et al.*, 1970; Whitehouse & Holborow, 1971; Holborow, Hemsted & Mead, 1973). The antibodies are detected by indirect immunofluorescence on cryostat tissue sections, and the characteristic staining is seen in the smooth muscle fibres of rat stomach and blood vessel walls. It has been suggested that this is due to antibodies against smooth-muscle actomyosin, since extracts of human myometrium containing this protein are effective in absorbing out the antibody (Farrow, Holborow & Brighton, 1971; Holborow, 1972).

Many sera which show smooth muscle antibody (SMA) activity on immunofluorescence

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also give a polygonal pattern of fluorescence in the liver (Johnson, Holborow & Glynn, 1966; Farrow *et al.*, 1971), or glomerular fluorescence (Whittingham, MacKay & Irwin, 1966), or both.

Some positive sera also stain bile ducts in liver sections and the apical portions of epithelial cells in the stomach and kidney. The question therefore arises whether this spectrum of staining patterns is evidence that in conditions where 'smooth muscle antibody' is produced this represents a variable range of antibodies against antigenically different intracellular contractile proteins related to smooth muscle actomyosin.

We have therefore prepared rabbit antisera against six different contractile proteins extracted from human smooth muscle and have examined the staining patterns they give on immunofluorescence. This paper reports the distribution of smooth muscle actomyosin, myosin, actin, tropomyosin, heavy meromyosin and light meromyosin in various organs and tissues.

MATERIALS AND METHODS

Pregnant and non-pregnant human uteruses were obtained freshly from the operating theatre and kept frozen at −70°C. They were thawed immediately before use, cut into pieces and homogenized.

Extraction procedures

Actomyosin was extracted with 0·6 M KCl and subsequently precipitated by 10 volumes of water (Becker, 1972). The precipitate was redissolved in 0·6 M KCl and re-precipitated. This was done three times and the final solution clarified by centrifugation at 30,000 ***g*** for 1 hr. To purify the actomyosin further, the solution in 0·6 M KCl was precipitated by adding an equal volume of distilled water, redissolving in 0·6 M KCl and repeating the procedure twice more. Myosin and the C and F proteins most likely to be present as contaminants are soluble in 0·3 M KCl.

Actin was extracted in de-ionized distilled water at 4°C from acetone-dried tissue powder prepared from the homogenate (Iyengar and Weber, 1964) and purified by passage through a column of Sephadex G-200 (Adelstein, Godfrey & Kielley, 1963). The second peak contained the actin, which aggregated readily in 0·1 M KCl and bound added myosin specifically.

Tropomyosin was extracted with 1·0 M KCl according to the procedure of Bailey (1948) but with two modifications: firstly, isoelectric point precipitation was performed at pH 4·1; and secondly ammonium sulphate precipitation was repeated three times, the fraction precipitating between 53 and 60% saturation being collected (Carsten, 1968; Carsten, 1971).

Myosin was extracted with KCl at low ionic strength (I 0·075) (Huriaux, Hamoir & Oppenheimer, 1967). Crystallized trypsin (Seravac Laboratories) was added in a weight ratio 1:200. The digestion period was prolonged to 40 minutes because of the greater resistance of smooth muscle myosin to trypsin digestion (Hamoir, 1973). Heavy meromyosin and light meromyosin were isolated from the digest by ammonium sulphate precipitation; heavy meromyosin was recovered between 45 and 50%, and light meromyosin between 50 and 70% ammonium sulphate saturation.

The various extracted protein solutions were run electrophoretically in 6–8% sodium dodecyl sulphate polyacrylamide gel (Weber & Osborn, 1969) and the separated protein bands stained with Coomassie Blue (25%). Only one band was observed with heavy meromyosin, tropomyosin and actin; the other preparations gave two or more bands.

Immunization

Immune sera against the extracted proteins were raised in rabbits by means of a single subcutaneous injection of 2 mg protein per rabbit emulsified in Freund's incomplete adjuvant. Animals were bled 21 days later.

