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Received September 21, 1964. P.S.E.B.M., 1965, v118.

Histo-Immunological Detection of Collagen Fractions. (29838)

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Demonstration of the antigenicity of collagen(1,2) and application of the fluorescent antibody technique(3) have made possible the detection of collagen antigens in reticulin, as well as in collagen fibers of many organs (4,5,6). Since these studies were performed with crude and heterogeneous preparations of connective tissue, we then used more highly purifed collagen preparations to obtain more specific antibodies, and preliminary results were reported(7). In the present study, by means of a fluorescent labeling technique, an attempt is made to demonstrate more precisely the localization of individual collagen "fractions" within connective tissue structures.

Materials and methods. An acetic acid extract of collagen(2) and more highly purified neutral salt, citrate-soluble and insoluble fractions prepared from leg tendons of adult chickens by the procedures of Jackson(8), were used as antigens. Schedule of heterologous sensitization of adult rabbits and guinea pigs, and a description of the serological tests applied to check the production and specificity of corresponding antibodies, have been published(7). Globulin, or the gamma globulin fraction, was separated from anti-collagen antisera by a salt fractionation procedure, and dissolved to a concentration of 20 mg of protein per ml; it was then conjugated with fluorescein isothiocyanate(9). Unconjugated fluorescein was removed by dialysis against phosphate-buffered saline (pH 7.1) at 4°C. Nonspecific fluorescence was removed by prior absorption twice with a powder prepared from rat or guinea pig liver, followed by centrifugation, or by passage through a column of Sephadex G 25 (10); the material was then lyophilyzed. Samples of unconjugated globulin antibody, also adjusted to a concentration of 20 mg of protein per ml, were lyophilyzed and used for control blocking reactions.

Blocks from several organs containing different types of connective tissue (leg tendon, larynx cartilage, tibial bone, skin, cornea, sclera, stomach, intestine, comb, kidney, liver, testis and aorta) were obtained from adult Leghorn chickens about 2 to 4 months old. A portion of each block was fixed in 4% formol and submitted to silver impregnation, and other small pieces were frozen rapidly and cut at 4 to 8 μ, in a cryostat at —20°C.

Direct or indirect Coons' techniques(3) were applied to the unfixed sections or to sections fixed previously with 90% alcohol or acetone. For these purposes the conjugated globulin fraction or the whole immune serum was used, followed, in the latter case, by the corresponding sheep globulin anti-rabbit globulin fraction (obtained from Microbiological Assoc.). Control reactions were performed in both cases: 1) with serum or globulin fraction, both from normal adult rabbits or guinea pigs; 2) with other immune sera such as rabbit sera anti-chicken testis; 3) by a blocking test with unconjugated anti-collagen sera or its globulin fraction; 4) with immune sera treated previously with 80 to

* This work was done during the tenure of a postdoctoral fellowship from Consejo Nacional de Investigaciones Cientificas y Técnicas, Buenos Aires, Arg.

100 mg/ml of lyophilyzed collagen fraction; and 5) by an extraction procedure of the soluble collagen antigen from tissues. The last was performed by treatment of fresh sections with water and 0.08 M NaCl for 40 to 120 minutes prior to application of the direct or indirect immunofluorescent technique.

