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DEVELOPMENT AND CHARACTERIZATION OF MONOCLONAL ANTIBODIES TO HUMAN TYPE III PROCOLLAGEN

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Summary. Hybridomas which secrete monoclonal antibodies against human type III procollagen have been developed. By an enzyme-linked immunosorbent assay, three of the monoclonal antibodies have been determined to be against non-helical extensions of the molecules while two of the antibodies are against helical portion of the molecules which is sensitive to bacterial collagenase action. These findings have been further confirmed by carrying out immunoreaction of the proα-chains transferred on nitrocellulose paper from sodium dodecyl sulfate polyacrylamide gels. These monoclonal antibodies have been found to be suitable reagents for immunohistochemical studies as well as for immunoassays of type III procollagen and collagen.

INTRODUCTION

Collagen, one of the most predominant proteins in the body, primarily functions as a supporting element in a variety of connective tissues. Existence of at least five genetically and structurally distinct collagens designated types I to V have been established (1-3). All of these collagens are composed of three polypeptide chains (α-chains) in triple-helical form. Collagens are synthesized in the precursor form termed procollagens which have non-helical extensions at both amino and carboxy terminal ends of the α-chains. A number of functions have been postulated to the non-helical extensions of the procollagen molecules (see review 3).

Conventionally derived polyclonal antibodies have been used as immunochemical reagents in the elucidation of structure, mechanism of biosynthesis and secretion and distribution of collagens in the tissues (reviews 3-5). However, the use of monoclonal antibodies for such investigations will be advantageous not only because they are monospecific to a single antigenic determinant but

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also because it is possible to derive monoclonal antibodies using only partially purified immunogens. The production of monoclonal antibodies to chick types I and II collagens has been reported by Linsenmayer et al.(6,7). We have developed monoclonal antibodies to human types I and III procollagens and collagens and type IV collagen (Abstract 8). In this communication, we describe characterization of monoclonal antibodies specific to non-helical extensions and also to helical pepsin resistant portion of human type III procollagen. These antibodies are anticipated to be useful reagents for histochemical studies, immunoassays and also structural and biosynthetic investigations of type III procollagen.

MATERIALS AND METHODS

Isolation of type III procollagen. Human type III procollagen was isolated and purified from the culture filtrates of SV40-transformed human skin fibroblasts (GM637, obtained from the Inst. Med. Res., Camden, NJ) by ammonium sulfate precipitation, ethanol precipitation, followed by DEAE - cellulose chromatography (9,10).

Production of hybridomas. Balb/c mice (Charles River) were immunized subcultaneously with 50 µg of type III procollagen in phosphate buffered saline (PBS) with complete Freund's adjuvant. The second and third injections consisting of 50 µg of procollagen in PBS, with and without incomplete adjuvant respectively, were given intraperitoneally at 4 weeks intervals. Three days after the third injection the spleens were removed from the mice and spleen cells were hybridized with a mouse myeloma cell line as follows: Approximately $1x10^8$ spleen cells obtained from one immunized mouse were fused with $1x10^7$ myeloma cells (SP2/0Ag14) described by Shulman et al. (11) obtained from the Salk Institute, Cell Distribution Center, San Diego. Polyethylene glycol-1000 (Accurate Chem. and Sci. Co.) was the fusion agent and the procedure used was of Galfre et al. (12). From a single hybridization, cells were dispersed into 2x96 well microtiter plates in HY medium (13) with 20% fetal calf serum. Half of the medium was replaced with HY containing HAT (14) for the next three days and every third day then on. After 3-4 weeks enough growth of the hybridomas was observed in the microtiter plates. The culture supernatants from these hybridomas were screened for the presence of antibody by an enzyme-linked immunosorbent assay (ELISA). Positive hybridomas were cloned by limited dilutions in HY medium in 96 well microtiter plates.

