feeding too (*13*). Thus, in the Israeli formations *Vermetus* and *Dendropoma* diverge in both habitat and feeding techniques, but in the Bermudan formations the local *Dendropoma* occupies both habitats and possibly utilizes both feeding methods.

Intertidal reefs with features similar to those in Israel and Bermuda are known from the Cape Verde Islands (*14*) and Fernando de Noronha Island (*15*). They should probably not be expected where fringing coral reefs occur: the habitat of vermetid reefs in subtropical waters is very close to that of fringing coral reefs in tropical waters. In Bermuda, where winter surface temperatures are higher than in Israel (*16*), coral reefs do occur, but they do not reach the surface, and microatolls are located at the seaward edge of these reefs, where one could have expected a fringing coral reef.

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17. Contribution No. 528 from the Bermuda Biological Station. Supported in part by NSF grant GB-6404 to the Bermuda Biological Station and in part by NSF grant GB-6230 to N. G. Hairston, University of Michigan, Ann Arbor. Thanks are due to R. N. Ginsburg for allowing me to collect samples from the exposures he made.

12 June 1974; revised 24 September 1974

Radioimmunoassay for Human Procollagen

Abstract. *Rabbit antiserums were produced against the procollagen molecule secreted into the medium of cultured human skin fibroblasts. The isolated antigenic, amino terminal portion of the procollagen molecule was purified, labeled with iodine-125, and used in a radioimmunoassay which detected nanogram quantities of the same antigen. With the assay, immunologically identical molecules were detected in the culture medium of different strains of human fibroblasts and in normal human serums. Serums from human cord blood contained a 12-fold higher concentration of the antigen than serums from adults, while serums from other vertebrates gave reactions to incomplete cross-reactivity or nonreactivity.*

Cultured human skin fibroblasts synthesize a procollagen molecule (molecular weight, 360,000) composed of three polypeptide chains (*1, 2*). The first 200 residues at the amino termini of each of the three chains (the propeptides) are in a globular conformation (*3*) stabilized by interchain disulfide bonds between half-cystine residues. The remainder of the molecule is in a triple-helical array stabilized by noncovalent interactions between the chains. An enzymatic excision of the amino terminal globular sequences occurs after procollagen is secreted from the cell. One product of the excision is tropocollagen (molecular weight, 285,000), the triple-helical molecule which aggregates to form collagen fibers.

Procollagen accumulates in the medium of cultured human diploid skin fibroblasts because the enzymatic excision of the globular sequences occurs slowly and appears to be a rate-limiting step in this system (*2*). Rabbits injected with culture medium from these cells develop antibodies directed only against antigenic determinants in the

