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AN IMMUNO-HISTOLOGICAL STUDY OF CONNECTIVE TISSUE

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Studies of connective tissue are germane to the investigation of the rheumatic diseases, and many different technical procedures have been used in the study of the histogenesis, metabolism, and pattern of reaction of connective tissue to noxious influences. Recently, immuno-histochemical procedures based on the method evolved by Coons and his colleagues (Coons and Kaplan, 1950; Coons, Leduc, and Kaplan, 1951) have been applied to the search for evidence of antibodies fixed to pathologically altered connective tissue and to the investigation of the antigenicity of connective tissue.

The present report concerns an immuno-histochemical analysis of connective tissue in which antibodies were used for the detection and localization of antigenically active components of connective tissue. Experiments will be described which have led to the conclusion that a difference in antigenic content enables reticulin fibres, which form the framework of the reticulo-endothelial system, and certain other connective tissue fibres, which are found in association with proliferating connective tissue, to be differentiated by immuno-histochemical means. The reagents used were antisera, prepared by injecting homogenates of human tissue into rabbits, and contained antibodies capable of combining specifically with antigenic elements in the tissue from which the homogenates were prepared. When serum globulin separated from such antisera is labelled with a fluorescent substance and then allowed to react with sections of human organs, the sites of specific fixation of labelled antibodies by their antigens are easily identified by their fluorescence when the tissue sections are examined under the fluorescence microscope. By using different preparations of antibody globulin, it is possible to differentiate some connective tissue components from others by virtue of the differing specificities of their content of antigenic material. In earlier work along these lines the sites of specific fluorescence produced in tissue sections by anti-human-glomerulus globulin labelled with fluorescein by the method of Coons and his co-workers (Coons and Kaplan, 1950) were compared with those produced in serial sections of tissue by anti-human-synovium globulin similarly labelled (Scott, 1957). An additional fluorochrome, lissamine rhodamine B 200 (R B 200), has recently been introduced (Chadwick, McEntegart, and Nairn, 1958). Since R B 200 emits an orange fluorescence and fluorescein a green fluorescence on bombardment by ultra-violet light, it is now practicable to expose tissue antigens simultaneously to two contrastingly labelled antibodies.

Materials and Methods

The basic immuno-histochemical manipulation used in the present investigation was the exposure of tissue sections to a mixture of anti-human-glomerulus globulin and anti-human-synovium globulin, each labelled with one or other of the two fluorochromes, fluorescein or R B 200.

The preparation of anti-human-glomerulus and anti-human-synovium antisera, their conjugation with fluorescein, and the preparation of tissue sections from quick-frozen unfixed blocks of tissue have already been described (Scott, 1957). The technique of conjugating antisera with R B 200 and the examination of tissue sections exposed to contrastingly labelled conjugates will be described in the report of a preliminary investigation of the use of contrastingly labelled conjugates to be published shortly.

Treatment of Tissue Sections.—Although the detection of antigens in a tissue section by labelled antibody involves an immunological process, it is convenient to use the histological term "staining" to denote both the treatment of tissue sections with conjugated antibody and the fluorescence seen in treated sections. In deciding whether staining was specific or non-specific, the criterion used was that specific staining should be prevented by pre-treatment of a tissue section with the

corresponding unconjugated antibody globulin, before its exposure to a conjugate, but not with unconjugated normal rabbit globulin.

The term inhibition is used here to refer to specific blocking by unconjugated antibody globulin of the staining produced by a conjugate.

Tests of Specificity.—These were performed in the following manner:

One of two sections from a block of quick-frozen unfixed tissue was exposed to antibody globulin for 18 hours, and the other to unconjugated normal rabbit globulin for the same period. The unconjugated globulins were rinsed off the sections with buffered saline (pH 7·0-7·2). After they had been rinsed the sections were washed for 5 minutes, excess saline was carefully removed, and the sections were exposed to the appropriate conjugate for 30-45 minutes. The conjugate was then rinsed off the sections which were washed in three changes of buffered saline. Sections were mounted in glycerol containing 10 per cent. buffered saline.

