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Serological and Immunofluorescent Study of Soluble Collagen in Tissue Culture of Fibroblasts (33538)

L. LUSTIG, H. COSTANTINI AND R. E. MANCINI (Introduced by Hans Popper)

Departamento de Histologia, Facultad de Medicina, Universidad de Buenos Aires, Argentina

The soluble fraction of collagen fibers appears before the insoluble fraction. Electron microscopic studies suggested that anticollagen antibodies inhibit the development of an intracellular substance, a probable precursor of collagen (1), in tissue culture of fibriblasts. According to morphological and biochemical studies the endoplasmic reticulum and microsomes are the sites of collagen synthesis (2, 3). The pericellular or extracellular organization of collagen fibers, however, is controversial (4, 5). It was previously suggested (6, 7), on the basis of immunofluorescence investigations, that soluble collagen is found in the fibroblasts of embryonic connective tissue, while the insoluble fraction develops extracellularly. The present study attempts to determine more precisely the specificity and the histological localization of soluble collagen antibodies and their relation of cell fibrillogenesis in cultures of fibroblasts.

Material and Methods. Preparation of collagen. Purified neutral salt-soluble, citrate-soluble, and insoluble fractions of collagen were prepared from leg tendons of adult chickens, according to Jackson (8). Other antigens, such as neutral salt-soluble collagen of bovine sclera and an acetic acid extract of carp swimming bladder prepared, following Jackson (8) and Gallop (9), respectively, served as control. All antigens were lyophilized. In the neutral salt-soluble collagen of chicken tendon, total sugars were determined by the anthrone method (10), proline by the Chinard technique (11), and hydroxyproline according to Logan (12), after collagen had been hydrolyzed with 6 *N* HCl for 24 hr at 100°.

Immunization. Four adult rabbits were immunized with the neutral salt-soluble collagen of chicken tendon. The total dose of collagen given in 45 days was 21 mg/animal. The antigen was first injected intracutaneously with complete Freund's adjuvant; after 2 weeks' interval a similar dose without adjuvant was injected subcutaneously. Ten days later, three successive daily injections were given by intraperitoneal, subcutaneous, and intravenous routes and repeated after a 1-week interval. The animals were bled 12 days after the final injection.

Serological tests. Complement fixation (13), agar double diffusion (14), tanned red cell agglutination (15), and immunoelectrophoresis (16) were utilized. Direct and cross reactions were used to verify: (a) the serological specificity of the neutral salt-soluble collagen by comparing it with the citrate-soluble and insoluble fraction of chicken tendon; (b) the species specificity of the same antigen, by comparing it with the neutral salt collagen-soluble fraction of bovine sclera and with an acetic acid extract of ichthyocol;

TABLE I. Specificity of Anticollagen Antibodies (Direct and Cross Reactions).[a]

Sera	Antigens	Titer
Complement fixation test		
Normal rabbit serum	NSC of chicken tendon	Negative
	CSC of chicken tendon	Negative
	IC of chicken tendon	Negative
	NSC of bovine sclera	Negative
	AAE of ichthyocol	Negative
Antiserum, against neutral salt-soluble collagen of chicken tendon	NSC of chicken tendon	1:1600
	CSC of chicken tendon	1:130
	IC of chicken tendon	1:120
	NSC of bovine sclera	1:45
	AAE of ichthyocol	Negative
	Normal chicken serum	Negative
Antiserum after absorption with NSC of chicken tendon	NSC of chicken tendon	Negative
Antiserum after absorption with CSC of chicken tendon	NSC of chicken tendon	1:1400
Antiserum after absorption with IC of chicken tendon	NSC of chicken tendon	1:1400
Hemagglutination test		
Normal rabbit serum	NSC of chicken tendon	Negative
Antiserum	NSC of chicken tendon	1:15000
Antiserum after absorption with NSC of chicken tendon	NSC of chicken tendon	Negative
Agar double diffusion test		
Normal rabbit serum	NSC of chicken tendon	Negative
	CSC of chicken tendon	Negative
	IC of chicken tendon	Negative
Antiserum	NSC of chicken tendon	1 Line
	CSC of chicken tendon	Negative
	IC of chicken tendon	Negative
	Normal chicken serum	Negative
Antiserum after absorption with NSC of chicken tendon	NSC of chicken tendon	Negative

[a] Abbrev.: NSC = neutral salt-soluble collagen fraction; CSC = Citrate-soluble collagen fraction; IC = insoluble collagen fraction; and AAE = acetic acid extract.

and (c) the presence of contaminants, using normal chicken serum against the antineutral salt-soluble collagen serum. The antigens were used in the complement fixation and Ouchterlong tests at a concentration of 0.5 mg/ml and 20 mg/ml in buffer, pH 7.2, respectively. Only direct reactions were utilized in the tanned red cell agglutination test, at an antigen concentration of 0.02 mg/ml. Normal rabbit serum and the antineutral salt-soluble collagen serum, previously absorbed with neutral salt-soluble collagen, citrate-soluble, and insoluble collagen fractions served as controls. The absorption procedure was performed by incubating the antiserum with 5 mg/ml of the lyophilized antigens at 37° for 60 min. The mixture was then left 24 hr at 4° and centrifuged. Immunoelectrophoresis of the antiserum was carried out according to Scheidegger (16); the antigen was homogenized in a Mullard MSE ultrasonic disintegrator at 60 W, 25 kcps, 4°; the neutral salt-soluble fraction was added in the central trough, at a concentration of 60 mg/ml. The patterns were allowed to develop for 24 hr at room temperature, and slides were then washed and stained with amido-Schwartz. As controls serum of normal chicken (60 mg/ml of lyophilized serum), antiserum after absorption with neutral salt-soluble collagen, and normal rabbit serum were used.

