SOLUBLE COLLAGEN IN HUMAN SERUM*

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(Received February 26, 1968)

SUMMARY

Soluble collagen-like components in whole human serum and in serum fractions obtained after filtration on Sephadex G-200 or after sucrose density gradient centrifugation were analyzed by micro-immunodiffusion and by immunoelectrophoresis. Antiserum to human skin collagen served as the reagent for the detection of the antigen.

The collagen-like components were shown to exist in at least 2, and possibly 4, molecular forms, which shared a common antigenicity, but differed in size, density and electrophoretic mobility. One of the components was eluted from the gel in the same area as the larger protein molecules of serum, while the other component appeared in the area of the smaller serum protein molecules.

The use of antiserum to human skin collagen preparation to detect multiple collagen-like components in human serum offers a new and simplified method for further investigations in the area of collagen metabolism.

INTRODUCTION

Collagen, one of the major protein constituents of the body, has attracted the interest of numerous investigators[1-3]. Studies with skin and with the aortic intima from normal individuals have shown that as the age of the individual increases there is a corresponding increase in the amount present of the highly cross-linked insoluble form of collagen, as measured by the hydroxyproline assay technique (refs. 1, 2). The rate of increase is accelerated in certain disease states such as atherosclerosis[1] and sun-damaged skin[2].

The presence of a collagen-like substance in human serum has been demonstrated by hydroxyproline assay and by immunological assay[4,5]. Physical techniques

of analysis, such as analytical ultracentrifugation and electrophoresis, have shown that the collagen exists in at least 2, and possibly 3, molecular forms[6,7].

In the present investigation, the immunological technique of Frey *et al.*[5] was used to study the collagen-like components in human serum. The serum also was fractionated by gel filtration and by gradient centrifugation; investigation of the sub-fractions showed that while the collagen-like components shared a common antigenicity, they differed in size, density and electrophoretic mobility.

MATERIALS AND METHODS

Preparation of soluble collagen

Human skin powder (lyophilized) was prepared from fresh skin by the method of Lerner[8]; acid-soluble collagen was isolated from the powder by the method of Gallop and Seifter[9] using 1 g of skin powder/4 ml of extracting buffer. The total protein content of the acid-soluble collagen preparation was determined by the method of Lowry *et al.*[10]; its homogeneity was determined by analytical ultracentrifugation and by immunoelectrophoresis.

Preparation of antiserum

Equal volumes of human skin collagen preparation and Freund's adjuvant were mixed to form a stable emulsion, and 2 ml were injected either subcutaneously or into the rear footpad of young adult male rabbits once a week, for 3 consecutive weeks. Blood was withdrawn before the initial injection, and at 1-week intervals after the last injection.

Antisera to whole human serum were obtained from Hoechst Pharmaceuticals, Inc., Kansas City, Missouri.

Serological techniques

Micro-immunodiffusion and immunoelectrophoresis were done by the method of Crowle[11], except that Agarose (Seakem Agarose, Bausch and Lomb, Rochester, New York) was used instead of purified agar. The slides were incubated in a moist chamber at $4°$ for 5–7 days or until marked precipitin bands appeared. The slides were then washed with physiological saline for 24–48 h to remove unreacted protein, dried, and stained for protein using the triple stain described by Crowle[11].

Serum fractionation

(a) *Gel filtration*[12]. Twenty ml of whole human serum were applied to a refrigerated column of Sephadex G-200, 130 cm long with an I.D. of 5 cm. Total volume of the column was 1800 ml with a void volume of 650–700 ml; the flow rate was approximately 12 ml/h; and the eluate was collected in 10-ml fractions. Each fraction was tested for the presence of collagen-like components by its reactivity with rabbit antiserum to human skin collagen using micro-immunodiffusion. Fractions showing reactivity were pooled and concentrated by dialysis against Carbowax at $4°$ to approximately one-tenth their original volume. Each concentrated pool was subjected to immunoelectrophoresis, and tested for immunologic reactivity against rabbit antiserum to human skin collagen and antiserum to human serum.

