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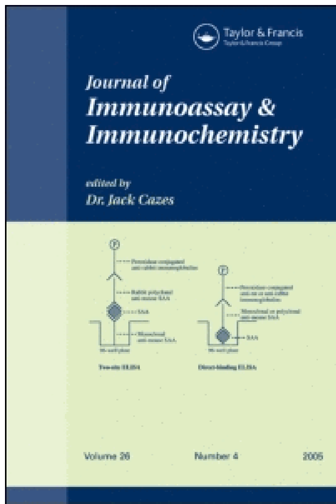

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RADIOIMMUNOASSAY FOR HUMAN TYPE II COLLAGEN

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ABSTRACT

Human articular cartilage type II collagen (h coll.II) was purified and used to develop a radioimmunoassay. The sequential saturation procedure allowed a sensitivity of 3 ng/tube. The intra and between assay coefficients of variation were less than 10 and 20% respectively in the linear part of the curve. The assay was highly specific for native human articular type II collagen. There was no cross-reactivity with other constituents of cartilage: human proteoglycans, fibronectin, laminin and hyaluronic acid did not interfere with the assay. No cross-reactivity existed with bovine collagen types I, III, IV. However, native collagens from human placenta (I, III, IV, V, VI), rat and calf skin type I collagens and bovine type II collagen produced a weak cross-reaction only at high doses. Concerning the latter, inhibition curves were not parallel. Parallelism of inhibition curves were observed for dilution of type II collagen, produced by human chondrocytes in three-dimensional culture. All of these characteristics indicate that radioimmunoassay of type II collagen is a very sensitive and specific method available for the study and quantification of type II collagen in in vitro experimental conditions.

KEY WORDS: (human articular cartilage type II collagen; extraction method; radioimmunoassay).

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INTRODUCTION

Collagen is a major component of the extracellular matrix of all cartilage and constitutes approximately 10% of the wet weight and 40-50% of the dry weight of the tissue (1). The major collagenous component of hyaline cartilage is type II collagen which forms a network of fibrils essential for the retention of proteoglycan aggregates. Type II collagen represents approximately 90% of the total cartilage collagen (2). In addition to type II, several minor collagens have also been identified and designated type IX (3,4), type X (5,6) and type XI (7,8). Type II collagen is composed of three identical alpha 1 (II) chains and is denoted [α -1 (II).]3. During the formation of the collagen molecule, the three chains form a right-handed helix to give a relatively stiff rod-like structure of 300 by 1.5 nm (9). Proline and hydroxyproline comprise about one fifth of the amino-acids in collagen. These chains possess chromatographic and electrophoretic characteristics similar to the alpha 1 chains of type I collagen. In addition α-1 (II) can be differentiated from α-1 (I) on the basis of a five-fold increase in the hydroxylation of lysyl residues and a ninefold increase in the content of hydroxylysine-linked carbohydrate (2). The different types of collagen are very similar in their physical properties and molecular shapes and show a considerable homology in the amino-acid sequence of their component chains (10). Despite these

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similarities, antibodies specific for type I, II and III collagens can be raised in rats (11), rabbits (12) and mice (13). Different methods have been used to quantify collagen in cell cultures of human tissue samples. Hill et al.(14) used total collagen in tissues by extraction of tissue with hot 5% trichloracetic acid (TCA). Although collagen is a relatively poor immunogen compared to globular proteins, radioimmunoassays have been developed for collagens and procollagens (15,16). Several authors have developed enzyme-linked immunosorbent assay (ELISA) tests for rat type I, II, III collagens (17) or mice type I,II,III collagens (18). In this work, a radioimmunoassay for human type II collagen has been developed to investigate its synthesis by human chondrocytes in culture.

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MATERIALS AND METHODS

Human type II collagen extraction

Human type II collagen was extracted from knee cartilage by the method of Herbage et al. (19) slightly modified. Briefly, normal human cartilage obtained at autopsy was homogenized in a liquid nitrogen (N2) with Mikro-Dismembrator (Braun, Belgium). The proteoglycans were extracted with sodium hydroxide (NaOH) solution (48 hrs, 4°C). The insoluble residues containing collagen were washed with 0.5M acetic acid (pH 2.8) and digested by pepsin from pig stomach mucosa (U.S. Biochemical Corporation) as

described by Miller (20). Cartilage powder was added to the pepsin solution (1 gr/l) in 0.5M acetic acid (pH 2.8). After constant stirring for 48 hrs at 4°C, pepsin was inactivated by addition of 0.6 vol of 1M Tris base (pH 10) to the supernatant obtained after centrifugation (27000 g, 20 min) and solubilized collagen was precipitated by addition of sodium chloride (NaCl) to a final concentration of 2.4 M (18 hrs, 4°C). The precipitated collagen was collected by centrifugation at 13000 g for 20 min, solubilized in 0.1M acetic acid and dialysed overnight against 0.01M di-Potassium hydrogen phosphate (K2HPO4). The precipitate was collected by centrifugation for 20 min at 13000 g, dissolved and dialysed against 0.1M acetic acid and lyophilized. The purity of type II collagen was assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis at 7.5% as described by Laemmli (21) with or without 50 mM dithiothreitol (DTT).