ELISA. The ELISA used here was Gosslau and Barrach's (15) modification of Engvall and Perlmann's (16) procedure with some more modifications. Microtiter plates were coated for 2-24 h with 100 µl of 10 µg/ml concentrations of type III procollagen in coating buffer at 4°C. The coated wells were washed with 1% bovine serum albumin (fraction V, Sigma) in PBS and then covered with 100 µl of culture supernatants for 2 h at 25°C. After washing the wells three times with PBS, 100 µl of 1/500 dilution of peroxidase conjugated IgG fraction of rabbit antimouse IgG (Cappel Laboratory) in 1% BSA in PBS were added to each well and the plates were incubated at 25°C for 1 h. This antibody cross-reacted with mouse IgM. After washing the wells three times with PBS they were covered with 100 µl of 5-aminosalicylic acid reagent (17) for 1 h. The absorbance of the reagents was determined at 450 nm in Multiskan microplate reader (Flow Laboratory) after stopping the reaction by adding 100 µl of 1 N NaOH to each well.

Production of high concentration-antibodies and their further characterization. For the large scale antibody production cloned hybridoma cells were injected intraperitoneally into pristane-primed Balb/c mice (18). Ascites fluid was collected from the mice bearing tumors of hybridomas in ascitic form, 10-15 days after the injections. After removing the cells and debris by centrifugation, the clear fluid contained large concentration of the hybridoma antibody. Antibodies were also concentrated from large volumes of culture supernatants by $(NH_4)_2SO_4$ fractionation (19). The antibodies concentrated from the culture supernatants were tested by double immunodiffusion (20) against antibodies specific to mouse κ and λ light chains (Miles Laboratory) and mouse IgG (γ-chain) and IgM (μ-chain) from Cappel Laboratories.

Antibody specificity. Antibody specificity was first tested by ELISA using human types I and III procollagens and types I, III, IV and V collagens. To determine whether the antigenic determinants of the molecule resided in the helical part or the non-helical extensions of the procollagen molecule, the following experiment was performed. Type III procollagen was treated with bacterial collagenase (Sigma) further purified according to Peterkofsky and Diegelmann (21), for 1 h at 37°C. Microtiter plates were coated with collagenase-treated or non-treated procollagen for ELISA test.

Transfer of procollagen α-chains to nitrocellulose paper and detection of antigenic determinants. Type III procollagens (25-30 µg/well) were electrophoresed on 6% polyacrylamide SDS slab gels according to Laemmli (22). Procollagen chains from SDS-gels were then transferred electrophoretically to nitrocellulose paper (Biorad) and immunoreactivity of the monoclonal antibodies with the transferred polypeptide chains was tested using Towbin's procedure (22) with a few modifications. Peroxidase conjugated IgG fraction of rabbit antimouse IgG (Cappel) was used at 1:250 dilution as the second antibody and instead of o-dianisidine, diaminobenzidine reagent (24) was employed to detect the binding of peroxidase conjugated antibody.

Indirect enzyme-linked immunostaining. Immunohistochemical staining for light microscopy was carried out on cryostat sections (6 µm) of human skin frozen in Tissue-Tek II O.C.T. compound. The sections transferred on microscope slides were treated successively with 10% rabbit serum in PBS for 1 h, a drop of appropriate antibody dilutions in 10% rabbit serum in PBS for 1 h and the peroxidase conjugated rabbit antimouse IgG serum (1:80 dilution) for 1 h. Between each of the above treatments, the sections were washed thoroughly with PBS for 1 h. The sections were then treated with a reagent containing benzidine dihydrochloride and Safranin O (25). After dehydrating by washing through ethanol and xylene, the sections were mounted in Permount (Fisher Sci. Co.). For staining cells in culture, the cells were grown in chamber slides. After washing 3 times with PBS, the cells on the slides were stained using the same procedure used for staining the tissue sections.

RESULTS AND DISCUSSION

Selection of antibody-producing hybridomas. From the fusion of spleen cells from one of the mice immunized with type III procollagen, with myeloma cells SP2/0 Ag 14, hybridomas developed in 186 wells in the microtiter plates. ELISA-screening indicated secretion of antibodies against type III procollagen by 15 of these hybridomas. However, stable cloned hybridomas could be developed from only five of the original hybridomas. One positive clone from each of the five original hybridomas was used for further studies.

TABLE I OUCHTERLONY IMMUNODIFFUSION ANALYSIS OF THE MONOCLONAL ANTIBODIES

Monoclonal Antibody	Antimouse IgM, μ chain specific	Anti-κ light chain specific	Antimouse IgG, γ chain specific	Anti λ light chain specific
704-3	-	+	+	-
707-4	-	+	+	-
710-5	-	-	+	-
711-2	-	+	+	-
712-5	+	+	-	-
715-1	-	+	+	-

Culture supernatants of hybridomas concentrated by $(NH_4)_2\ SO_4$ precipitation were tested against the specific antimouse immunoglobulin antibodies by Ouchterlony double immunodiffusion; +, precipitin line formed; -, no precipitin line.