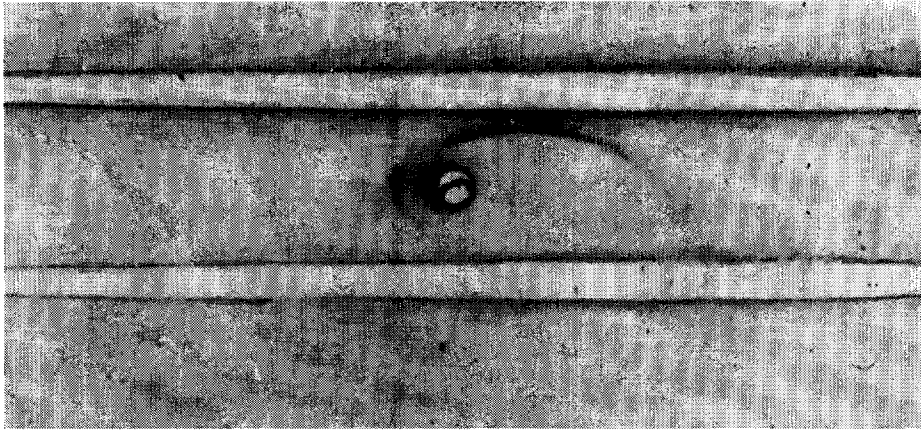(b) *Gradient centrifugation*[13]. Human serum, 0.5 ml, was layered over a pre-

Fig. 1. Immunoelectrophoretic analysis of human skin collagen preparation. Center well, collagen preparation; upper trough, antiserum to human skin collagen preparation (anti-HSCP); lower trough, antiserum to whole human serum (anti-HS).

formed gradient of sucrose ranging from 10 to 40%, and centrifuged at 35 000 rev./min for 20 h in a Model L2 Beckman centrifuge equipped with a SW 39 rotor. After centrifugation, the solution was harvested from the bottom of the tube by drop collection into tubes (5 drops/tube). These fractions were tested for reactivity with antiserum to human skin collagen by means of immunoelectrophoresis.

RESULTS

By the eighth week after the rabbits were injected with the emulsion of human skin collagen preparation and Freund's adjuvant, a measurable level of precipitating antibodies was found in the blood; the maximum level was reached by the eighteenth week. Serum prepared from blood withdrawn during this latter period was used throughout the present investigation, and is referred to as antiserum to human skin collagen (anti-HSCP).

When the original human skin collagen preparation was subjected to immuno electrophoretic analysis, a single well-defined band of reactivity appeared between it and the anti-HSCP located in the upper trough. A weak band of reactivity appeared between the human skin collagen preparation, and rabbit antiserum to whole human serum (anti-HS) located in the lower trough (Fig. 1).

Serum from each of 5 human control subjects was examined for collagen-like components by immunoelectrophoresis. The serum was placed in the center well, anti-HSCP in the upper trough, and anti-HS in the lower trough. Two components were visible between the whole serum from subjects A and B and the trough containing anti-HSCP, while three were visible in the whole serum from subjects C, D, and E (Fig. 2). The anti-HS served as a guide to the location of the major protein components of serum, albumin and the globulins (Fig. 2).

The amount of soluble collagen-like material in the various serums, as determined by micro-immunodiffusion, ranged in terms of serum dilution from 1:8 to 1:32.

