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Antigenic Properties of a Non-collagenous Reticulin Component of Normal Connective Tissue

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Summary. Rabbit antisera raised against a saline-insoluble non-collagenous reticulin component (NCRC) of pig and human kidney gave immunofluorescent staining of basement membranes, stroma of liver and kidney and newly formed blood vessels in pig, rat and human tissue. The staining patterns closely resembled those reported for anti-reticulin antibodies, and both species-specific and species-shared determinants could be distinguished. Although the antisera reacted least with rat glomeruli in cryostat sections, rabbit immunoglobulin localized persistently but harmlessly in the renal glomeruli of rats given the antisera intravenously. Absorption with NCRC of sera from patients with gluten-sensitive enteropathy in most cases removed the anti-reticulin antibody characteristic of this group of diseases.

INTRODUCTION

Although the principal structural proteins of connective tissue—collagen and reticulin—are readily distinguishable by their histological appearances, differentiation on a chemical basis is less well established. It was recently shown (Pras and Glynn, 1973) that a non-collagenous protein could be isolated by dispersion in water from normal tissue from which saline-soluble material had been extracted. This protein was distinguishable from collagen on amino acid analysis and by its resistance to collagenase. The purpose of this paper is to report the antigenic properties of this protein, which appears to be a component of reticulin.

MATERIALS AND METHODS

Tissues

Pig kidney and liver were obtained 1 hour after the animals were killed. Renal cortex was separated and stored with the liver at −20°.

Human kidneys were obtained at autopsy from the victim of a train accident (age 26) and a case of carbon monoxide poisoning (age 22), 24 and 8 hours after death respectively. The cortex was separated and stored at −20°.

Preparation of immunizing antigen

Non-collagenous reticulin component (NCRC) was isolated by water dispersion of the

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saline-insoluble proteins obtained from the different organs as previously described (Pras and Glynn, 1973). The protein was precipitated within 15 minutes after isolation by the addition of NaCl to a concentration of 0·15 M and stored at −20°, or for short periods at 4° up to 2 days.

Immunization

Rabbits were given 1 ml of an emulsion containing 1 mg of NCRC in Freund's complete adjuvant subcutaneously in five sites. The animals were bled after 4 weeks, boosted after 6 weeks and bled again 10 days after the second injection.

Fluorescent conjugates

Goat anti-rabbit globulin was conjugated with fluorescein isothiocyanate at a labelling ratio of 25 μg of FITC: 1 mg of protein for 90 minutes at 20°. Free dye was removed on a column of Sephadex G-25.

Sheep anti-human immunoglobulin fluorescein conjugate was obtained from Wellcome Reagents.

Human sera containing antibodies to reticulin demonstrable by immunofluorescence (Seah, Fry, Hoffbrand and Holborow, 1971) were obtained from cases of coeliac disease and dermatitis herpetiformis.

Indirect immunofluorescent technique. Cryostat sections 6 μm thick were cut from snap-frozen blocks of the following unfixed tissues: pig kidney and liver; rat kidney, liver, stomach, skeletal muscle, nerve, adrenal and granulation tissue (developing at the site of deep skin incision); guinea-pig kidney, liver, spleen; human kidney and rectal biopsies, appendix and skin. The sections were taken on to Multispot slides (O'Neill and Johnson, 1970), dried immediately with a fan and treated with diluted antisera (1:40–1:100) for 20 minutes. After a brief wash in phosphate-buffered saline they were stained for 20 minutes with conjugate diluted according to the results of chessboard titration (usually 1:30), washed finally for 1 hour and mounted in buffered glycerol. The ability of antisera to bind complement was tested by adding fresh human serum to the diluted sera, which were then applied to the tissue sections in the usual way and staining was carried out with fluorescein-conjugated anti-human βI_A-1_C. Appropriate controls were included as previously described (Johnson and Holborow, 1973).

Inhibition studies. Sera were incubated with saline-insolubilized NCRC for 1 hour at 37° followed by centrifugation at 15,000 rev/min (Spinco 40 rotor). Centrifuging at lower speeds was ineffective because of the tendency of the antigen to re-disperse in the presence of serum.