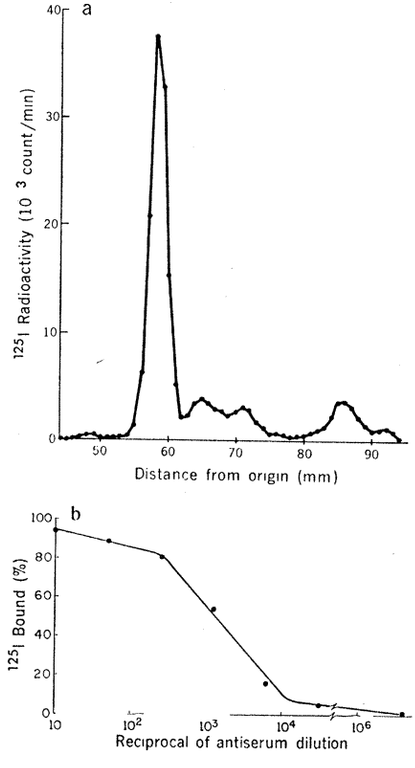

Fig. 1. (a) Electrophoresis of ^{125}I-labeled antigen on polyacrylamide gel containing SDS and urea. The sample was dissolved in the running buffer for electrophoresis and denatured by heat; electrophoresis and measurement of radioactivity in gel slices were performed as described (*1*). (b) Titration of antiserum to iodinated antigen. All dilutions were made with a standard buffer containing 0.025*M* phosphate, *p*H 7.6, 0.1 percent ovalbumin, and 0.03 percent Triton X-100. Duplicate tubes contained dilutions of adsorbed rabbit antiserum (in 0.1 ml), 0.1 ml of ^{125}I-labeled propeptide (50,000 count/min), 0.1 ml of 5 percent normal rabbit serum, and 0.2 ml of buffer. Tubes were incubated for 2 hours at 37°C and then for 24 hours at 4°C. Sheep antiserum to rabbit 7*S* globulin (0.075 ml), 0.1 ml of EDTA (0.1*M* in buffer), and buffer to a final volume of 1.0 ml were added. Tubes were incubated for 1 hour at 37°C and 4 hours at 4°C and then centrifuged at 5000*g* for 10 minutes. Supernatants were added to Aquasol (New England Nuclear), and the radioactivity was measured in a scintillation spectrometer. Rabbit antiserum was omitted from control tubes.

amino terminal sequences of the propeptides (*4*). To isolate the antigenic portion of the molecule, we treated the culture medium with collagenase to digest away the triple-helical segment of procollagen; the collagenase-resistant globular portion of the molecule (the propeptide fragment) was then recovered and purified (*5*). The fragment has a molecular weight of 75,000, it consists of three disulfide-linked polypeptide chains with an amino acid composition different from that of tropocollagen, and it reacts specifically with rabbit antiserums to the culture medium.

We now report the use of the antiserum and the antigen in a radioimmunoassay (RIA) that specifically detects polypeptide sequences unique to the precursor of human collagen. Cells of a human diploid skin fibroblast strain (American Type Culture Collection, CRL 1121) were grown to confluence in a medium containing 10 percent fetal calf serum (*1*). To eliminate most of the latter protein, we washed cell layers with phosphate-buffered saline and then covered them with serum-free medium for 24 hours. The serum-free medium was then removed and used for the immunization of rabbits (*4*), and the antiserums were adsorbed with fetal calf serum prior to use in the RIA. The propeptide fragment was isolated and purified as reported, except that we used a lower concentration of collagenase and a modified final ion exchange step to eliminate a minor contaminant from the final product (*5*). The purified propeptide fragment was labeled with ^{125}I by the chloramine-T method (*6*). For the conditions used, and assuming that the recovery of the iodinated protein is 100 percent, the labeled fragment had an average specific activity of 7 $\mu c/\mu g$. To test the effect of the iodine labeling on the structure of the fragment, we subjected a sample to electrophoresis on a 5 percent polyacrylamide gel in a buffer consisting of sodium dodecyl sulfate (SDS), urea, and phosphate (*1*). Most of the radioactivity was recovered as a single species 58 mm from the origin (Fig. 1a), and the remainder was distributed between three other species of lower molecular weight. Purified antigen containing [^3H]tryptophan but no ^{125}I was subjected to electrophoresis simultaneously on a parallel gel (not shown), and in this instance all the radioactivity was recovered in a peak at 58 mm. Gel electrophoresis of the iodinated antigen performed several weeks after iodination showed that a larger fraction of the total radioactivity was in the three lower-molecular-weight species. From these observations we conclude that the lower-molecular-weight species are split from the propeptide fragment as a consequence of the iodination procedure. The freshly iodinated antigen was successively reacted with immune rabbit serum and sheep antiserum to rabbit 7*S* globulin. When the immune precipitate was solubilized in SDS and subjected to electrophoresis on an SDS-polyacrylamide gel, the profile of radioactivity obtained was identical to that of Fig. 1a, showing that all the ^{125}I-labeled molecules were immunoreactive.

A representative titration of the adsorbed rabbit antiserum against the ^{125}I-labeled propeptide fragment is shown in Fig. 1b. More than 90 percent of the input radioactivity was specifically bound at the lowest serum dilutions. In the absence of antiserum, less than 1 percent of the radioactivity was nonspecifically bound. The slope of the curve between 20 and 80 percent bind-