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An autoradiography has been performed by applying NaI ^{125} labelled type II collagen on polyacrylamide gel electrophoresis 7,5% in presence of dithiothreitol (DTT). Afterwards, the gel has been revealed by contact with radiographic film during 48 hours. Human proteoglycan radioimmunoassay (22) showed this collagen to be free of contaminating proteoglycans.

Hydroxyproline determination

The concentration of all collagen solutions was obtained using the method of Bergman (23) which is based on the assay of hydroxyproline.

Radioimmunoassay for human type II collagen

Anti human type II collagen antiserum: Guinea pig antisera were obtained using the Vaitukaitis et al. method (24) of multiple intradermal injections of an emulsion made up of 0.5 ml complete Freund's adjuvant containing 150 µg to 200 µg of human type II collagen. Ten booster administrations were then given at two-weekly intervals, using the same procedure and emulsion of human type II collagen.

Labelling and tracer purification: The purified antigen was labelled with 125 Iodine using the iodogen method (25). 5 µg of type II collagen was dissolved in 25 µl of 0.05M phosphate buffer, sodium chloride (NaCl) 0.3M, 0.05% sodium azide (NaN3) pH 7.5 (labelled buffer) and 1 mCi of sodium 125 Iodine previously buffered at the same pH with 25 µl of labelled buffer. A solution of iodogen (0.5 µg/50 µl) dissolved in dichloromethane was allowed to stay in the bottom of a small polypropylene tube. After 5 min of shaking at room temperature the oxidation reaction was stopped by addition of 500 µl of 0.25M Potassium Iodide (KI). The mixture was chromatographed on Sephadex G 50 (30 x 0.8 cm) in 0.05M phosphate buffer, pH 7.5 containing 0.3M sodium chloride (NaCl), 0.05% sodium azide (NaN3) and bovine serum albumin 5 g/l. This buffer will be referred to as "incubation buffer". Human type II collagen was eluted in the first peak. Additional purification was obtained by filtration on Sephadex G 200 (60 x 0.9 cm, 12 ml/h) in the same buffer.

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Incubation conditions

The assay was carried out according to a sequential saturation proceedure. In the first step, 0.2 ml of incubation buffer containing either increasing amounts (0, 0.5, 1, 2, 5, 10, 20, 50, 100, 200, 500 ng) of unlabelled human type II collagen, or the sample to be assayed were incubated at 4°C for 24 hrs with 0.1 ml antiserum at a dilution 1/5000. In the second step, the tracer (20.000 cpm in 0.1 ml buffer) was added to the medium for a further 24 h incubation. Free-labelled type II collagen was separated from that bound to antibody by double antibody precipitation (26). After centrifugation following the addition of 2 ml of incubation buffer containing 300 mg/l of microcrystalline cellulose and 5 ml/l Tween 20, the precipitate was counted.

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Assay specificity

Other human collagens: Several other human type I, III, IV, V (Sigma, St Louis, USA) and VI collagens (kindly given by Professor C.M. Lapiere, Laboratory of Dermatology, Liege, Belgium) were investigated for possible cross-reactivity in the type II collagen radioimmunoassay. Human type III collagen was isolated and purified from human skin and types I, IV, V and VI from human placenta.

Other non-human collagens: Type I collagen was extracted from rat skin in our laboratory by salt precipitation method (27). Calf type I collagen extracted from skin was obtained from Sigma (St

Louis, USA). Bovine collagen types I, II, III and IV were kindly supplied by Professor C.M. Lapierre (Laboratory of Dermatology, Liege, Belgium). All collagens described above were investigated for possible cross-reactivity in their native or denatured forms. Denatured collagens were obtained by heating an aliquot of the respective native collagen in a 70°C water bath for 45 min.

Other cartilage constituents: Several articular cartilage constituents were investigated for possible cross-reactivity in h coll.II radioimmunoassay: purified fibronectin (28), human cartilage proteoglycans (22), human laminin (kindly supplied by Prof. Foidart, Laboratory of Biology, Liege, Belgium) and human umbilical cord hyaluronic acid (Sigma, St Louis, USA).

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Recovery

Recovery was determined by comparing the results obtained by radioimmunoassay with the known quantities of purified type II human collagen added to the assay system. Several amounts of purified type II collagen (0, 0.5, 1, 2, 5, 10, 20, 50, 100, 200, 500 ng/tube) were added to one ml of foetal calf serum or culture medium (DMEM, Dulbecco's Modified Eagle Medium) containing 1% of Ultroser G. (Gibco, France). The collagen assay was performed using 100 μl of the preparation.