Direct Staining Experiments.—A drop of one or other of the conjugates was applied to a tissue section and allowed to react with it for 2 to 18 hours. Tests of specificity were run in parallel with direct staining experiments.

Mixed Staining Experiments.—The staining produced by a mixture containing equal parts of an anti-glomerulus globulin labelled with fluorescein (F anti-glomerulus) and an anti-synovium globulin labelled with R B 200 (R anti-synovium) was compared with that produced by a mixture containing R anti-glomerulus and F anti-synovium. In other experiments the staining produced by a mixture of the two immune globulins contrastingly labelled was compared with that produced by a mixture in which one of the immune conjugates had been replaced by a normal rabbit globulin appropriately labelled. For example, the staining produced by an F anti-glomerulus + R anti-synovium mixture was compared with that produced by an F anti-glomerulus + R normal rabbit globulin (N R G) mixture.

Cross-Inhibition Experiments.—Sections were pretreated for 18 hours with an unconjugated anti-glomerulus globulin (or unconjugated anti-synovium globulin) and then exposed to an anti-synovium (or anti-glomerulus) conjugate.

Observations

The appearances produced as a result of mixed staining experiments are summarized in Table I. A range of colours from green through yellow to orange or orange-red was seen in sections treated with a mixture of the two antibodies contrastingly labelled, but the colour produced by a particular mixture in any one histological component of connective tissue was constant. When the various histological components of connective tissue were grouped according to the colour of their staining it became evident that there were three broad groups of tissue antigens.

TABLE I

REACTIONS OF CONNECTIVE TISSUES WITH MIXTURES OF ANTIGLOMERULUS AND ANTI-SYNOVIUM CONJUGATES

Histological Components		F anti-glomerulus + R anti-synovium	R anti-glomerulus + F anti-synovium
Basement Membranes	Renal glomeruli	G	O–R
	Thyroid acini	G	O–R
	Renal tubules	Y	O–R
Vascular Media	Muscular arteries and arterioles	G	O–R
	External iliac artery	G and Y	O and Y
	Aorta (thoracic)	Y	Y
	Pre-capillaries	O	G–Y
Reticulin	Synovial cell layer	Y–O	Y–G
	Splenic and hepatic reticulin	Y–G	Y–O
	Periacinar reticulin, thyroid gland	Y	Y
	Peritubular reticulin in kidney	as BM	Y and O
	Pericapillary reticulin	Y	Y
	Junction of media and adventitia of arteries	Y	Y
Fibrous Tissue	Fibrils in adventitia of vessels	O	G
	Fibrils amongst collagen of joint capsules	O	G
	Fibrils in synovial tissue	O	G
	Fibres in proliferating fibrous tissue	O	G
	Fibroblasts (cytoplasmic processes)	O–Y	G

G—green fluorescence
Y—yellow fluorescence
O—orange fluorescence
O-R—orange-red fluorescence
G-Y—green the predominating colour
Y-O—yellow the predominating colour
Y-G—yellow the predominating colour
O-Y—orange the predominating colour
BM—basement membranes

When mixtures of R+F anti-glomerulus or R+F anti-synovium were used, differential staining of the various components of connective tissue was not seen—all were stained yellow.

One of these groups contained antigens which were stained green by F anti-glomerulus + R anti-synovium mixtures and orange-red by R anti-glomerulus + F anti-synovium mixtures. Antigens reacting with the staining mixtures in this way were found in the renal glomerular capillaries (Figs 1 and 2) and arteriolar capillaries in the lung, the media of muscular arteries and arterioles, and the basement membrane of the acini of glands. They were also found in the vasa vasorum of the aorta, but were not present as constituents of the aortic media itself. Antigens of this group were not constantly seen in the basement membranes of renal tubules. When stained by F anti-glomerulus + R anti-synovium mixtures, the renal tubular basement membranes usually appeared yellow: thickened renal tubular basement membranes in a kidney showing changes of polyarteritis nodosa were seen, however, as thick green lines after staining by the same mixture (Fig. 3). When sections of normal kidney were stained by R anti-glomerulus + F anti-synovium mixture, the tubular basement membranes usually showed a fine orange inner membrane lying closely applied both to tubular cells and to an outer yellow membrane.