Fluorescence microscopy. Reichert equipment incorporating quartz–halogen illumination with cardioid dark-ground condenser, Balzer FITC-3 primary filter and Kodak-Wratten 12 secondary filter was employed. Agfa-Gevaert CT18 reversal film was used for the production of colour transparencies.

Immunoelectron microscopy

Tissues were fixed in 1 per cent paraformaldehyde in 0·1 M phosphate buffer at pH 7·2–7·4 containing 7·5 per cent sucrose for 30 minutes at 4°. Cryostat sections were treated with antiserum followed by peroxidase-conjugated goat anti-rabbit serum and prepared for electron microscopy as described by Webb and Dorling (1973).

In vivo *studies*

Rats were given a single intravenous injection of 0·5 ml of undiluted rabbit antiserum to pig kidney NCRC and killed after 10 minutes, 24 hours, 3 and 7 days, and 3, 7, 12 and 16 weeks. The kidneys were snap-frozen and cryostat sections stained with fluorescein-conjugated antiserum to rabbit globulin.

RESULTS

DISTRIBUTION OF ANTIGEN IN VARIOUS ORGANS

Antisera to NCRC isolated from pig or human renal cortex both produced staining by immunofluorescence of connective tissue structures in all organs and tissues examined: stromal reticulin (Fig. 1a, b); basement membranes (Fig. 2); connective tissue between epithelial cells in various endocrine glands (Fig. 3); muscle cells (Fig. 4a); and kidney tubules (Fig. 2), but did not stain collagen in skin or other organs. The staining thus resembled the reticulin pattern described by Cruickshank and Hill (1953) who employed anti-organ sera to study the distribution of antigens in connective tissue by immunofluorescence. No staining was obtained with anti-βI_A-1_C conjugate in sections treated with antisera to which complement had been added.

The pattern of immunofluorescent staining corresponded in a striking manner with the pattern obtained by the silver impregnation method for reticulin (Fig. 4b). However there were two exceptions where silver-positive material did not react with the antibody. The first of these was the stroma of young granulation tissue present in a 4-day-old healing wound in rat skin although young, newly formed blood vessels present in the same tissue were stained (Fig. 5a, b). Immunoperoxidase electron microscopy confirmed that in granulation tissue the material binding the antibody was confined to the vicinity of capillaries and small arteries. At higher magnification the immunoperoxidase staining was amorphous and closely associated with the surface of smooth muscle cells in the arteriolar wall (Fig. 5c). Immunoelectron microscopy also confirmed that collagen fibres were unstained (Fig. 5d). The second silver positive site which failed to show specific immunofluorescent staining with the antiserum was the connective tissue sheath around nerve fibres.

SPECIES SPECIFICITY OF THE ANTISERA

Rabbit antisera against pig NCRC stained stromal and basement membrane reticulin in tissues from a variety of mammalian species including rat, mouse, guinea-pig and man in addition to pig. Basement membranes were stained in all the organs tested from all these species, including outlines of capillaries and sinusoids as well as the adventitia of blood vessels. No differences were noted between species with but one exception—glomerular basement membrane was clearly stained in all species except the rat, where staining was only weak.

The staining obtained with antiserum against pig kidney NCRC was completely prevented by adsorption with NCRC prepared from pig kidney and liver. However, the addition of human kidney NCRC to antiserum to pig NCRC, while preventing immunofluorescent staining of reticulin in sections of human tissues, did not affect specific staining of rat or guinea-pig tissues. This indicates the presence of both species-specific and shared antigenic determinants in the reticulin preparations obtained from the various species.