Human type II collagen in culture medium of human chondrocytes

Human chondrocytes were cultivated in three-dimensions as described by Bassleer et al.(29). Briefly, human chondrocytes were cultivated in Dulbecco's Modified Eagles Medium (DMEM, Flow

Laboratories) supplemented with 1% of Ultroser G (Gibco, France), and ascorbic acid (50 μg/ml). Hyaline cartilage taken from the macroscopically normal part of human femoral heads immediately after surgery was cut into small fragments which were then digested by clostridial collagenase 1 mg/ml (Boehringer, Mannheim GmbH, Germany), in carbonate-bicarbonate buffer (1 mM adjusted to pH 7.4) for 24 h. Chondrocytes were then separated from their matrix. The cellular suspension was centrifuged (1500 rpm, 45 min) and the pellet was suspended in culture medium. After 6 successive washings and centrifugations (1500 rpm, 5 min) cells were put into suspension (10^6 cells/10 ml flask - Sovirel -, containing 2 ml of culture medium). Flasks were placed in a gyratory shaker (100 rpm). Cultures were maintained at 37°C in air with 5% added $CO2$.

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RESULTS

Pepsin-solubilized collagen isolated from human cartilage was analysed by SDS-PAGE. The fraction precipitating at 2.4 M NaCl, pH 7.5 contained bands migrating in the position of α 1 (I) collagen chain and β 1.1 and γ bands from type I collagen. The β and γ bands revealing the presence of covalent bindings between two or three α chains. The other bands characteristic of the other collagens were not present (fig. 1A). The collagen preparation was used for labelling and as a standard. Human labelled type II collagen was applied to a Sephadex G 200 column. Two major peaks of radioactivity were obtained, one in the void volume, the other

at the elution volume of salts. This low molecular weight material was not immunoreactive. Only the first peak reacted with human type II antiserum. The first peak submitted to SDS PAGE and revealed by autoradiography showed a similar electrophoretic pattern to unlabelled type II collagens (fig. 1A-c). The first peak was used as tracer (fig. 1B).

Validation

The figure 2 shows a standard curve obtained by sequential saturation. Sequential methodology increases the sensitivity of type II collagen assay (4 fold) as shown by respective position of curves obtained by equilibrium and sequential saturation conditions (fig. 2). Denatured human type II collagen does not cross-react in the assay. Parallelism of the inhibition curve is observed for increasing amounts of culture medium conditioned by human chondrocytes (fig. 2).

Limits of detection

The smallest amount of unlabelled type II collagen capable of significantly reducing the binding of labelled type II collagen to antibody in the absence of unlabelled h coll.II was 3 +/- 0.45 ng/tube (n=20). The mean binding reduction expressed in percentage is 6,4 +/- 1% (M +/- SD).

Precision and reproducibility

The precision profile of type II collagen assay was illustrated in fig. 3a. The coefficient of variation (CV) of a measurements in the same assay (SD/M x 100) was calculated for

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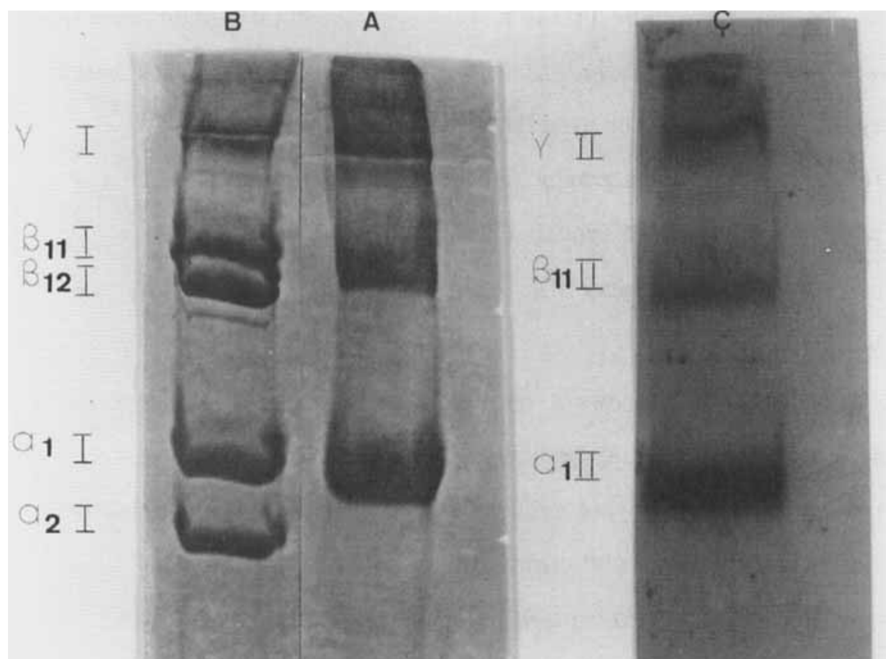

Figure 1A : Sodium dodecylsulfate/polyacrylamide gel electrophoresis of reduced pepsin-solubilized human articular type II collagen prepared by neutral-salt precipitation (7,5 % polyacrylamide slab gel, coomassie blue stained) (a). Rat skin type I collagen extracted by acid-salt precipitation and reduced by dithiothreitol (DTT) (b). Autoradiography of reduced Na I^{125}-labelled human type II collagen (c).

