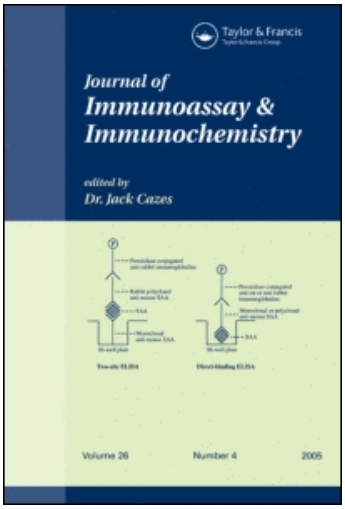

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Radioimmunoassay of Proteoglycans

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RADIOIMMUNOASSAY OF PROTEOGLYCANS

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ABSTRACT

Human articular cartilage proteoglycan monomers (PG) were purified and used for developing a radioimmunoassay. Its analytical sensitivity is 0.6 ng/tube and its clinical sensitivity is 20 µl/tube for serum and 0.12 µl/tube for synovial fluid. The intra- and between-assay variation coefficient are less than 10 and 20 %, respectively, in the linear part of the curve. There is a complete cross reaction with costal, vertebral disk and tracheal cartilage PGs and the PGs extracted from vein and artery. Concerning the latter, inhibition curves are not parallel. No cross reaction exists with PGs from various fetal tissues and small PGs from bone. However, large PGs from bone produce a weak cross reaction. Furthermore, the assay is species specific since cartilage PGs from dog, rat, chicken and calf embryos either do not or weakly cross react in the assay. Other constituents of cartilage : type II collagen, fibronectin, chondroitin sulfate and hyaluronic acid do not interfere with the assay. The antigenic determinants are localized on the protein core of the PG, as shown by the lack of cross reaction with glycosaminoglycans and PG treatment with various enzymes.
KEY WORDS : (human articular cartilage proteoglycans ; radioimmunoassay ; antigenicity; fetal tissues proteoglycans).

INTRODUCTION

Proteoglycans (PG) are constituents of the fundamental substance of connective tissue, particularly cartilage. They consist of a protein core ($1-2 \times 10^5$ daltons) to which a large number of

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glycosaminoglycans, chondroitin sulfate (CS) and keratan sulfate (KS) are covently bound. The monomeric PG has a mean molecular weight of 2.3 x 10^6 daltons (0.5 to 5 x 10^6); small oligosaccharide chains are also present in human cartilage PG : the O and N-linked oligosaccharides. One of the first units of the protein core is a mannose containing a glycoprotein region responsible for binding to HA. At the other core extremity is another unit, variable in length and containing CS. Between these two units is a third unit, lacking mannose , to which KS and oligosaccharides are attached (1). The first unit has a particular conformation permitting the association of one linear molecule of hyaluronic acid (HA) in a non-covalent manner. This attachment is stabilized by a glycoprotein link (GPL). The whole forms a PG-HA-GPL complex with a molecular weight between 60 and 150 x 10^6 daltons (2). Cartilage is involved in longitudinal growth and in various osteoarticular disorders either directly as in osteoarthritis (OA) or indirectly because of synovial inflammation as in rheumatoid arthritis (RA). We have developed a radioimmunoassay for human articular PG in order to investigate its metabolism in healthy and sick patients.

MATERIAL AND METHODS

Extraction and isolation of the proteoglycans

Human articular cartilage was obtained by surgery from patients with either femoral neck fracture or total hip prosthesis. Concerning the latter, macroscopically normal cartilage zones

were selected while local areas of degeneration were discarded. Human costal cartilage and femoral bone midshaft were obtained by autopsy. Other human tissues were prepared from a 23 week-old human fetus.

Human cartilage was extracted by the method of Sajdera and Hascall. (3) using 4 M guanidine hydrochloride (Gu 4M). Protease inhibitors (4) are added to all buffers. In order to isolate PGs, three steps are used : the first step involves equilibrium density gradient centrifugation in CsCl and yields a 1.75 g/ml fraction which contains PG (A_1) . This fraction is then ultracentrifuged three times without CsCl in order to isolate, in the bottom quarter , the macromolecular PG-HA-GPL (A_1S_1 complex) (6). Further purification is accomplished by a dissociative density gradient centrifugation which separates the A_1S_1 fraction into its three components : one of them, the $A_1S_1D_4$ fraction, which is found at the top of the gradient (density $\leq$ 1.45 g/ml), corresponds to small PGs with GPL (7). Another fraction, $A_1S_1D_1$ (density $\geq$ 1.55 g/ml) is loaded onto a Sepharose CL 2B column. The material eluted at $0.1 \leq Kd \leq 0.7$ is collected. It does not contain GPL (8) . In addition to having a characteristic molecular weight (internal volume of the Sepharose 2B with $0.1 \leq Kd \leq 0.7$) and the ability to bind HA, the $A_1S_1D_1$ material could be biochemically identified as PG. Following its isolation in a fraction with an average density of 1.61 g/ml, the protein and uronic acid levels are 11.0 % and 13.8 % respectively (9). These

