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STUDIES ON RETICULIN. I: SEROLOGICAL AND IMMUNOHISTOLOGICAL INVESTIGATION OF THE OCCURRENCE OF COLLAGEN TYPE III, FIBRONECTIN AND THE NON-COLLAGENOUS GLYCOPROTEIN OF PRAS AND GLYNN IN RETICULIN

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Summary.—Collagen Type III, fibronectin and a non-collagenous reticulin component (NCRC) are all secretory products of fibroblasts and other mesenchymal cells. Antisera to human collagen III (isolated from the placenta) human plasma fibronectin, and NCRC (from spleen) have been used to examine possible antigenic relationships between these proteins both by serological studies and by comparison of the distribution of the proteins in various tissues using immunofluorescence. For other comparative purposes, the distribution of collagen and of reticulin in the same tissues was also sought using conventional histological methods.

Serologically, the 3 antisera showed distinct specificities. However, when used on sections all 3 antisera gave closely similar staining patterns broadly resembling that of histological "reticulin", although minor differences were noted to be characteristic of the distribution of the respective antigens in certain tissues and in cell monolayers.

The results support previous suggestions that histological "reticulin" is not a single entity but is a compound fibrous structure containing collagen Type III, fibronectin and at least 1 additional non-collagenous glycoprotein (NCRC).

HISTOLOGISTS use the term reticulin for "reticular connective tissue which becomes black when impregnated with the double salts of ammonium and silver" (Foot, 1928). However, the chemical basis of this silver reactivity is unknown and its specificity has been questioned (Puchtler and Waldrop, 1978). Immunohistological studies have shown that the distribution of collagen Type III closely resembles that of silver-impregnable reticulin (Gay *et al.*, 1975*a*, *b*; Nowack *et al.*, 1976) and this co-distribution has led to suggestions that collagen Type III and reticulin are one and the same—a proposal perhaps supported by the fact that collagen is rich in cysteine which binds silver avidly.

Other immunohistological studies have shown that histological reticulin is not a discrete entity, but a fibrillar material related not only to collagen Type III, but also to other non-collagenous glycoproteins. These include a water-soluble non-collagenous reticulin component (NCRC), and fibronectin, which have both been shown to co-distribute with histological reticulin (Pras and Glynn, 1973; Pras *et al.*, 1974; Stenman and Vaheri, 1978).

However, whilst the NCRC preparations are stained black by silver impregnation (Pras and Glynn, 1973), neither

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collagen Type III nor fibronectin has been shown to be argyrophilic. The possible immunological relationships between collagen Type III, NCRC and fibronectin have not previously been examined in detail by serological methods, nor have the distributions of these antigens been directly compared with each other and with silver-impregnable reticulin in the same tissues. We therefore now report immunochemical and immunohistological studies of the inter-relationships between these 3 antigens.

METHODS

Fibronectin was prepared from human plasma by affinity chromatography using gelatin (BDH Chemicals) bound to sepharose 4B (Pharmacia Ltd), and monospecific fibronectin antisera raised in rabbits and characterized as described previously (Scott *et al.*, 1981*a*, *b*). Preparations of a non-collagenous reticulin component of human spleen were prepared by the method of Pras and Glynn (1973), and found to have a hydroxyproline content of <1%. Rabbit antisera to NCRC (anti-NCRC) were raised as described previously (Pras *et al.*, 1974). Native collagen Type I and III were prepared from human placenta, and collagen Type II from human lumbar intervertebral disc. These collagens and their specific guinea-pig antisera (anti-CI; anti-CII and CIII) were prepared as described by Beard, Lea and Ryver (1979) and Beard *et al.*, (1980).

Immunoelectrophoresis (IEL)

IEL was performed as described by Scheidegger (1955) using 1·5% agarose (indubiose) in barbitone (0·05M) EDTA (0·005M) buffer, pH 8·2.

Serological studies

Assays for anti-fibronectin reactivity.—(a) Haemagglutination assay. Human erythrocytes (Group O Rh+) were tanned and coated with fibronectin (or human IgG (Lister Institute) as control) according to the method of Herbert, 1978).

A 1% cell suspension was used, and haemagglutination reactions performed in microtitre plates (Sterilin Limited) with phosphate-buffered saline (PBS), pH 7·2, as diluent.

(b) Enzyme-linked immunosorbent assay (ELISA). Microtitre plates (Dynatech UK Limited) were coated with fibronectin (2 g/ml) in 0·1M sodium bicarbonate, pH 9·8, for 1 h at 37° and then for 16 h at +4°. Test sera were diluted in PBS containing 0·05% v/v Tween 20 (Koch-Light Laboratories Limited) (PNS-Tween 20) to prevent nonspecific binding. Peroxidase-labelled sheep anti-human immunoglobulin (polyvalent) was provided by Dr P. D. Weston (Wellcome Research Laboratories, Beckenham, Kent) and used at a working dilution of 1/10,000 in PBS Tween 20. The substrate solution was prepared by dissolving 34 mg of O-phenylenediamine (Sigma Chemical Company) and 15 ml of hydrogen peroxide 30% w/v (BDH) in 100 ml of phosphate–citrate buffer, pH 6·8 (24·3 ml 0·1M citric acid (BDH), 25·7 ml 0·2M di-sodium hydrogen orthophosphate (BDH) and 50 ml distilled water). In other respects the method was similar to that described by Engvall and Perlmann (1971). Non-immune animal sera gave low-titre positive reactions in this system. The results quoted in Table II have been corrected by subtraction of pre-immunization titre from immune titres.

