

Recognition of YKL-39, a human cartilage related protein, as a target antigen in patients with rheumatoid arthritis

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Abstract

Objective—To investigate whether autoimmunity to YKL-39, a recently cloned cartilage protein, occurs in patients with rheumatoid arthritis (RA).

Methods—Autoantibody to YKL-39 was assayed by enzyme linked immunosorbent assay (ELISA) and western blotting in serum samples from patients with RA, systemic lupus erythematosus (SLE), and healthy donors, using recombinant YKL-39 protein. This reactivity was compared with that against a YKL-39 homologue, YKL-40 (human cartilage gp-39/chondrex), which has been reported to be an autoantigen in RA.

Results—Autoantibody to YKL-39 was detected in seven of 87 patients with RA (8%), but not in serum samples from patients with SLE or healthy donors. YKL-40 reactivity was found in only one of 87 RA serum samples (1%), with no cross reactivity to YKL-39.

Conclusion—The existence of anti-YKL-39 antibody in a subset of patients with RA is reported here for the first time. Further, it was shown that the immune response to YKL-39 was independent of that to YKL-40. Clarification of the antibody and T cell responses to autoantigens derived from chondrocyte, cartilage, or other joint components may lead to a better understanding of the pathophysiology of joint destruction in patients with RA.

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Recently, two new cartilage related proteins were identified. The first, YKL-40 (also called human cartilage gp-39 or chondrex) is a 40 kDa protein primarily produced by human chondrocytes and synovial fibroblasts. Although its sequence homology places it in the chitinase family, no chitinase activity has been confirmed.⁴ In vitro studies have shown that YKL-40 accounts for 33% of the total protein produced by cultured chondrocytes.⁵ YKL-40 mRNA expression was detected in the cartilage of joints affected by RA, but not in normal human cartilage.⁴ Furthermore, it has been reported that serum concentration of YKL-40 correlates with the degree of joint destruction.^{6,7} Verheijden *et al* reported that YKL-40 derived peptides were selectively recognised by peripheral T cells in patients with RA.⁸ In addition, it was shown that immunisation with YKL-40 could induce RA-like arthritic lesions in a mouse model. These findings suggest that YKL-40 may be an autoantigen derived from cartilage in RA.

YKL-39, also identified recently, subsequent to the reporting of YKL-40,⁵ has 52% amino acid and 56% nucleotide sequence homology to YKL-40, and thus also belongs to the chitinase family.⁵ YKL-39 is speculated to have no chitinase activity because it lacks the active site glutamate, which is essential for the activity of chitinases.⁵ YKL-39 mRNA expression is detected mainly in chondrocytes and, to lesser extent, in synoviocytes, lung, and heart.⁵ YKL-39 accounts for 4% of chondrocyte secreted proteins in vitro. Because this protein is closely related to YKL-40, it is likely to have similar immunogenicity. To date, however, no data are available on its immunological significance.

In this study we used a recombinant fusion protein to investigate whether YKL-39 was an autoantigen in RA, and demonstrated, for the first time, the production of anti-YKL-39 autoantibody in patients with RA. Furthermore, we showed that anti-YKL-39 antibody production was independent of the anti-YKL-40 response, suggesting distinct recognition of these two closely related cartilage proteins by the immune system in patients with RA.

Materials and methods

PATIENTS

Eighty seven Japanese patients with RA (77 women, 10 men; mean (SD) age 57.5 (13.4) years) and 47 Japanese patients with systemic lupus erythematosus (SLE; 45 women, two

Rheumatoid arthritis (RA) is a chronic disorder characterised by synovial inflammation in multiple joints. In addition to hyperplasia of the synovial lining, affected joints show distinct lymphocytic infiltration. The reported association of certain HLA haplotypes, such as HLA-DR4 and DR1, with susceptibility to RA, suggests involvement of antigen-specific immune responses in its pathogenesis. It has been postulated that such HLA haplotypes present RA related antigens to their specific T cells, probably triggering a cascade of autoimmune responses.

The target antigens in RA remain elusive. No exogenous antigens have been identified as definite causative agents and the most likely candidates are components within or in close proximity to the joint itself. In this context, various joint related antigens, such as type II collagen, have been reported to cause autoimmune responses in RA in vivo or in vitro.¹⁻³

men; mean age 41.9 (13.5) years), diagnosed according to the revised criteria of the American College of Rheumatology^{9,10} were enrolled in this study. All patients were being treated at the hospital of the University of Tokyo or the hospital of St Marianna University, School of Medicine. Serum samples were obtained from the patients and from 121 healthy donors matched for age and sex. The study protocol was approved by the human ethics review committee of both institutions and a signed consent form was obtained from each participant.