Further characterization of the antibodies. The specific immunoglobulin class of the antibodies was determined by testing their immunoprecipitability with specific antibodies using double immunodiffusion. Results are presented in Table I. Four antibodies were IgG type with κ light chains and one (712-5) was IgM type with κ light chains.

Specificity of the antibodies. Table II shows the results of the ELISA test carried out to determine cross reactivity of the monoclonal antibodies.

TABLE II SPECIFICITY OF MONOCLONAL ANTIBODIES BY ELISA TEST

Collagens used for coating	Monoclonal antibodies 704-3	707-4	710-5	711-2	712-5	715-1
Type I collagen[a]	-	-	-	-	-	-
Type III collagen[a]	+	-	+	-	-	-
Type I procollagen[b]	-	-	-	-	-	-
Type III procollagen[b]	+	+	+	+	+	+
Type IV collagen[a]	-	-	-	-	-	-
Type V collagen[a]	-	-	-	-	-	-

[a], purified from human placenta according to Glanville et al. (26) and Trelstad (27).

[b], purified from the culture supernatants of skin fibroblasts (GM37, Inst. Med. Res., Camden, N.J.) as reported (10).

TABLE III EFFECT OF BACTERIAL COLLAGENASE TREATMENT OF TYPE III PROCOLLAGEN ON THE REACTIVITY OF THE PRODUCTS WITH THE MONOCLONAL ANTIBODIES.

MONOCLONAL ANTIBODIES	ELISA test readings of absorbance at 490 nm	
	Non-treated	Collagenase-treated*
704-3	0.50	0.10
707-4	0.62	0.60
710-5	0.56	0.12
711-2	0.68	0.59
712-5	0.60	0.54
715-1	0.61	0.58

*SDS-PAGE of the protein after the collagenase treatment indicated that more than 90 percent of the procollagen was hydrolyzed.

Antibodies (704-3 and 710-5) reacted both with type III procollagen and type III collagen indicating that the antigenic determinant of these antibodies were located in the pepsin-resistant part of the molecule. The other four antibodies reacted with type III procollagen and not type I procollagen or other collagen types. These antibodies were thus to non-helical extension of the molecule, which was further confirmed from the collagenase resistance of the antigenic determinant (Table III). Bacterial collagenase cleaves the helical portion of the molecule into tripeptides but does not cleave the helical extensions. Binding of antibodies 704-3 and 710-5 was considerably reduced after collagenase treatment confirming that the antigenic determinant was in the helical region.

Although type III procollagen used for immunizations and for ELISA test was more than 95% pure as determined by gel electrophoresis and bacterial collagenase sensitivity, further confirmation of the antibody specificity against non-helical extensions of type III procollagen was carried out as follows: The immunoreactivity of the antibodies was tested on proα(III) chains transferred to nitrocellulose paper from SDS-PAGE. As seen in Fig. 1, proα(III) chains did react with the antibodies 711-2, 712-5 and 715-1 but