* Terminology introduced by Heinegard (5).

values are in agreement with Roughley and White's data (10) for a D_1 preparation from human articular cartilage. $A_1S_1D_1$ has an amino acid composition composed of four major amino acids : Glu (127 %), Gly (117 %), Ser (116 %) and Pro (95 %). This is in agreement with the composition given by Roughley et al.(11). In addition , the galactosamine/Glucosamine ratio for $A_1S_1D_1$ is 1.91, which is in accordance with Bayliss and Venn (12). Finally, following chromatography on DEAE cellulose in 8 M urea, the $A_1S_1D_1$ fraction was eluted in a single peak at a sodium chloride concentration higher than 1M as found Antonopoulos et al. (13).

This material was used for antibody immunization, for labelling and for use as a standard.

Radioimmunoassay of the Proteoglycans

Anti-proteoglycan antiserum : Rabbit antisera were obtained using the Vaitukaitis et al. method (14) of multiple intradermal injections of 500 µg of PG in a suspension of 1 ml of complete Freund's adjuvant. Four booster doses of 500 µg of PG in solution without Freund's adjuvant were administered followed by an intramuscular injection of Coryne Bacterium Parvum at time intervals of one month.

Labelling and tracer purification : the purified antigen was labelled with ^{125} iodine using chloramine T (15) : 100 µg of $A_1S_1D_1$ dissolved in 25 µl of 0.05 M phosphate buffer , pH 7.5, was added to 1 mCi of sodium ^{125} iodine previously buffered at the same pH with 25 µl of 0.5 M phosphate buffer. Then , 25 µl of a solution of 22 mM chloramine T was added and after

30 seconds of shaking at room temperature the oxidation reaction was terminated by the addition of 100 µl of 12.5 mM sodium metabisulfite and 100 µl of 60 mM sodium iodide. The mixture was subsequently chromatographed on Sephadex G 50 M (25 x 1.6cm) in 0.05 M Sorensen phosphate buffer, pH 7.5, containing 0.15 M sodium chloride and bovine serum albumin (BSA) 5 g/l. This buffer will be referred to as incubation buffer.

The peak in the excluded volume corresponding to labelled PG (PG^*) was rechromatographed on Sepharose 2B (40 x 1 cm) in sodium acetate buffer, 0.5 M, pH 7.0. The fractions with $0.1 \leqslant Kd \leqslant 0.7$ were pooled and utilized as purified tracer. The radioactivity of this fraction was bound to anti-PG antibody and contained no degradation products of the iodination reaction (9).

The specific activity of the label was 4.6 $\pm$ 0.9 µCi/µg corresponding to 4.8 $\pm$ 0.9 atoms of ^{125}I per molecule of PG.

Incubation conditions : incubation was carried out in a volume of 400 µl containing the following : 100 µl of a dilution of the tracer (15-20,000 cpm) ; 100 µl of a 1/5,000 dilution of the antiserum or of a non-immune rabbit serum (control) ; and 200 µl of incubation medium containing either the substance to be measured or unlabelled antigen in amounts of 0, 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, 50, 100, 200 and 500 ng of $A_1S_1D_1$ per tube. After 4 days at 4°C the PG^*-antibody complexes were separated from the free labelled antigen by double precipitation : 100 µl of a 1:100 dilution of normal rabbit serum (NRS) and 100 µl of a 1:20 dilution of anti-rabbit IgG serum, obtained from a donkey, were added to the contents of the tubes and left for two days at

4°C. After centrifugation following the addition of 2 ml of incubation buffer containing 200 mg/l of microcristalline cellulose and 2.5 ml/l Tween 20, the precipitate was counted.

Assay specificity

Articular proteoglycan complexes : the PG, which does not react with hyaluronic acid (PG') , was purified by Heinegard and Hascall method (6) and was also tested in the radioimmunoassay. Two types of complexes were also studied : PG-HA-GPL (A_1 complex) purified in the void volume of a Sepharose 2B and PG-GPL ($A_1S_1D_4$)

Other cartilage constituents : several articular cartilage constituents were investigated for possible cross reaction in PG radioimmunoassay : purified fibronectin (16), human type II collagen (17), shark cartilage chondroitin sulfate (Calbiochem San Diego, USA) and human umbilical cord hyaluronic acid (Sigma St Louis USA). Furthermore, human type I and III collagens, isolated and purified from human skin (18) were also tested in the assay.