The sera were tested by double diffusion in agar containing 0·6 M KCl, and by indirect immunofluorescence as previously described (Farrow *et al.*, 1971). For the latter 6 μm cryostat sections of unfixed snap-frozen rat organs were used, and the sera were screened for antibodies at a dilution of 1:30. The sera were also tested on monolayer tissue culture

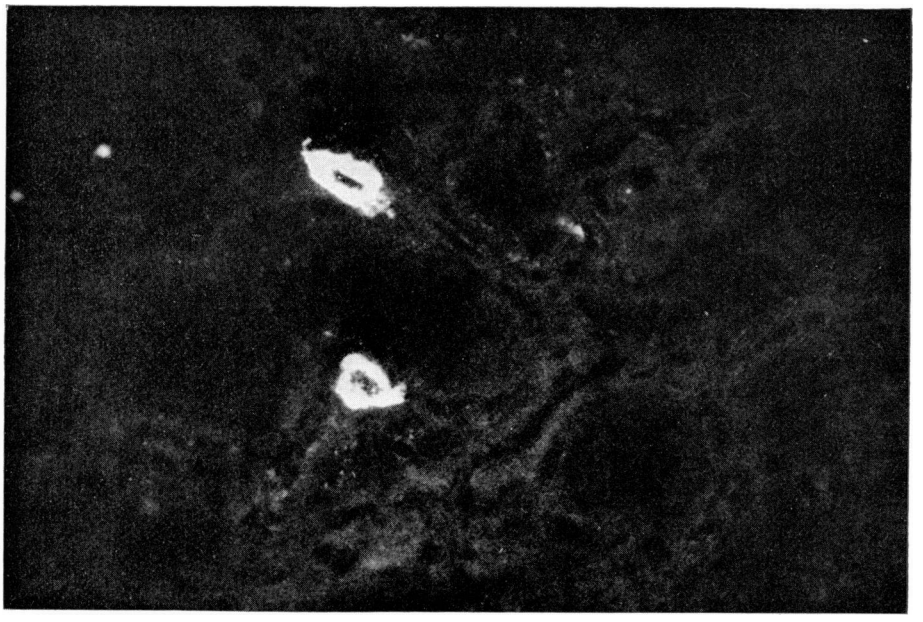

FIG. 1. Anti-tropomyosin antiserum staining two small blood vessels in rat kidney. Indirect immunofluorescence. (Magnification ×240.)

preparations of chick embryo fibroblasts and of HeLa cells taken after 24 hr growth. A goat anti-rabbit Ig FITC conjugated antiserum (Nordic) of tested specificity was used at a dilution of 1:30, which gave no background staining of substrates when normal rabbit serum was used as the middle layer.

Absorptions were carried out by adding one drop of protein solution containing 0·125 mg to one drop of antiserum and eight drops of saline.

Immunodiffusion in agar was used to test for antibodies reacting with human serum proteins and normal saline extracts of human uterus.

RESULTS

Precipitation tests

In double-diffusion in agar, the anti-tropomyosin sera gave a single line of precipitation against tropomyosin, and no precipitate against the other proteins. Anti-actomyosin