PREPARATION OF cDNA FOR HUMAN CARTILAGE 39 kDa PROTEINS AND PLASMID VECTOR CONSTRUCTION

RNA was extracted from HCS-2/8 chondrosarcoma cells,² according to the acid-guanidine-chloroform-phenol method,¹¹ and then converted to cDNA by reverse transcription and random hexamer priming. The DNA fragment encoding either YKL-40 or YKL-39 was amplified by a polymerase chain reaction (PCR) from the cDNA sample. Primers for YKL-39 and YKL-40 were as follows: YKL-39: forward: 5'-AGTTACGGATCCCATCATCATCATCATCATGCCTACAAACTGGTTTGC TAC-3', reverse: 5'-GTACGCTCTAGATCA CAAGGAGCCAAGGCTTC-3'; YKL-40: forward: 5'-ATCGGATCCCATCATCATCATCATCATATTACAAACTGGTCTGCTACTA-3', reverse: 5'-GTACGCTCTAGACTACGTTG CAGCGAGTGCAT-3' (the underlined letters indicate DNA restriction enzyme recognition sequences and italicised letters indicate sequences that encode a histidine tag). PCR was performed under the following conditions: 35 cycles of 94°C for 1.5 minutes, 58°C for 2 minutes, and 72°C for 2 minutes. The amplified DNA was then cloned into a plasmid vector, pMAL-c2 (New England Biolabs, Inc, Beverly, MA) that produced a fusion protein with a maltose binding protein (MBP). The sequences were checked by the dideoxy method (377 DNA Sequencing System, Perkin Elmer/Applied Biosystems, Foster City, CA). Histidine tagged MBP was prepared as a negative control by inserting the amplified DNA fragment that contained the multicloning site of pMAL-c2 using the following primers: forward: 5'-ATCGGATCCCATCATCATCATCATCATATTCCCTCTAGAGTCGACCTGCA-3' and reverse: 5'-ATCGTTAGTACTCAACC AAGTCATTC-3'.

EXPRESSION OF RECOMBINANT PROTEINS

Escherichia coli transformed with each of these recombinant plasmids were used for production of the recombinant proteins by adding isopropyl β-D-thiogalactopyranoside. Harvested cells were lysed in 8 M urea/phosphate buffered saline (PBS) pH 7.4, sonicated, and then centrifuged. His-tagged proteins were purified from the supernatant by affinity chromatography (His trap, Amersham Pharmacia Biotech AB, Uppsala, Sweden). Purified proteins were dialysed against PBS.

ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA) ELISA was performed according to the procedure described by Matsui *et al.*¹² Serum samples were preincubated for one hour with similarly purified MBP with the 6 × His-tag to absorb non-specific binding of serum samples to MBP. After reacting serum samples with coated recombinant proteins, horseradish peroxidase (HRP) labelled protein G was used to detect the bound antibodies. Bound protein G was visualised by enzymatic reaction of HRP with *o*-phenylenediamine. The results of each optical density (OD) value were calculated according to the following formula and were expressed in arbitrary units. Unit = (OD value for each serum sample × 100)/((mean of OD value for healthy serum sample) + (3 × standard deviation of OD value for healthy serum sample)): OD value = (value in A492 in YKL-39 or YKL-40 coated well) - (value in A492 in MBP (control) coated well). Samples measuring over 100 units were defined as positive.

WESTERN BLOTTING

Western blotting was performed as described by Matsui *et al.*¹² Briefly, the proteins, separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), were transferred to a nitrocellulose membrane. The membrane was then incubated with the samples diluted at 1:100 or an anti-MBP antibody (Toyobo, Osaka, Japan) as a positive control and further reacted with HRP labelled protein G. Bound protein G was visualised by enzymatic reaction of HRP with diaminobenzidine.