Non articular PG :

a) PGs extracted from adult costal cartilage to the $A_1S_1D_1$ stage according to Heinegard and Hascall method (6)

b) human fetal tissues : bone, skin, vertebral disk, trachea, artery, umbilical vein, oesophagus, kidney, liver, heart, cornea and lung from fetus were abundantly washed in NaCl 0.15 M with protease inhibitors. They were then ground in liquid N2 and extracted under dissociative conditions at 4°C

for 24 H with 4 M guanidinium hydrochloride (Gu 4M) containing proteinase inhibitors (4) and buffered with 0.05 M tris HCl pH : 7.0 . The extracts were centrifuged at 10.000 g and the supernatants were dialyzed with distilled water. The precipitate that eventually formed was discarded and the soluble material was freeze-dried.

c) human adult subperiosteal bone

After the removal of adhering soft tissues and periostum, the piece of midshaft bone was washed with 0.15 M NaCl containing proteinase inhibitors, powdered in liquid N2 and again washed before being submitted to sequential extractions (19). The first extract (4M guanidinium HCl, 0.05 M Tris pH 7.4 with proteinase inhibitors but without EDTA) and the second extract (the same cocktail to which 0.5 M EDTA was added) were centrifuged and the supernatants were concentrated by dialysis and lyophilization. The two extracts were then chromatographed on Sepharose CL 6B in 4 M guanidinium HCl 0.05 M Tris pH 7.4. The void volume of the first extract and the material with $0.07 \leqslant Kd \leqslant 0.32$ of the second extract were collected, dialyzed with distilled water and lyophilized. They constitute material containing large and small bone PGs, respectively.

Animal PG : cross reactivity with purified PG (A_1D_1) from rat and fetal calf costal cartilage, from dog articular cartilage and from chick embryo pelvic rudiment cartilage was studied.

Enzymatic digestion : our preparation ($A_1S_1D_1$) was submitted to various enzymes :

- Bovine pancreatic trypsin (Serva) and sheep pancreatic chymotrypsin (Mann Research Laboratory) : a solution of $A_1S_1D_1$ (2 mg/ml) in 0.05 M Tris-HCl, pH 7.6 + 1 mM $CaCl_2$ was incubated for 17 hours at 37°C with 4 µg/ml of trypsin and 8 µg/ml of α-chymotrypsin (7,20). The reaction was stopped by the addition of 50 µl/ml 10^{-4}M phenylmethyl sulfonyl fluoride (PMSF).
- Papain (Millipore Corporation, Bedford, Massachusetts USA) : 250 µl of a solution of $A_1S_1D_1$ (1 mg/ml) in 0.01 M phosphate buffer, pH 6.8 containing 0.15 M NaCl, 1 mM EDTA, 6 mM cystein and 5 mM mercaptoethanol, was incubated with 0.1 mg of papain for 18 hours at 37°C. The reaction was terminated by the addition of iodacetic acid to a final concentration of 2 mM.
- Clostridium perfringens neuraminidase (Boehringer): a solution of $A_1S_1D_1$ (1.5 mg/ml) in 0.05 M NaAc/HAc, pH 5.1 , was incubated for 17 hours at 37°C with 3.9 µg/ml neuraminidase. The reaction was terminated by successive freezings.
- Proteus vulgaris chondroitinase ABC (Serva) : a solution of $A_1S_1D_1$ (1.5 mg/ml) in 0.1 M NaAc/HAc, pH 7.1, was incubated for 1 hour at 37°C with 0.1 unit/ml of ABC chondroitinase (21).

Increasing dilutions of the digests expressed in terms of ng PG initially exposed to the enzyme were incubated, and inhibition curves were compared to those obtained from our reference preparation.

Accuracy

To one ml of OA synovial fluid (SF), several amounts of purified PG ($A_1S_1D_1$) were added : 100, 200, 500, 1000, 2000,

5000 and 10000 µg. The PG assay was performed using 0.5 µl of SF. To one ml of serum, several amounts of purified PG were added : 200, 400, 1000, 2000, 4000, 10000, 20000 ng. The PG assay was performed using 25 µl of serum.

RESULTS

Inhibition curves are illustrated in fig. 1, 2 and 3. Many assays have been performed from which assay characteristics may be defined.

Limit of detection (22)

The smallest amount of unlabelled PG capable of significantly reducing the binding of PG* to antibody in the absence of unlabelled PG was 0.6 ± 0.3 ng/tube, i.e. 0.30 ± 0.15 fmole/tube (n = 16). Clinical sensitivities, defined as the smallest volume of biological fluid to significantly reduce the binding of PG* to antibody, were 20 µl/tube and 0.12 µl/tube respectively, for serum and SF.

Precision

The precision profile of the PG assay is illustrated in fig.4. The relative error of measurement of the PG concentration (variation coefficient) is plotted against the corresponding analyte concentration (23).

