A sensitive assay for the measurement of serum chondroitin sulfate 3B3(-) epitope levels in human rheumatic diseases

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Abstract

Objective

To develop a sensitive assay to quantitate serum 3B3(-) levels in patients with rheumatoid arthritis (RA) and osteoarthritis (OA) as well as levels in control sera.

Methods

An enzyme-linked immunosorbent assay (ELISA) was developed using the monoclonal antibody (MAb) 3B3 to detect a chondroitin sulfate (CS) epitope in the sera and synovial fluid (SF) of RA and OA patients. Keratan sulfate levels were measured in the same biological fluids using the 5D4 monoclonal antibody.

Results

The detection limit for our 3B3(-) assay was 2 g/L. Most OA sera sample curves run on the 3B3 assay were parallel (87.5%) to the standard curve and detectable (90.0%). RA sera sample curves were 87.1% detectable and 85.2% parallel. The 3B3(-) epitope was detectable in 60% of control sera and of these 66.7% of sample curves were parallel to the standard curve. All RA and OA SF had detectable quantities of 3B3(-). For the 3B3 assay, the OA and RA sera levels were significantly higher than for control sera (P=0.03, P=0.04 respectively). We found a significant correlation in a subset of paired OA sera and SF 3B3(-) concentrations. No correlation was found between age, joint activity scores, HAQ and CRP in RA patients and sera 3B3(-) and 5D4 levels.

Conclusion

We have validated this assay for the quantification of 3B3(-) epitope in RA and OA serum. Levels of this epitope are significantly higher in sera from RA and OA patients than controls. 3B3(-) levels in RA sera were found to correlate with disease duration.

Key words

Chondroitin sulfate, keratan sulphate, arthritis - rheumatoid, osteoarthritis, enzyme-linked immunosorbent assay.

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Introduction

Rheumatoid arthritis (RA) and osteoarthritis (OA) are common diseases which cause significant disability and progressive loss of joint function (1). There are limited means for assessing cartilage degradation and repair, or for monitoring the response of cartilage to therapeutic interventions.

During normal cartilage turnover, matrix components such as proteoglycan (PG) and collagen are released into the synovial fluid (SF), and subsequently into the bloodstream and urine. In pathological conditions, turnover of matrix components is accelerated and synthesis is impaired (2). The result is the release of increased amounts of degradation fragments and newly synthesized molecules that may not be properly incorporated into the matrix (2).

It may be possible to monitor events, by following changes in the levels of biochemical markers of the metabolism of the cartilage matrix, such as carboxy terminal type II procollagen peptide (pCOL II-C) (3) and urinary collagen cross-links (4).

Monoclonal antibodies (MAb) against altered PG or PG fragments may also be used as biochemical markers. MAb have been developed against all parts of the PG molecule. Enzyme-linked immunosorbent assays (ELISAs) have been developed using the MAb 3B3, which is specific for a terminal 6-sulfated chondroitin sulfate (CS) epitope (5). Chondroitinase (CASE) treatment of PG generates this CS epitope, 3B3 (+), as well as removing steric interference caused by other CS GAGs. The naturally occurring 3B3(-) mimotope is slightly different as it has a saturated glucuronic acid residue, instead of an unsaturated one as found with 3B3(+). The MAb 3B3 does not seem to discriminate between saturated and unsaturated residues (6). It has been suggested that increased expression of this unusual CS epitope is a measure of new matrix synthesis or renewal (7). It is regarded as an anabolic epitope (8) as it is expressed in embryonal (9) and malignant tissue and synthesized in adult cartilage during early OA as an attempted repair response (10). We have modified the 3B3 assay to increase the sensitivity and verify this by showing that the 3B3(-) epitope can be quantified in serum in a reproducible manner.

Subjects and methods

Patients and controls

The population was derived from OA and RA patients being assessed in studies of disease activity and severity (Bath, UK). Sera and SF samples were obtained from both groups and frozen at -70°C.