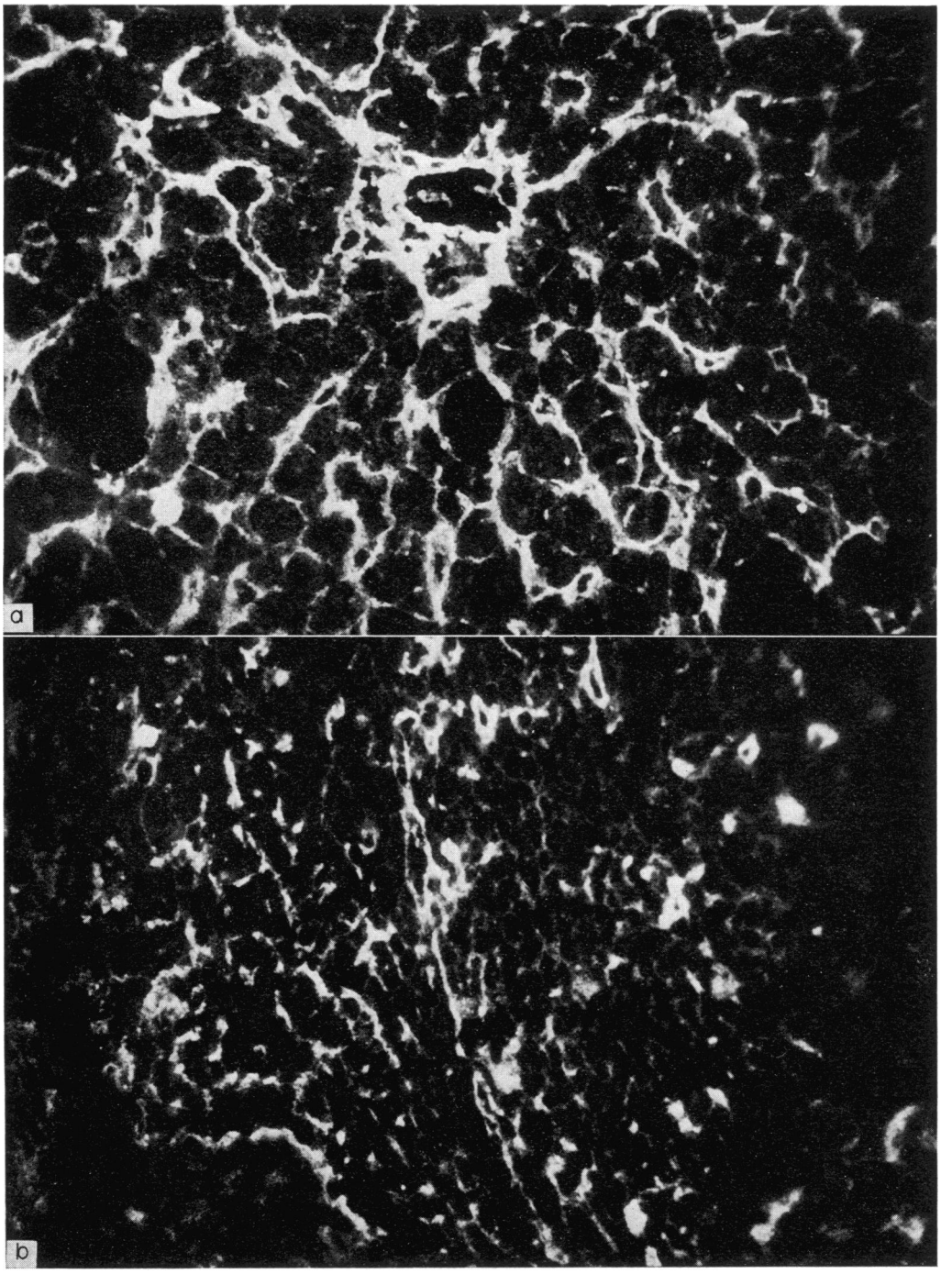

FIG. 1. (a) Cryostat section of guinea-pig liver, treated with rabbit anti-pig NCRC and fluorescein-labelled anti-rabbit immunoglobulin showing fluorescent staining of stromal connective tissue, and adventitia of a small vessel. (b) Guinea-pig spleen, showing similar stromal staining. (Magnification ×216.)