The relative error is less that 10 % for amounts of unlabelled PG ranging from 5 to 200 ng/tube. The Gaddum (24) index (λ) in the linear portion of the curve was 0.08.

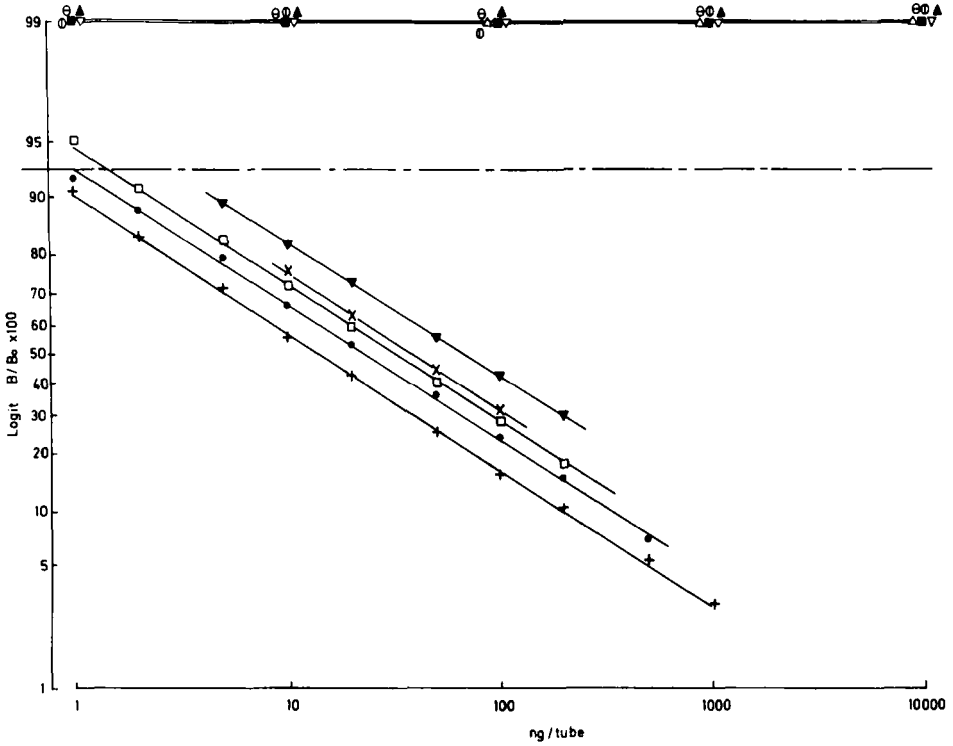

Figure 1 : Inhibition curves in logit-log representation obtained with different types of proteoglycans (PG) (●) and related substances. Hyaluronic acid (◍); chondroitin sulfate C (Δ) ; and human type II (▲), I (θ) , III (■) collagens and fibronectin (▽) do not inhibit the PG* antibody reaction. PG, which do not form aggregates with HA (+), human PG-HA-GPL (A1S1) (▼) or PG-GPL (A1S1D4) (□) , show complete cross-reactivity with human articular PGs : their inhibition curves are parallel.