All OA and RA patients were diagnosed and classified according to accepted criteria (11, 12). Disease onset was defined as the time of onset of joint symptoms and disease duration as the time from onset of symptoms to the time of sera collection. Control sera samples were collected from hospital and laboratory staff and normal volunteers participating in other studies. This study was approved by The Local Institutional Ethics Committee. A summary of patient characteristics is presented in Table I.

Clinical methods

A 44 tender joint count was undertaken by the one observer and a Ritchie articular index calculated (13) for the RA patients. The total (44) swollen joint count was determined. An assessment of joint damage was derived in this group of RA patients by obtaining a measure of joint damage by an assessment of the range of joint mobility of the wrists, elbows, shoulders, hips, knees and ankles (range 1-30: Table II). The Health Assessment Questionnaire was used as an assessment of function. The serum CRP level was determined by nephelometry.

Chemicals and antibodies

Bovine serum albumin (BSA) and para-nitrophenol phosphate (pNPP) disodium tablets were obtained from Sigma Chemical Company (Sydney, Australia). Flat-bottom 96-well MaxiSorp Immunoplates were obtained from Nunc (Denmark). Dimethylmethylene Blue Chloride (DMMB) was purchased from Serva (Germany). Chondroitinase ABC (CASE) from Proteus

Table I. Patient characteristics and clinical data.*

	Sera			Synovial Fluid	
	Control	OA	RA	OA	RA
Sample No.	30	30	31	30	15
Age (yrs), mean ± SD	34 ± 11	70 ± 10	56 ± 11	69 ± 9	53 ± 19
Gender					
Female	9	15	23	11	8
Male	21	15	8	19	7
Disease duration (yrs)					
Range	-	5	12	5	11
Median	-	0.6-30	2-26	0.3-30	2-46
NSAID (%)		70%	65%	73%	67%
DMARD (%)	-	-	52%	-	60%
Prednisolone (%)	-	-	10%	-	20%

* OA:Osteoarthritis; RA:Rheumatoid Arthritis; SF: synovial fluid; NSAID:non-steroidal anti-inflammatory drug; DMARD: disease-modifying anti-rheumatic drug.

Table II. Quantification of peripheral joint range of movement for rheumatoid arthritis subjects.

Joint	Score	Range of movement
Wrist[1]	0	> 90° (normal)
	1	45° - 90°
	2	< 45°
Elbow[2]	0	FF < 5° or F > 130°
	1	FF 5°-30° or F 90°-130°
	2	FF > 30° or F < 90°
Shoulder[3]	0	180°
	1	150°-179°
	2	90°-149°
	3	< 90°
Hip[4]	0	> 110°
	1	91°-110°
	2	60°-90°
	3	< 60°
Knee[5]	0	FF < 5° or F > 135°
	1	FF 5°-10° or F 90°-134°
	2	FF 11°-15° or F 60°-89°
	3	FF > 15° or F < 60°
Ankle[6]	0	> 60°
	1	30°-59°
	2	< 30°
Total Score	(0-30)	

All patients with a partial or total joint replacement were given the maximum score for that joint.
[1]Dorsiflexion or palmar flexion; [2]FF = fixed flexion, F = flexion (from full extension to full flexion); [3]active flexion or abduction; [4]range of flexion; [5]F = range of flexion from the extension lag to full flexion; [6]full dorsiflexion to full plantar flexion.

vulgaris was purchased from Seikagaku Kogyo Co, Ltd (Japan).

The MAbs 3B3 and 5D4 were kindly supplied by Professor Bruce Caterson (Connective Tissue Biology Laboratories, Cardiff University, Wales, UK). Alkaline phosphatase-conjugated goat anti-mouse IgM and IgG was obtained from Sigma Chemical Company (Sydney, Australia).

CASE-treated PG derived from normal human cadaveric articular cartilage, PG-CASE, was used as the standard in these assays (14). The total amount of sulfated GAG in the PG-CASE standard was measured using the DMMB dye-binding assay (15). All results are expressed in (g/L sulfated GAG).

