EXTENDED REPORT

Characterisation of cartilage intermediate layer protein (CILP)-induced arthropathy in mice

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Objectives: To characterise cartilage intermediate layer protein (CILP)-induced arthropathy in mice.**Methods:** The first and second halves of the nucleotide triphosphate pyrophosphohydrolase (NTPPHase) non-homologous region of human CILP were prepared as recombinant proteins (C1 and C2, respectively), including three overlapping fragments of C2 (C2F1, C2F2, and C2F3). C57BL/6 mice were immunised with these proteins to induce arthritis. In addition, a separate group of mice were immunised repeatedly with the mixture of C1 and C2 to see the effect of chronic immunisation. Arthritis developed in the mice, and cellular and humoral immune responses against CILP were analysed.**Results:** Immunisation with C2 and with the mixture C2F1/C2F2/C2F3 caused the severest arthritis to develop in mice. Immunisation with one of C1, C2F1, C2F2, or C2F3 caused milder arthritis, even though each of the fragments carried T cell epitopes. Immunisation either with C1 or C2 alone evoked cellular and humoral immune responses to both the C1 and C2 proteins. Further, the repeated immunisation with the C1/C2 mixture caused tendon calcification and bone irregularity, together with decreased NTPPH activity.**Conclusions:** The results show that multiple T cell epitopes are needed for the development of CILP-induced arthritis, and present the characteristic new model of mild arthropathy accompanied by extra-articular calcifications. An immune response to putative murine CILP/NTPPH may be involved in the ectopic calcifications in the arthritic mice.

Extracellular matrix of the cartilage is mainly composed of type II collagen and proteoglycans. Autoimmunity to such major components of the cartilage has been shown to cause severe and acute destructive polyarthritis in animals, such as type II collagen-induced arthritis (CIA)^{1,2} and proteoglycan core protein-induced arthritis.³⁻⁵ They have been investigated, in part, as a model of rheumatoid arthritis (RA) in humans. However, it is not fully understood whether autoimmunity to other minor components of cartilage causes such destructive arthritis as RA to develop. Recently, immunisation of mice with a cartilage derived chitinase family protein human cartilage glycoprotein 39 (HC gp39) was reported to develop severe polyarthritis,⁶ whereas immunisation with YKL-39, a similar cartilage related protein homologous to HC gp39, develops only mild arthritis.⁷ These findings indicate that the characteristics of immune mediated arthritis/arthropathy depend on the individual antigens immunised.

Cartilage intermediate layer protein (CILP) was cloned as a cartilage related protein which resides in the middle layer of the cartilage matrix, with a homologous region to porcine nucleotide triphosphate pyrophosphohydrolase (NTPPHase) in its carboxyl terminal half.⁸⁻⁹ It has been suggested that the CILP/NTPPH might participate in extracellular inorganic pyrophosphate (PPI) production.¹⁰ Expression of CILP is reported to be up regulated during aging^{8,11} and also in patients with the early stages of osteoarthritis.^{8,9} We recently reported that CILP was recognised as an autoantigen in patients with osteoarthritis and RA, and that immunisation of mice with recombinant CILP proteins developed mild but chronic polyarthritis.¹²

In this present study we attempted to characterise the arthropathy induced by CILP immunisation more extensively.

MATERIALS AND METHODS

Mice

Female 8-10 week old C57BL/6 mice were obtained from Nippon SLC Inc (Tokyo, Japan). Animal care was in accordance with the institutional guidelines.

Preparation and isolation of recombinant CILP proteins

As previously described, complementary DNA fragments that encoded the first half (cDNA^{C1}) and the second half (cDNA^{C2}) of the non-NTPPHase homologous region of CILP were obtained from mRNA of human articular chondrocytes.¹² The C2 region was further divided into three regions (C2F1: 396 bp, C2F2: 399 bp, C2F3: 351 bp), and their respective cDNA fragments were obtained from cDNA^{C2} (fig 1).

Production and purification of the CILP-βGal fusion proteins and the CILP-MBP fusion proteins were performed as described previously.^{13,14} Briefly, each of the cDNA fragments that encode the C1, C2, C2F1, C2F2, and C2F3 regions were subcloned into plasmid expression vectors pTEX-eHis, which produced β-galactosidase (βGal) fusion proteins or into pMAL-eHis, which produced MBP fusion proteins, respectively.