Figure 1B : Elution profiles on Sephadex G200 (60x1 cm; 12 ml/h) of tracer (●______●) and unlabelled type II collagen detected by hydroxyproline assay (O______O) or by RIA (▲ -.-.-. ▲). (*_______*) represent the binding of the tracer of each fraction to antiserum diluted 1/5000 as in assay method.(■-------■) represent the non specific binding in absence of antiserum, replaced by 0,1 ml of incubation buffer, the other step of the assay method being kept.

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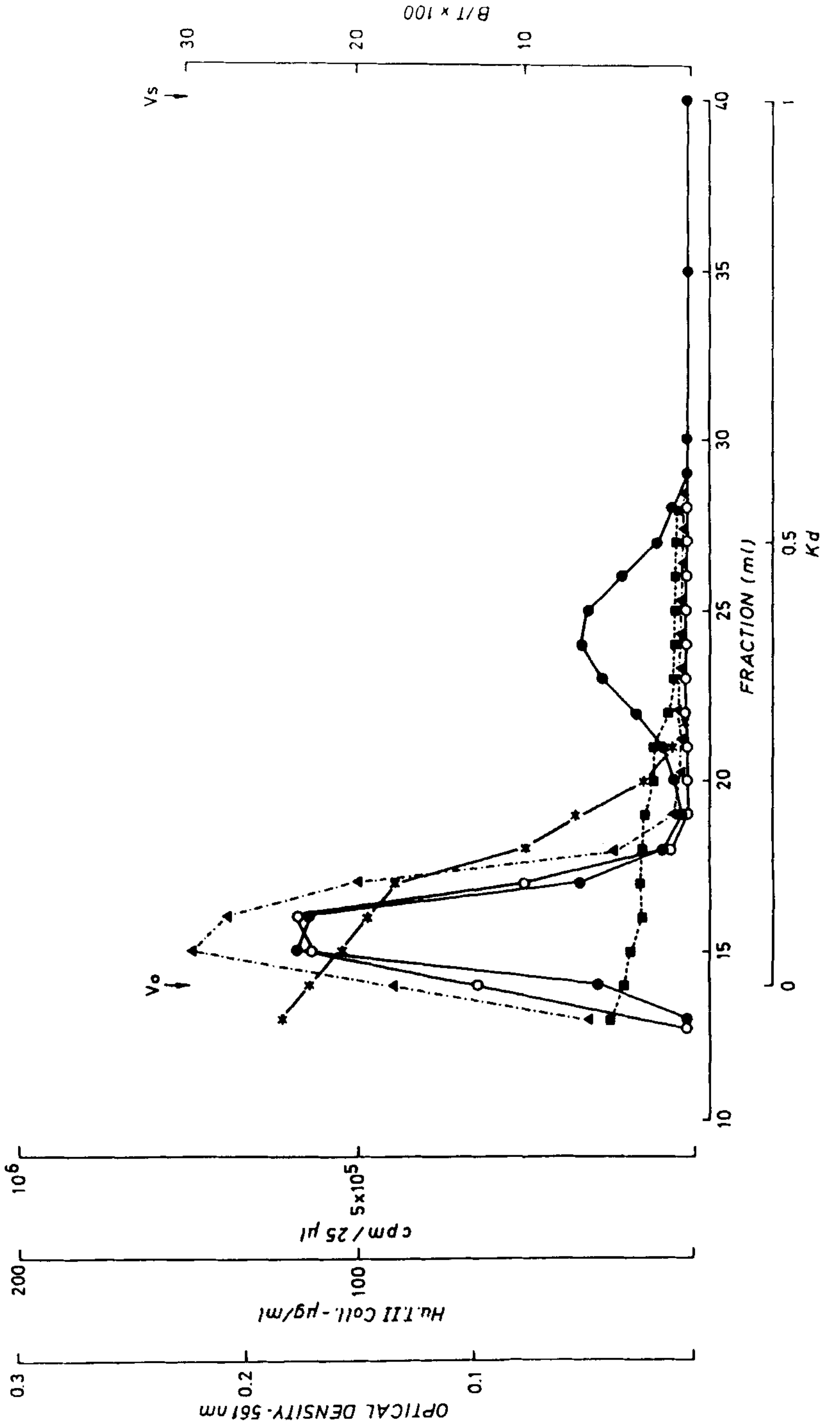