IMMUNOPRECIPITATION

Anti-YKL-39 autoantibodies were purified from pooled serum samples from anti-YKL-39 autoantibody positive patients as described previously.¹² The purified anti-YKL-39 autoantibodies were then incubated with protein G-Sepharose (Amersham Pharmacia Biotech AB). Whole cell lysate of HCS-2/8, chondrosarcoma cell lines, preabsorbed with protein G-Sepharose, was then incubated with the anti-YKL-39 autoantibody-bound protein G-Sepharose. After washing, bound proteins were separated by 12.5% SDS-PAGE and transferred onto the nitrocellulose membrane as above. Finally, the precipitated YKL-39 was detected by western blotting using rabbit anti-YKL-39 polyclonal antibodies which were prepared by immunisation of rabbits with the recombinant YKL-39 and subsequent adsorption with MBP.

Results

DETECTION OF AUTOANTIBODIES TO YKL-39 AND YKL-40

To investigate whether there was an autoimmune response to YKL-39, a recently cloned cartilage protein, in patients with RA, we constructed a recombinant YKL-39/MBP fusion protein and analysed autoantibody titres by ELISA and western blotting. We first prepared recombinant fusion proteins of YKL-39 and YKL-40. As shown in fig 1A, fusion proteins

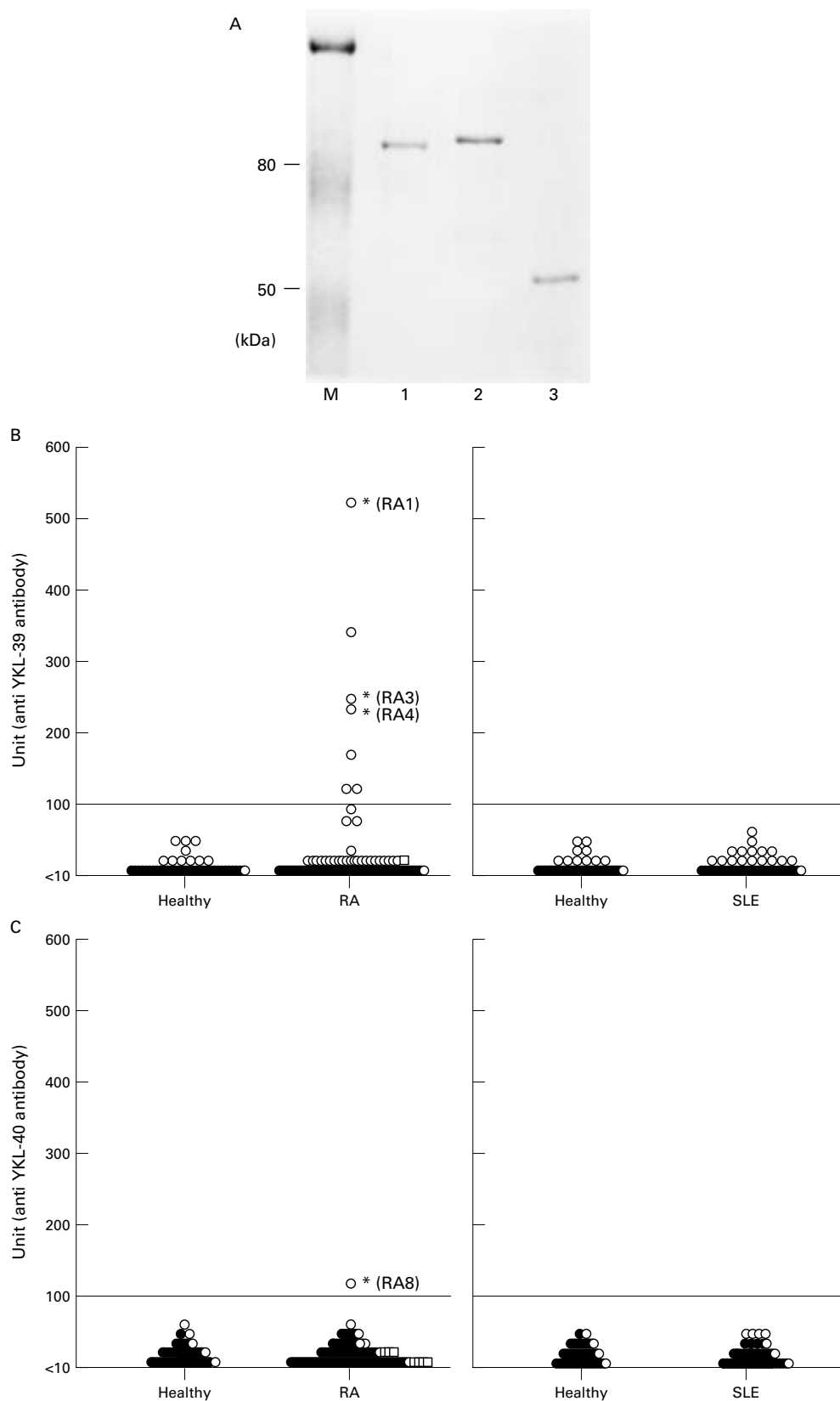


Figure 1 Detection of anti-YKL-39 antibody. (A) Purified fusion proteins, YKL-40 (1), YKL-39 (2), and maltose binding protein (MBP) (3) were electrophoresed on a 10% SDS-PAGE gel and detected by Coomassie brilliant blue staining. (B, C) Detection of autoantibodies to YKL-39 (B) and YKL-40 (C) in serum samples of patients with RA and SLE and healthy control subjects by ELISA. Each symbol indicates a single person. Serum samples from healthy donors matched for age and sex were used as controls. Units of antibodies to YKL-40 in anti-YKL-39 positive sera (open squares in C) and to YKL-39 in anti-YKL-40 positive sera (open circles in B). Data are expressed in arbitrary units as described in "Materials and methods".

with expected molecular weights were detected by SDS-PAGE and subsequent Coomassie brilliant blue staining. The sizes of recombinant YKL-39 and YKL-40 coupled with MBP were ~82 and ~81 kDa, respectively, and

that of control MBP was ~51 kDa, as the latter protein contains the LacZ α gene product. Using these recombinant proteins, we tested serum samples from a panel of patients with RA and SLE, representative joint specific and