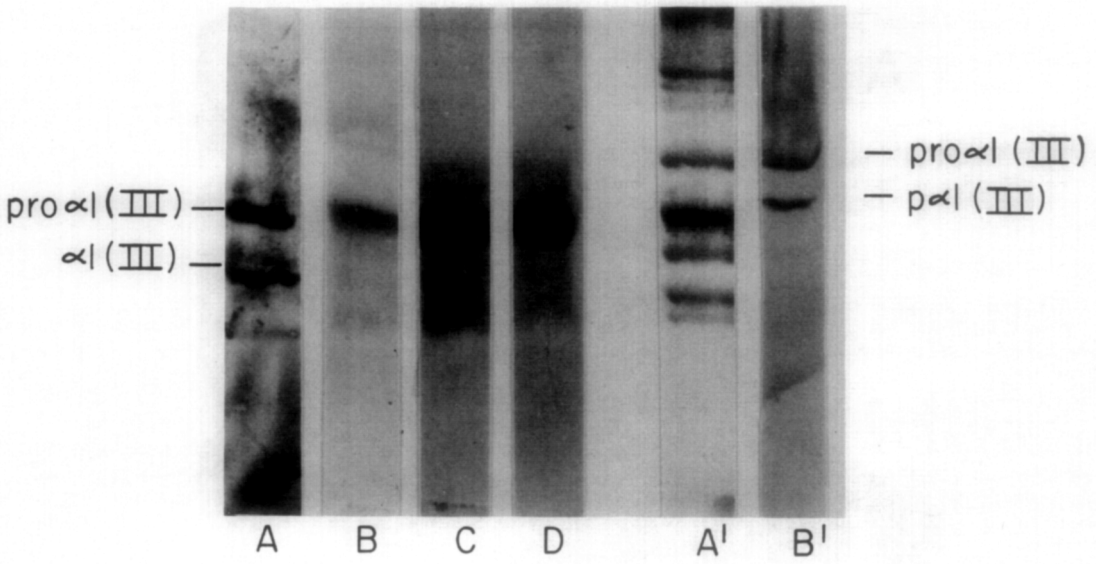

Fig. 1 Binding of the monoclonal antibodies to proα1(III) chains on nitrocellulose blots. A, blot of proα1(III) chains from SDS-PAGE stained with amido black. B, C, and D blots same as A but reacted with the antibodies 711-2, 712-5 and 715-1, respectively, followed by peroxidase conjugated antimouse IgG and diaminobenzidine reagent. A', blot of partially purified procollagens stained with amido black. B', same as A' but reacted with antibody 715-1.

not with 704-3, 707-4 or 710-5. Since the results of ELISA test using bacterial collagenase-treated procollagen suggested that 704-3 and 710-5 antibodies are against helical portion of the molecule, non-reactivity of α-chains separated by SDS-PAGE with these antibodies was expected. However, non-reactivity of 707-4 antibody with proα(III) chains indicated that its antigenic determinant is probably conformational dependent.

Immunohistology using the monoclonal antibodies. Neither 707-4, 711-2, 712-5 nor 715-1 reacted with the human skin sections when tested by the indirect immunochemical staining. Fibrillar networks derived from type III collagen are thought to retain at least some proportion of the procollagen sequence (see Rev. 2). Therefore, non-reactivity of these antibodies suggested that their antigenic determinants are located on the non-helical extension of the molecule which is not retained in the fibrils or fibers of the collagens in the dermis. However, antibodies 704-3 and 710-5 which are against the helical portion of the molecule, reacted with the dermis of the skin as expected (Fig. 2A). The staining was more intense in the papillary