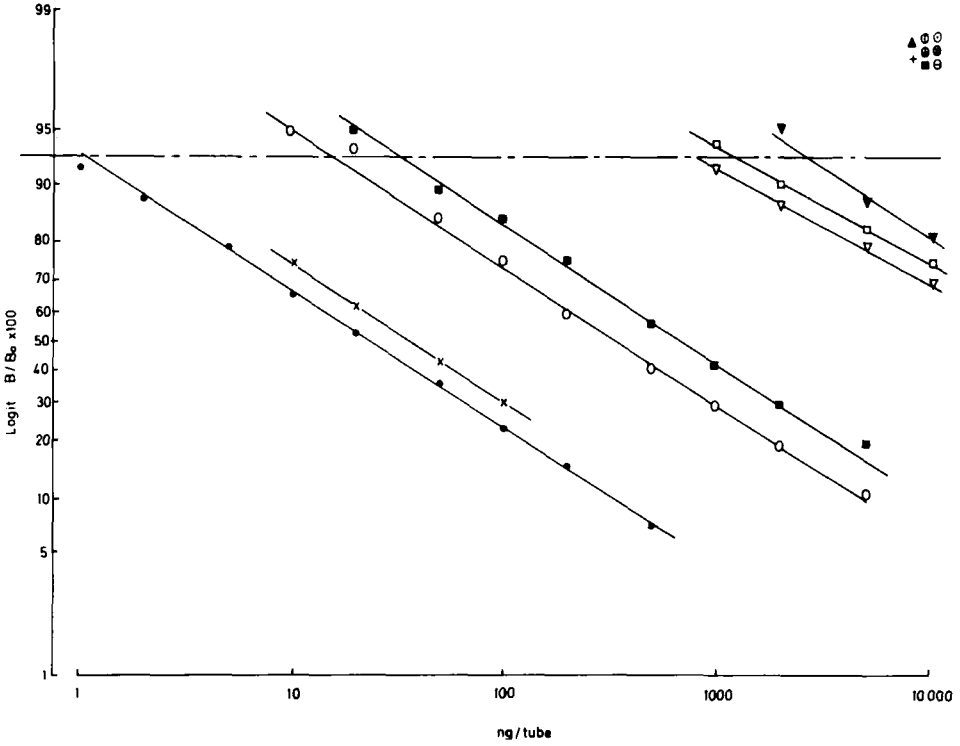

Figure 2 : Inhibition curves in logit-log representation obtained with proteoglycans (PG) of various human tissues : articular cartilage (●), costal cartilage (X), trachea (0), vertebral disk (■), artery (▽), vein (□) and bone large PG (▼). Other fetal tissues such as skin, cornea, oesophagus, lung, liver, kidney, heart and bone small PG do not inhibit the binding of PG* to antibodies up to the amount of 20 µg/tube.
Only arterial PGs show a non parallel cross reaction.

Reproduction

The relative errors of PG concentration measurement (between assay variation coefficient) from one assay to another (n=16) correspond to ± 20 % for amounts of unlabelled PG ranging from 2 to 500 ng/tube (fig. 4).

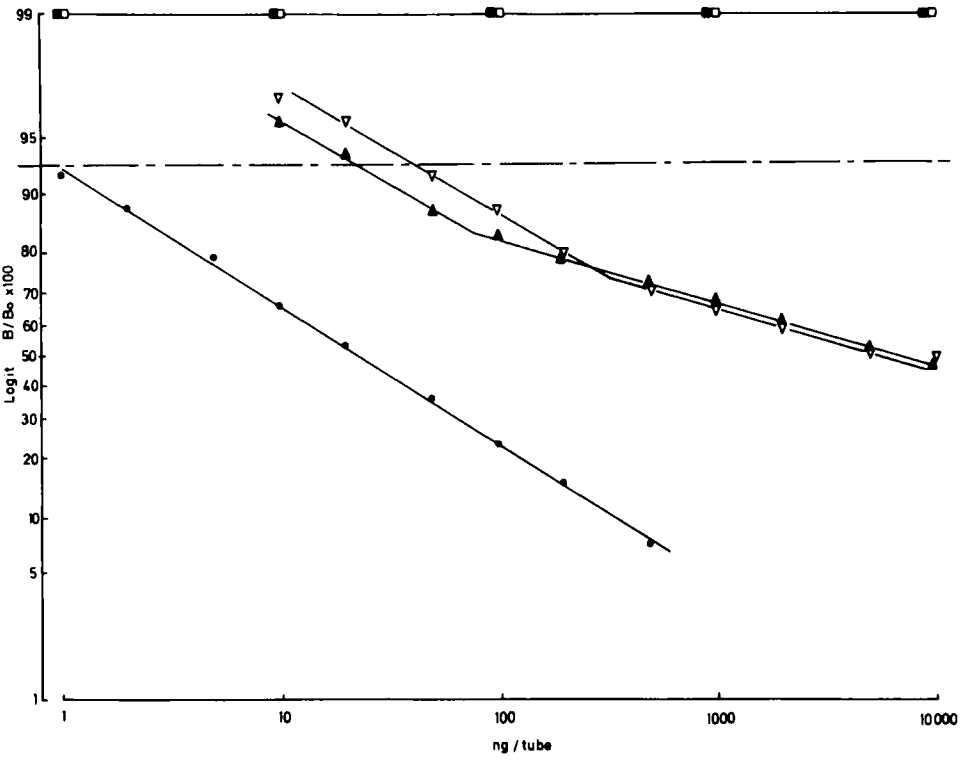

Figure 3 : Inhibition curves in logit-log representation obtained with proteoglycans (PG) of different species. There is a weak and incomplete cross-reaction between PGs from dog (▲) and fetal calf (▽) costal cartilage and human articular PGs (●). PGs from rat (□) and chick embryo cartilage (■) do not inhibit the PG*- antibody reaction.

Accuracy

Accuracy was defined as the relationship between known amounts (X) added to the serum or the SF and the amounts of PG measured in these media by radioimmunoassay (Y).