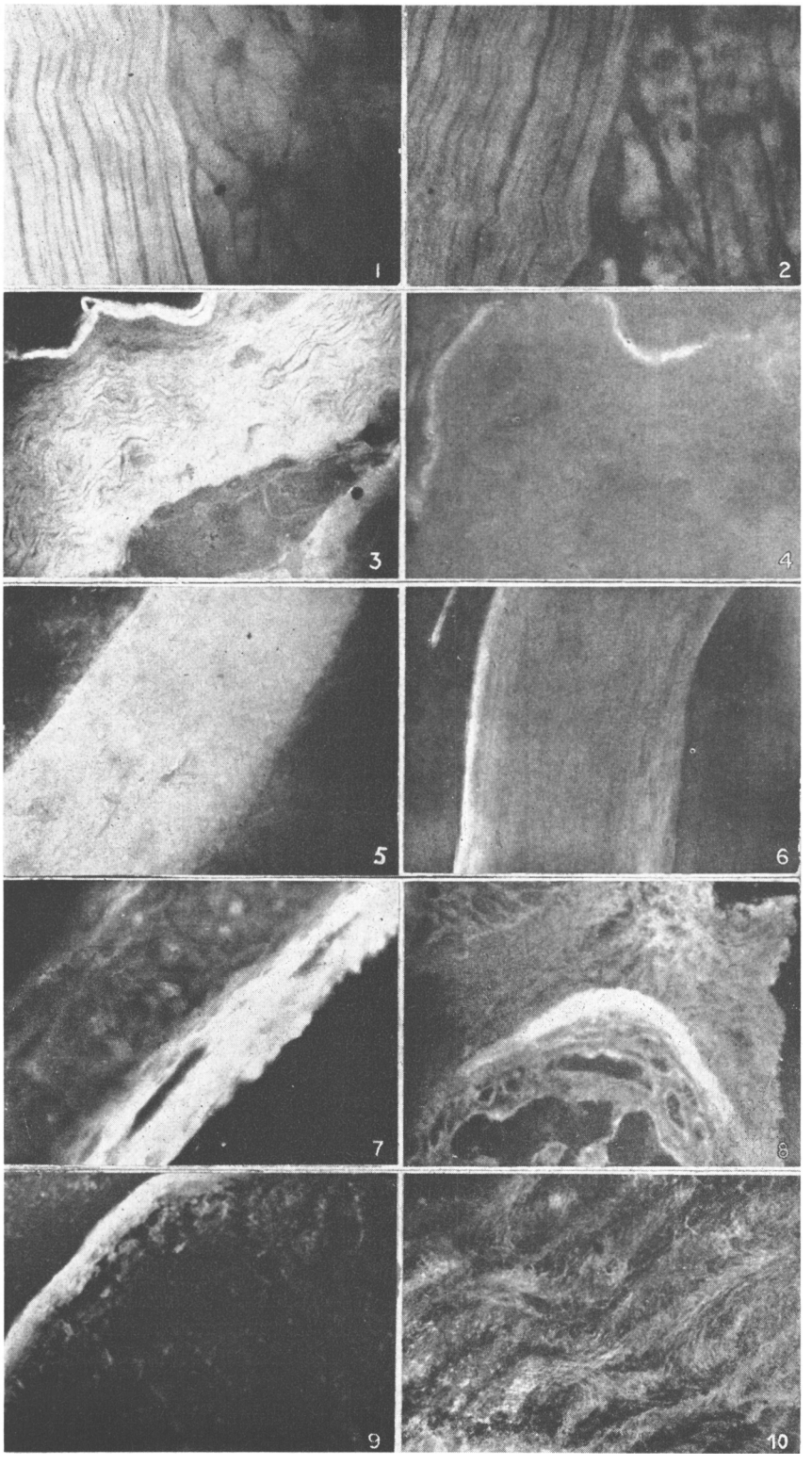
For examination of the slides a Leitz-Ortho Lux fluorescent microscope with appropriate filters was used; the sections were mounted with buffered glycerin, or post-fixed with acetone, dried and mounted with Permount.

Results. Determination of proline and hydroxyproline contents of soluble and insoluble collagen fractions as well as details concerning antibody levels and specificities have been published(7). Histo-immunological results were better with fresh frozen sections than with those fixed previously with 95% alcohol or acetone. Compared with direct mounting in glycerol, post-fixation with acetone both preserved and gave clearer fluorescent images and well-defined structures. Conjugated globulin treated with Sephadex exhibited a higher intensity of the specific fluorescence and less unspecific staining of the background when compared with the same globulin purified by treatment with liver powder. The indirect Coons' technique revealed, in general, the same localization of specific fluorescence as the direct method, but the intensity was higher in many cases. Except for the blocking test, control reactions to check the specificity of these techniques were negative; blocking did not always abolish fully the specific fluorescence. Unsatisfactory results were obtained with the extraction procedure; this procedure altered completely the distribution of fluorescence and often enhanced the unspecific staining.