Induction of arthritis

Fifty micrograms each of the single or mixed CILP-βGal fusion proteins was mixed with Freund's incomplete adjuvant (FIA) at 1:1 and injected into the right footpad of eight mice subcutaneously. Eight control mice received the same amount of the fusion partner of βGal alone with FIA. After 14 days the mice were immunised again with the same amounts of the protein subcutaneously into the root of the

Abbreviations: BSA, bovine serum albumin; CIA, collagen-induced arthritis; CILP, cartilage intermediate layer protein; ELISA, enzyme linked immunosorbent assay; FCA, Freund's complete adjuvant; FIA, Freund's incomplete adjuvant; HC gp, human cartilage glycoprotein; MBP, maltose binding protein; NTPPHase, nucleotide triphosphate pyrophosphohydrolase; OD, optical density; PBST, phosphate buffered saline-Tween-20; PPI, inorganic pyrophosphate; RA, rheumatoid arthritis; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; SI, stimulation index

Figure 1 Recombinant CILP proteins used in this study. (A) Scheme showing the human CILP and recombinant CILP proteins prepared in this study. The first and second halves of the NTPPHase non-homologous region of the human CILP molecule (C1 and C2) and three regions that overlapped and covered the C2 region (C2F1, C2F2, and C2F3) were prepared as fusion proteins. (B) Recombinant CILP proteins produced as a maltose binding protein (MBP) fusion protein. Each of the C1, C2, C2F1, C2F2, and C2F3 regions was expressed as an MBP fusion protein. Purified protein samples were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto a nitrocellulose membrane, and stained with Ponceau-S.

tail. Further, as a separate experiment, a mixture of C1- β Gal and C2- β Gal was used for repeated immunisations. Briefly, eight mice were immunised with 50 μ g of the mixture with an equal weight of Freund's complete adjuvant (FCA; Sigma-Aldrich, USA) intracutaneously, and this was then followed by four boosting immunisations on the root of the tail with 50 μ g of the same proteins using FIA on days 7, 28, 49, and 70.

Scoring of arthritis

The incidence of arthritis was assessed daily. Clinical severity of arthritis was graded according to a published scoring method.^{15, 16} All the mice were observed for at least 90 days after the first immunisation.

Enzyme linked immunosorbent assay (ELISA)

The reactivity of serum samples obtained from the immunised mice for the CILP-MBP proteins was determined by ELISA as previously described.¹³ Briefly, each well of a multititre plate (Cook; Dynatech, Alexandria, VA) was coated with 2 μ g per well of CILP-MBP or bovine type II collagen in 100 mM carbonate buffer (pH 9.4) at 4°C overnight. Thereafter, the plates were washed five times in phosphate buffered saline (PBS, pH 7.4) containing 0.05% Tween-20 (PBST), and incubated in PBST containing 3% bovine serum albumin (BSA) for 1 hour at room temperature, and then washed again. To absorb reactivity against bacterial proteins and the fusion partner, the murine serum samples were preincubated in PBST-3% BSA with bacterial lysate containing non-recombinant pMAL-eHis products, and then incubated in the coated wells in triplicate for 16 hours at 4°C. After washing, the plates were incubated for 1 hour with horseradish peroxidase conjugated antimouse IgG antibodies (Zymed Laboratories, Inc), washed again, and allowed to react with an *o*-phenylenediamine of a substrate of horseradish peroxidase. The reaction was terminated by addition of 9N H₂SO₄, and absorbance at 492 nm was measured by Titertek Multiscan (Biorad). For each sample, the value of the optical density (OD) for MBP was subtracted from the OD for the CILP-MBP proteins to obtain OD values for CILP (OD sample). The reactivity of each serum with the CILP proteins was expressed in units according to the following formula: binding units of samples = (OD sample/(mean OD of normal sera+3 SD of normal sera)) $\times$ 100. Normal serum samples were obtained from non-treated mice.