To determine the location of the multiple components of soluble collagen-like material in serum relative to the location of the various serum proteins, the serum
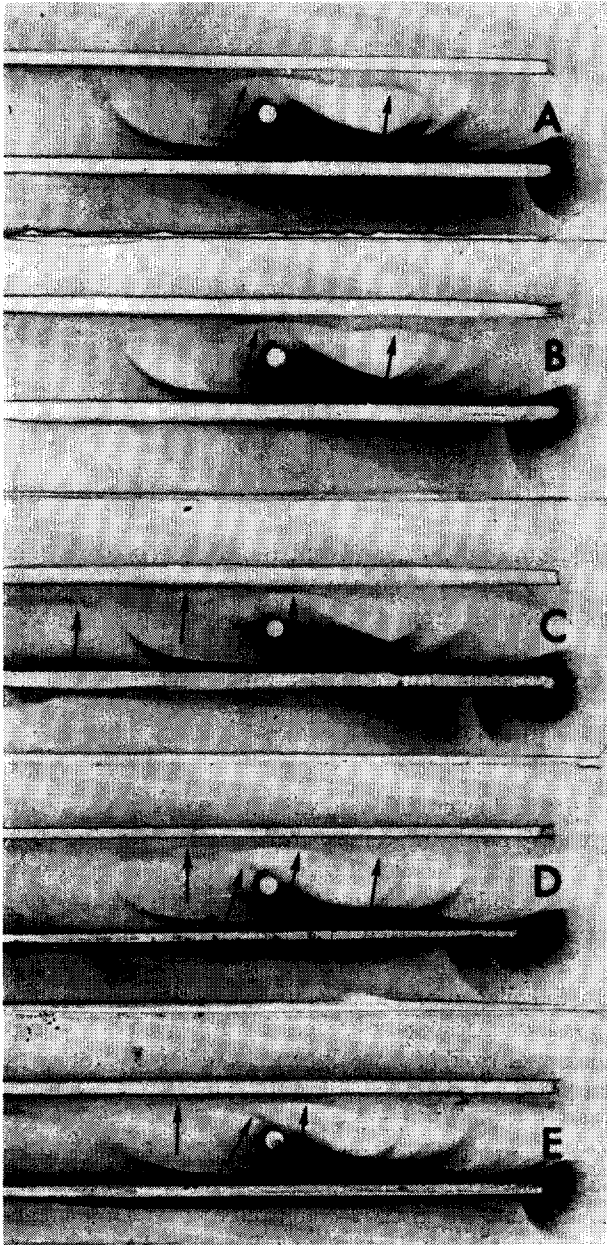
Fig. 2 Immunoelectrophoretic analysis of serum from control human subjects for collagen-like components. Center well, human serum; upper trough, anti-HSCP; lower trough, anti-HS.

first was fractionated on Sephadex G-200. The eluate was collected in 10-ml fractions, and each fraction was analyzed by micro-immunodiffusion against anti-HSCP to determine which fractions of the eluate contained the soluble collagen-like components. The eluate collected between 600 and 1400 ml was found to contain 2 areas of reactivity (Fig. 3). Consecutive 10-ml fractions from this portion of the eluate were

collected in aggregates to form 7 separate pools. The solution in each of the pools was concentrated, and subjected to immunoelectrophoresis, with anti-HSCP in the upper trough, and anti-HS in the lower trough. The first area of reactivity, pool 1, coincided with the elution of serum proteins that are largest in molecular size[12], e.g., β-lipoprotein and macroglobulins. The second area of reactivity, pools 4–6, contained serum proteins that are smaller in molecular size, e.g., albumin and globulins. Pools 2, 3 and 7, even though concentrated, showed no reactivity to anti-HSCP.

Before gel filtration, immunoelectrophoresis of the whole serum showed 3 soluble collagen-like components reactive with anti-HSCP (Fig. 4, C). After gel filtration the soluble collagen-like components contained in the 4 pools (Fig. 3; 1, 4–6) did not differ in electrophoretic mobility (Fig. 4; D, E and F) from each other, although, based on their position of elution from the Sephadex column, they must vary in size.

Whole serum layered over a sucrose density gradient was separated into 16 different fractions by centrifugation. Immunodiffusion of the fractions against anti-HSCP showed 2 areas of reactivity, fractions 2–3 and 8–15 (Fig. 5).

Immunoelectrophoresis of the reactive fractions against anti-HSCP showed a single precipitin band in fractions 2–3 (Fig. 6, A), and a second precipitin band in fractions 8–15 (Fig. 6, B).

The components in the isolated fractions displayed a different electrophoretic mobility from those in the intact serum.

DISCUSSION

In the present investigation, soluble collagen-like components with a common antigenicity to human skin collagen were demonstrated in human serum by immunoelectrophoresis. Fractionation of the serum either by filtration on Sephadex G-200 or by sucrose density gradient centrifugation, and analysis of the various fractions by immunoelectrophoresis indicated that at least 2 different soluble components are present. The components differ in molecular size and density, as well as in electrophoretic mobility. The finding of at least 2 components agrees with LeRoy et al.[4] who used the chemical determination of hydroxyproline in the eluates from a chromatographic separation, as a measure of soluble serum collagen.