Enzyme-linked immunosorbent assay (ELISA)

The methods used were those described by Thonar *et al.* (16) with modifications. The assay buffer, Tris Incubation Buffer (TIB), consisted of 0.1% BSA, 0.1% Nonidet P40, 0.01M Tris HCl and 0.15M NaCl (pH7.4).

Wells (excluding outer wells) were coated with 100 L of 500 g/L PG-CASE in 20 mM sodium carbonate buffer (pH 9.6). Plates were then covered with Parafilm, incubated overnight at 4°C, then incubated at 37°C for a further 2 hours. During this incubation period, an incubation mixture (MAb and antigen) was prepared as follows. To 1.5 mL Eppendorf tubes, 150 L of serially diluted PG-CASE (128-2 g/L) in TIB was added. Two blank tubes were made with TIB. Serum and SF samples were serially diluted from 1/4 to 1/16, with further dilutions if needed. To all of the tubes except one of the blanks (the zero tube), 150 L 1/250,000 3B3 in TIB was added. Tubes were vortex mixed and incubated at 37°C for 35 min. During this time, the wells were washed 4 times with TIB, and blocked with 1% BSA in TIB for 30 min at 37°C. After blocking, wells were washed a further 4 times. 100 L from each inhibition mixture tube was added to each of duplicate wells. Wells were covered and incubated at 37°C for 1h. Wells were washed 4 times with TIB. 100 L of 1:2000 alkaline phosphatase conjugated goat

anti-mouse IgM (diluted in TIB) was added to each well and incubated at 37°C for 1h. Wells were again washed four times with TIB. 100 L of pNPP solution (one 5 mg pNPP tablet in 10 mL diethanolamine buffer, pH 9.8) was added to each well for colour development, incubated at 37°C for 90 min. The reaction was stopped by adding 50 L 2M NaOH to each well. The plate was read at 405 nm on a Biorad 3550 microplate reader (Sydney, Australia). Absorbance values were converted to percentage inhibition, by the formula:

% Inhibition = 100 - [(OD(Bound) - OD(NSB)] / (OD(Bo) - OD(NSB)] x 100]

where NSB = non-specific binding and Bo = binding at zero PG-CASE.

Percentage inhibition was then plotted against standard concentrations of PG-CASE (Microsoft Excel 5.0).

The functional sensitivity of the assay was determined from the 20% coefficient of variation of the standard curve used as the detection limit (17). The detection limit for the 3B3 assay was found to be 2 g/L. The intra-run variation was calculated by using 6 samples of sera and SF and determining the percentage coefficient of variation (CV) for each duplicate dilution using the same plate. The inter-run variability was calculated by determining the CV of the epitope concentrations for one serum and one SF sample over 6 runs. Intra-assay variation was 0.3 - 9.1% CV for sera, and 1.1 - 11.5% CV for SF. The inter-assay variation was 7.5% CV for sera, and 11.8% CV for SF.

The 5D4 ELISA was also optimised by the above method. The well coating concentration of PG-CASE was changed to 250 g/L and the 5D4 dilution used was 1/500,000. The detection limit was 2 g/L. The intra-assay variation was 4.5 - 11.5% CV for sera, and 1.2 - 6.4% CV for SF. The inter-assay variation was 8.7% CV for sera, and 11.1% CV for SF. A bovine nasal cartilage PG was also tested as an alternative standard.

Statistical analysis

Differences between patient (and control) groups were determined by subjecting this data to a non-parametric two-tailed Mann-Whitney test using GraphPad PRISM (Ver. 2.0) (GraphPad Software, Inc., San Diego, California). P values less than or equal to 0.05 were considered significant.

Correlations between epitope concentrations and clinical and disease variables were undertaken using Spearman's correlation coefficient, (GraphPad PRISM). To test whether sample curves were parallel to the standard, multiple linear regression using the SPSS statistical package was applied to each curve and the 95% confidence interval for the slope was calculated. Only those curves with more than one detectable dilution point were used.