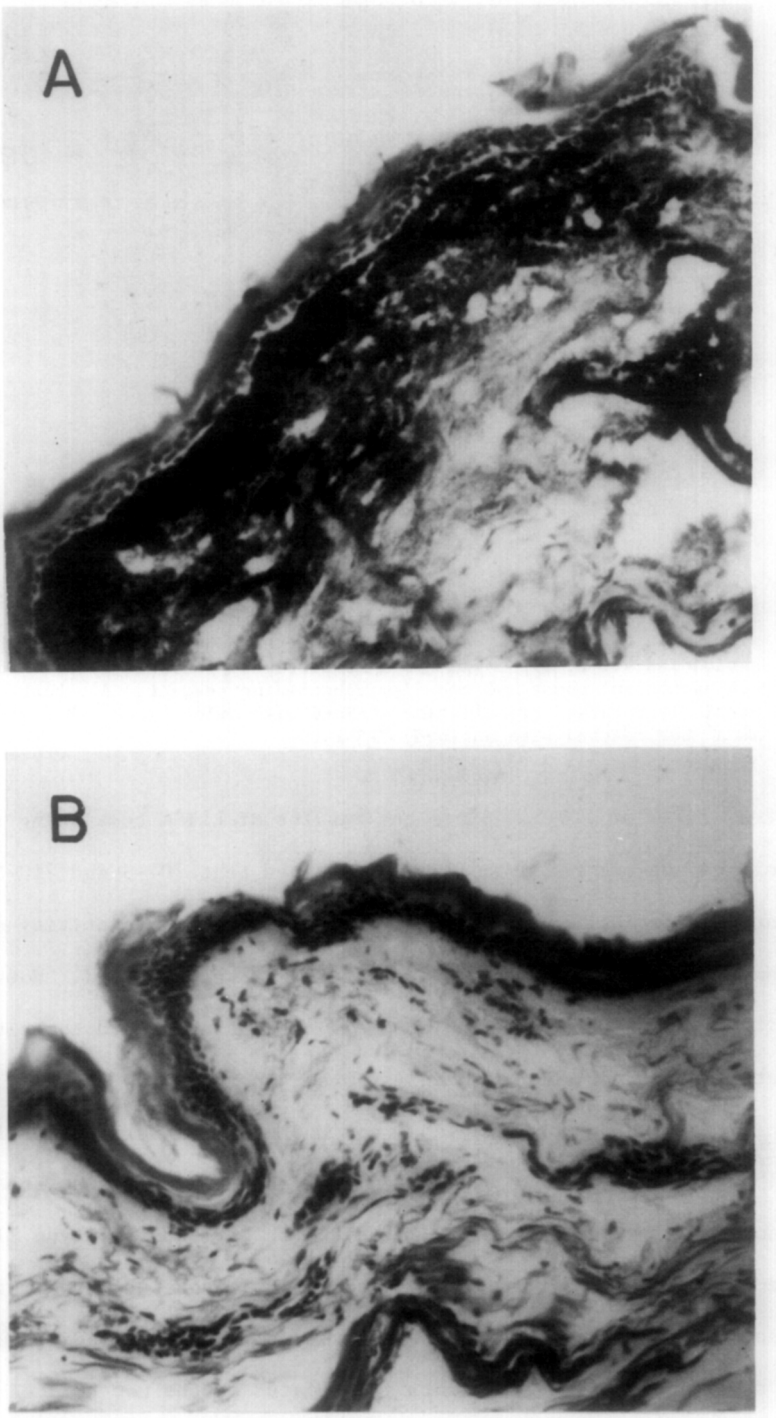

Fig. 2 Indirect immunohistochemical staining of human skin sections (A & B) and human skin fibroblasts in culture (C & D). A and C reacted with ascites fluid containing 710-5 and 712-5 antibodies, respectively. B and D (controls) reacted with ascites fluid from a mouse bearing SP2/0 Ag14 tumor.