The weak reactivity between anti-HS and human skin collagen suggests that relatively large amounts of antigen are required to produce antibody to collagen, as has been reported previously by Watson et al.[14]. Frey[15] has reported that horse anti-HS produced by l'Institut Pasteur is reactive with human skin collagen; however, only one reactive component was noted.

In the patterns of reactivity seen in Fig. 2, the most intensely stained precipitin band is located approximately symmetrical to the origin and is common to all of the serums analyzed. This component, upon the basis of electrophoretic migration, appears to be similar to the precipitin band between the original immunizing antigen and its antiserum, Fig. 1. A serum component, which stained only faintly (Fig. 2, D), migrated toward the anode in a manner similar to serum β-globulin. The other components detected in the serum (Fig. 2, C, D and E) migrated toward the cathode and varied somewhat from serum to serum. The use of different samples of collagen in the preparation of the antiserum and the analysis of a number of other serums gave the same results. The immunological detection of multiple collagen-like components in
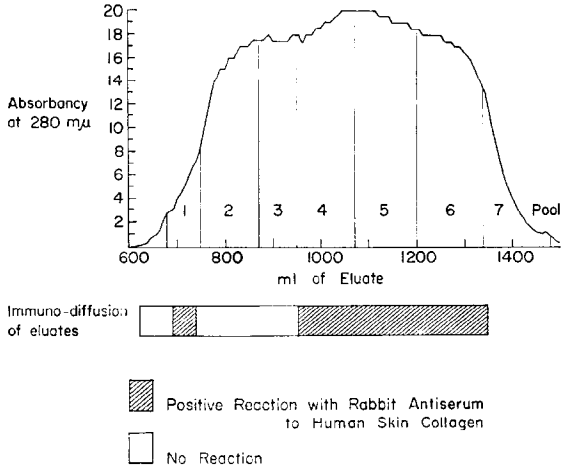
Fig. 3. Fractionation on Sephadex G-200 of normal human serum. The cross-hatching in the bar below the elution pattern shows which pool fractions of the eluate reacted with rabbit antiserum to human skin collagen preparation.
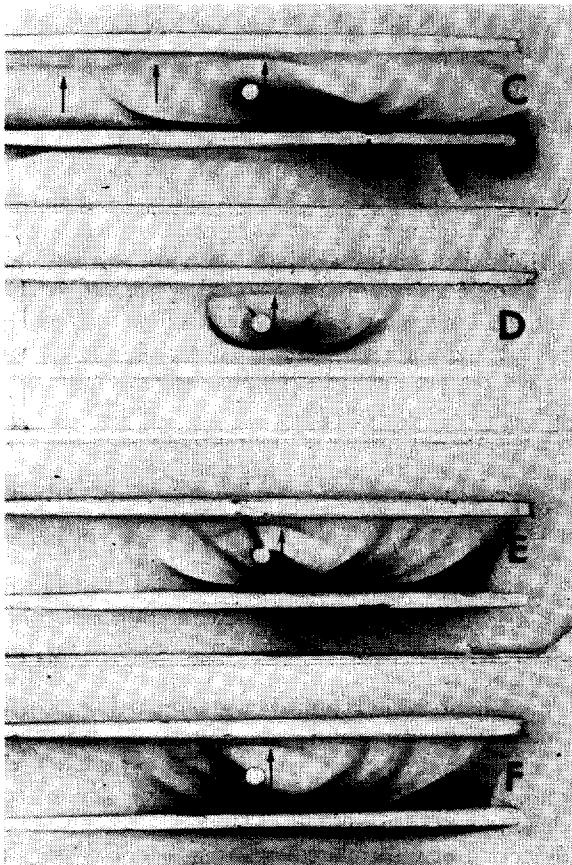