Results

3B3(-) and 5D4 KS levels for patient groups

Typical PG-CASE standard curves for our 3B3 and 5D4 ELISAs can be seen in Figures 1A and 1B, respectively. Scatterplots of 3B3(-) and 5D4 KS epitope concentrations in OA and RA sera and SF, as well as serum from subjects without joint disease (controls) are shown in Figures 2 and 3, respectively. Most of the samples assayed by 3B3 were detectable and parallel to the standard curve (Table III). Not as many control sera samples were detectable when compared to OA and RA sera (18/30 versus 27/30 and 26/31, respectively). Of the control sera that were detectable, only 66.7% were parallel to the standard curve. OA and RA sera had very similar 3B3(-) concentrations, which were significantly higher than control serum 3B3(-) levels ($P = 0.03$ and $P = 0.04$, respectively).

Serial dilutions of SF samples from RA and OA patients were found to be parallel to the standard curve. The RA SF 3B3(-) levels were 4.5 times higher than RA sera, as compared to 5.3 times for OA SF 3B3(-) versus OA sera 3B3(-).

All samples assayed by 5D4 were detectable and parallel to the standard curve. The KS levels for OA SF were 12.8 times higher than OA sera, whilst RA SF KS concentration was 8.6 times higher than RA sera. In individual samples, KS concentrations were 53-129 times higher than 3B3(-). The OA and RA sera 3B3(-) levels were approximately twice that of control sera levels. The 5D4 KS concentration in OA and RA sera were also very similar, although only RA sera KS levels were

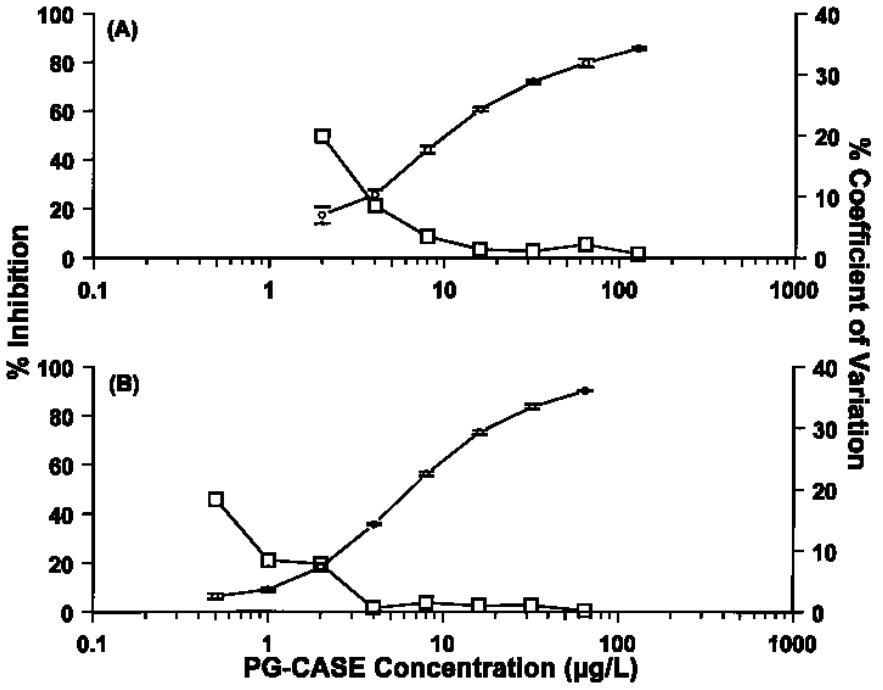

Fig. 1. The PG-CASE standard curve when run on 3B3 (**A**) and 5D4 (**B**) ELISAs. Circles represent the PG-CASE standard curve. Squares represent the CV curve. Error bars are one SD. The detection limit was 2 g/L for the 3B3 assay, and 0.5 g/L for the 5D4 assay.