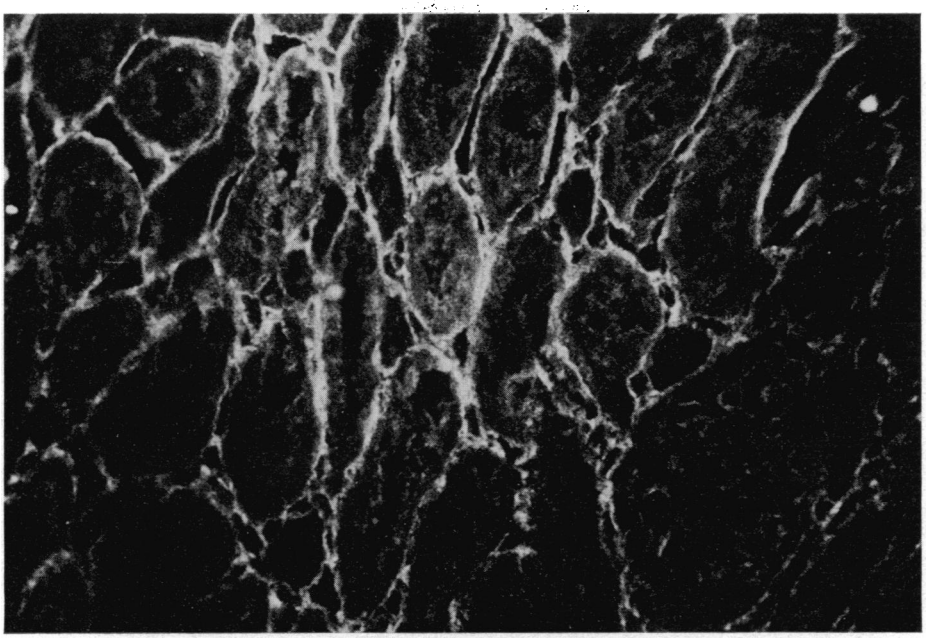

FIG. 2. Guinea-pig kidney, showing basement membrane staining in glomerulus, and staining of basement membrane and/or intertubular connective tissue.

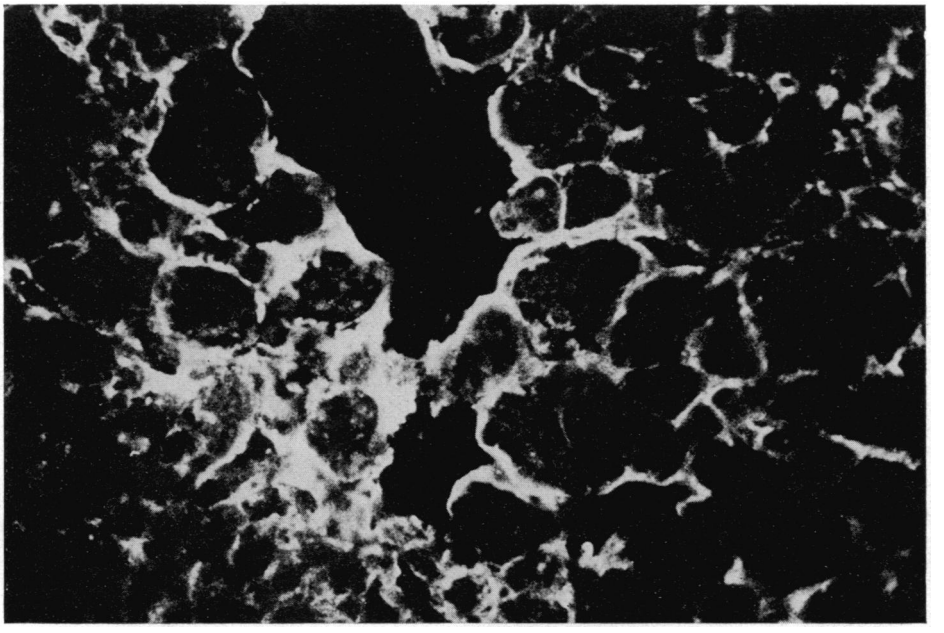

FIG. 3. Guinea-pig adrenal, showing stromal staining.