Haemagglutination assay for anti-collagen reactivity.—Tanned sheep erythrocytes were coated with collagen Types I, II or III, as described by Beard *et al.* (1979). Antigen-coated sheep erythrocytes were suspended at 2·5% v/v in PBS containing 0·4% normal rabbit serum as stabilizer. Sera giving haemagglutination titres greater than 1/4 were considered positive.

Serological assays for NCRC reactivity.—These studies were not performed. The saline-insoluble NCRC preparations presented numerous technical difficulties when attempts were made to use them in serological assay systems.

Immunofluorescence

Tissue sections.—Cryostat sections (6 μm) of rat oesophagus, stomach, spleen, small intestine, large intestine, liver, kidney, skeletal muscle, and normal human skin, stomach, small-intestine and thyroid tissues were air-dried for indirect immunofluoresence (IF) studies, as described by Johnson, Holborow and Dorling (1978).

Cell monolayers.—Subcultures of human (foetal) skin fibroblasts (HSF) and of human pharyngeal carcinoma cells (HEP2) were grown as monolayers on sterile multispot slides (Hendley (Essex) Ltd) by incubation (37°) in culture medium (RPMI 1640 (1%) with L-glutamine (1%), penicillin streptomycin (1%), foetal calf serum (5%) and 0·1% non-essential amino acids, pH 7·2 (all obtained from Gibco Europe). Slides were removed at various times (24 h, 48 h, *etc.*) and the cells fixed in prechilled (−10°) acetone for 3 min. Fixed slides were washed in PBS and used in indirect IF studies.

Fluorescein–isothiocynate-conjugated species-specific (anti-rabbit or anti-guinea-pig) anti-immunoglobulin antisera (polyvalent) with fluorescein: protein ratios ranging between 3·6 and 4·2 were obtained from Wellcome Reagents,

and used at end-points determined by block titration. Details of microscopy and photography were as before (Pras *et al.*, 1974).

Absorption studies.—Anti-fibronectin, anti-NCRC and anti-collagen III were retested in the various assays, after pre-incubation for 1 h at room temperature with continuous mixing with either fibronectin, NCRC preparation or collagen III. Each antigen was used at a concentration in PBS which completely abolished the IF staining given by its specific antiserum on tissue sections. Absorbed antisera were spun at 1500 *g* for 15 min and the supernatant used.

Histology

Immunofluorescent staining of tissue sections and cell monolayers was compared with parallel histological stains. The methods of Gomori (1937) and of Gordon and Sweets (1936) for reticulin, and Van Gieson's method for collagen were used.

RESULTS

Immunoelectrophoretic analysis

On immunoelectrophoresis, anti-fibronectin gave a precipitin arc when run against normal human plasma or serum (in confirmation of previous findings—see Fig. 1 of Scott *et al.*, 1981*b*) or against purified plasma fibronectin. On the other hand, anti-NCRC and anti-collagen III failed to give any visible reaction with serum or plasma proteins.

Serological analysis

Each antiserum gave a sufficiently high haemagglutination or ELISA titre against its corresponding antigen (see Tables I–III) to allow cross-absorption experiments to test for cross-reactivity. No agglutination occurred of cells coated with IgG (control). The haemagglutination technique could not be used in studies involving NCRC antigen as "coat" on the red cells. This was because this antigen is soluble only in very low ionic-strength solutions which cause red-cell lysis. Attempts to use other inert particles (latex, polystyrene, *etc.*) as carrier particles for this antigen were unsuccessful because of technical difficulties. However, NCRC could be used for absorbing its ipsilogous antiserum, or the other 2 antisera, in haemagglutination or ELISA tests or before use in immunofluorescence.

When fibronectin (FN) reactivity was compared with that of anti-NCRC by a haemagglutination technique (Table I), it was found that the titre of anti-FN against cells coated with FN was not affected by prior absorption with NCRC antigen. On the other hand, the titre of anti-NCRC against cells similarly coated was also appreciable and could similarly be absorbed with either FN or NCRC. An essentially similar result was obtained using the ELISA assay and microtitre plates coated with fibronectin (Table II). The incorporation of anti-collagen III into this assay showed that this antiserum had

TABLE I.—*Haemagglutination titres (reciprocal) against fibronectin-coated erythrocytes*

Antiserum	Unabsorbed	Absorbed with Fibronectin	Absorbed with NCRC
Anti-fibronectin	16,000	0	16,000
Anti-NCRC	2,048	16	0
Normal animal sera	0	0	0

TABLE II.—*ELISA titres against micro-titres plates coated with fibronectin*

Antisera	Unabsorbed	Absorbed with Fibronectin	Absorbed with NCRC	Absorbed with Collagen III
Anti-fibronectin	9	2	7	9
Anti-NCRC	5	3	3	5
Anti-collagen	2	1	2	1
Contol sera (normal guinea-pig, normal ribbit sera)	0	0	0	0

N.B.—Results expressed as tube numbers, where Tube I contained serum diluted at 1/200 and subsequent tubes contained doubling dilutions of Tube 1.