It is suggested that antigens of this group react only with antibodies present in anti-glomerulus conjugates, but not represented in antisera prepared against synovial tissue. This suggestion is based on the demonstration that renal glomerular capillaries, the basement membrane of thyroid acini, glomerular arterioles, and the media of muscular arteries, did not react with anti-synovium antibodies in direct staining experiments or in cross-inhibition experiments (Table II). Two antigenically active components are recognizable around the acini of the thyroid gland (Fig. 4). One of these, the basement membrane, reacts only with anti-glomerulus conjugates, the other, which is for convenience referred to here as periacinar reticulin, reacts with anti-glomerulus and with anti-synovium conjugates. The appearances seen after staining sections of human kidney with R anti-glomerulus + F anti-synovium mixtures suggest that a similar juxtaposition of basement membranes and peritubular reticulin occurs in relation to renal tubules. Goodman, Greenspon, and Krakower (1955), however, have produced evidence that renal tubular basement membranes contain antigens which react with antibodies to glomerular basement membranes but possess additional antigenic specificities not detected by such antibodies. Whether the basement membranes of renal tubules are identical with the basement membranes of renal glomerular capillaries or not must at present remain an open question.

Recently Krakower and Greenspon (1958) have suggested that large elastic arteries owe their ability to stimulate the production of a nephrotoxic serum to their vasa vasorum rather than to any specific constituent of the media or subendothelial tissue. This observation implies that the media of large

TABLE II

CROSS-INHIBITION EXPERIMENTS

Histological Component		Treatment of Tissue Sections			
		NRG ↓ F anti-glom.	Anti-synovium ↓ F anti-glom.	NRG ↓ F anti-syn.	Anti-glomerulus ↓ F anti-syn.
Basement Membranes	Renal glomeruli	+	+	−	−
	Thyroid acini	+	+	−	−
	Renal tubules	+	+	−	−
Vascular Media	Muscular arteries and arterioles	+	+	−	−
	Pre-capillaries	+	−	+	−
Reticulin	Synovial cell layer	+	−	+	−
	Splenic reticulin	+	−	+	−
	Periacinar reticulin thyroid gland	As BM	−	+	−
	Junction of media and adventitia of arteries	Not visible	Not visible	Not visible	Not visible
	Peritubular reticulin in kidney	As BM	−	+	−
Fibrous Tissue	Fibrils in adventitia of arteries	+	−	+	−
	Fibrils in subsynovial tissue	+	−	+	−
	Fibrils amongst collagen of joint capsules	+	−	+	−
	Fibres in proliferating fibrous tissue	+	−	+	−
	Fibroblasts (cytoplasmic processes)	+	−	+	−

\+ = Specific staining present.
BM = Basement membranes.
− = Staining absent.

Sections were pre-treated with unconjugated normal rabbit globulin (NRG) or with an unconjugated portion of one of the immune globulins and then exposed to a conjugated portion of the other immune globulin.

vessels and glomerular capillary basement membranes are antigenically distinct and may perhaps bear some relation to the present observation that in the aorta the "basement membrane antigen" was found only in the vasa vasorum.