In the intercellular fibrillar substance of connective tissue structures of various organs of adult animals, there appeared specific fluorescence corresponding to the presence of antigens associated with both soluble and insoluble collagen fractions. This was never detected within the fibroblasts or other cells of the connective tissue, in the matrix or within the cells of hyaline cartilage or bone, within muscle cells nor in the epithelium and parenchymal cells of the organs studied. Specific fluorescence was observed intercellularly in collagen fibers, reaching a maximum in the collagen bundles (Fig. 1-9). Negative results were obtained in the autofluorescent elastic fibers, the reticular fibers, basement membranes, or the interfibrillar mucopolysaccharides appearing in the mucoid layer of the comb and in the media of aorta (Fig. 10). Some differences, as judged by the intensity raised by the fluorescence, were noticed in the results obtained with the anti-collagen antibodies in a given organ and in different types of connective tissue. Bright fluorescence appeared with antibodies to the material in acetic acid extracts, and moderate fluorescence with anti-collagen antibodies prepared to salt, citrate-salt or insoluble collagen fractions. Moreover, fluorescence was higher generally in tendon (Fig. 1-2), aponeurosis, perichondrium (Fig. 7), periosteum (Fig. 8), capsules (Fig. 9), fibrous septa and matrix of cornea and sclera (Fig. 5-6) than in less fibrous structures such as dermal skin (Fig. 3-4), adipose layer, interstitial networks (Fig. 9), intima and adventitia of aorta (Fig. 10), submucosa of gastrointestinal tract, and in the collagen fibers of the mucoid layer of the comb.

Discussion. Results presented here indicate that by histo-immunological techniques one may detect materials which correspond to soluble and insoluble collagen fractions. The validity of microscopical evidence is supported by the fact that fundamental immunological and serological requirements were achieved, *i.e.*, purified antigens, satisfactory immunological response and specificity of the induced antibodies in direct and cross reactions(7). Poor antigenicity of the collagen proteins is apparent, not only as concerns the low titers of antibodies against the fraction soluble in acetic acid as shown by others(2), but also of those against soluble and insoluble fractions. In contrast with the direct reactivity of the insoluble fraction, the cross reactions among the soluble fractions(7) suggest certain antigenic similarities, and accord with the known chemical similarity of these fractions.

These immunological properties of soluble and insoluble collagen fractions are rein-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forced by their visualization in the connective tissue structures using the immunofluorescent technique.

Our results tend to indicate that the anti-collagen antibodies had reacted with antigens only in the collagen fibers of connective tissue of the organs studied, and not with components of the elastic fibers, interfibrillar mucopolysaccharides or other tissue proteins. The stronger reactions revealed by the well-packed collagen fibers and bundles of fibrous tissues, as compared with the weak one exhibited in the thinner and less numerous collagen fibers of loose and mucoid type of connective tissue, could be explainable by a variable concentration of antigens. The negative results shown by the reticular fibers and basement membranes might be due also to a variable and lesser concentration or in fact these may be antigenically dissimilar. The specific staining of reticulin as well as collagen fibers obtained by others(6) could be due to the greater sensitivity of the methods employed: injection into rats of rabbit anti-rat collagen serum and subsequent treatment of sections with anti-rabbit globulin. This greater sensitivity is obtained despite the heterogeneous nature of the acetic acid extract used as the collagen antigen, and despite the considerable dilution of the antibodies injected intravenously. Nevertheless, that reticulin fibers seem to be antigenically different from collagen is deduced from experiments using an immunofluorescent technique with labeled antibodies against rat glomeruli or lung tissue(11). Fluorescence was seen in the reticulin fibers of many organs and in basement membranes, but it was not observed at sites where collagen fibers are present. The same conclusion has been arrived at by others, based on discrepancies observed in the reactions between antibodies contained in antiglomerular and antisynovium conjugates and connective tissue fibrils(4). It has been reported also that anti-rat glomeruli and anti-lung reticulin antibodies react with a wide variety of naturally occurring reticulins, but not with those present in areas of granulation tissue(5). It is interesting that reticulin fibers have some histological and chemical differences(12) as well as similarities with collagen, and that their electron microscopical appearance is similar. In contrast with the above mentioned findings on collagen or reticulin using unidentified mixtures of antigenic substances, (the antibodies to which may not be to the proteins under investigation), our results obtained with purified collagen fractions indicate that the corresponding antibodies are directed only to collagen fibers. This is backed by parallel observations with a silver impregnation technique, by the negative results shown by control reactions and by results after prior treatment of the antisera with the corresponding antigens. Since the presence of material corres-

FIG. 1. Leg tendon (left) and adjacent skeletal muscle (right). Section incubated with labeled globulin anti-insoluble collagen fraction. Specific fluorescence appears only in the tendon fibers. × 50.