TABLE III.—*Haemagglutination titres (reciprocal) for anti-collagen (C) reactivity*

Antiserum	Collagen I-coated erythrocytes	Collagen II-coated erythrocytes	Collagen III-coated erythrocytes
Anti-FN	2	64*	0
Anti-NCRC	0	256*	0
Anti-CIII	0	0	256
Anti-CII	ND†	512	ND†
Normal sera	0	0	0

* Titre not reduced by prior absorption with collagen II.
† Not done.

no significant reactivity with fibronectin (Table II).

Haemagglutination tests using cells coated with collagen Type I, II or III (Table III) confirmed the absence of cross-reactivity between collagen III and fibronectin and showed a similar absence of cross-reactivity between collagen III and NCRC. An unexpected finding was that of the titres given by both anti-FN and anti-NCRC against cells coated with collagen Type II. This reactivity was not removed by prior absorption of the antisera with a preparation of collagen Type II. No satisfactory explanation was found for this observation.

Immunofluorescence

Anti-fibronectin, anti-NCRC and anti-collagen III gave immunofluorescence (IF) staining patterns on rat and human tissue sections and cell monolayers which were consistent with those previously reported (Pras *et al.*, 1974; Nowack *et al.*, 1976; Stenman and Vaheri, 1978; Scott, Delamere and Walton, 1981*a*; Scott *et al.*, 1981*b*) (Table IV). Comparison of the IF staining patterns given by these antisera, showed general similarities, but some differences, allowing the antisera to be distinguished from one another. The IF staining given by each antiserum was completely abolished by absorption with its corresponding antigen, but not by the other 2 antigens. Partial absorption with low concentrations of fibronectin removed certain features of the IF pattern originally given by anti-fibronectin on tissue sections (kidney glomerulus, Fig. 1a; liver sinusoids, Fig. 2a; oesophageal and muscularis mucosae, Fig. 3a); all became negative, so that the anti-fibronectin staining pattern was converted to an anti-NCRC like pattern. Higher concentrations of fibronectin resulted in complete absorption of antifibronectin staining.

Tissue sections

The patterns seen on rat tissue are detailed in Table IV. Whilst anti-fibronectin-stained rat glomerular basement membrane (Fig. 1a), anti-NCRC (Fig. 1b) and anti-collagen III (Fig. 1c) did not. Anti-fibronectin gave extensive staining of liver sinusoids (Fig. 2a), and whilst anti-collagen III (Fig. 2c) did not stain sinusoids, intermediate activity was shown by anti-NCRC (Fig. 2b), which gave restricted sinusoidal staining. This was always less extensive than that seen with anti-fibronectin, but more extensive than that given by anti-collagen III. Anti-fibronectin, anti-collagen III and anti-NCRC gave remarkably similar IF staining patterns on the other tissues used, except that the perimysium of muscle bundles within the lamina propria of rat oesophagus, whilst being stained by anti-fibronectin (Fig. 3a) and anti-NCRC (Fig. 3b), was not stained by anti-collagen III (Fig. 3c) (or by Van Gieson's fluid), and the broad connective-tissue bundles of the stomach submucosa, which are continuous with the connective tissue of the muscularis mucosae and are stained by Van Gieson's fluid or by anti-collagen III (Fig. 5b), were not stained by anti-NCRC or by anti-fibronectin.