Some histological components of connective tissue were stained yellow both by F anti-glomerulus + R anti-synovium mixtures and by R anti-glomerulus + F anti-synovium mixtures. Structures reacting to the staining mixtures in this way formed the second group of antigens. Antigens of this group were found in the epithelial cells of renal glomeruli (Fig. 2), in the cell layer of the synovium, and in splenic and hepatic reticulin. They were also found at boundaries within connective tissue, in the sarcolemma of muscle, in pericapillary reticulin, and in the periacinar reticulin of the thyroid gland and pancreas, where they could be distinguished from the basement membrane. A yellow line was constantly present at the junction of the media and adventitia of muscular arteries and arterioles (Fig. 5) and immediately outside the media of "pre-capillaries", vessels in which the tunica media was present, but in which no internal elastic lamina was seen (Fig. 6). "Pre-capillaries" were especially numerous in the sub-synovial layer of synovium in which the changes of rheumatoid arthritis were present and in rheumatoid tendon nodules; they were also found in normal sub-synovium and in the tela-choroidea and choroid plexus. There was a gradual change in the nature of the media of elastic arteries as the vessels were followed peripherally. The media of the aorta contained antigens of the second group only, but the media of the external iliac artery contained antigens of the first and second groups.

The yellow staining produced in mixed staining experiments does not necessarily imply that contrastingly labelled anti-synovium and anti-glomerulus antibodies had become fixed to one antigenic component of connective tissue. Two components, one reacting with fluorescein labelled antibodies and the other reacting only with R B 200 labelled antibodies might well appear as a single yellow fibre if they happened to lie sufficiently close together. However, the results of cross-inhibition experiments (Table II) increase the probability that the yellow staining of capillaries, sarcolemma, periacinar reticulin in the thyroid, and splenic and hepatic reticulin in mixed staining experiments, was due to the fixation by those antigens of antibodies from each of the contrastingly labelled conjugates. The results of cross-inhibition experiments indicated that those structures which were stained yellow in mixed staining experiments reacted with antibodies present in both conjugates. Unconjugated anti-synovium antibodies, for example, prevented the staining of capillaries and splenic reticulin by anti-glomerulus conjugates. It is suggested, therefore, that antibodies to antigens of the second group are represented both in anti-synovium and anti-glomerulus antisera.

Antigens of the third group, which were stained green by R anti-glomerulus + F anti-synovium mixtures and orange by F anti-glomerulus + R anti-synovium mixtures, were found in vascular adventitia and in the capsule of joints, where they appeared as very fine lines closely applied to bundles of collagen. Collagen, which was readily recognized by virtue of its blue autofluorescence, did not react with either of the antibody globulins. The processes of fibroblasts in the cerebral lepto-

All photographs are fluorescence photomicrographs of unfixed tissue sections treated with fluorescent globulin solutions. They were taken on Super Anscochrome 35-mm. film.

Fig. 1.—Human kidney treated with F anti-glomerulus. Blue autofluorescence of tubule cells. All specific fluorescence is green. (Water immersion objective. Dark-ground illumination. 5-min. exposure × 160.)

Fig. 2.—Human kidney treated with a mixture of F anti-glomerulus and R anti-synovium. Specific green fluorescence in glomerular capillary basement membrane, yellow fluorescence in glomerular epithelial cells and orange fluorescence in periglomerular connective tissue. Blue autofluorescence of cells of renal tubules. (Water immersion lens. Dark-ground illumination. 7-min. exposure × 160.)

Fig. 3.—Human kidney from a case of polyarteritis nodosa treated with F anti-glomerulus + R anti-synovium. Fibrous tissue of interstitium shows specific orange fluorescence and thickened tubular basement membranes show specific green fluorescence. (Water immersion lens. Dark-ground illumination. 7-min. exposure × 240.)

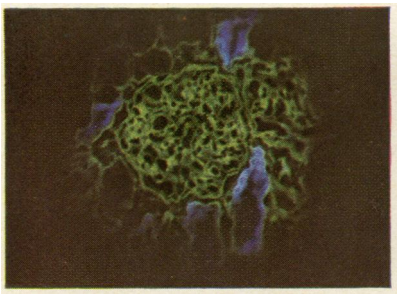
Fig. 4.—Human thyroid—a disrupted section, stained with F anti-glomerulus + R anti-synovium. The yellow periacinar reticulin has been pulled away from the green basement membrane around one acinus. (Water immersion objective. Dark-ground illumination. 3-min. exposure × 270.)