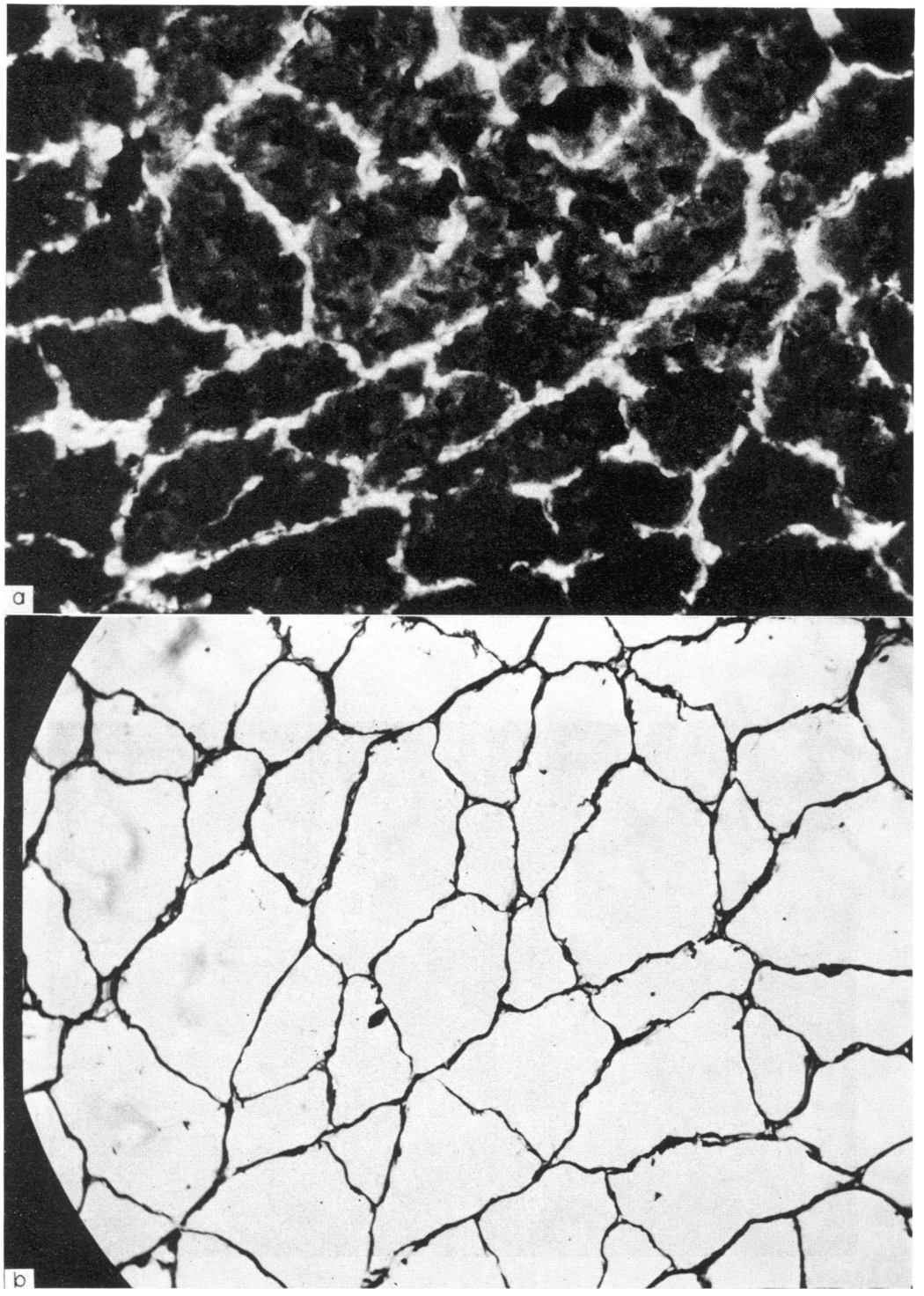

FIG. 4. (a) Rat skeletal muscle, showing staining of endomysium. (b) Rat skeletal muscle, stained by the silver impregnation method. (Magnification ×216.)