Figure 1B

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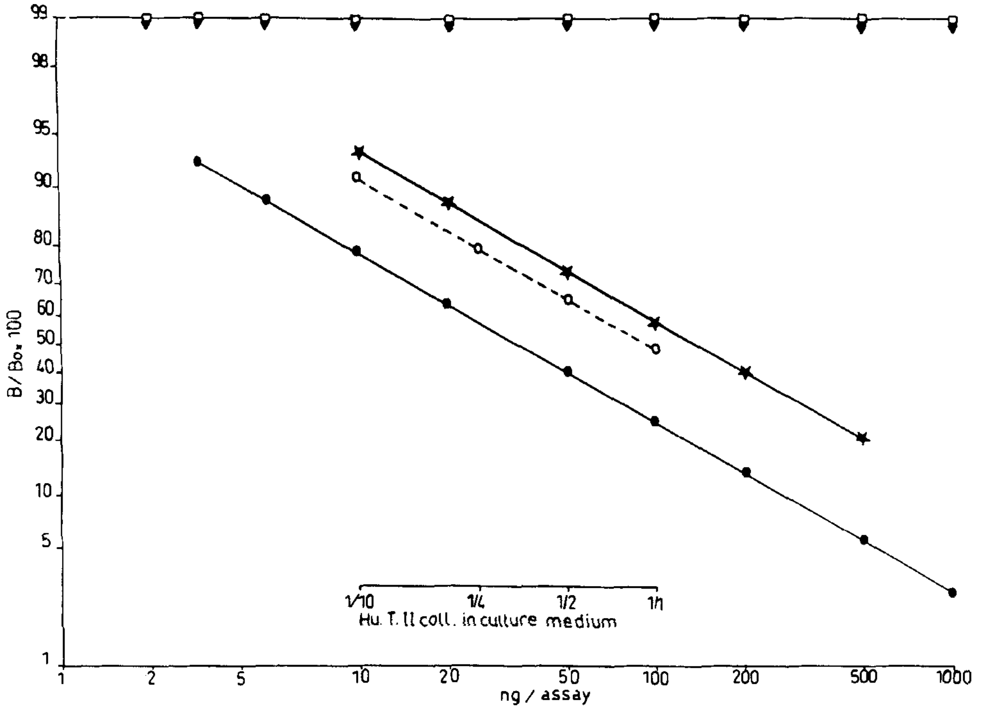

Figure 2 : Human type II collagen RIA using sequential saturation method (●——●) or equilibrium method (✶——✶). Parallelism of inhibition curves is observed for dilution of culture medium of human chondrocytes (O-----O). No cross-reaction were observed with denatured human type II collagen (□——□) and different constituents of cartilage (▽——▽)(as human laminin, fibronectin, hyaluronic acid or proteoglycans).