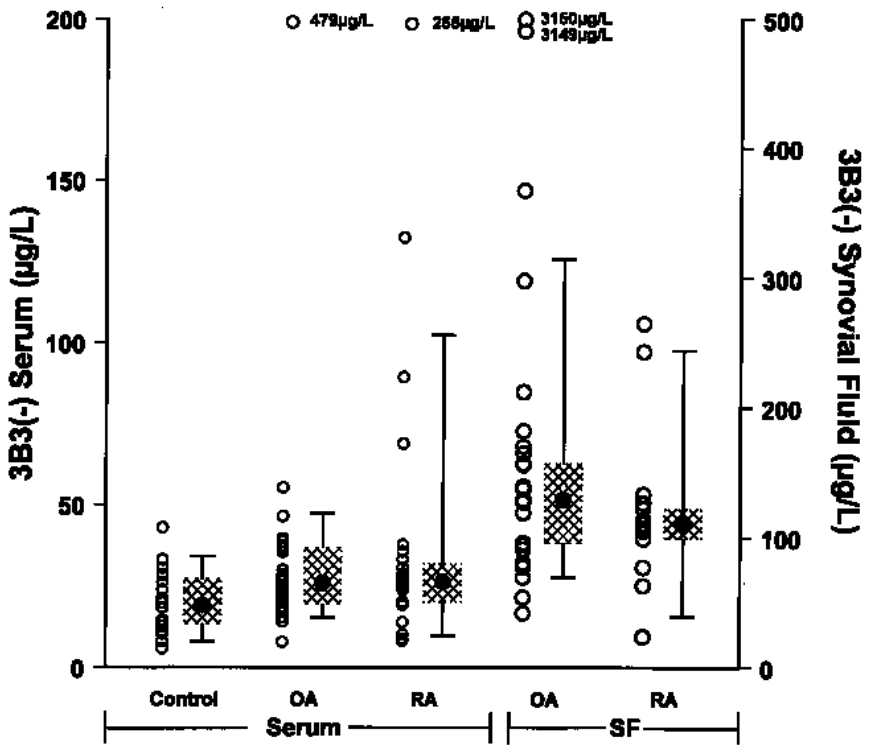

Fig. 2. Scatterplots of 3B3(-) epitope concentrations measured with the MAb 3B3 in control, OA and RA sera (small circles), as well as OA and RA SF (large circles). Box plots show the median concentration plus interquartile range, with the error bars showing the 10th and 90th centiles.

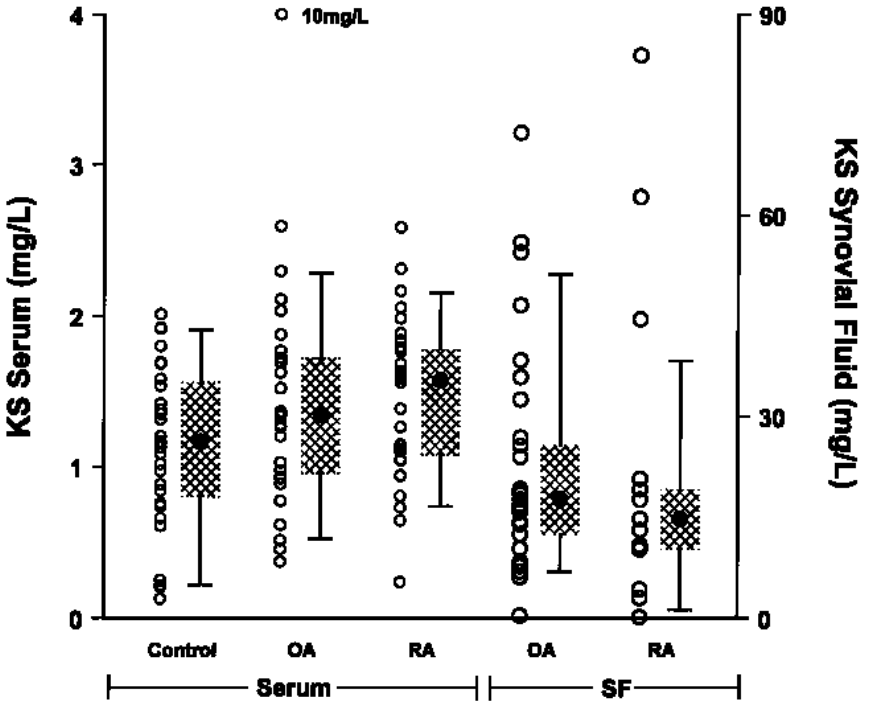

Fig. 3. Scatterplots of the KS epitope concentrations measured with the MAb 5D4 in control, OA and RA sera (small circles), as well as OA and RA SF (large circles). Box plots show the median concentration plus interquartile range, with the error bars showing the 10th and 90th centiles.

significantly higher than control sera ($P < 0.05$). There was no difference between RA and OA SF 3B3(-) or 5D4 KS levels.