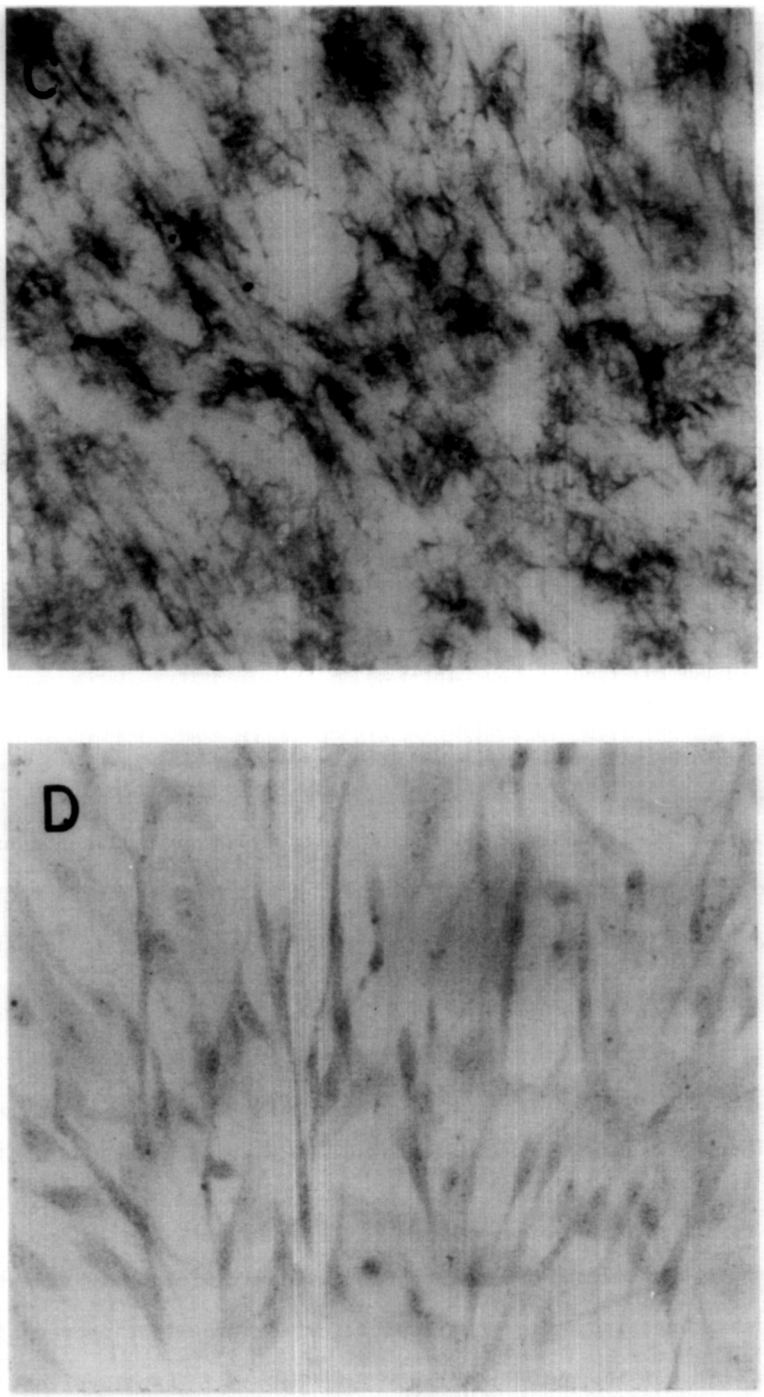

Fig. 2 continued

region. All the six monoclonal antibodies described here reacted with the extracellular matrix of the human skin fibroblasts in culture (example, Fig. 2C).

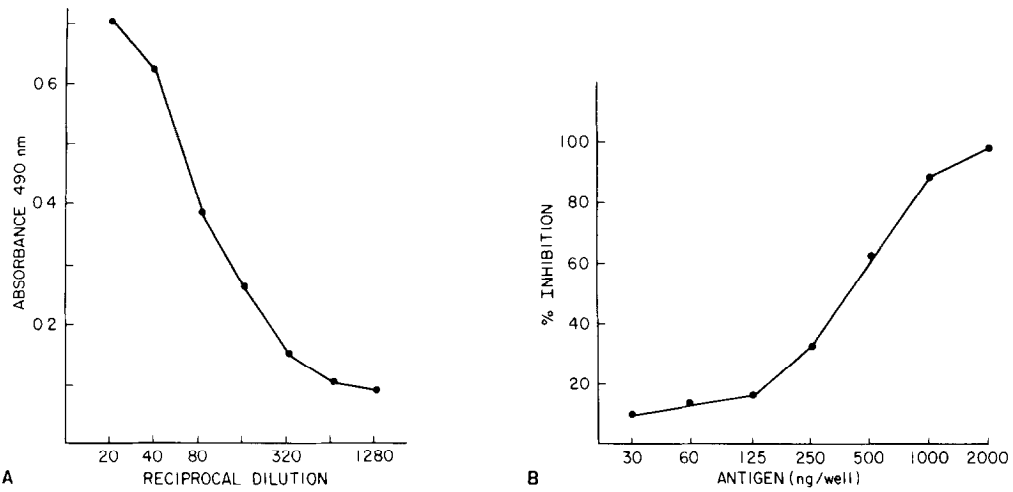

Fig. 3 Titration of antibodies and antigens by ELISA. A, titration of monoclonal antibody 712-5 in microtiter wells coated with type III procollagen (5 µg/ml of coating buffer). B, inhibition of the antibody binding to solid phase to type III procollagen by different concentration of type III procollagen. Various concentrations of human type III procollagens were incubated with equal volumes (50 µl) of 1:200 dilution of 712-5 antibody in the microtiter plates coated with 5 µg/ml of type III procollagen.

Use of the monoclonal antibodies to assay type III procollagen. The monoclonal antibody 712-5 was employed to set up type III procollagen assays. The titration of the ascites fluid containing 712-5 antibody by ELISA technique is shown in Fig. 3A. Using 1/200 dilution of the ascites fluid, competition by various concentrations of soluble procollagen with the solid phase antigen for antibody binding site is presented in Fig. 3B. This assay showed 50% inhibition of the binding of antibody to solid phase antigen with 3 µg/ml of type III procollagen, and could detect 0.1-0.2 µg of type I procollagen/ml.

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