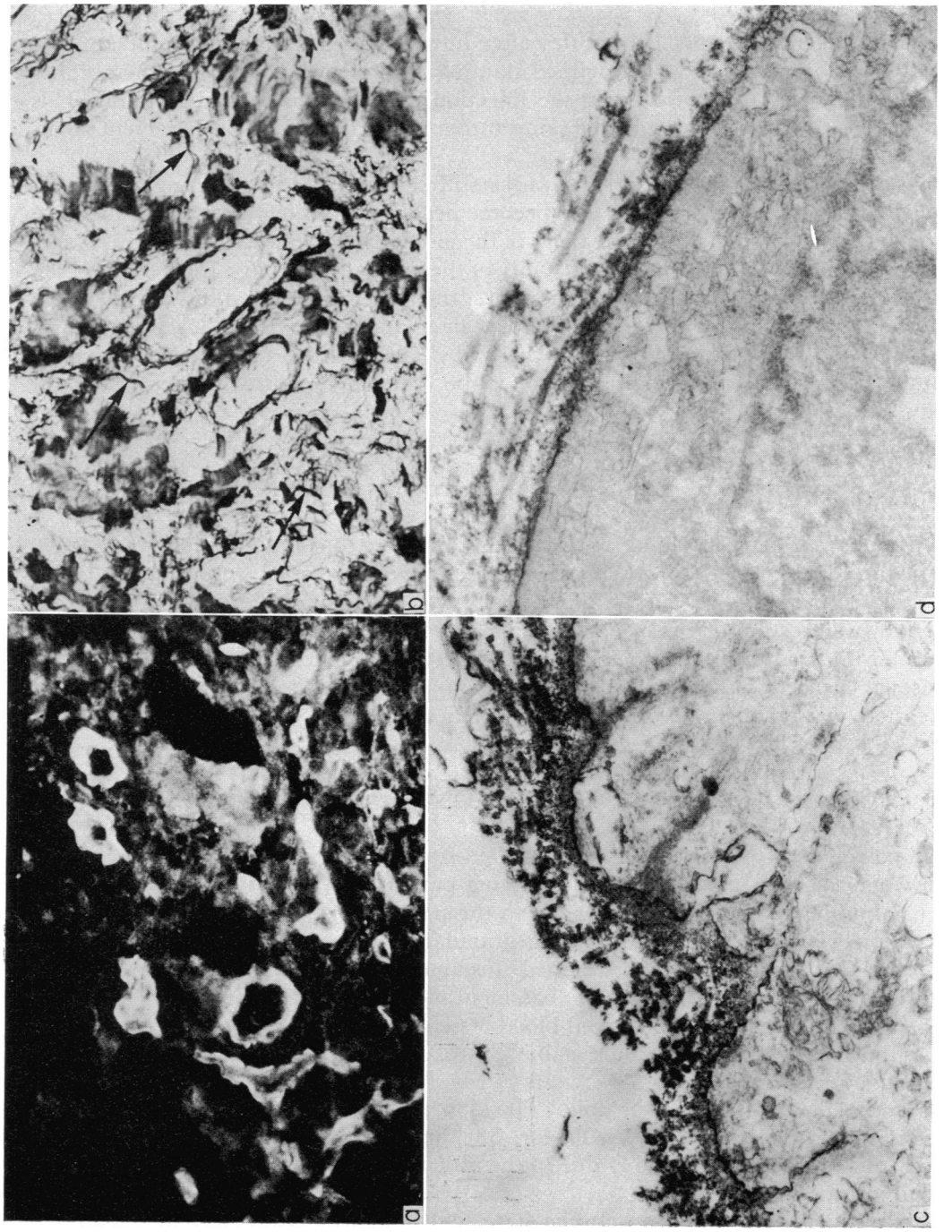

FIG. 5. (a) Four-day-old granulation tissue in a healing rat skin wound. Only blood vessels are specifically stained with anti-NCRC antiserum. (Magnification × 144.) (b) Silver impregnation of a similar section showing fine black fibres in the stroma (arrowed) as well as around blood vessels. (Magnification × 144.) (c) Immunoelectron microscopy of part of a smooth muscle cell in the wall of a small blood vessel as shown in (a). The stained material is amorphous and associated with the surface of the smooth muscle cell. (Magnification × 19,500.) (d) Another field as in (c), but showing unstained collagen fibres. (Magnification × 19,500.)

In vivo STUDIES

In kidneys taken from rats 10 minutes after intravenous injection of rabbit anti-pig kidney NCRC, bright and sharply defined staining of glomerular basement membranes was seen, which was absent following the injection of non-immune rabbit serum. This contrasted with the poor staining by the same antiserum of rat glomerular basement membrane *in vitro* as described above.

Glomeruli in kidneys taken after 12 weeks still showed positive staining for rabbit immunoglobulin, but after 16 weeks had become negative. Histological staining failed to reveal any evidence of pathological changes in the glomeruli of these animals at any stage. There was thus no indication of nephrotoxicity of the antibody although it persisted in the rat kidney for as long as 3 months. Furthermore, the hyperimmune rabbits in which the antisera were raised did not have proteinuria or haematuria despite the large amount of antibody to reticulin in their blood, and when they were killed 8 months after the start of immunization, their kidneys were found to be completely normal by conventional histology.