Cellular proliferation to the fragments of CILP

Mononuclear cells were separated from the mice spleens and draining lymph node cells by a standard gradient centrifugation method with Ficoll-Paque (Pharmacia Biotech AB, Sweden). The mononuclear cells were maintained in RPMI medium supplemented with heat inactivated 10% fetal calf serum. Proliferative responses of the mononuclear cells against the CILP-MBP proteins or MBP alone were quantified by the ³H labelled thymidine uptake assay, as described previously.¹² The cell proliferation was used to calculate a stimulation index (SI) using the following formula: SI = (cpm stimulated by the fusion protein)/(cpm stimulated by MBP of the fusion partner alone). All experiments were performed in triplicate.

Histopathological analysis

At the end of the study the mice were killed and their hind feet were removed, fixed in 10% formalin, and decalcified for 24 hours. Subsequently, they were dehydrated in graded alcohol concentrations, and then processed for embedding in paraffin wax. Each section throughout the joint was cut at 6 μ m on a microtome and stained with haematoxylin and eosin or safranin O.

NTPPHase activity

The enzyme activities in the serum samples of the mice were determined as described previously.¹⁷ Briefly, 10 μ l of serum sample diluted in 90 μ l of 10 mM Tris-HCl buffer (pH 7.8) was mixed with an equal volume of 2 mM thymidine monophosphate *p*-nitrophenyl ester (Sigma Aldrich) in 50 mM sodium carbonate buffer (pH 9.8). Then the solution was incubated for 1 hour at 37°C. The reaction was stopped by addition of 800 μ l 0.1N NaOH, and absorbance was determined at 410 nm. All measurements were done in duplicate.

Radiographic analysis

Conventional radiographs were obtained on the day on which clinical symptoms appeared (day 21) and at a later stage (day 100) using *x* rays (Hitachi Co, Japan) at 2 mA and 35 kV for 100 ms, while the mice were under anaesthesia.

Statistical analysis

Results were analysed by Bonferroni/Dunn Student's *t* test. All statistical analyses were performed using personal

Figure 2 Arthritis developed by the immunisation of CILP proteins. (A) Arthritis developed by immunisation with C1-βGal, C2-βGal, or βGal alone as a control. The second immunisation was performed on the 14th day after the first immunisation. Error bars show mean (SD). (B) Arthritis developed by immunisation with C2-βGal, C2F1-βGal, C2F2-βGal, C2F3-βGal, or βGal alone as a control. (C) Arthritis developed by immunisation with C2-βGal, mixture of C2F1-βGal, C2F2-βGal, and C2F3-βGal, or βGal alone as a control.

computer based statistical software. Values are shown as mean (SD).

RESULTS

Expression of recombinant CILP fusion proteins

According to the reported nucleotide sequence of human CILP,⁹ we prepared recombinant CILP proteins (fig 1). Specifically, in addition to the recombinant CILP proteins fused with βGal (C1-βGal, C2-βGal, C2F1-βGal, C2F2-βGal, and C2F3-βGal), as described in our previous study,¹² here we additionally prepared the same panel of recombinant CILP proteins, which were identical but fused with MBP (C1-MBP, C2-MBP, C2F1-MBP, C2F2-MBP, and C2F3-MBP, fig 1B). This enabled us to detect immune reactions to CILP in mice immunised with the CILP-βGal proteins, avoiding detection of immune reaction to βGal. Each of the produced and purified fusion proteins showed a single band with its expected molecular weight by SDS-PAGE separation (fig 1B and data not shown). Thus they were considered to be of sufficient quality to use in the following experiments.

Arthritogenicity of the recombinant CILP proteins

We previously demonstrated the arthritogenicity of CILP by immunising mice with recombinant CILP proteins. Extending the study, we here investigated whether dominant arthritogenic epitope(s) exist or not. Specifically, we immunised C57BL/6 mice with C1-βGal, C2-βGal, and βGal alone separately and evaluated the occurrence of arthritis in each group. We found that the C2 region was more potent in inducing arthritis than the C1 region (fig 2A). The arthritis score provided by the C2-βGal immunisation was nearly equal to that obtained by immunisation of the C1-βGal/C2-βGal mixture (data not shown). Thus we concluded that major arthritogenic epitope(s) existed in the C2 region.