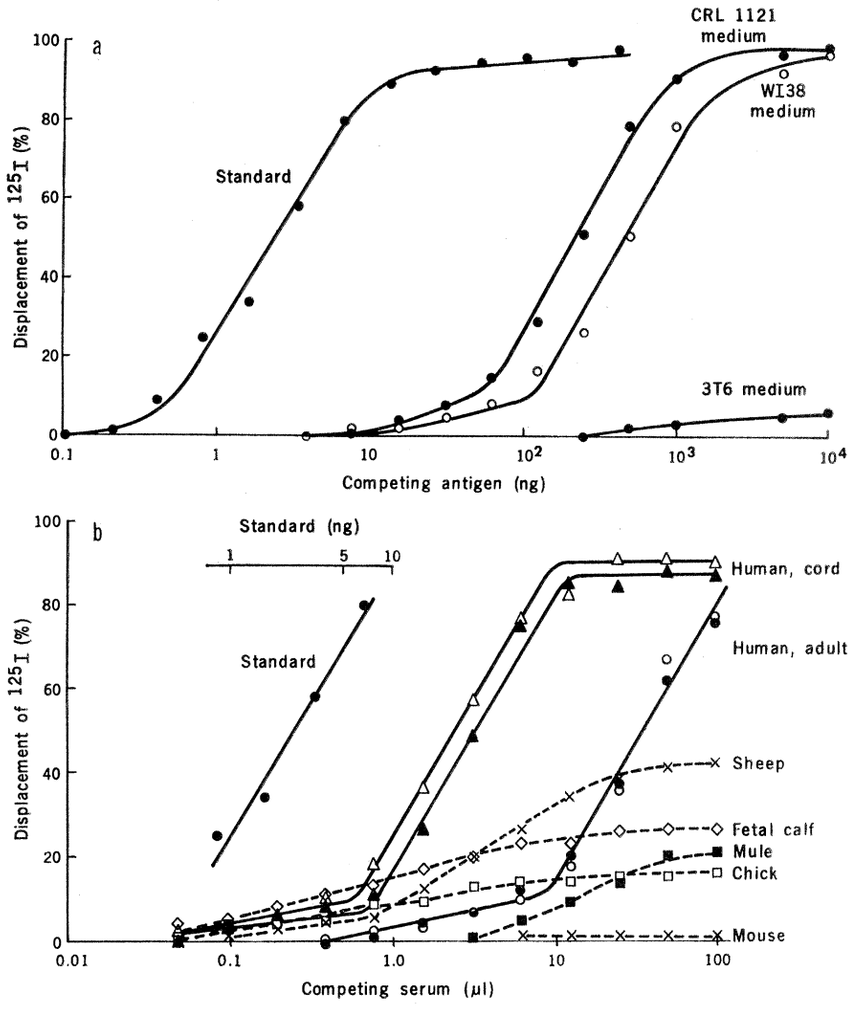

Fig. 2. Radioimmunoassay: binding displacement curves. Duplicate tubes contained unlabeled competing antigens (in 0.1 ml), 10^{-3} dilution of adsorbed rabbit antiserum (in 0.1 ml), 0.1 ml of 5 percent normal rabbit serum, and 0.1 ml of buffer. Tubes were incubated at 37°C for 2 hours; 0.1 ml of ^{125}I-labeled propeptide (50,000 count/min) was added; and tubes were then incubated at 37°C for 1 hour and at 4°C for 36 hours. Then 0.075 ml of sheep antiserum to rabbit 7*S* globulin and 0.1 ml of 0.1*M* EDTA were added and volumes were brought to 1 ml with buffer. Tubes were incubated at 37°C for 1 hour and at 4°C for 4 hours, and centrifuged as described. Supernatants were decanted into Aquasol, as were the washed pellets after solubilization in 0.5 ml of 0.5*M* acetic acid. Radioactivity was measured as described. In one control sample competing antigen was omitted, and in another, rabbit antiserum and competing antigen were omitted. Samples were counted to a 99 percent confidence level, and duplicate tubes had a maximum standard error from the mean of 0.2 percent. (a) The standard curve was derived by additions of the purified, uniodinated propeptide fragment. Other curves were derived by additions of media from cultured fibroblasts. (b) Competition curves were generated by additions of the respective serums. The standard curve was taken from the experiment of (a).

ing shows that the antiserum is of suitable avidity for the detection of small differences in amounts of input antigen. Curves identical to that of Fig. 1b were obtained with unadsorbed antiserums. We have titrated antiserum produced against the purified propeptide fragment, and the slope of the curve between 20 and 80 percent binding was identical to that of Fig. 1b.