Correlations between 3B3(-) and 5D4 KS levels with patient clinical indices

RA sera 3B3(-) levels were found to correlate with disease duration ($r = 0.432$; $P < 0.02$). No significant correlations were found between age, joint swelling and tenderness scores, HAQ, joint damage score and CRP with epitope concentration for RA sera.

There was a tendency to avoid the use of corticosteroids in the RA group of patients in this study. Only 10% of the patients having serum assays were receiving oral corticosteroids while 20% of patients with a synovial fluid effusion that was aspirated were receiving corticosteroids.

Correlations between paired OA sera and SF samples

There appears to be two populations of OA patients. All patients show a similar sera 3B3(-) concentration, but one population has much higher SF 3B3(-) concentrations than the other. These two trendlines can be seen in Figure 4. There was a significant correlation between the 3B3(-) concentrations in sera and SF for both groups ($r = 0.94$, $P = 0.007$ for high SF; $r = 0.69$, $P = 0.002$ for low SF). There were insufficient paired RA sera and SF samples to make the same comparison.

Bovine nasal cartilage standard

Another PG standard was used, which is a CASE-treated bovine nasal cartilage PG. Although the detection limits for the 3B3 assay were found to be slightly lower (1.8 g/L) than when using PG-CASE (2 g/L), more sample dilution curves were parallel with the human antigen standard curve than the bovine. Thus, the PG-CASE human antigen was retained as the standard.

Discussion

We have developed a highly sensitive inhibition ELISA which detects and quantifies the 3B3(-) epitope in human sera. This assay is of sufficient sensitivity that most sera from patients with

Table III. The 3B3(-) and KS epitope concentration range for the disease groups studied.

	Control	Serum OA	Serum RA	Synovial fluid OA	Synovial fluid RA
CS 3B3(-) epitope, g/L					
Median	10.7#	25.3#	25.3#	133	114
5th centile	1.0	1.0	1.0	49	23.9
95th centile	38	188	271	3,147	243
Range	(1.0 - 43)	(1.0 - 350)	(1.0 - 479)	(42 - 3150)	(23.9 - 265)
% Detectable	60%	90%	87.1%	100%	100%
% Parallel*	66.7%	87.5%	85.2%	100%	100%
KS 5D4+ epitope, g/L					
Median	1,162#	1,345	1,582#	17,220	13,671
5th centile	170	418	482	401	114
95th centile	1,961	5,926	2,530	63,267	(62,682)
Range	(127 - 2011)	(372 - 9999)	(238 - 2585)	(371 - 72234)	(114 - 83826)

* Those with only one dilution point above the standard curve were not included. Medians were determined by using all samples that had percent inhibitions above the detection limit. Those with undetectable epitope concentrations were assigned a value of 1 g/L (half the detection limit).
+All samples were detectable and parallel.
#OA and RA sera levels of 3B3(-) were significantly higher than control sera 3B3(-) (P=0.03 and P=0.04, respectively). Only RA sera had significantly higher 5D4 levels than control sera ($P < 0.05$).

OA and RA will be detectable using a 1/4 dilution. We have also measured 3B3(-) levels in SF, as well as an oversulfated KS epitope (by 5D4 ELISA) in both sera and SF.