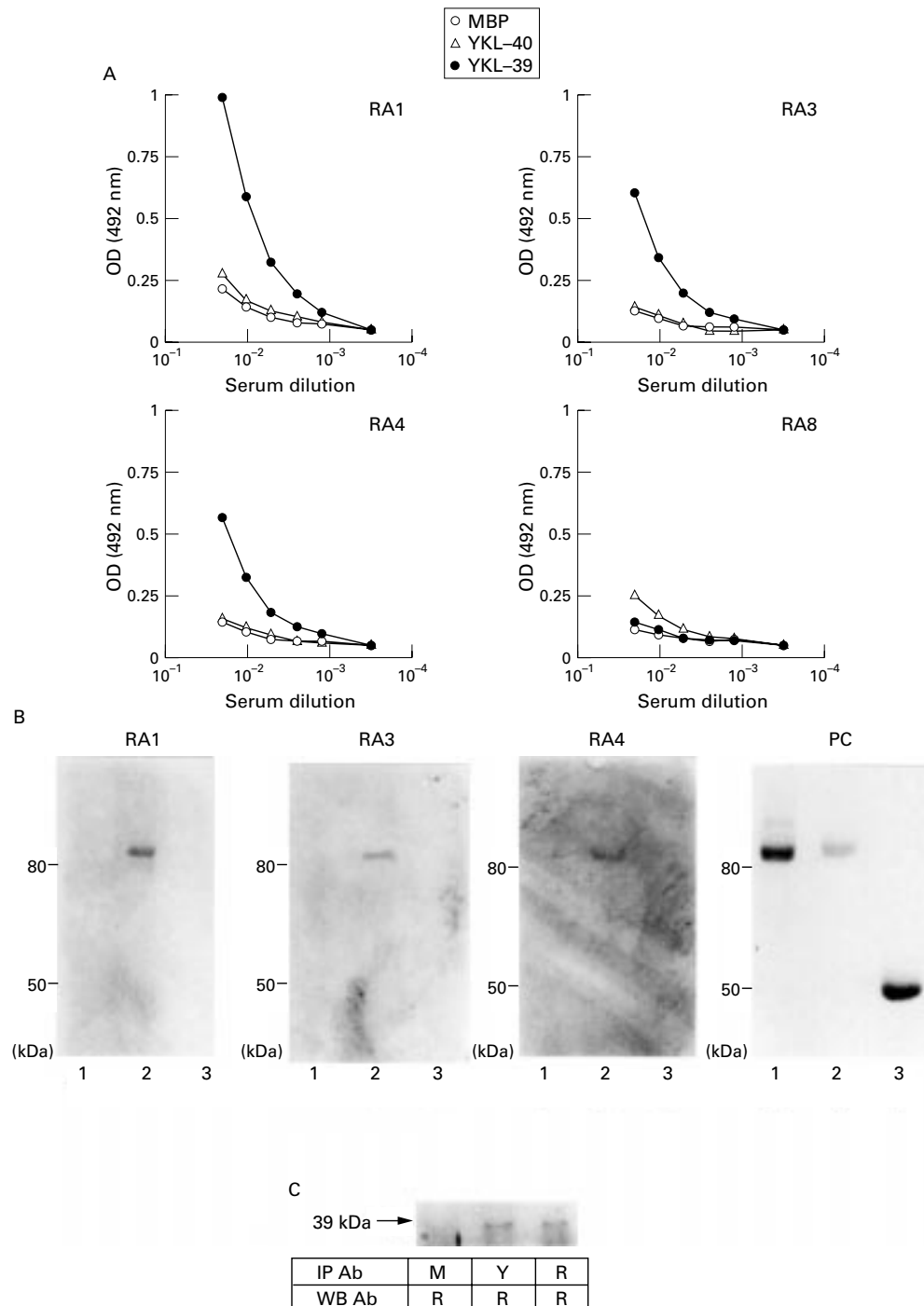


Figure 2 Specificity of autoantibodies to YKL-39 and YKL-40. (A) Antibodies to YKL-39 and YKL-40 were determined by ELISA using serially diluted serum samples. Selected samples are indicated by asterisks in figs 1B and C. Each OD value from maltose binding protein (MBP), YKL-40, and YKL-39 is indicated. (B) YKL-39 positive sera were processed by western blotting to assess reactivity to YKL-40. Recombinant proteins, YKL-40 (1), YKL-39 (2), and MBP (3) were electrophoresed on a 10% SDS-PAGE gel and then western blotted using serum samples from YKL-39 antibody positive patients with RA or anti-MBP antibody as a positive control (PC). (C) Antibodies to YKL-39-MBP fusion protein and those to MBP alone as a negative control were purified from pooled anti-recombinant YKL-39 positive serum samples separately. Then, native YKL-39 in the cell lysate of HCS-2/8 was immunoprecipitated by these purified antibodies and by rabbit anti-YKL-39 polyclonal antibodies as a positive control. The precipitated native YKL-39 was then detected by western blotting using the rabbit anti-YKL-39 antibodies. IP Ab = antibodies used for immunoprecipitation; WB Ab = antibodies used for western blotting; M = antibodies purified by MBP alone; Y = antibodies purified by the recombinant YKL-39 fused with MBP; R = rabbit anti-YKL-39 polyclonal antibodies which were prepared by immunisation of rabbits with the recombinant YKL-39-MBP fusion proteins and subsequent adsorption with MBP.

Table 1 Clinical data of the patients with RA. Results are means (SD)

	Age (year)	Disease duration (year)	Stage*	RF† (U/ml)	ESR‡ (mm/1st h)	CRP‡ (mg/l)
Anti-YKL-39 antibody negative patients (n=80)	56.2 (13.4)	14.3 (14.1)	3.0 (1.1)	163 (293)	36.5 (25.6)	19 (22)