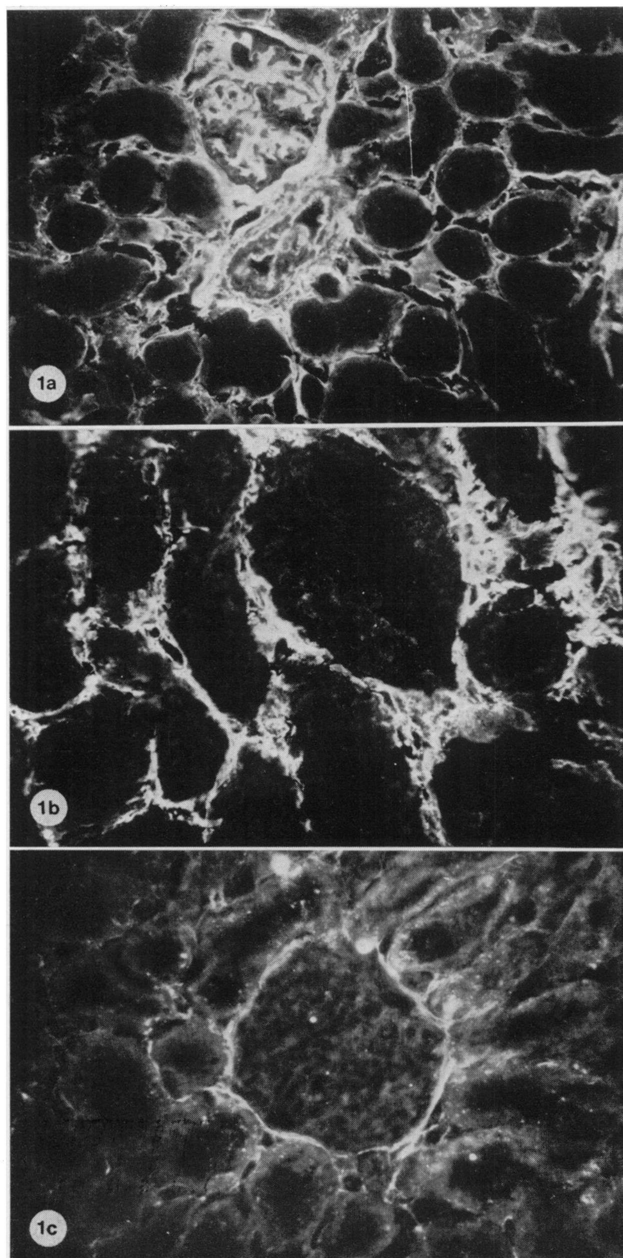

FIG. 1(a).—Anti-fibronectin staining of cryostat sections of rat kidney. Note bright fluorescent staining of glomerular basement membrane, and Bowman's capsule (periglomerular), as well as peritubular fluorescence. × 150.

FIG. 1(b).—Anti-NCRC staining of rat kidney section. Weak staining within the glomerulus, but prominent periglomerular and peritubular staining. × 240.

FIG. 1(c).—Anti-collagen III staining of kidney sections. Similar to anti-NCRC staining (1b) with no staining within the glomerulus. × 240.

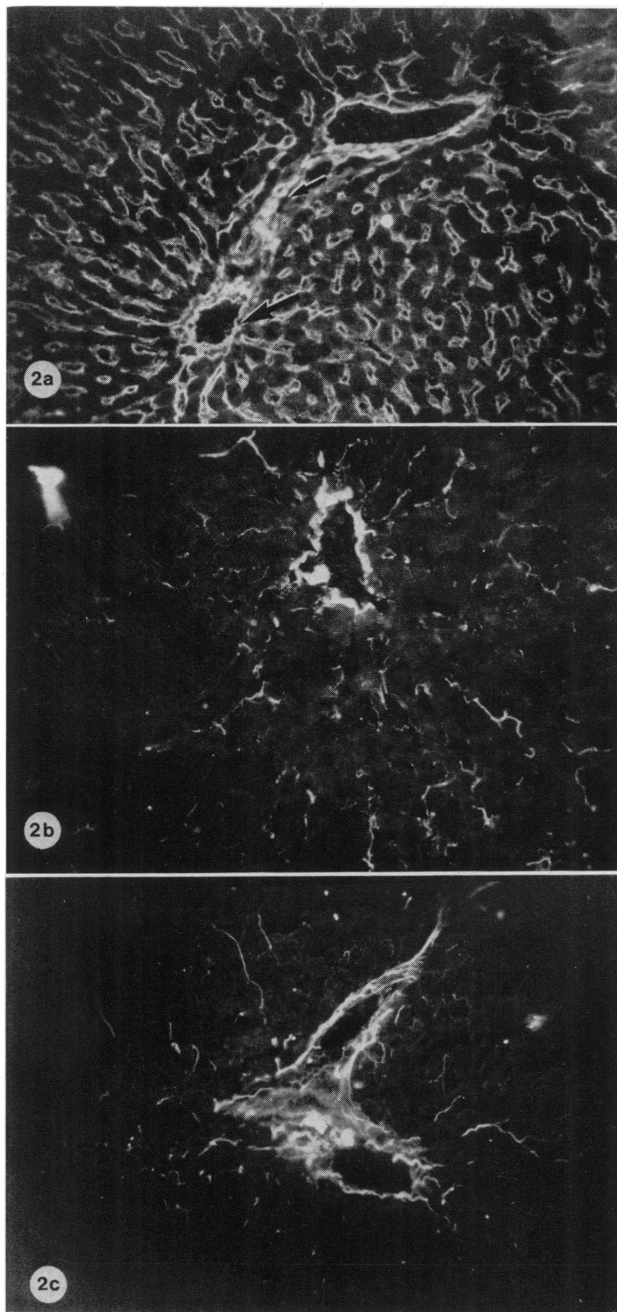

Fig. 2(a).—Anti-fibronectin staining of rat liver section. All sinusoids and adventitial tissue around the portal tracts (arrowed) are brightly stained. × 240.

Fig. 2(b).—Anti-NCRC staining of rat liver sections. Staining of adventitial tissue around portal tracts and discontinuous staining of sinusoidal lining in places. × 150.

Fig. 2(c).—Anti-collagen III staining of rat liver sections. Similar to anti-NCRC staining but less extensive staining of sinusoid lining. × 150.