Fig. 5.—Human tela choroidea treated with R anti-glomerulus + F anti-synovium. Intima of the artery not visible. Blue autofluorescence of internal elastic lamina. Specific orange-red fluorescence of media and yellow fluorescence at junction of media and adventitia. (Water immersion lens. Dark-ground illumination. 10-min. exposure × 160.)

Fig. 6.—Human tela choroidea treated with R anti-glomerulus + F anti-synovium. A "pre-capillary" with no intima visible, specific orange fluorescence of media, yellow pericapillary reticulin. (Water immersion lens. Dark-ground illumination. 10-min. exposure × 160.)

Fig. 7.—Human leptomeninges treated with a mixture of R anti-glomerulus + F anti-synovium. Processes of fibroblasts stained green. (Water immersion objective. Dark-ground illumination. 12-min. exposure × 270.)

Fig. 8.—Human muscle—section from a case of dermatomyositis. Blue autofluorescence of muscle. Specific green fluorescence of fibrosed interstitium and specific yellow fluorescence of a thick-walled capillary. (Water immersion objective. Dark-ground illumination. 10-min. exposure × 60.)



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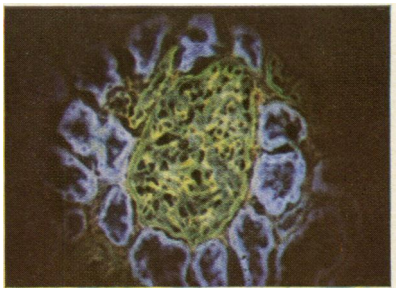

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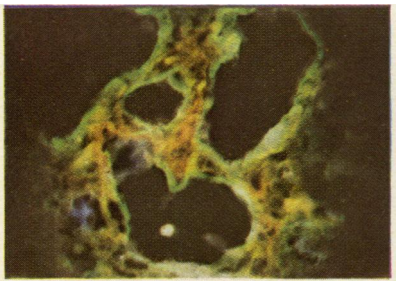

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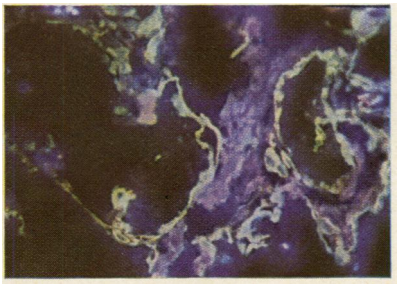

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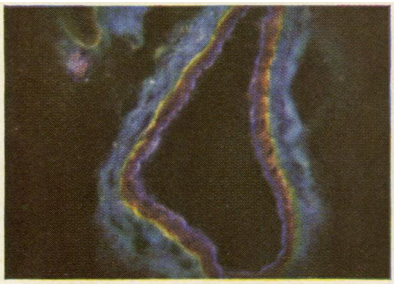

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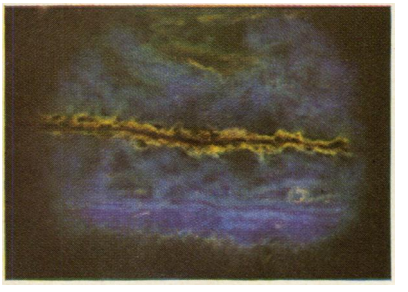

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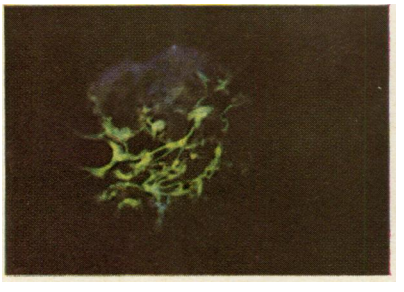

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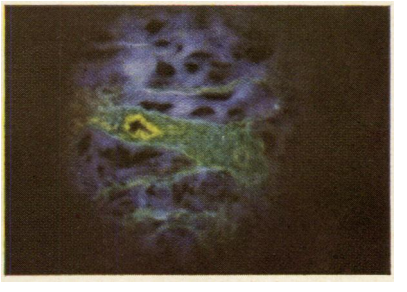

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meninges (Fig. 7) and in granulation tissue contained antigens of the third group. Green staining was produced by mixtures of R anti-glomerulus and F anti-synovium in fibrous tissue found amongst degenerated muscle bundles in a biopsy specimen from a case of dermatomyositis (Fig. 8), in sclerosed glomeruli of kidneys showing senile nephrosclerosis, and in the interstitium of a kidney showing the changes of polyarteritis nodosa (Fig. 3). Mixtures of F anti-glomerulus and R anti-synovium produced orange staining in the same sites.