The intra- and inter-run CVs for the 3B3 and 5D4 ELISAs are comparable to those found by Sharif *et al*. (18). Of those samples with detectable 3B3(-) levels, a high proportion of these serial dilution curves were parallel (60-100%; Table III). Parallelism between sample and standard curves is very important for this type of assay where accurate quantification of epitope in biological fluids is needed. Control sera showed the lowest 3B3(-) levels. More control sera sample curves were non-parallel as compared to RA and OA sera sample curves. This is likely to be due to the serial dilution curves being close to the detection limit of the assay. Non-parallelism may also be due to interfering factors, such as matrix components, or to differential binding of 3B3 MAb to different sized PG fragments.

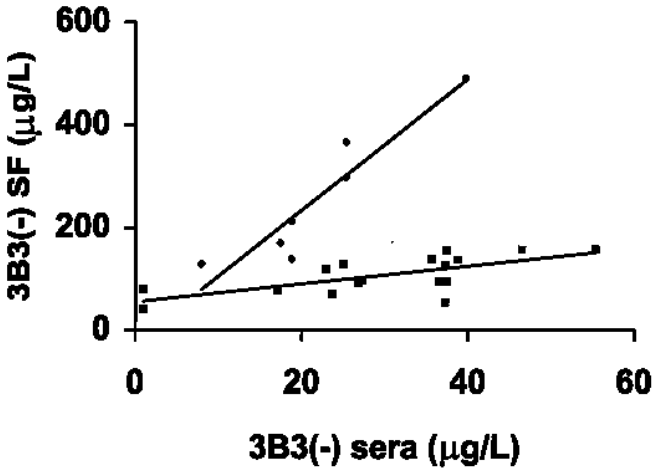

Fig. 4. Significant correlation between paired OA sera and SF 3B3(-) concentrations. Two populations of OA patients can be seen,as indicated by the trendlines. Large squares represent patients with low SF (r = 0.9370; P=0.007). Small squares represent patients with high SF (r = 0.6900; P=0.002).

Detection limits for the 3B3 ELISA were recently reported by Ratcliffe *et al*. (19). They reported a detection limit of 0.5 g/L, which was 20 times lower than their previous assay and four-fold lower than our current assay. They did not report their method for deriving the detection limit. The standard that they used was also a human aggrecan digested with CASE. The antibody dilution used was 1/400,000, which is slightly higher than ours, and their antigen coating concentration, incubation temperature and incubation times were also slightly different.

Previously reported ELISAs have quantified 3B3(-) epitope levels in human SF from a variety of normal, arthritic and injured joints (18, 20, 21, 22-25), as well as canine sera and SF (26, 27). There is a wide range of reported values. It is not possible to directly compare our results with those of other workers due to different standards used for some assays, different fragment sizes of the PG in the SF, differing rates of clearance of PG fragments and differing clinical subsets.

As assessed by 3B3(-) levels, there may be two subsets of OA patients (Fig. 4). It appears that there is one group of patients with high SF and high serum levels with a second group with lower SF levels. The higher sera levels of 3B3(-) in RA and OA patients compared with the control population may be due to the younger age of the controls. The controls were not age matched with the diseased group subjects.

In the recent paper by Middelton *et al*. (28), their method for assaying serum 3B3(-) concentrations is based on the method of Hazell *et al*. (22). The lower limit of detection was 1.6 g/L which is similar to our assay. They do not define sensitivity but quote a value of 1 g/L. They do not indicate whether parallelism of the samples when serially diluted occurs with the standard curve. This is essential if meaningful results are to be obtained and their very high inter-run CV of 47% for 3B3(-) contrasts with our result of 8.7% for

sera. With such a high inter-run CV, all of the patient assays, including all the control samples, would have to be run on the same assay. If this were not the case then association with the clinical parameters of the patients would have been meaningless between patients. A CV over 20% eliminates this assay from clinical practice. All the patients in this paper had early disease at first presentation (median disease duration was 6 months) and at first presentation serum 3B3(-) levels were significantly lower than control values and remained significantly lower at all time points up to 36 months. In contrast, we have found in our patients with a median disease duration of 12 years, that serum 3B3(-) values were significantly higher than control values.