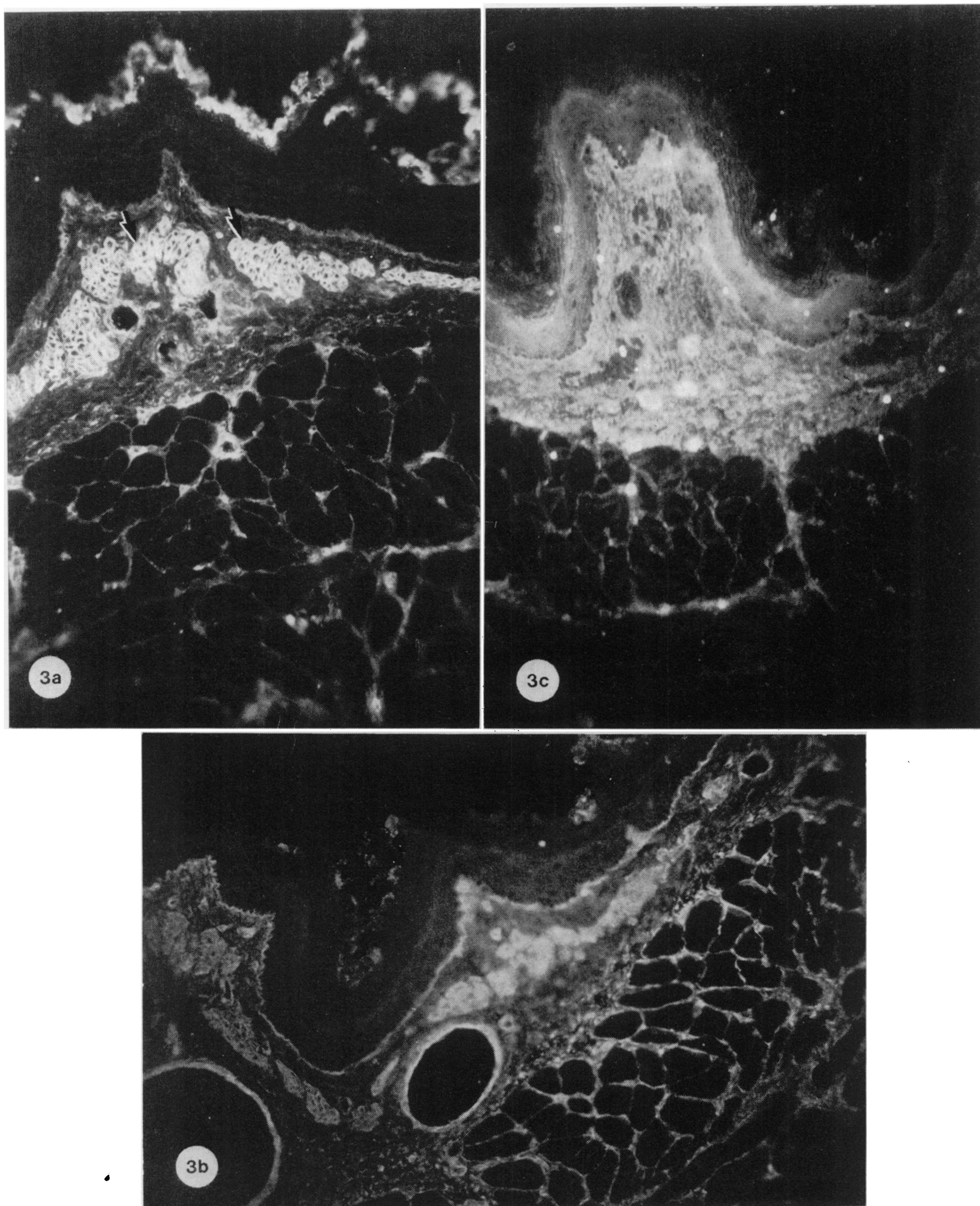

FIG. 3(a).—Anti-fibronectin staining of sections of rat (thoracic) oesophagus. Bright staining of perimysium of muscle bundles in the lamina propria (arrowed) and of connective tissue in the muscularis mucosa and submucosa. × 180.

FIG. 3(b).—Anti-NCRC staining of rat oesophagus; similar to that given by anti-fibronectin (Fig. 3a). × 180.

FIG. 3(c).—Anti-collagen III staining of rat (thoracic) oesophagus sections. Muscle bundles within the lamina propria are conspicuously unstained. × 180.