Next, we tried to identify the arthritogenic epitope(s) in the C2 region. Specifically, we immunised mice with C2F1-βGal, C2F2-βGal, and C2F3-βGal separately and evaluated arthritis. Figure 2B shows that the arthritis score obtained by immunisation of each of the three fusions was much lower than that obtained by C2-βGal immunisation, even though the arthritis score of the C2F2-βGal immunised mice was significantly higher than that of the βGal immunised mice as a negative control. The arthritis scores of the C2F1-βGal or C2F3-βGal immunised mice were similar to that of the negative control (fig 2B). This indicates that multiple epitopes in the C2 region are required for development of the arthritis. To confirm this, we immunised mice with a mixture of C2F1-βGal, C2F2-βGal, and C2F3-βGal and compared the arthritis scores with that of the C2-βGal immunised mice. Figure 2C shows that the mice immunised with the mixture developed arthritis with a score which was comparable with that of the C2-βGal immunised mice.

T cell proliferative response against CILP fragments

We investigated T cell epitope distribution on CILP in these arthritic mice as follows. The proliferative response of spleen cells from non-treated or immunised mice to all five fragments was measured by the [³H]thymidine incorporation assay. To avoid the effects of βGal, we used MBP fusions as detecting antigens. As a result, spleen cells from the C1-βGal immunised mice showed a proliferative response to both C1-MBP and C2-MBP (fig 3A). Similarly, spleen cells from the C2-βGal immunised mice showed a proliferative response to both C1-MBP and C2-MBP (fig 3B). It thus was confirmed at a cellular level that the immune response to the C1 region spread to the C2 region and vice versa. Interestingly, the C2-βGal immunised mice showed a much stronger proliferative response against C2-MBP than that against C1-MBP in the C1-βGal immunised mice (fig 3B). In addition, cellular

Figure 3 The cellular responses to the CILP proteins. The cell proliferation was used to calculate a stimulation index using the following formula: $SI = (\text{cpm stimulated by the fusion protein}) / (\text{cpm stimulated by MBP of the fusion partner alone})$. Error bars show mean (SD).

responses to each fragment of C2—that is, to C2F1-MBP, C2F2-MBP, and C2F3-MBP, were comparable with that to C2-MBP in the C2 immunised mice (fig 3C).

Humoral responses against CILP fragments

Next, we investigated humoral immune responses to the CILP proteins. Firstly, we measured titres of anti-C1 and anti-C2 antibodies both in the C1-βGal and C2-βGal immunised mice. C1-MBP and C2-MBP fusion proteins were used as a capturing antigen in the ELISA, to avoid detecting anti-βGal antibodies. As a result, antibodies to both C1-MBP and C2-MBP were detected in each of the C1-βGal and C2-βGal immunised mouse groups (fig 4). In addition, the anti-C1 and anti-C2 antibodies did not cross react with each other (data not shown). This indicates that the immune response to the C1 region spread to the C2 region and vice versa. We further investigated the location of B cell epitope(s) on the C2 region using three small fragments of C2F1-MBP, C2F2-MBP, and C2F3-MBP, which collectively covered the whole C2 sequence (fig 1). As a result, we found that the C1 and C2 immunised mice were positive for antibodies against each of the three fragments; thus all the C2F1, C2F2, and C2F3 fragments were shown to carry B cell epitopes (data not shown).

Figure 4 Antibody titres to the C1 and C2 regions in the CILP immunised mice. Pooled serum samples of each group of mice ($n=8$) immunised either with C1-βGal, C2-βGal, or βGal alone were used to detect antibodies to C1-MBP and C2-MBP in an ELISA. Error bars show mean (SD).

Histochemical and radiographic characterisation of the CILP-induced arthropathy

As described in our previous study, immunisation with CILP-βGal developed only mild arthritis in all four tested mouse strains,¹² which was in contrast with the severe erosive arthritis usually seen in CIA. To clarify further the arthritogenicity of CILP, especially in long term immunisation, we immunised mice with a mixture of C1-βGal and C2-βGal five times by day 70. As a result, mild synovitis—that is, proliferation of synovial cells and infiltration of mononuclear cells (figs 5A and B) and a thin and degraded cartilage layer were seen (figs 5C and D).

In these repeatedly immunised mice we found two interesting radiographic findings. Firstly, the CILP-βGal, but not βGal, immunised (control) mice developed irregular bone cortex in the metatarsals (fig 6C, arrow head). Staining with safranin O confirmed the irregularity and thickness of the bone cortex, as a representative case of calcaneum was shown (fig 7A). Secondly, the CILP-βGal, but not βGal, immunised mice developed calcification of the tendocalcaneus (fig 6C, arrow). This was also confirmed histochemically as mild (fig 7B). Further, irregular bone formation was also seen (fig 7C).