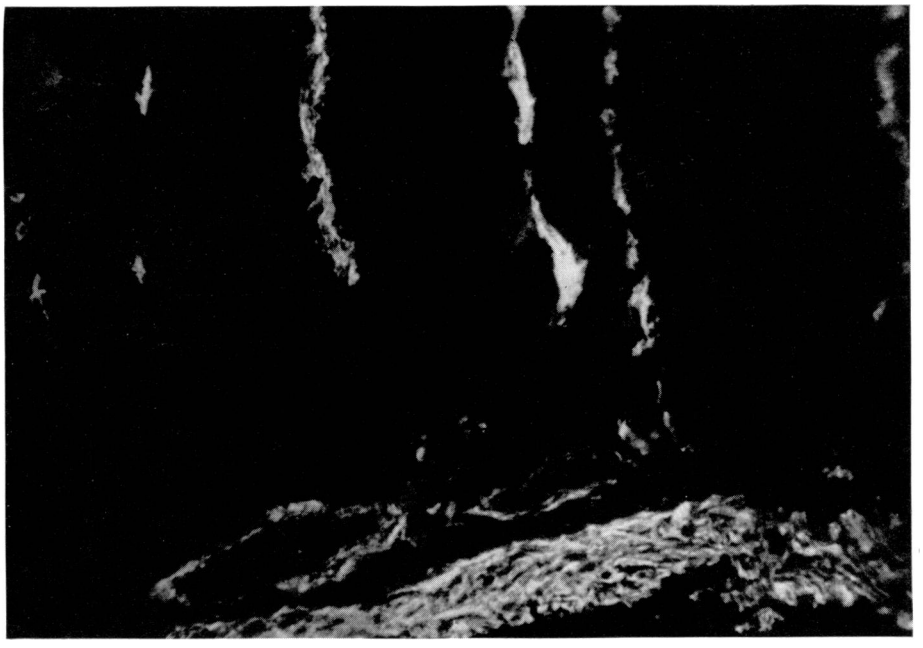

FIG. 2. Anti-myosin antiserum staining smooth muscle fibres in mucosa, muscularis mucosa and blood vessel walls in rat stomach. (Magnification × 192.)

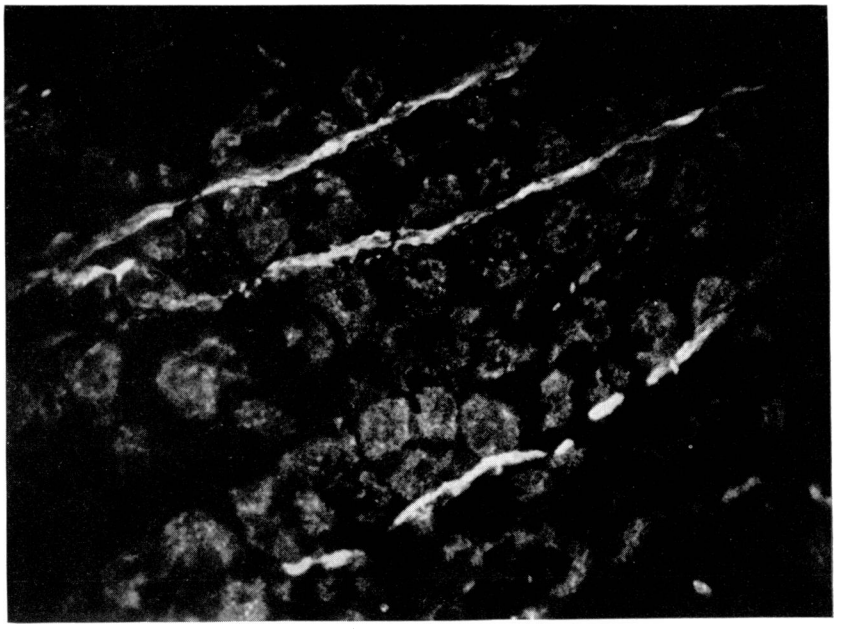

FIG. 3. Anti-actin antiserum staining smooth muscle fibres in rat stomach mucosa. (Magnification × 192.)

and anti-myosin sera both gave single lines with both actomyosin and myosin. Anti-heavy meromyosin gave a single line with heavy meromyosin only, but the best anti-light meromyosin serum produced a faint line against tropomyosin that showed continuity with the stronger line this serum gave with light meromyosin. The precipitation lines took 8–10 days to develop at 4°C, and several re-fillings of the antiserum wells were necessary. With the actin–anti-actin system no precipitates were obtained.