As shown in fig. 5, there is a good correlation between X and Y. This is indicated by the regression lines : Y = 1.04 X

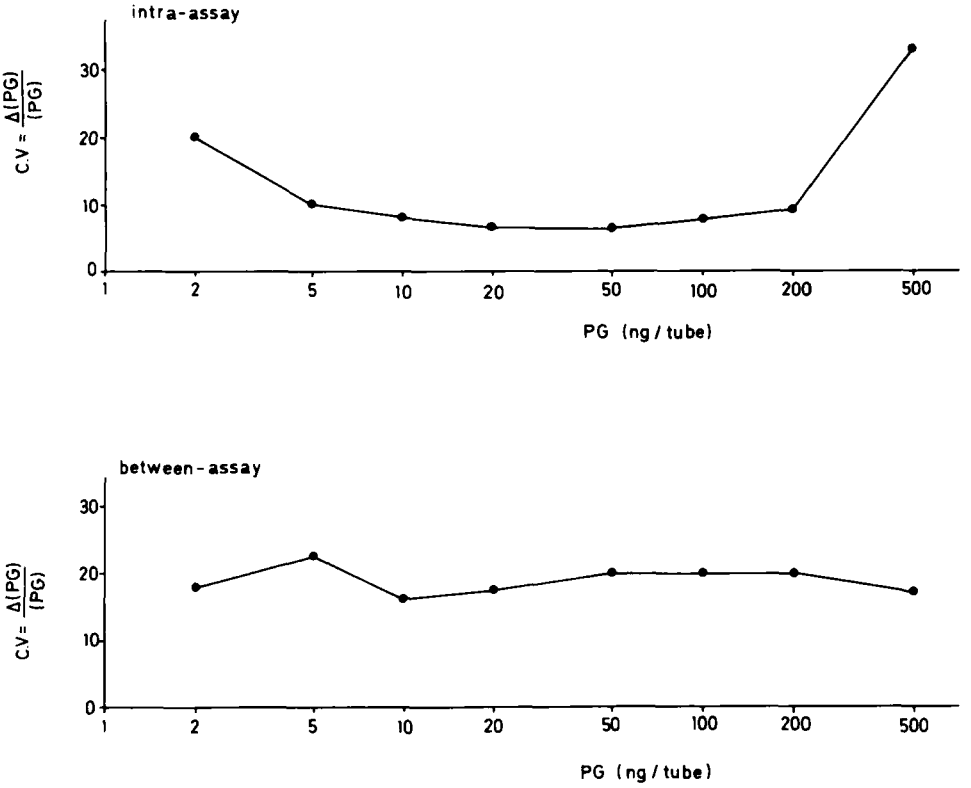

Figure 4 : Profiles of precision and reproduction represented by the intra-assay and the between-assay variation coefficients.

+ 101 (r = 0.999) for SF and Y = 1.01 X + 529 (r = 0.999) for serum.

Specificity

The PGs which were unable to bind to hyaluronic acid (PG'), PG-GPL ($A_1S_1D_4$), and PG-HA-GPL (A_1) complexes, showed complete cross reactivity. $A_1S_1D_4$ had almost the same immunoreactivity as the reference preparation, while PG' was 1.65 time more immunoreactive and A_1,3 times less immunoreactive on a per

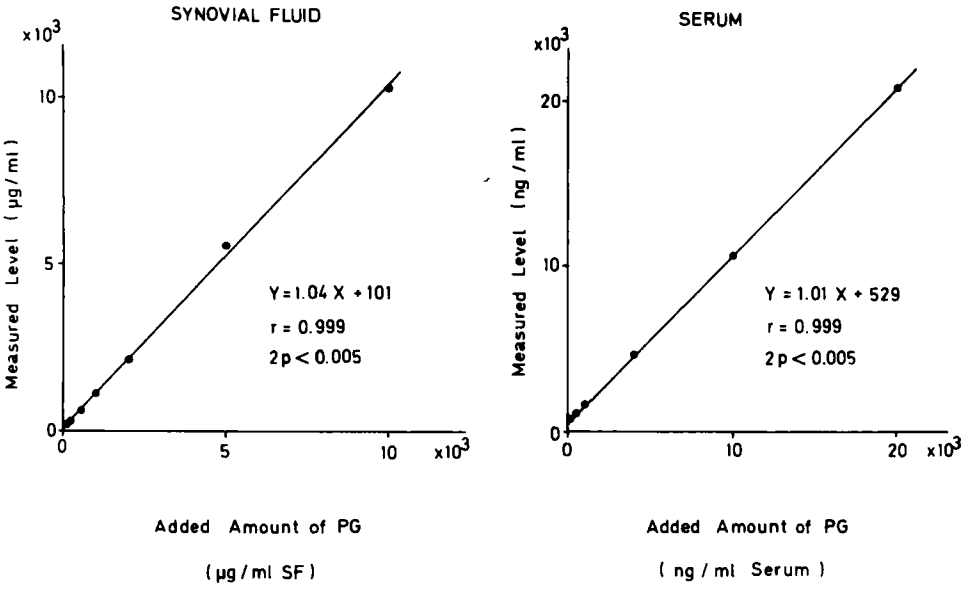

Figure 5 : Recovery test in synovial fluid and in serum. There are excellent correlations between PG levels measured by the assay and amounts added to both SF and serum.

weight basis (Fig. 1). Furthermore, there was no cross-reaction between the human articular PG preparation and other constituents of human cartilage:type II collagen (up to 10 µg/tube), chondroitin sulfate C (up to 1 mg/tube), hyaluronic acid (up to 10 µg/tube) and fibronectin (up to 20 µg/tube)(Fig. 1). Type I and III collagens do not react in PG radioimmunoassay when the added dose equals 10 µg/tube. There was a complete cross-reactivity between human PGs extracted from articular, costal, tracheal cartilage, and vertebral disk (fig. 2).