FIG. 2. Same as 1, incubated with normal globulin. No fluorescence in either structure. × 50.

FIG. 3. Dorsal skin, incubated with labeled globulin anti-insoluble collagen fraction. Specific fluorescence is seen in the middle region of dermis. Spontaneous fluorescence clearly appears in the keratin layer. × 50.

FIG. 4. Same as 3 incubated with labeled normal globulin. Spontaneous fluorescence in the keratin layer. × 50.

FIG. 5. Tangential section of the sclera incubated with labeled globulin anti-neutral soluble collagen fraction. Specific fluorescence is seen in the collagen fibers of the matrix; spontaneous fluorescence appears in the epithelium. × 50.

FIG. 6. Same as 5 incubated with labeled normal globulin. Spontaneous fluorescence in the superficial layer of the epithelium. × 50.

FIG. 7. Tangential section of larynx cartilage incubated with labeled gamma globulin anti-insoluble collagen fraction. Specific fluorescence is shown in the thick fibers of the perichondrium and is negative in the cartilage matrix and chondrocytes. × 200.

FIG. 8. Section of growing vertebral bone incubated with labeled globulin anti-neutral-soluble collagen. Specific fluorescence in the fibrous periosteum. No fluorescence is seen in the bone trabeculae and in the surrounding skeletal muscle. × 50.

FIG. 9. Cross section of testis, incubated with labeled globulin anti-neutral-soluble collagen fractions. Specific fluorescence appears in the albuginea. × 190.

FIG. 10. Cross section of aorta, incubated with labeled globulin anti-neutral-soluble collagen fraction. No fluorescence is seen in the reticular fibers and interfibrillar spaces. Only slight spontaneous fluorescence is present in the elastic fibers. × 200.

ponding to both soluble and insoluble collagen fractions was demonstrated biochemically in the connective tissue of many organs, a reaction occurring with antibodies against the collagen fractions as well as with those against the acetic acid extract was to be expected. Consequently, only those fractions such as the neutral salt-soluble and the insoluble fractions, which exhibit no cross immunological reactions appear suitable for the histo-immunological detection of collagen. It is suggested that the technique used in the present study may provide another approach for the microscopical localization of the precursors of collagen structures during development of connective tissue.

Summary. The soluble and insoluble fractions of collagen from chicken leg tendon were used as antigens to induce corresponding antibodies in adult rabbits and guinea pigs. Antibodies were checked immuno-serologically and the globulins or its gamma fraction labeled with fluorescein isothiocyanate and purified with Sephadex. Adequately controlled Coons' technique was used in order to detect the presence of the antigen in different types of connective tissue of adult chickens. It was observed: 1) as previously demonstrated, there was cross reaction between the neutral-soluble and the citrate-soluble fractions, whereas a direct reaction occurred with the insoluble collagen fraction, 2) the fluorescent antibodies against these different fractions reacted similarly with the collagen fibers and bundles but not with reticulin and elastic fibers, mucopolysaccharides, basement membranes, nor with matrix of cartilage and bone, 3) intensity of fluorescence was higher with the fibers of dense connective tissue as compared with loose or mucoid type of connective tissue.

We express our sincere thanks to Dr. Sam Seifter for his helpful criticism in revising the text and to Dr. Joseph Seifter for the introduction of this paper.

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Received September 25, 1964. P.S.E.B.M., 1965, v118.

The Presence of Prolactin Inhibiting Factor (PIF) in Extracts of Beef, Sheep and Pig Hypothalami.* (29839)

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Talwalker, Ratner and Meites(1) clearly demonstrated the presence of a factor in rat hypothalamic extracts which inhibited the synthesis and release of prolactin *in vitro*. This was provisionally designated as prolactin inhibiting factor (PIF). Schally, Meites, Bowers and Ratner(2) showed that preparations of hypothalamic luteinizing hormone re-

* Supported by USPHS grants AM 07467-01 and AM 06413-02.