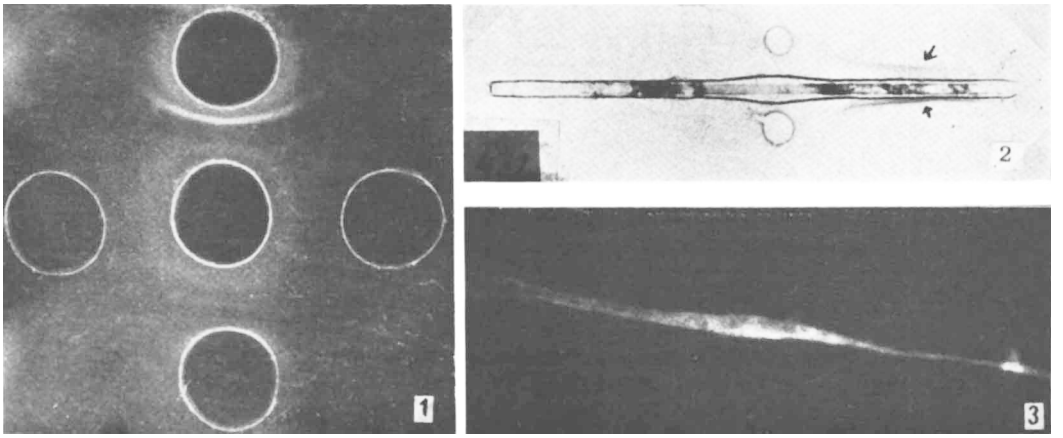

FIG. 1. Ouchterlony technique: antineutral salt-soluble collagen serum of chicken tendon in the central well; neutral salt-soluble collagen of chicken tendon in the upper well; citrate-soluble and insoluble collagen of the same source in the right and lower wells; neutral salt-soluble collagen fraction of bovine sclera in the left well. A single precipitin line is seen between the immune-serum and its specific antigen.

FIG. 2. Immunoelectrophoresis: following electrophoresis of the antineutral salt-soluble collagen serum (upper and lower wells), the specific antigen was added in the central trough; arrows indicate the precipitin lines of the antigen–antibody reaction in the cathodic region.

FIG. 3. Indirect fluorescent antibody technique, using an antiserum against neutral salt-soluble collagen of chicken tendon, followed by antirabbit globulin conjugated with fluorescein isothiocyanate. Bright fluorescence is seen in the cytoplasm of a differentiated fibroblast (48-hr cultures, ×1000).

Immunofluorescence. Direct and indirect Coons' techniques (17), with modifications previously reported (6), were applied. Stainings with normal rabbit serum, blocking in the direct technique, and anticollagen serum after absorption with neutral salt-soluble, citrate-soluble, and insoluble collagen fractions in the indirect technique served as controls. For tissue cultures of fibroblasts, skin of 9–11-day-old chick embryos was trypsinized and placed on coverslips in Leighton tubes at 37–38°, for 24–48–96 hr in a medium consisting of 60% Hanks' salt solution, 20% normal human serum, 10% chick embryo extract, and 10% lactalbumin hydrolysate. Acetone was the fixative.

Results. The neutral salt-soluble collagen showed on chemical analysis: hydroxyproline 97/1000 residues, proline 105/1000 residues, proline: hydroxyproline ratio 1.08, total sugars 5.5 moles/100mg. Serological tests showed titers with the antiserum against neutral salt-soluble collagen fraction, and negative results with homologous serum proteins which might have been present as contaminants and with the antiserum absorbed with the neutral salt-soluble collagen (Table I). The antiserum was species specific also because of the low or negative titers obtained with soluble fractions of bovine collagen and ichthyocol (Fig. 1). The collagen fraction

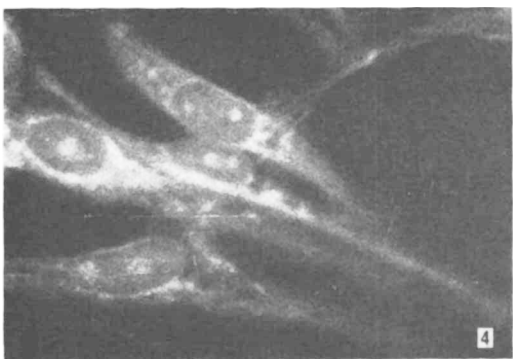

FIG. 4. Direct antibody fluorescent technique using antineutral salt-soluble collagen globulin conjugated with fluorescein isothiocyanate. Bright fluorescence is observed in the perinuclear region of the cytoplasm of fibroblasts (72 hr cultures, × 1000).

seemed to cross react with insoluble and citrate-soluble collagen of chicken tendon only slightly, since the titer of the antiserum remained high after the absorption with citrate-soluble and insoluble collagen fraction of chicken tendon. The precipitin band observed in the Ouchterlony test, with the neutral salt soluble collagen fraction, persisted up to an antiserum dilution of 1:16. The antibodies exhibited the same electrophoretic mobility as gamma globulin; the antigen–antibody reaction appeared as a single precipitin line (Fig. 2). Control experiments, with absorbed antiserum and normal rabbit serum were negative.