PGs extracted from fetal artery and vein show a complete but not parallel cross-reaction.

PG extracts (up to the amount of 10 µg/tube) from fetal skin, cornea, lung, oesophagus, kidney, heart and liver did not significantly inhibit the binding of labelled PG to antibodies (fig. 2).

Small MW adult bone PG does not significantly cross react with cartilage PGs, whereas high MW adult bone PGs induce a weak but significant inhibition for the highest quantities assayed : 5 and 10 µg. At the latter dose, high MW adult bone PGs are 800 times less immunoreactive than the reference preparation of cartilage PG (fig. 2).

The PG from rat costal cartilage and chick embryo pelvic rudiment cartilage showed no reactivity even with amounts of 20 µg/tube. However, PG extracted from dog articular cartilage and fetal calf costal cartilage showed inhibition of the binding of labelled human PG to antibodies. The inhibition curves were not parallel to those obtained with unlabelled human PG. As calculated from the 50 % inhibition point, the immunoreactivity of fetal calf and dog PG was 210 and 215 times less, respectively, than that of human PG (fig. 3).

The action of different enzymes on PG makes the localization of the antigenic site possible. Neither neuraminidase nor chondroitinase ABC had any effect on PG immunoreactivity. Trypsin-chymotrypsin destroyed 95 % of the ability of the reference preparation to inhibit binding to the antibody. Papain caused complete loss of reactivity (fig. 6).

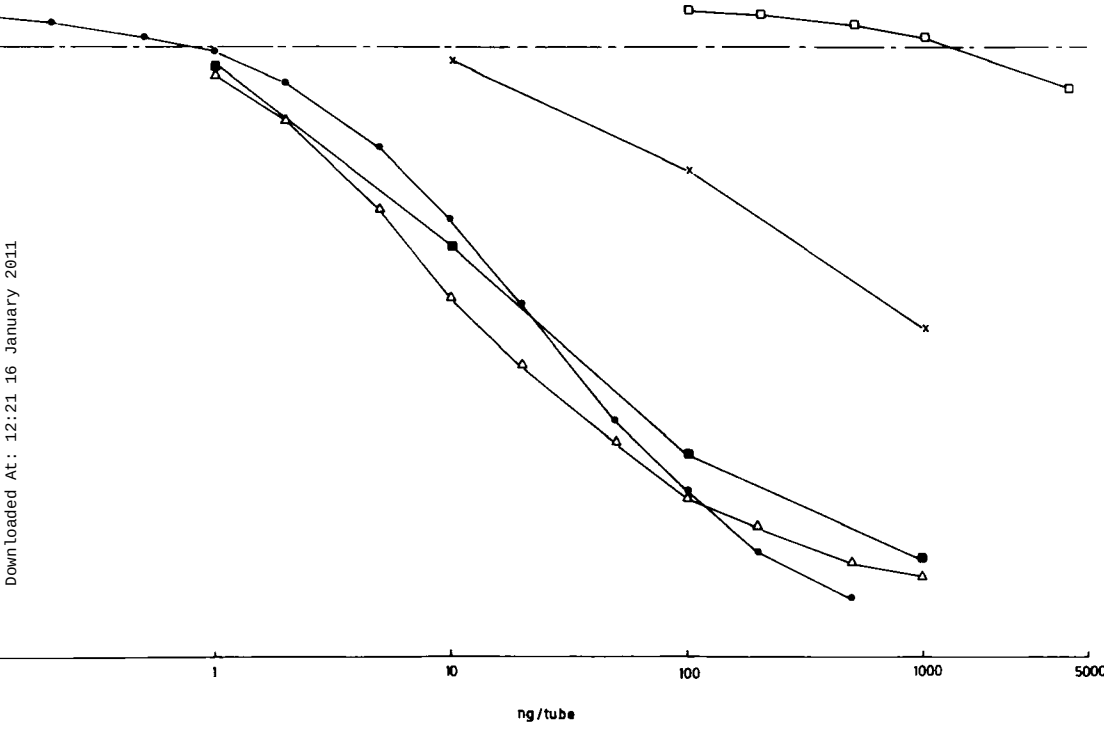

Figure 6 : Inhibition curves of the labelled PG-antibody reaction induced by unlabelled purified PGs (●) and PGs treated with neuraminidase (■), chondroitinase ABC (Δ), trypsin-chymotrypsin (X) and papain (□).