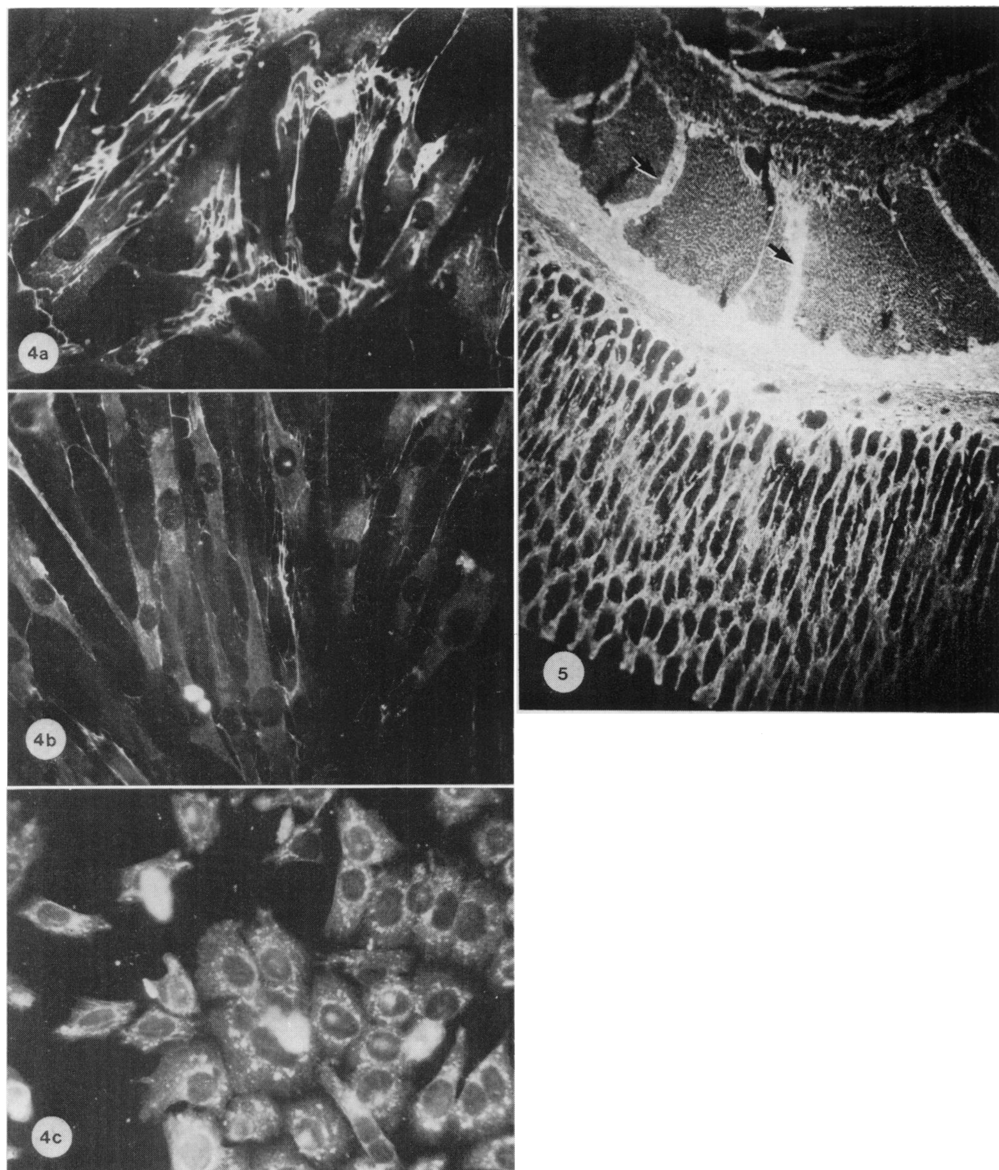

FIG. 4(a).—Fibroblast monolayer (48h culture). Anti-fibronectin gives cytoplasmic (diffuse) staining and bright staining of extracellular fibres. × 167.

FIG. 4(b).—Fibroblast monolayer (48h culture). Anti-NCRC gives some cytoplasmic staining and minimal staining of extracellular fibres. × 167.

FIG. 4(c).—HEp2-cell monolayer (48h culture). Anti-NCRC stains perinuclear granules; nuclei unstained. × 167.

FIG. 5.—Anti-collagen III antibody gives more extensive staining of submucosa of rat stomach than does anti-fibronectin or anti-NCRC. Anti-collagen III stains thick bundles (arrowed). × 267.

Differentiation of fibronectin and the NCRC antigen in cell culture

Both fibronectin and the NCRC antigen were present in human fibroblast cultures. Fibroblasts, within 24 h of plating on to cover slips, showed cellular immunofluorescence with anti-fibronectin and anti-NCRC antisera. At a later stage (48 h) fibronectin (Fig. 4a) was deposited extracellularly as fibrillary arrays in the extracellular matrix and in confluent cultures fibronectin was predominantly extracellular. In contrast, the immunofluorescent reactivity with NCRC antigen was predominantly cellular at all stages of growth of fibroblasts in culture, although from 48 h onwards there was some fibrillary staining of the extracellular matrix (Fig. 4b). Human epithelial cell carcinoma (HEp2) cells in culture gave bright staining of perinuclear vacuoles with anti-NCRC (Fig. 4c), but antisera against fibronectin and collagen III were conspicuously negative when tested on this cell line.

TABLE IV.—*Immunohistological results* (*rat tissues*)

Features	Antisera			Histology	
	Anti-FN	Anti-NCRC	Anti-CIII	Silver staining	Van Gieson stain
Kidney					
Glomerulus	+ (Fig. 1a)	Weak + (Fig. 1b)	− Fig. 1c)	Weak +	−
Peritubular & periglomerular	+	+	+	+	+
Liver					
Sinusoids	+ (Fig. 2a)	+ (Fig. 2b)	+ Fig. 2c)	+	−
Portal tract adventitia	+	+	+	+	+
Stomach					
Muscularis mucosa	+	+	+	+	+
Submucosa—thick bundles	−	−	+ (Fig. 5)	+	+
—endomysium	+	+	+	+	−
Interstititial fibres between stomach glands	+ (fine)	+	+	+	+
Oesophagus					
Perimysium of muscle bundles in lamina propria	+ (Fig. 3a)	+ (Fig. 3b)	− (Fig. 3c)	+	−
Muscularis mucosa and submucosa	+	+	+	+	+
Skeletal muscle					
Epimysium	+ (coarse)	+ (fine)	Weak +	+	−
Perimysium	+	+	+	+	+