Indirect and direct immunofluorescence techniques rendered similar results. The cytoplasm of the fibroblasts showed specific fluorescence (Figs. 3, 4). Granules or small homogeneous masses were seen in the perinuclear region and/or in the cytoplasmic processes, particularly of the mature fibroblasts characterized by their spindle shape. Undifferentiated cells showed fluorescence only in the prominent nucleoli. Tissue cultures maintained for 48–96 hr had more mature fibroblasts, with more fluorescence and more thin pericellular fibrils stained with Azan trichrome and impregnated by silver. In control slides, normal rabbit serum, blocking and absorption of the immunoserum with the specific antigen yielded negative or negligible reactions. Absorption of the antiserum with citrate-soluble and insoluble collagen did not remove the specific fluorescence. A positive reaction in the nucleoli of the undifferentiated fibroblasts occurred occasionally with normal rabbit serum.

Discussion. Immunofluorescence techniques are useful to localize soluble collagen fractions in cultured fibroblasts, as previously shown for the connective tissue of chicken embryos (6, 7). The higher titer of the anti-neutral salt-soluble collagen serum, as compared with our previous studies (18) may reflect the more efficient sensitization. The low titers in cross serological reactions (complement fixation test), may suggest some common antigenic reactivity between citrate-soluble and insoluble collagen fractions from chicken tendon.

The antibody against neutral-soluble collagen binds with the cytoplasm of the fibroblasts in immunofluorescence, suggesting an intracellular localization of the antigen. The specificity of this localization is supported by the control tests, particularly the absorption with specific antigen and the citrate-soluble and insoluble collagen fractions. Thus neutral salt-soluble collagen, a presumed precursor of the insoluble fraction (5), is apparently formed within mature fibroblasts when differentiation and formation of fibrils starts. This synthesis appears related to the thin fibrils surrounding or in continuity with the cytoplasmic processes of mature fibroblasts. These morphological observations agree with previous data based on ultracentrifugation, radioautography and electron microscopy revealing (19–23) labeled proline and hydroxyproline in the microsome fraction, polysomes, endoplasmic reticulum and Golgi complex.

Summary. To localize the synthesis of collagen precursor within fibroblasts, antibodies against purified neutral salt-soluble collagen fraction of chicken leg tendon were induced in adult rabbits. The antibodies against this presumed precursor of insoluble mature collagen had, (a) a high titer against specific antigen on complement fixation (1:1600), and on hemagglutination (1:15,000); (b) a single precipitin band in agar double diffusion and in immunoelectrophoresis; and (c) high specificity as to species and collagen fraction in cross reactions with citrate-soluble and insoluble collagen of chicken tendon, with neutral salt-soluble collagen of bovine sclera, and with an acetic extract of ichthyocol. Neutral salt-soluble collagen was localized by immunofluorescence in the cytoplasm, and processes of mature cultured fibroblasts, but not in undifferentiated cells.

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Conditions of Transfer of Morphine Tolerance by Brain Extracts* (33539)

G. UNGAR AND L. GALVAN

Departments of Anesthesiology and Pharmacology, Baylor University College of Medicine, Houston, Texas 77025

In a previous publication (1) we described experiments in which tolerance to morphine could be induced in mice by treating them with extracts of brain taken from animals rendered tolerant to the drug by repeated exposure. The present paper reports further examples of this transfer of tolerance and discusses the probable causes of failure in attempts made to replicate the experiments (2, 3).

Materials and Methods. The brain extracts used were prepared by the procedures previously described (1). Sprague-Dawley rats were made tolerant to morphine by a series of three daily subcutaneous injections of morphine sulfate at doses progressively increased from 10 to 100 mg/kg during a period varying from 14 to 20 days. On the last day, 4–10 hr after the last injection, the rats were decapitated and their brains were immediately frozen on dry ice. Extracts were made by dialyzing a homogenate of brain, concentrating the dialyzate and partitioning it with phenol. The active material was precipitated out from the phenol phase with 20 vol of cold acetone. The precipitate, dissolved in distilled water at the concentration of the equivalent of 2 g of wet weight of brain per ml, was the preparation used in most experiments. Procedures used for further purification of the active material will be mentioned below.

The extracts were injected intraperitoneally into male Swiss albino mice (20–25 g). Control animals were either left uninjected or were treated with similarly prepared extracts of brain taken from normal, nontolerant rats. It was established previously that the latter preparations did not induce tolerance to morphine.

Analgesic effect was tested by the method

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