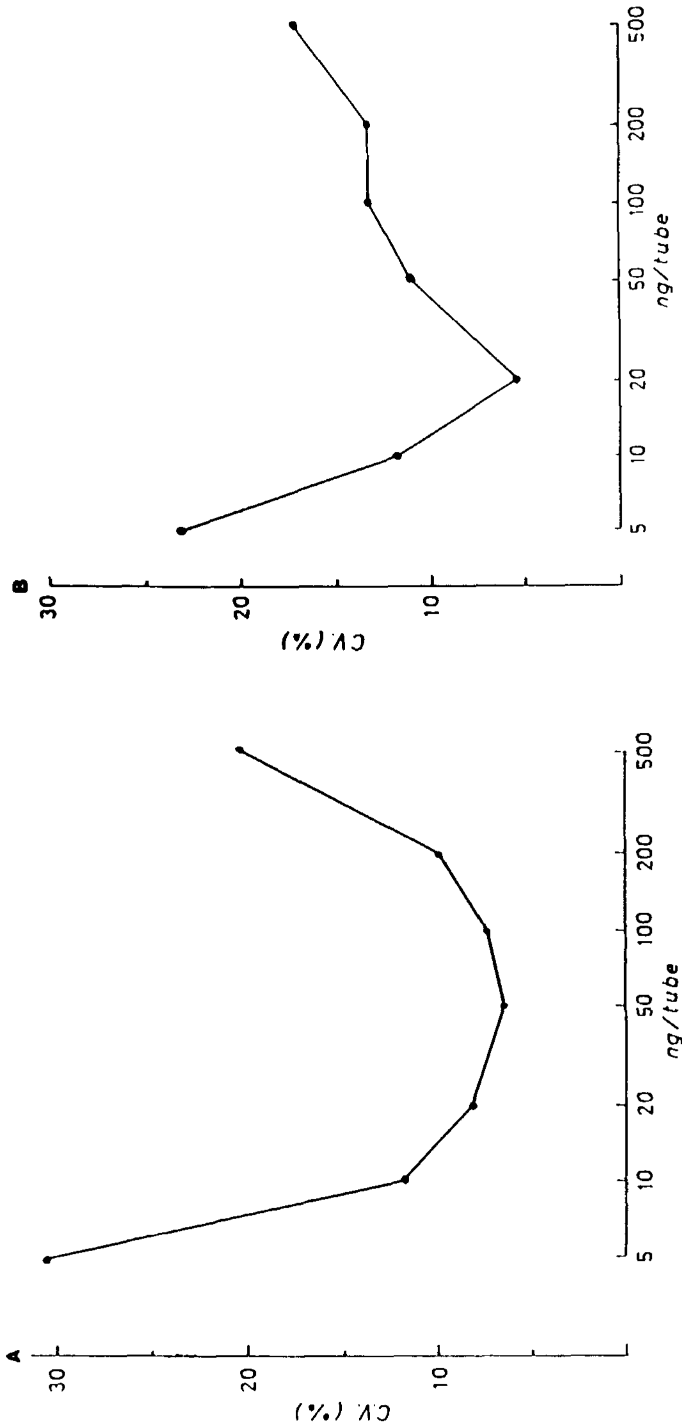

Figure 3a-b: Profiles of precision (a) and reproducibility (b) represented by intrassay and between assay coefficient of variation using sequential saturation method.

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each concentration of the standard curve. Intra-assay CV was less than 10% for amounts of unlabelled human type II collagen ranging from 5 to 200 ng/tube. The gaddum (30) index in the linear part of the curve was 0.09.

The between-assay CV of type II collagen concentration (29) measurements was less than 15% for amounts of unlabelled type II collagen ranging from 10 to 200 ng/tube (fig. 3b).

Recovery

Recovery was defined as the relationship between known amounts (X) of h coll.II added to buffer, fetal calf serum and culture medium of human chondrocyte and the amounts of h coll.II measured in these media by RIA (Y). As shown in figure 4, there was a good correlation between X and Y. This is indicated by a slope very close to 1 and by the coefficient of correlation (r) of 0.99 and 1.

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Specificity

There was no cross-reactivity between the h coll.II preparation and other constituents of human cartilage: human articular preparation (up to 2000 ng/tube), human laminin (up to 2000 ng/tube), human fibronectin and hyaluronic acid (up to 2000 ng/tube). Bovine collagen types I, III and IV did not interfere in h coll. II radioimmunoassay, even when the added dose equalled 2000 ng/tube. Moreover, although bovine type II collagen induced a significant inhibition, the curve was not parallel to those obtained with unlabelled human type II collagen. As calculated

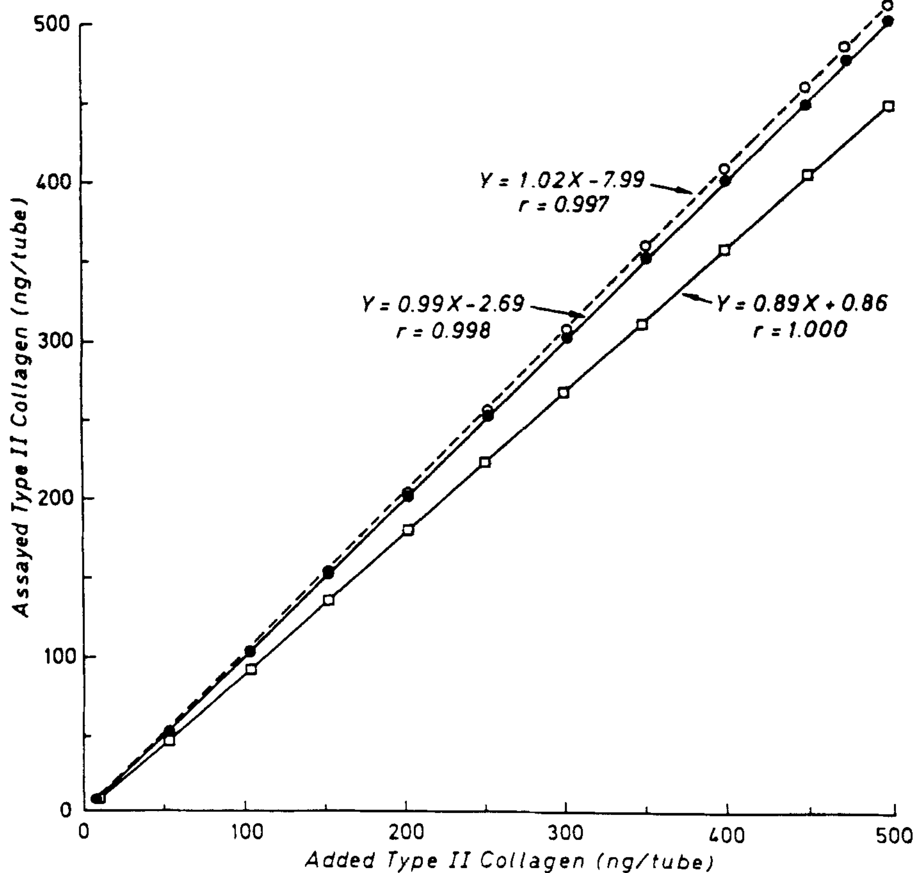

Figure 4 : Recovery test in incubation buffer (●———●) fetal calf serum (O-----O) and culture medium (DMEM + 1 % Ultroser G) (◇———◇).