Anti-YKL-39 antibody positive patients (n=7)	63.3 (10.9)	15.8 (11.6)	3.3 (1.0)	128 (112)	42.8 (22.9)	11 (12)

*Stage, radiographic staging of the joint destruction by the criteria reported by Steinbrocker *et al*¹⁴
†RF = rheumatoid factor; ESR = erythrocyte sedimentation rate; CRP = C reactive protein.

systemic autoimmune diseases, respectively. Figure 1B summarises the results of ELISA for YKL-39 in serum samples from 87 patients with RA and 47 patients with SLE. Seven of 87 patients with RA were positive for the anti-YKL-39 antibody. In contrast, no serum samples from patients with SLE or healthy control subjects were positive.

Considering that YKL-40 has been reported to be an autoantigen in RA, and that YKL-39 and YKL-40 share 52% amino acid sequence homology, overlapping of antigenicity of the two proteins might be expected. We thus compared the reactivity of the serum samples from patients with RA with recombinant YKL-40. Only one sample reacted with recombinant YKL-40 protein (fig 1C). Thus autoimmunity to YKL-39 seemed to be more prevalent than to YKL-40. In addition, the anti-YKL-40 positive serum did not recognise recombinant YKL-39 (figs 1B and C).

To confirm further the specificity of anti-YKL-39 and anti-YKL-40 antibodies we performed ELISA using serially diluted positive serum samples and western blotting. ELISA results showed that OD values to recombinant YKL-39 increased with increasing serum concentration, whereas reactivity to recombinant YKL-40 and MBP alone was not increased in any anti-YKL positive serum samples. Figure 2A shows representative cases. This was also the case in the anti-YKL-40 positive serum sample. Furthermore, western blotting results also indicated that anti-YKL-39 reactivity was independent of anti-YKL-40 reactivity (fig 2B).