Plaas *et al*. (29,30) have found that there may not be a close correlation between direct quantification of the terminal disaccharide GlcA 1,3GalNAc-6S, the mimotope for 3B3(-) and quantification based on immunoassays. This may relate to variable density of the epitope on the solid phase and altering binding to the monoclonal antibody, blockage of expression of the 3B3(-) epitope by other PG epitopes or other serum molecules and multiple interactions between the pentavalent IgM monoclonal and multiple 3B3(-) epitopes on PG fragments. Nevertheless there are many examples of a divergence between the quantification of a biochemical analyte due to different analytical methods, for example, deoxypyridinoline as measured by HPLC and N-telopeptide as measured by immunoassay. These differences may still translate into clinically useful results and one assay may be more applicable to one disease state than another (31).

For the 5D4 KS ELISA, every sample, whether it was sera or SF, had detectable and parallel KS serial dilution curves. The intra- and inter-run CVs for the 5D4 ELISA are comparable to those found by Thonar *et al.*, Spector *et al.* and Sharif *et al.* (16, 32, 33). Detection limits for 5D4 ELISAs have not previously been published. The 3B3(-) epitope content of serum samples was found to be 50-120 times lower than KS epitope as detected by the MAb 5D4. It is not possible however to compare the two assays. There may be variable epitope density of the 3B3(-) and 5D4 epitopes on PG fragments. There may also be variable binding of the respective monoclonals to the PG epitopes.

Oversulfated KS has been measured in both sera and SF using the 5D4 assay (16, 18, 21, 24, 25, 35-38). We found that KS levels in RA sera were significantly higher than control sera, but there was no significant difference between OA and control sera levels. Mehraban *et al*. showed that KS levels in both RA and OA sera were significantly higher than those for control sera (36), whilst Spector *et al.* (32) found that RA sera KS concentrations were significantly lower than KS levels in normal sera, and OA and control sera had similar levels.

The variability in the serum KS levels previously reported in different patient groups by investigators emphasises the need for optimization of the assay and ensuring that there is parallelism between patient sera and standard curves. There was no difference between OA and RA SF 3B3(-) or KS concentrations, which is consistent with Belcher *et al.* (21). There are many reports for the 5D4 KS ELISA, but very little agreement between them. The likely explanation is that each assay was developed independently, with differing standards as well as other assay variables. The patient groups also differ.

No correlation was found between 3B3(-) and 5D4 KS epitope concentration within patient groups. Hardingham commented that increased 3B3(-) in SF was not accompanied by similar increases in KS epitope (37). The 3B3(-) epitope and the 5D4 KS epitope are markers of different cartilage metabolic processes and the lack of a relationship between the levels of these epitopes in SF or serum is perhaps not surprising.

Like Middelton *et al.* (28),in our cross-sectional RA study, no relationship was found between age, gender, disease activity, joint damage, HAQ or serum CRP levels and sera 3B3(-) levels. There was also no association between 5D4 KS epitope concentrations and clinical parameters. In contrast, Spector *et al.* found that 5D4 KS levels in RA sera positively correlated with CRP (32). There was a significant positive correlation between disease duration and 3B3(-) concentration for the RA sera samples tested. No correlation was found between disease duration and 5D4 KS epitope concentration for any disease grouping. Thonar *et al.*, Spector *et al.* and Seibel *et al.* (16, 32, 38) did not find a relationship between serum KS levels with age or gender, whilst Campion *et al*. found a correlation with gender, where serum KS levels were significantly higher in men than women (39).

Expression of the 3B3(-) epitope occurs in tissues undergoing rapid growth and development (6), as well as increased chondrocyte division and PG metabolism (6,7). From recent studies, it has been suggested that the expression of 3B3(-) epitopes reflects in part the hypermetabolic response of the chondrocyte, which may increase matrix synthesis and repair matrix damage (37). If this epitope is not specific for cartilage synthesis, nevertheless measurement of the sera levels of this epitope may provide a means of assessing cartilage turnover.

The improved optimisation of this 3B3(-) ELISA now enables rheumatologists to apply this assay to defined groups of patients with arthritis.

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