TABLE V.—*Human cell monolayers*

Cell monolayers, duration of culture (and site of reactivity)	Antisera			Histology	
	Anti-FN	Anti-NCRC	Anti-CIII	Silver staining	Van Gieson stain
Fibroblasts					
24h (cellular)	+ (diffuse)	+ (diffuse)	−	−	−
24h (extracellular)	−	−	−	−	−
48h (cellular)	+ (diffuse)	+ (diffuse)	+	−	−
48h (extracellular)	+ (Fig. 4a)	+ (restricted)	−	−	+
HEp2					
48h (cellular)	−	+ (perinuclear granules) (Fig. 4c)	−	−	−
48h (extracellular)	−	−	−	−	−

Histological staining of tissue sections showed some difference between silver impregnation and anti-collagen III anti-NCRC or anti-fibronectin staining (Tables IV and V). The silver-staining pattern most closely resembled anti-fibronectin immunofluorescent staining, but silver staining of fibroblast monolayers at confluence (48 h) did not show impregnation of extracellular fibres. Silver staining in all tissues was much more extensive than staining given by Van Gieson's fluid (Table IV) which was confined to much coarser (collagen) fibres.

DISCUSSION

The antigens we have examined all originate as secretory products of fibroblasts and other connective-tissue cells. They nevertheless differ markedly one from another in their physio-chemical properties. For example, fibronectin occurs in the tissues as an insoluble cell-associated protein but also as a soluble form in plasma. These 2 forms are immunologically virtually indistinguishable but do show certain biochemical differences (Ruoslahti and Vaheri, 1975; Keski-Oja, Mosher and Vaheri, 1977; Ruoslahti and Engvall, 1978).

NCRC, on the other hand, is present only in the tissues and no immunologically detectable material is demonstrable in plasma using antiserum to NCRC derived from tissue sources. NCRC is recovered from tissues by aqueous extraction. The isolated antigen's solubility is very sensitive to increase in salt concentration, precipitation occurring even at low ionic strengths. Tests for the purity of this antigen by either electrophoretic or immunochemical methods are therefore negated by its lack of solubility in the salt-containing media required for these techniques.

In recent years it has been shown that the various organs and tissues of the body contain genetically and biochemically different types of collagen. The "interstitial collagens" (Gay, Gay and Miller, 1980) comprise Types I, II and III collagen. Of these, Type I is found in bone and dense fibrous tissue (tendon, dermis *etc.*), Type II collagen in cartilage, and Type III in fine reticular networks in many organs. Type IV collagen is found in basement membranes. Type III collagen can only be isolated after soluble proteins have been extracted from homogenates of tissue (in our case placenta) with strong salt solutions and dilute acid and the insoluble residue has been subjected to prolonged pepsin digestion. The Type III collagen is then isolated by fractional salt precipitation. It will therefore be evident that collagen Type III differs again in solubility characteristics from the 2 preceding antigens and it, of course, is also absent from plasma.

Despite these differences, the closely similar distributions of these antigens in the tissues which we have observed may be due either to the antigens being: (a) structurally very closely related to one another; or (b) impure with one antigen "contaminating" another in the preparation used for immunization, or (c) complexed with one another in the tissues and therefore co-distributed.

With regard to the first of these possibilities, NCRC and fibronectin could be clearly distinguished from collagen Type III serologically. On the other hand, the serological analyses provided some evidence of cross-reactivity between the anti-NCRC and anti-FN used. The anti-fibronectin was raised against an immunologically and electrophoretically pure protein. Similar prior testing of the NCRC antigen employed to produce the batch of antiserum used was not possible for the technical reasons referred to above.

It is therefore conceivable that the particular NCRC extract used to prepare this batch of antiserum may have been "contaminated" with some fibronectin (presumably tissue fibronectin). This is consistent with our previous (unpublished) observation that the pattern of IF staining given with antisera raised to different batches of NCRC antigen varies slightly from batch to batch. Variation is particu-

larly noted in the extent and intensity of sinusoidal staining in liver sections. Patterns have been observed to range from the bright and extensive staining given by anti-fibronectin to a more restricted pattern like that seen with anti-collagen Type III (see Figs 2a, b and c). Nevertheless, any "contaminating" anti-fibronectin activity in the anti-NCRC antiserum must have been very weak since this antiserum, when treated by immunodiffusion or immunoelectrophoresis, gave no precipitin reaction with the fibronectin present in plasma or serum. In addition, prior absorption of the anti-NCRC antiserum with fibronectin did not substantially alter the pattern of fluorescence given by the unabsorbed antiserum. Lastly, against particular cells (see below) anti-NCRC showed unique reactivity.

The close similarity of staining given by these antisera was especially marked using heterologous (rat) tissues (see Figs 1–3) but was also seen using many normal human tissues (not illustrated). In contrast minor but clear points of differentiation were noted when they were applied to human cell monolayers. For example, their application to fibroblasts grown for varying periods revealed different time-courses of production and secretion of the respective antigens by the same cells. In addition, reactivity with the perinuclear granules of the HEP-2 cell-line was restricted to NCRC (see Table V and Figs 4a, b and c).