DISCUSSION

Even now there is still no radioimmunoassay (RIA) for human native proteoglycans (PG) though many immunochemical studies have been done with cartilage PGs from ox, rat, pig and chick embryo. RIA or ELISA have been proposed by some groups for bovine PG (25,26,27,28) , rat (29), pig (30) and chick embryo (31,32). These different groups utilize polyclonal or monoclonal antibodies reacting against either monomeric PGs or purified fragments of them. Their studies are essentially directed to the analysis of immunochemical structure of cartilage PG.

Concerning human material, Caterson et al.(33) obtained monoclonal antibodies which recognize an antigenic determinant in keratan sulfate being raised against human keratan sulfate-protein core. Champion et al.(34) obtained polyclonal antibodies reacting against human adult and fetal articular cartilage PGs. These authors,by studying cellular immunity to PGs and their ability to preabsorb antibody activity (34), demonstrate that adult PGs express all fetal antigenic determinants and have additional determinants not seen in fetal PGs.

We purified human articular PGs and proposed a RIA for this cartilage constituent. Preliminary communications have been published concerning this subject (8,9,35). In this article, a complete description of a RIA for human articular PGs is given and validated from the point of view of specificity , sensitivity, precision, reproduction and accuracy.

Assay specificity has now been investigated : constituents of cartilage other than PGs , such as human type II collagen, fibronectin, hyaluronic acid, in addition to skin type I and type III collagens and chondroitin sulfate C do not cross react in cartilage PG RIA.

Among human fetus tissue extracts, only cartilaginous tissues (trachea, vertebral disk) show a complete and parallel cross-reaction with adult cartilage PGs. Furthermore, adult costal cartilage PGs show the same immunoreactivity. Artery extract shows a complete but not parallel cross-reaction. In 1980, Gardell et al.(36) demonstrated that PGs from bovine aorta reacted with antisera which had been raised against the hyaluronic acid-binding region and chondroitin sulfate peptides from bovine cartilage PGs. These facts suggest a common partial antigenicity between the PGs of these two kinds of tissues. We found no cross reaction between cartilagenous PG and the extracts of other fetal tissues. No comparison is possible since no data is found in the litterature concerning this subject with the exception of the study by Rennard et al.(37) that shows no cross reaction between non cartilaginous PGs and rat cartilage PGs using the ELISA method.

Small PGs (80-120 10^3 daltons) from adult bone do not react with the antiserum raised against cartilaginous PG. These results seem to be in agreement with Fisher et al.(19) who, using the ELISA method for small bone PGs, showed an absence of cross reaction between fetal calf small bone PGs and extracts

of a series of tissues such as cartilage. On the contrary, we showed that large bone PGs (10^6 daltons) weakly react with cartilaginous PGs in our radioimmunoassay. This was also described by Fisher et al.(19) who found that antibodies raised against bovine cartilage PGs cross reacted with large bovine bone PG. However, these authors found no detectable amount of large PGs in bovine adult bone which was contrary to our findings. We extracted an appreciable amount of large PGs from human adult bone permitting us to realize inhibition curves and chromatographies.

Furthermore, our antiserum shows a species specificity since rat and chick embryo PGs do not cross react and those from dog and fetal calf show a non parallel cross reaction, being approximately 200-fold less immunoreactive than human PG.

Antibodies are directed against the protein core of the PG as shown by the lack of cross reaction by chondroitin sulfate C and treatment with various enzymes. Only treatment with proteolytic enzymes (papain, trypsin + chymotrypsin) abolishes or diminishes immunoreactivity as opposed to treatment with chondroitinase ABC or neuraminidase. Many authors (26,31,38) have also shown that the antigenic determinant of bovine PG are located within the protein core and are not related to the glycosaminoglycan chains. Other characteristics of our RIA, such as sensitivity , are excellent with 0.6 ng/tube, i.e. 0.3 f mole/tube approximately. Precision and reproduction are satisfactory since the relative error concerning the

estimation of PG levels is less than 10 % in the range of 5 to 200 ng/tube. For the between-assay variation coefficient it is approximately 20 % in the range of 2 to 500 ng/tube.

Finally, accuracy is excellent since the correlation between added amounts of PG to biological fluids and measured levels by the RIA have a correlation coefficient equal to 0.999 for se-rum and SF. The slope is near the value "1".

All these characteristics indicate that radioimmunoassay of human aggregating PG monomers is a very sensitive and specific method available for the study and estimation of PG in several in vivo physiological and pathological conditions and in in vitro experimental conditions.

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