Antigens of the third group, like those of the second group, were found to react with antibodies present in anti-glomerulus and anti-synovium conjugates. Antigens of these groups were stained specifically by both conjugates in direct staining experiments. In mixed staining experiments, however, antigens of the third group reacted preferentially with antibodies present in anti-synovium conjugates, whereas antigens of the second group did not. These observations suggest that antigens of the second and third groups while not antigenically identical are antigenically related. This conclusion is supported by the results of a preliminary investigation which was concerned with the techniques of the application of contrastingly labelled conjugates in immuno-histochemical studies of connective tissue, and with the interpretation of the results of experiments in which sections were exposed to two different immune conjugates contrastingly labelled. In that investigation, to be described in more detail elsewhere, anti-synovium and anti-glomerulus conjugates were found to react with splenic reticulin and with fibrils in vascular adventitia, but a difference was demonstrated in the stability of union between antigens present in vascular adventitia and antibodies present in anti-glomerulus conjugates on one hand and in anti-synovium conjugates on the other. There was no difference of this kind between the reactions of antibodies present in the two antibody globulins and antigens in splenic reticulin.

Discussion

The observations made during the course of the present investigations have been interpreted as meaning that three connective tissue antigens, or groups of antigens, can be recognized by immuno-histochemical means. For convenience these groups of antigens will be referred to as basement membrane antigens, reticulin antigens, and fibrous tissue antigens. The basement membrane antigens are distinct from the other two groups of antigens in that they react only with anti-glomerulus conjugates. The reticulin and fibrous tissue antigens react with both conjugates but differ from each other in that the fibrous tissue antigens react preferentially with anti-synovium conjugates, whereas reticulin antigens do not. The observation that anti-glomerulus conjugates reacted with reticulin does not necessarily imply that the basement membranes of renal glomeruli contain reticulin. The preparation of isolated glomeruli used for the immunization of animals contained glomerular parietal capsules as well as glomerular capillary tufts. The difference between the reaction of anti-glomerulus conjugates and anti-synovium conjugates with structures containing the fibrous tissue antigen, or antigens, might imply that the relevant anti-glomerulus antibodies were behaving as cross-reacting antibodies, but is also explicable by the postulate that traces of renal interstitium present in the crude homogenates of isolated renal glomeruli stimulated the production of antibodies having only poor combining power. The immuno-histochemical classification of connective tissue cannot be correlated with classifications based on conventional histological techniques. There is no certainty that the structures described here as basement membranes are the argyrophilic basement membranes seen in sections cut from fixed paraffin-embedded blocks of tissue. The structures detected by silver "staining" techniques may be those described here as periacinar reticulin. The difficulty usually experienced in the impregnation of renal glomerular basement membranes with silver does imply that the glomerular capillary basement membranes and other argyrophilic basement membranes of conventional histology are not identical. One of the histological criteria for reticulin is its argyrophilia and by this criterion basement membranes in general are found to contain reticulin, but the renal glomerular capillary basement membranes do not.

The classification of connective tissue by immunological methods must eventually be correlated with data obtained by chemical and physico-chemical analysis of isolated components of connective tissue. The information available at present is insufficient to permit any such correlation.

Summary

The results are reported of an immuno-histochemical analysis of connective tissue based on the use of two different antisera each labelled with one of two contrasting fluorochromes, fluorescein or lissamine rhodamine B 200.