Immunofluorescent staining

All the antisera stained smooth muscle cell cytoplasm in blood vessel walls (Fig. 1), stomach (Figs 2 and 3), duodenum, intestine, colon, lung, ureter and bladder. The intensity

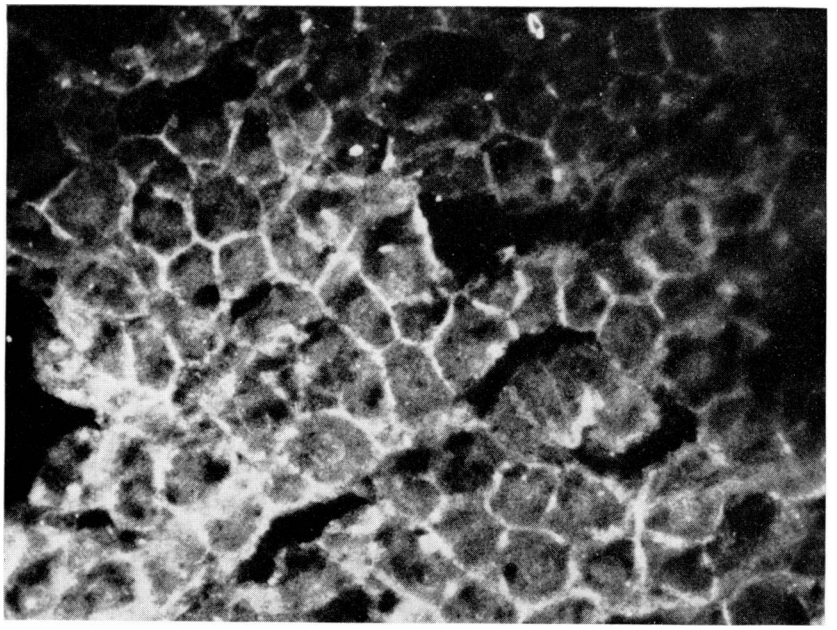

FIG. 4. Anti-heavy meromyosin antiserum; polygonal pattern on rat liver sections. (Magnification ×192.)

of the fluorescence varied from serum to serum. The strongest staining of smooth muscle fibres was seen with anti-myosin serum, and in descending order with anti-actomyosin, anti-actin, anti-tropomyosin and anti-light meromyosin. The weakest staining was with anti-heavy meromyosin. The appearances did not differ morphologically from the smooth muscle staining seen with patients' sera. When tissue sections of rat heart and skeletal muscle were used instead, the only specific fluorescence was in vessel walls, except with anti-actin, which gave some additional staining with heart muscle.

As well as staining smooth muscle fibres, all the sera also produced staining of liver cells in the region of their cell membranes (Fig. 4). The pattern was closely similar to the polygonal pattern of fluorescence of hepatocytes often seen with human sera containing SMA.

Sera against one of the extracted proteins, actin, also stained renal glomeruli (Fig. 5).

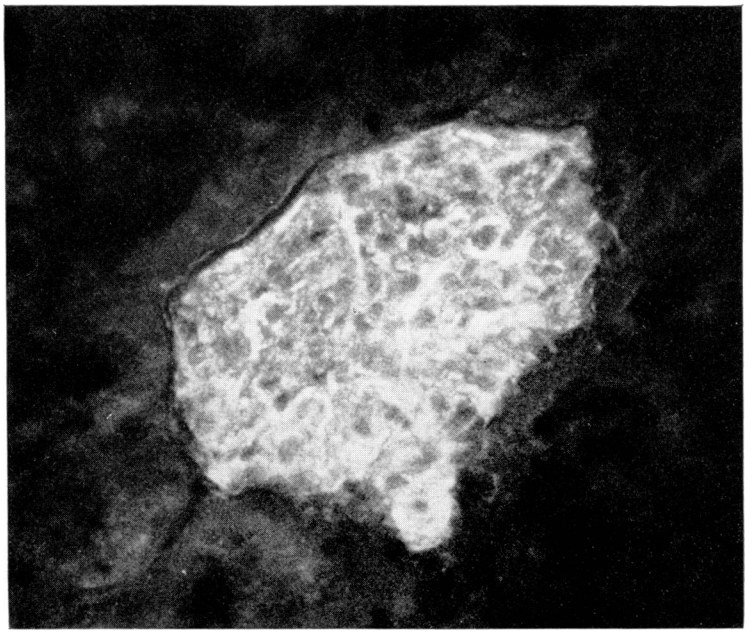

FIG. 5. Staining of rat renal glomerulus by anti-actin antiserum. The adjacent arteriole is also stained. (Magnification × 192.)