In addition, we immunoprecipitated a native form of YKL-39 from the cell lysate of HCS-2/8 using the purified anti-YKL-39 autoantibodies. As a result, the native YKL-39 protein was found to be precipitated by them (fig 2C). This indicates that the autoantibodies to YKL-39 can bind to the native form of YKL-39 as well as to the recombinant YKL-39.

CLINICAL FEATURES AND ANTI-YKL-39 ANTIBODIES

We compared clinical and laboratory data from anti-YKL-39 antibody positive and antibody negative patients with RA. Table 1 summarises the results. We found no significant differences between the two patient groups in age, disease duration, radiographic staging of the joint destruction, serum rheumatoid factor titres, erythrocyte sedimentation rates, and serum C reactive protein concentration. Also, other biochemical laboratory variables and blood cell counts tested did not differ between the two groups generally (data not shown).

Discussion

We have shown in this study the presence of autoimmunity to YKL-39, a chondrocyte derived protein, in a subgroup of patients with RA, using a recombinant YKL-39 fusion protein. We also found that anti-YKL-39 autoantibody was more prevalent than autoantibody to a homologous protein, YKL-40, which has been reported to be an autoantigen in RA.

The anti-YKL-39 autoantibody was found in 8% of patients with RA—a relatively low frequency. Thus the presence of the anti-YKL-39 autoantibody would not be a sensitive marker for diagnosis of RA. However, as no patients with SLE were positive for anti-YKL-39 antibody, production of anti-YKL-39 antibody is likely to result from specific recognition of antigens derived from a joint component in RA rather than being non-specifically produced in autoimmune disorders. Although concentrations of YKL-39 in joint fluid have not been analysed, we would expect more YKL-39 peptide secretion into the joint cavity when the cartilage matrix is degraded. However, no significant correlation was found in this study between the anti-YKL-39 antibody titre and joint destruction as evaluated by radiographic findings. Further, it is not determined whether the anti-YKL-39 autoantibodies are specific for RA or not. To clarify these points, a larger sample, including patients with other joint disease, needs to be analysed.

We have also shown in this study that the autoantibody responses to YKL-39 and YKL-40 were independent. This finding suggests that even though these two closely related cartilage derived proteins share homologous amino acid sequences, recognition of these autoantigens by T cells is distinct, without cross reactivity. Analysis of the T cell response to YKL-39 is in progress in our laboratory.

Anti-YKL-39 antibody was more prevalent (7/87 patients; 8%) than anti-YKL-40 antibody (1/87 patients; 1%) in our study. In a previous study YKL-39 was reported to occupy only 4% of the secreted proteins from cultured chondrocytes, whereas YKL-40 was more dominant (33%). None the less, YKL-39 seemed to be more antigenic than YKL-40. For the cellular response to YKL-40, Verheijden *et al* reported that, at most, 44% of patients with RA exhibited a peripheral T cell response to YKL-40 derived peptides,⁸ though the response to the whole YKL-40 molecule was not analysed. Taken together, these results suggest that the immune response to YKL-40 may be shifted towards cellular rather than humoral immunity. Alternatively, the major antigenic determinants of YKL-40 may be conformation dependent and thus not appropriately config-

ured in bacterially expressed recombinant YKL-40. However, commercially available anti-YKL-40 antibodies, which were obtained by immunisation of rabbits with purified native YKL-40, clearly bound to our recombinant YKL-40 (data not shown). This indicates that at least a part of the epitopes on the native YKL-40 molecule was expressed on the recombinant YKL-40. Thus the second possibility would be less likely.

We obtained recombinant fusion proteins of YKL-39 and YKL-40 from HCS-2/8, a chondrosarcoma cell line, which was reported to produce various chondrocyte related proteins.¹³ This cell line was confirmed in previous studies to conserve chondrocyte characteristics. Thus HCS-2/8 should be a useful tool to investigate possible immunological roles of chondrocytes, including antigenicity of their secreted proteins, as described here.

In conclusion, we have described, for the first time, the presence of autoimmunity to YKL-39, a recently cloned cartilage derived protein, in patients with RA. Identification and characterisation of such joint derived autoantigens will be helpful in understanding the pathogenesis of joint destruction in RA.

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