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from the 25% inhibition point, the immunoreactivity of bovine type II collagen was 70 times less than that the human type II collagen.

Rat and calf skin type I collagen induce a weak inhibition for the highest quantities assayed: 2 and 3 μg/tube. At the latter

dose, rat and calf collagen are respectively 350 and 850 times less immunoreactive than the reference preparation (fig. 5).

Human collagen types V and I did not significantlty inhibit the binding of labelled human type II collagen to antibodies (fig. 6): however, calculated at 10% inhibition, the immunoreactivity of human collagen types III,IV and VI was 500 times less than that of human type II collagen. When all these collagens were denatured by heating, they did not interfere with human type II collagen assay.

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DISCUSSION

Many chemical assays for collagen have been developed. According to Goldberg and Green (31) the hydroxyproline/proline ratio is relatively specific for collagen, but does not distinguish between different types of collagen. A number of chromatographic (32) and electrophoretic (33) methods have been developed to determine both the amount and the type of collagen. Furthermore immunochemical studies have been done for immunocytochemical studies of type II collagen in tissue (34). Solid phase radioimmunoassay (RIA) was developed for the detection of serum IgG antibodies to collagen type (36). RIA or ELISA have been proposed by some groups for rat, bovine, calf or human collagens (11, 12, 13, 14, 15, 16, 17, 18, 36). To the best of our knowledge this is the first description of a radioimmunoassay for human type II collagen. Human type II collagen was first purified and a sensitive RIA for this cartilage constituent was developed.

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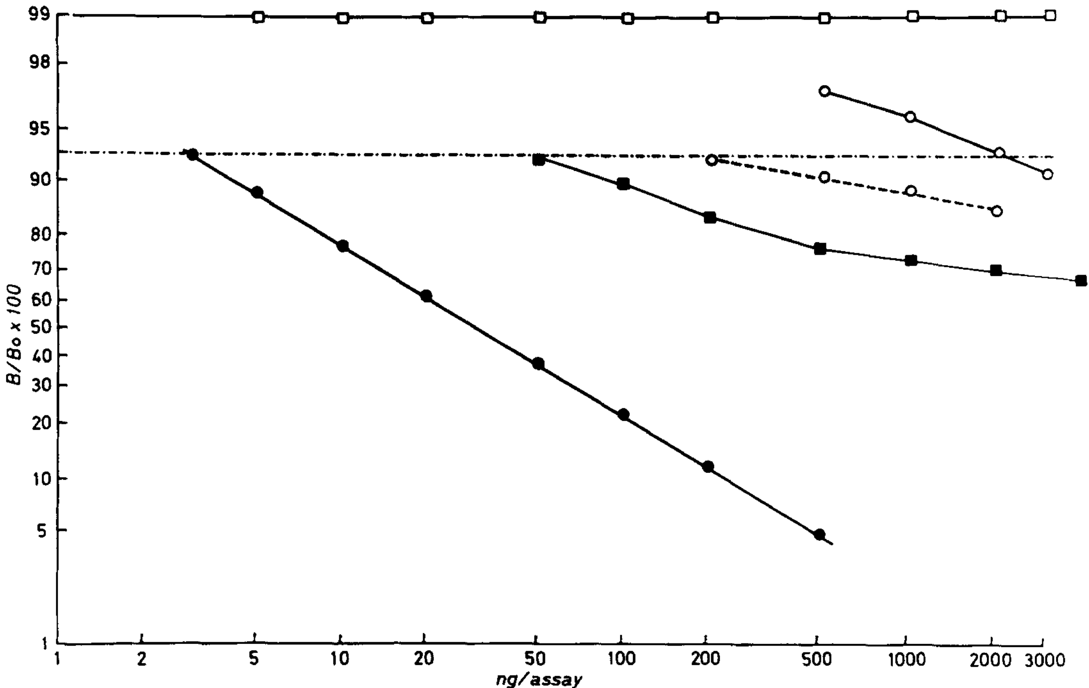

Figure 5 : Inhibition curves in logit-log representation obtained with various mammalian collagens. Only rat (O———O), calf type I (O-----O) and bovin type II collagens (●———■) show a non parallel cross-reaction. Bovin type I,III,IV and denatured collagens (□———□) the binding of h coll.II to antibodies up do not inhibit the amount of 2 g/tube.