On balance, it was therefore concluded that each antigen does have distinctive specificity and that the similarity of pattern given by their respective antisera, could not be entirely accounted for either by structural similarity of the antigens nor by the mutual "contamination" of antigens by one another in the preparations used for immunization. Their co-distribution in the tissues and the broad resemblance of this pattern to that of histological reticulin made the third possibility (that of the antigens being complexed with one another in the tissues) seem the most likely.

In this regard, it has been suggested by several authors (for references see review by Anderson, 1976) that tissue glycoproteins (such as fibronectin and NCRC) play a structural role in the aggregation and orientation of collagen molecules to give rise to fibrillar structures recognizable microscopically as collagen or reticulin fibres. The steric arrangement of these fibrils and their cross-linking probably also involves the interaction of proteoglycans or glycosaminoglycans which are additional secretory products of connective-tissue cells. This concept is supported by observations made on fibroblasts maintained in culture. As we have confirmed, NCRC and FN are synthesized and secreted by fibroblasts *before* there is evidence of collagen production. But if cells are grown on until collagen synthesis also begins, the pericellular network of fibres then shows co-distribution of fibronectin and procollagen (Vaheri *et al.*, 1978).

It is well known that histologically identified reticulin and collagen are often intimately related, one merging into the other, especially in areas of fibrosis, such as in liver cirrhosis (McGee, 1977). In our hands, although NCRC, FN and collagen Type III showed considerable co-distribution with silver-impregnable reticulin in various tissues, collagen Type III was absent from some sites which gave a positive, silver-staining reaction (Table IV). Moreover, fibronectin and NCRC were identified extracellularly in fibroblast cultures *before* there was clearly identifiable reticulin by silver staining of the extracellular matrix. This is consistent with previous observations on the time-course of the appearance of histological reticulin in the extracellular matrix of cells maintained in tissue culture (Richards and Jacoby, 1976). These findings suggest that the "reticulin staining reaction" is probably due to the interaction of a number of components rather than to any one single component but more work is required to identify the components essential to silver reactivity.

Our results support previous sugges-

tions (Puchtler, 1964; Puchtler and Waldrop, 1978) that reticulin is probably a compound fibrous material containing Type III collagen (Gay *et al.*, 1975*a, b*); Nowack *et al.*, 1976; Remberger, Gay and Fietzek, 1978), fibronectin (Stenman and Vaheri, 1978; Scott *et al.*, 1981*a, b*) and NCRC (Pras and Glynn, 1973; Pras *et al.*, 1974). However, it should be stressed that our work does not exclude the possibility that other, as yet unidentified, tissue glycoproteins or proteoglycans and glycosaminoglycans may be additional components of these complex structures.

It is also possible that the *proportions* of structural glycoproteins, proteoglycans, and even the *type* of collagen may vary in the compound fibrils of the connective tissue of various organs, at various sites within the same organ, or as a result of pathological processes affecting the connective tissues. For example, the additional reactivity of anti-FN, as compared with anti-NCRC or anti-collagen Type III, in delineating basement membranes of blood-vessels and the glomeruli of the kidney (see Figs 1a, b and c), where collagen Type IV is known to be distributed, could be due to the interaction of fibronectin with structures containing Type IV collagen as well as with "reticulin"-containing Type III collagen. Similar observations were made by Scott *et al.* (1981*a, b*) in relation to the reactivity of anti-fibronectin with basement-membranes and reticular fibres in synovial tissue.

At first sight, the immunohistological staining pattern given by anti-FN might lead one to expect that fibronectin might show *in vitro* preferential or selective affinity for Types III and IV collagen while the distribution of NCRC might suggest more restricted affinity for Type III collagen alone. However, in practice, fibronectin has been found to bind *in vitro* to the native, and even more strongly to the denatured form of *all* types of collagen (for references, see Scott *et al.*, 1981*b*). As yet, little is known of the binding capacity of NCRC to different types of collagen.

The compound but possibly variable nature of reticulin suggested by our own and other work has a bearing on interpretation of the significance of occurrence of the auto-antibodies to "reticulin" which are encountered in the sera of some patients with gluten enteropathy (Seah *et al.*, 1971) and other conditions (Eade *et al.*, 1977). The so-called "R_1 pattern" of anti-reticulin reactivity described by Rizzetto and Doniach (1973) is similar in essence to the pattern we have observed to be common to anti-NCRC, anti-FN and anti-collagen Type III. Nevertheless, it has been claimed by Timpl, Wick and Granditsch, (1977) that "anti-reticulin" antibodies in the sera of children with coeliac disease are not directed against Type III collagen. It is clear therefore that the significance of R_1 patterns given by pathological sera should be interpreted with caution in relation to immunological specificity and the nature of the antigen involved.

In conclusion it has to be said that the underlying biochemical basis for our immunohistological observations is not yet fully apparent. Nevertheless, it is clear that NCRC and fibronectin almost certainly serve as structural glycoproteins of connective tissue and play an important role (possibly in association with other as yet undefined components) in the fibrillogenesis of "reticulin" and fibrous tissue. Further investigations therefore seem warranted of their role in the organization, disorganization and repair of the connective tissues in disease processes.

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