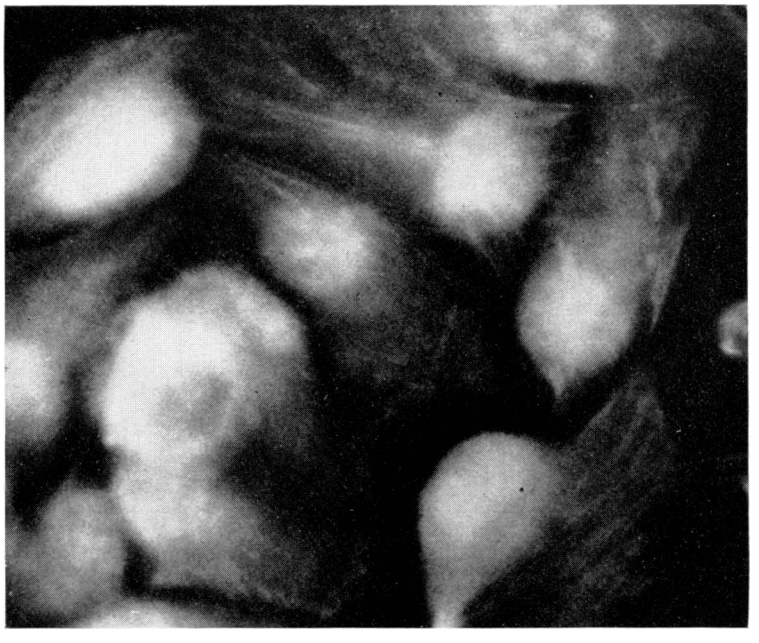

FIG. 6. Anti-actin antiserum giving intracellular filamentous staining of HeLa cells. The nuclear fluorescence is unrelated to the anti-actin activity and is seen with many rabbit sera. (Magnification × 384.)

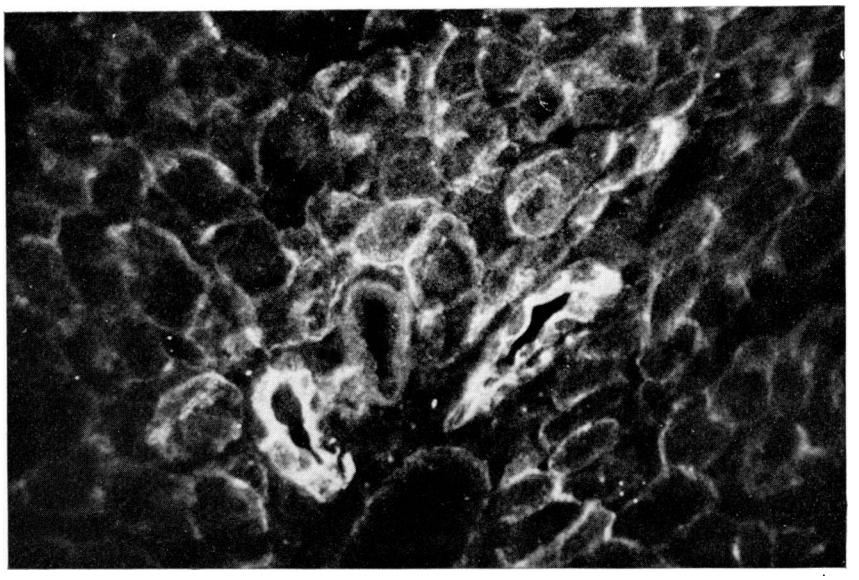

FIG. 7. Anti-heavy meromyosin antiserum staining a rat liver section. In the portal tract the vein (below) and the artery (above) are unstained, while the bile ducts on either side show cytoplasmic fluorescence. (Magnification × 192.)