For the RIA, an antiserum dilution of 10^{-3} was chosen, which corresponded to 55 percent binding of the input ^{125}I-labeled antigen. To obtain the standard curve, known quantities of the purified uniodinated antigen were incubated with the antiserum before addition of the ^{125}I-labeled antigen, and then the immune complexes were precipitated with sheep antiserum to rabbit 7*S* globulin. Binding of antibody sites by the unlabeled antigen was detected as a displacement of radioactivity from the immune precipitates to the supernatants. Scintillation counting of free antigen in supernatants or bound antigen in washed precipitates gave reciprocal results, and the data were plotted as percent displacement of radioactivity against the log of the number of nanograms of unlabeled antigen. The typical standard curve of Fig. 2a is linear between displacements of 20 to 80 percent, and the 20 percent displacement was given by only 0.8 ng of competing standard antigen. A 50 percent displacement corresponded to 2.5 ng, in good agreement with the calculated number of nanograms of ^{125}I-antigen bound in the assay in the absence of competing antigen. Antigen additions above 30 ng caused almost complete displacement of the bound radioactivity.

Because the rabbit antiserum was produced against the intact procollagen molecule in the culture medium of CRL-1121 fibroblasts, it was of interest to test this medium as a source of competing antigen in the assay. The competition curve obtained with the medium has a slope identical to the standard curve in the linear displacement range, indicating a reaction of antigenic identity (Fig. 2a) (*7*). This result also indicates that the RIA does not distinguish between the intact procollagen molecule and the propeptide fragment derived from it. Addition of 220 ng of lyophilized CRL-1121 medium was required to produce a 50 percent displacement of radioactivity, indicating that procollagen represented about 5 percent (by weight) of the input material.

To test the applicability of the assay for procollagen synthesized in culture by other human fibroblast strains, WI-38 human fetal lung fibroblasts were grown to confluence, and the culture medium was tested as a source of competing antigen. This medium also gave a reaction of identity (Fig. 2a). When culture medium from the collagen-synthesizing, 3T6 mouse fibroblast line (*8*) was tested, no significant competition was observed (Fig. 2a). The latter experiment shows that displacement curves of identity are not nonspecifically generated by medium from any cell culture.

Competition analyses were also performed using serums as the potential source of competing antigen (Fig. 2b). Human serums from adults and from cord blood of full-term newborns gave reactions of antigenic identity in the assay. As calculated from the 50 percent displacement points, the concentration of the antigen in human serums is 86 μg/100 ml for newborns and 7 μg/100 ml for adults. The curves obtained with sheep, fetal calf, mule, and chick serums had different slopes from that of the standard curve and showed maximal displacements of radioactivity of 18 to 40 percent. These are reactions of incomplete cross-reactivity (*7*) and indicate that the antigens in the serums of these species contain only partial homologies to the human propeptide sequences. Mouse serum did not compete at all for antibody sites in the assay. The result that different serums give reactions of identity, incomplete cross-reactivity, and nonreactivity argues against the interpretation that nonspecific substances in serums could have caused the displacements observed.

At present, the RIA does not identify the molecular form of the antigen in serums; it could be the intact procollagen molecule, a propeptide fragment, or both. The conversion of procollagen to tropocollagen in vivo could generate a propeptide fragment, for it appears that the required enzyme functions as an endopeptidase (*9*). Considering the large relative contribution of collagen to total body protein, and its role in morphogenesis, maintenance of tissue structure, and inflammation and repair, it need not be surprising that serums should contain procollagen protein, and that its concentration should be higher in serums from newborns than from normal adults. At the measured serum concentration of 7 μg/100 ml, procollagen protein could account for more than 0.2 percent of the hydroxyproline-containing, collagen-like protein detected at 3 to 5 mg/100 ml in plasma by an isotope dilution method (*10*). Most of this collagen-like material probably represents the C1q component of complement (*11*) and the rest, catabolic peptides derived from tropocollagen. Moreover, human C1q (*11*) shows no cross-reactivity with procollagen in the RIA.

The RIA provides the means for assessing homologies between genetically distinct procollagens synthesized by different cell types of the same species. As shown above, the RIA can also test cross-reactivities between procollagens from different species. Additionally, we anticipate that the assay may be of diagnostic and prognostic value when applied to serums and other body fluids from patients with diseases affecting collagen metabolism.

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15 July 1974