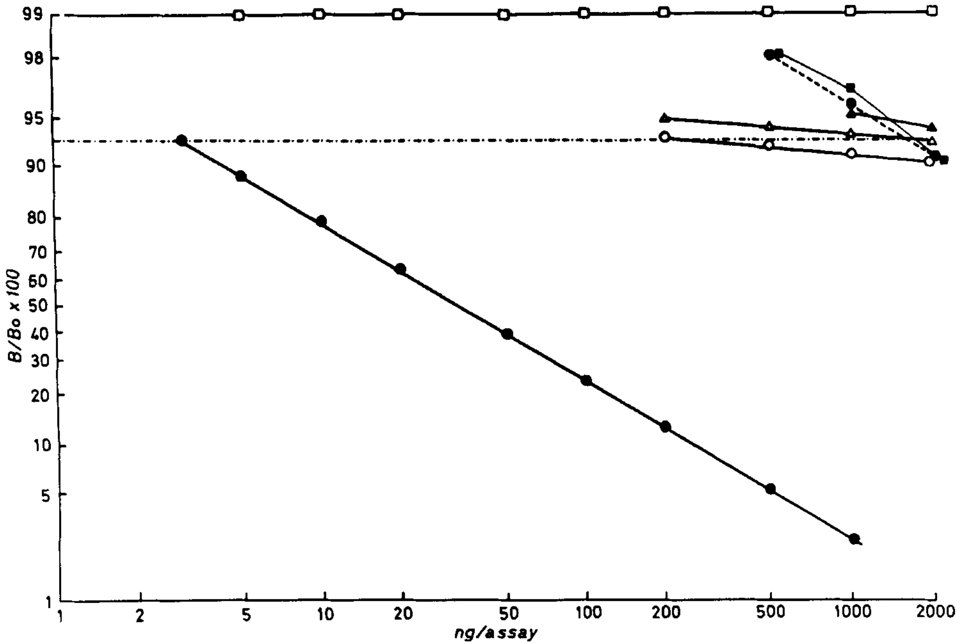

Figure 6 : Inhibition curves in logit-log representation obtained with various human type of collagen. Human type I (▲——▲), II (●——●), III (O——O), IV (●-----●), V (Δ——Δ), VI (■——■) collagens show a weakly non-parallel cross-reaction. Denatured collagen (□——□) do not inhibit h coll.II antibody reaction.

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Quality control indicated highly satisfactory specificity, sensitivity, precision, reproducibility and accuracy of the assay. We raised polyclonal antibodies in guinea pigs using 200 μg of the immunogen; other authors obtained antisera against calf, pig, chick or rat types II collagen in rabbits, rats, and guinea pigs (12,37) by immunization with rather high doses of native collagen (10 mg for rabbits, 0.5-0.750 mg for guinea pigs per injection) emulsified in Freund's Adjuvant. Many previous studies have indicated that the major antigenic determinant of calf or rat type II collagen is located in the helical region of the CNBr peptide α 1 (II) C.B. 11 (11,12). Minor antigenic sites are also located at the amino and carboxy terminal non- helical regions (11). In our hands, no cross reaction existed between native and denatured collagen type II. This constitutes an argument for the antigenic determinant being situated on the helicoidal part of the molecule. Similarly, Hahn et al.(12) have observed that only native triple helical rat collagen type I reacts with the respective antibodies. Denatured molecule did not react. However, antigenicity of the native molecule in the rat cannot be a function of the helical structure alone, but must also be dependent of a specific array of amino acid side chains contributed by the different types of alpha-chains comprising the collagen molecule. Assay specificity has been extensively investigated. Constituents of cartilage other than type II collagen (laminin, fibronectin, hyaluronic acid) and bovine collagen types I, III, IV collagen do not cross-react in

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cartilage type II collagen RIA. Only rat and skin type I collagen and human collagen types I, III, IV, V showed a weak, non-parallel cross reaction. Other authors have shown by hemagglutination and radioimmunoassays, that antibodies are generally specific for the type of collagen use for immunization (38,13) and showed also negligible cross-reaction with other types of collagen. In general, antisera to type II collagen show a high degree of interspecies cross-reactivity. Rabbit or guinea pig antisera raised against chick type II collagen cross-react in the immunofluorescence test with type II collagen of human, cow, dog, sheep, quail, mouse, rat and shark cartilage (39). In our hands, there is an incomplete cross reaction between bovine and human type II collagen.

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When labelled human type II collagen was applied to Sephadex G 200, two major peaks of radioactivity were obtained, only the first of which reacted with our antiserum. The second seem to be damaged collagen type II which lost its immunoreactivity. Finally, parallelism of the inhibition curves was obtained for increasing amounts of culture medium conditioned by human chondrocytes and the standard curve showing that this radioimmunoassay is well adapted to quantifying native type II collagen in culture medium and to detecting subtle differences induced by drugs or hormones.

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