FIG. 8. Longitudinally sectioned mucosal gland in rat duodenum stained with anti-heavy meromyosin. The apical portions of the epithelial cells show bright fluorescence. (Magnification × 192.)

The pattern of glomerular fluorescence was diffuse and not of anti-basement membrane type, and was indistinguishable from that seen with some SMA-positive human sera. Of the other antisera, only anti-heavy meromyosin gave glomerular staining, but the intensity was considerably less. The anti-actin sera also stained chick embryo fibroblasts and HeLa cell monolayers (Fig. 6). Specific fluorescence was seen as a fine filamentous network in the cytoplasm.

Apart from smooth muscle cells, the anti-heavy meromyosin sera stained the epithelial cells lining the bile ducts in the liver (Fig. 7), and also epithelial cells in the stomach duodenum and intestine. The fluorescence was concentrated at the apex of these cells (Fig. 8), but there was also linear staining near the base (Fig. 9). A similar pattern was found with anti-actin, but its intensity was weaker.

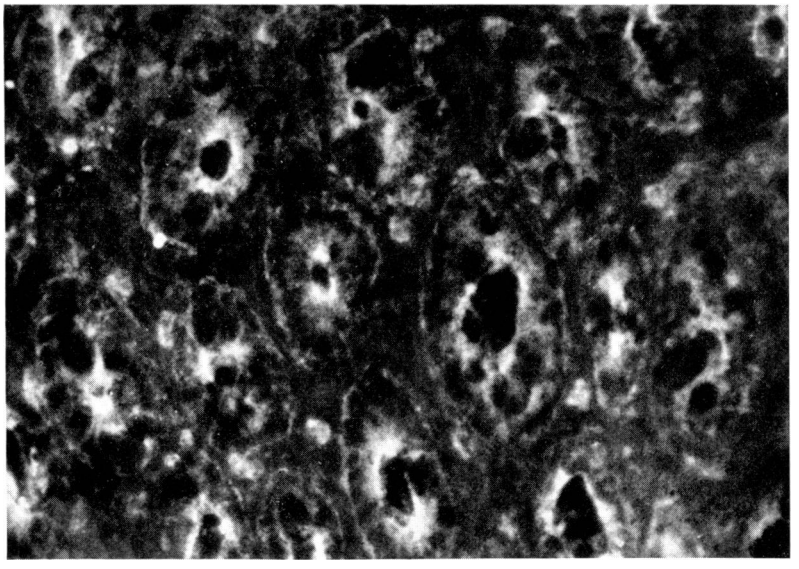

FIG. 9. Cross section of duodenum (as in Fig. 8) showing apical staining and linear basal staining with anti-heavy meromyosin antiserum. (Magnification × 168.)

Effects of absorption

The respective patterns of staining described above were always abolished by absorption with the appropriate antigens, including actin. In the case of the anti-actin sera, the ability of actin to absorb specific reactivity was abolished if the actin solution had been pre-treated with myosin, and the resulting insoluble complex removed by centrifugation. Pre-treatment of actin solution with tropomyosin and light meromyosin did not have this effect. Absorption with human serum or with saline extract of human uterus produced no effect.

DISCUSSION

Specificity of the antisera

That the antisera against these six different smooth muscle proteins showed differences in their respective staining patterns is evidence of differences in their specificities. The

patterns described were not affected by previous absorption with human sera or saline uterine extract, and were specifically removed only by adsorption with the appropriate antigens. Furthermore, polyacrylamide gel analysis of the various contractile protein antigens against which the antisera were raised showed only single bands in the cases of actin, heavy meromyosin and tropomyosin. The additional bands found in the myosin and actomyosin preparations may have included bands produced by the C and F proteins usually present in these extracts (Offer, Moos & Starr, 1973).

It may be noted that each rabbit received only a single injection of 2 mg of protein in Freund's incomplete adjuvant, and was bled 3 weeks later, and this schedule may well have favoured narrow specificity in the resulting antisera.