Fig. 4. Immunoelectrophoretic analysis of normal human serum, and of concentrates of the pooled fractions of the eluate after filtration on Sephadex G-200. Upper trough, anti-HSCP; lower trough, anti-HS; C, control human serum; D, pool 1; E, pool 4; F, pool 5.
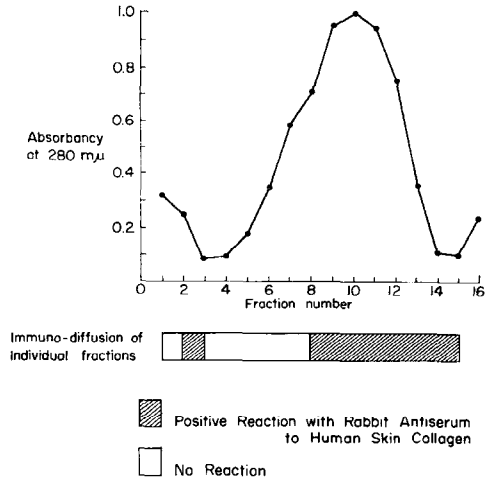
Fig. 5. Fractionation of normal human serum by sucrose density gradient centrifugation. The cross-hatching in the bar below the fractionation pattern shows which fractions reacted with rabbit antiserum to human skin collagen preparation.
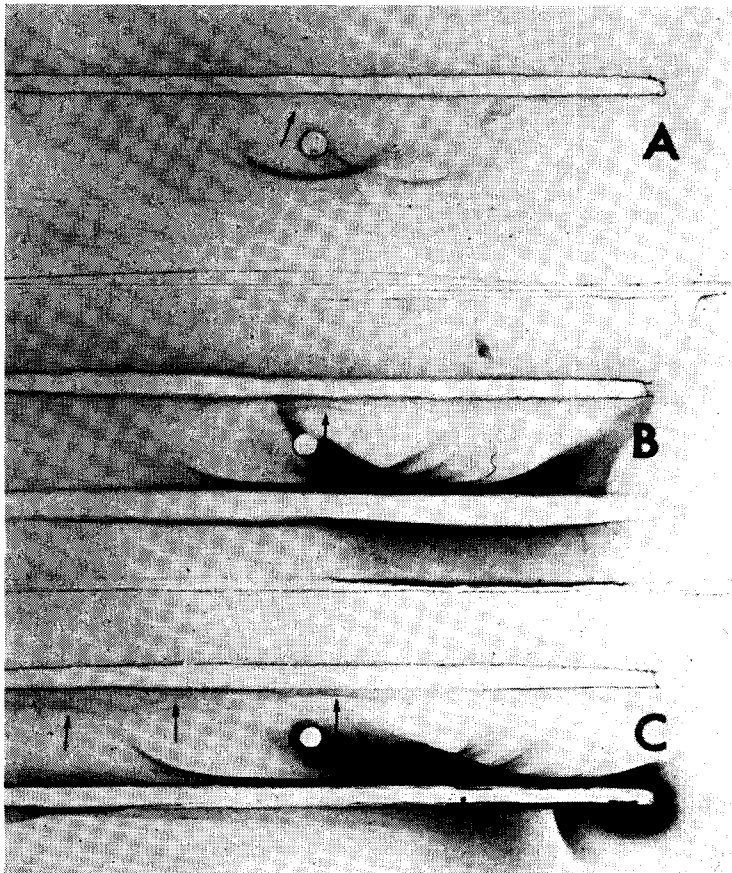

Fig. 6. Immunoelectrophoretic analysis of fractions of human serum after sucrose density gradient centrifugation, and of native human serum. Upper trough, anti-HSCP; lower trough, anti-HS; center-well, A, fraction 2; B, fraction 13; C, native human serum.

this series of human sera suggests that the sera differ from each other both qualitatively and quantitatively in their soluble collagen components. Micro-immunodiffusion, although useful in the detection and quantitation of the antigen, is less discriminating than immunoelectrophoresis in demonstrating the presence of multiple components.

Monomers, dimers and trimers of the collagen molecule have been reported previously by Nagai *et al.*[7], and Francois and Glimcher[16], using calf skin, and calf and cofish skin, respectively, as sources of collagen. Bornstein and Piez[17] have suggested that a shift in the ratio of the multiple collagen forms found in normal skin may indicate a pathological alteration in collagen metabolism. Harris and Sjoerdsma[2] showed a shift in the ratio of the "α and β" components of soluble human skin collagen in 2 patients with homocystinuria, while no change was evident in a variety of other chronic clinical conditions.

The use of anti-HSCP to detect multiple collagen-like components in human serum offers a new and simplified method for further investigations in the area of collagen metabolism in various normal and abnormal conditions.

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