It was found that basement membranes are antigenically distinct from reticulin on the one hand, and from a third group of connective tissue components on the other. Members of this third group

include fibroblasts, fibrils in the interstitium of organs, in vascular adventitia, in proliferating fibrous tissue, and lying between bundles of collagen in the capsule of joints. Reticulin was antigenically related to, but not antigenically identical with, members of the third group of connective tissue components.

Thus it appears that three connective tissue antigens, or groups of antigens, can be recognized by immuno-histochemical means. All three groups react with antisera prepared against human glomeruli, but only two, reticulin and the third or fibrous tissue group, react with antisera prepared against human synovium.

These findings do not necessarily imply that the renal glomerular basement membrane contains reticulin.

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REFERENCES

Chadwick, C. S., McEntegart, M. G., and Nairn, R. C. (1958). *Lancet*, **1**, 412.
Coons, A. H., and Kaplan, M. H. (1950). *J. exp. Med.*, **91**, 1.
——, Leduc, E. H., and Kaplan, M. H. (1951). *Ibid.*, **93**, 173.
Goodman, M., Greenspon, S. A., and Krakower, C. A. (1955). *J. Immunol.*, 75, 96.
Krakower, C. A., and Greenspon, S. A. (1958). *A.M.A. Arch. Path.*, **66**, 364.
Scott, D. G. (1957). *Brit. J. exp. Path.*, 38, 178.

Etudes immuno-histologiques du tissu conjonctif

RÉSUMÉ

On relate les résultats d'une analyse immuno-histochimique du tissu conjonctif, basée sur l'emploi de deux antisérums différents, chacun marqué par deux fluorochromes de contraste, fluorescine ou lissamine rhodamine B 200.

On a trouvé que la couche sous-épithéliale est distincte antigéniquement de la réticuline d'un coté et d'un troisième groupe de composants du tissu conjonctif de l'autre. Ce trosième groupe comprend des fibroblastes, des fibrilles dans les interstices des organes, dans la tunique adventice, dans le tissu fibreux proliférant et, situées entre des faisceaux de collagène, dans la capsule articulaire. Du point de vue antigénique, la réticuline était associée mais non pas identique aux éléments du troisième groupe de composants du tissu conjonctif.

Il semble donc, qu'on peut reconnaître par des moyens immuno-histochimiques, trois antigènes, ou groupes d'antigènes, du tissu conjonctif. Tous les trois groupes réagissent avec des antisérums préparés contre des glomérules humains, mais deux seulement d'entre eux, la réticuline et le troisième ou celui de tissu fibreux, réagissent avec des antisérums préparés contre la synoviale humaine.

Ces résultats n'impliquent pas nécessairement que la couche sous-épithéliale glomérulaire rénale contienne de la réticuline.

Investigación imuno-histoquimica del tejido conjuntivo

SUMARIO

Se relatan los resultados de un análisis imuno-histoquímico del tejido conjuntivo, basado en el empleo de dos antisueros diferentes, ambos marcados por dos fluorocromos de contraste, fluorescina o *lissamine rhodamine B* 200.

Se halló que la capa sub-epitelial se distingue antigenicamente de la reticulina por un lado y de un tercer grupo de componentes del tejido conjuntivo por el otro. El tercer grupo contiene fibroblastos, fibrillas en los intersticos de órganos, en la adventicia, en el tejido fibroso proliferante y, entre fasciculos de colágeno, en la cápsula articular. Desde el punto de vista antigénico, la reticulina fué asociada pero no idéntica con los elementos del tercer grupo de componentes del tejido conjuntivo.

Parece pues, que por medios imuno-histoquimicos se pueden reconocer tres antigenos, o grupos de antígenos, del tejido conjuntivo. Los tres grupos reaccionan con antisueros preparados contra glomérulos humanos, pero tan sólo dos, la reticulina y el tercer grupo, él de tejido fibroso, reacciona con antisueros preparados contra la sinovia humana.

Estos resultados no implican necesariamente que la capa sub-epitelial glomerular renal contenga reticulina.