Although no precipitating antibody could be demonstrated in the anti-actin sera, immunofluorescence revealed their specific reactivity, which was confirmed by the absorption experiments described above. Anti-actin antibody activity in antisera produced by immunization with muscle proteins has not been previously reported; the similarity of the rabbit anti-actin glomerular staining pattern and the glomerular staining seen with some human sera containing SMA, suggests that anti-actin is one of the anti-contractile protein autoantibodies that may arise in human disease, and supports recent work of Gabbiani (personal communication). In experiments not reported here we have shown that absorption with actin removes the ability to give a glomerular pattern of staining from such human sera.

Distribution of contractile proteins in tissue cells

Although all the rabbit antisera stained smooth muscle fibres in the various organs used as substrate, the intensity of the staining varied, anti-myosin giving the most brilliant and anti-heavy meromyosin the weakest. It was also noted that vessel walls in different organs varied in the brightness of fluorescence they showed with the different antisera, arterial wall smooth muscle in the kidney, for example, staining more brightly than in the liver.

The immunofluorescent methods used in this work shows that the contractile proteins present in smooth muscle are widely distributed in many other tissues also. Electron microscopy has already demonstrated that thick and thin filaments, thought to be myosin and actin respectively, are similarly distributed in several different cell types (Ishikawa, Bischoff & Holtzer, 1969; Rostgaard, 1972; Burton & Kirkland, 1972) and Becker (1972) using anti-actomyosin serum in immunofluorescent studies, has reported the presence of smooth muscle actomyosin in glomeruli, intertubular capillaries and endothelial cells.

Different types of cell differ with regard to their predominant contractile protein(s). In the case of the epithelial cells of the rat duodenal mucosa, for example, the contractile proteins most readily demonstrable by immunofluorescence are heavy meromyosin and (less brightly) actin. These two proteins are quite sharply localized to a band in the apical regions of these cells, and to a thinner linear structure near the base of each cell. This distribution is exactly that of the microfilaments studied by Wessells *et al.* (1971) in relation to their role in morphogenesis. The intracellular distribution of actin which we have observed also accords with conclusions from electron microscopical studies based on morphological appearances and the binding of heavy meromyosin (Ishikawa *et al.*, 1969; Rostgaard, Kristensen & Nielsen, 1972). In the chick fibroblasts and HeLa cells, the specific immunofluorescence with anti-actin sera clearly took the form of a filamentous intracellular network, closely similar to a pattern already noted in chick embryo cells treated with human SMA-

positive sera (Farrow *et al.*, 1971). The patterns of specific fluorescence seen with the rabbit antisera reproduce all the patterns we have described as occurring among human sera positive for SM antibody. Two striking examples are the bright glomerular staining in the kidney and the cytoplasmic staining of bile duct epithelial cells in the liver sections. The glomerular staining was seen only with the anti-actin and (less brightly) with the anti-heavy meromyosin sera, and acurately reproduced the diffuse glomerular staining which often accompanies the smooth muscle staining produced by sera from human chronic active hepatitis (Johnson *et al.*, 1955), infectious hepatitis (Farrow *et al.*, 1971) and infectious mononucleosis (Holborow *et al.*, 1973). The cytoplasmic, chiefly apical, staining of bile duct epithelial cells was best seen with the rabbit anti-heavy meromyosin sera, and reproduced identically a pattern we have noted with chronic active hepatitis sera and with rheumatoid arthritis sera. This bile duct staining is often accompanied by staining of similar apical distribution in the epithelial cells of the gastric mucosal glands and identical with that described above in the duodenal mucosal epithelium.

These results suggest that human sera giving smooth muscle staining by immunofluorescence do so because they contain auto-antibodies to various constellations of contractile proteins, including those which constitute the microfilamentous machinery of many cell types. The production of such auto-antibodies could be attributed to immunological involvement of individual contractile proteins in disease processes. The elucidation of processes which bring this about will be the subject of future studies.

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