RELATION OF ANTIGEN TO HUMAN ANTIBODIES TO RETICULIN

Sera from eleven patients found by immunofluorescence to contain antibodies reactive with reticulin (Seah *et al.*, 1971) when tested on sections of rat tissues, were incubated with NCRC isolated from normal human renal cortex and after centrifuging as described, retested on cryostat sections of rat kidney, stomach and liver. Complete inhibition or marked reduction of the characteristic reticulin staining pattern occurred in six of the seven sera from cases of coeliac disease, and in three of the four sera from cases of dermatitis herpetiformis. There was no effect on other auto-antibodies.

DISCUSSION

A non-collagenous protein component isolated from pig organs proved to be a potent antigen when injected into rabbits. The staining patterns obtained with the antisera by indirect immunofluorescence corresponded to the pattern of staining produced by silver impregnation with two exceptions—young granulation tissue, and connective tissue in peripheral nerve, both of which remained unstained by the antisera. Three reticulin patterns were recognized, involving: (1) basement membrane; (2) stroma of liver, kidney and other organs; and (3) newly formed blood vessels, all of which tissues are argyrophilic but may be distinguished by other staining procedures (Fullmer, 1965). Despite its argyrophilia a close relationship between newly formed collagen as seen in healing wounds and reticulin has been in doubt (Jackson and Williams, 1956). The immunological differences demonstrated by Taylor, Shepherd and Robertson (1961) and confirmed by our findings are probably due to the absence from young collagen fibres of the specific glycoprotein of reticulin.

A close antigenic relationship between basement membrane and stromal reticulin is further confirmed by the ability of reticulin derived from liver (rich in stromal reticulin) to inhibit completely the binding of antibody against renal cortex reticulin (rich in base-

ment membrane) and by the similarity of the amino acid analysis of the two preparations previously reported (Pras and Glynn, 1973).

Positive staining of basement membranes with the periodic acid–Schiff reagent and their metachromasia after sulphation (Fullmer and Lillie, 1958) indicate the presence of glycoprotein in basement membrane material in addition to the non-collagenous reticulin component, whereas stromal reticulin consists principally of the latter. The occurrence of mucopolysaccharide in glomerular basement membrane has been reported by Spiro (1967).

Our findings by immunofluorescence and immunoelectron microscopy indicate that NCRC is also present in endomysium, which stains positively by silver impregnation. The anti-reticulin antisera stained epithelial as well as mesothelial basement membranes in contrast with the findings of Scott (1967) who failed to observe this with his antisera. This may be due to differences between the antigens employed for immunization.

The extensive species cross-reactivity which we have observed suggests a phylogenetically primitive protein, although the inconsistency of inhibition across species barriers which we found showed the existence of both common and species-specific determinants in the preparations studied.

Failure of the heterologous antibody to cause renal damage in rats despite its high concentration and prolonged adherence to glomerular basement membrane might be related to inability of the antibody to bind complement, as demonstrated by the absence of staining for complement uptake by immunofluorescence. This differentiates the antibody concerned from other antibodies against basement membranes which show a similar histological localization and have been associated with nephrotoxicity (Cruickshank and Hill, 1953; Steblay and Lepper, 1961). These earlier studies, however, were carried out with cruder preparations containing water-insoluble materials that would have been excluded from the antigen employed in the present investigation. This more closely resembles the material obtained by extraction with alkali reported by Myers, Frei, Cohen, Rose and Richter (1966), antibodies to which were also not nephrotoxic.

It should also be noted that Misra (1974) has recently shown that among non-collagenous soluble antigens obtainable from glomerular basement membrane from several species, a sialoglycoprotein has nephrotoxigenic properties, while a smaller glycopeptide has not.

The ability of the non-collagenous reticulin component studied here to absorb the anti-reticulin antibody often present in human gluten-sensitive enteropathy is of particular interest, for it strongly suggests that the antigenic material (presumably in the gut), which for unknown reasons stimulates production of anti-reticulin antibodies in these patients, resembles NCRC in its chemical structure. Failure to absorb the human antibodies from all the patients' sera containing them, however, indicates that they can be directed at a wider range of antigenic determinants than those present in NCRC.

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