Pyrophosphate generated by NTPPHase, possibly including that of CILP/NTPPHase, is reported to be a major inhibitor of calcification.^{10 18–21} Therefore, we suggested that the NTPPHase activity of putative murine CILP in the human CILP immunised mice was impaired by an immune reaction, resulting in the calcification. To explore this hypothesis, we measured NTPPHase activity in the serum samples of the mice immunised repeatedly with C1/C2. Figure 8 shows that NTPPHase-specific activities were decreased in the sera of the mice significantly, compared with non-treated mice or mice repeatedly injected with βGal alone.

DISCUSSION

Our findings on CILP-induced arthritis in mice can be summarised as follows: firstly, the arthritis caused by the immunisation of CILP was long lasting but mild. This was seen both in mice receiving a single immunisation and in mice repeatedly immunised (data not shown). Secondly, multiple arthritogenic epitopes in the C2 region were required for development of the arthritis. Thirdly, immunisation of the C1 protein developed an immune reaction to the C2 region and vice versa, indicating development of autoimmunity to mouse CILP, which is supposed to exist but has yet not been

Figure 5 Histological analysis of the immunised paw by safranin O staining. Proliferation of the synovial tissue with mild inflammatory cell infiltration was seen (indicated by asterisks) in the midtarsal joint (A) and the ankle joint (B). Surface irregularity and cartilage degeneration are seen in the affected ankle joint (C). Magnified view of the rectangular area of (C) is shown in (D).

Figure 6 Radiographic findings in the CILP immunised mice. (A) Non-treated mouse; (B) β Gal immunised mouse; (C) mouse immunised with a mixture of C1- β Gal and C2- β Gal. Irregular thickness of the bone cortex (arrow head) and calcification of the tendocalcaneus (arrow) were seen.

identified. Fourthly, despite the mildness of the arthritis, calcification of the tendocalcaneus and irregularity of the bone cortex were seen. The decreased NTPPHase activity demonstrated here might be involved in the ectopic calcification.

On the first and second points, studies of animals immunised with joint related antigens have provided some clues to potential mechanisms for antigen mediated arthritis/arthropathy. The properties of such arthritis/arthropathy would depend on individual antigens, even though they are categorised as cartilage-specific. In the case of major components of cartilage—for example, immunisation of type II collagen, severe destructive arthritis develops.^{1 2 22} Similarly, proteoglycan induced severe arthritis.^{4 5} However, the arthritogenic nature of minor non-collagenous components has been rather unclear. As described previously, immunisation of mice with HC gp39 causes the development of moderate arthritis,⁶ whereas immunisation with YKL-39 develops mild arthritis.

We previously reported that immunisation of CILP produced relatively mild but longlasting arthritis.¹² In this study we performed repeated immunisation of CILP, but the severity of the arthritis did not change compared with that of mice receiving a single immunisation. So the arthritogenicity of CILP appeared to be much weaker than type II collagen or aggrecan, immunisation of which caused severe arthritis to develop in mice.³ Interestingly, dominant arthritogenic epitopes are documented in the cases of CIA and aggrecan-induced arthritis.^{2 3} We therefore first tried to identify such dominant arthritogenic epitopes in CILP. As shown here, the severity of C2 immunised mice is similar to that of C1/C2 immunised mice, and higher than that of C1 immunised mice (fig 2A). Thus the C2 region alone carries major arthritogenic epitopes. However, each of the separate C2F1, C2F2, and C2F3 fragments did not show such arthritogenicity, although a mixture of C2F1, C2F2, and C2F3 caused arthritis similar to that of the C2 immunised mice (figs 2B and C). This indicates that multiple epitopes are involved in the pathogenic immune

Figure 7 Histological manifestations of the immunised paw stained by safranin O. (A) The metatarsal cortex showed irregular thickness (arrows). (B) Calcification was developed within the calcaneal tendon (arrow), and the arrangement of tendon fibres was irregular and redundant (arrowhead). Asterisk shows the tip of the calcaneus. (C) Bone marrow was filled with osteal tissue and an osteal projection was found in the front of the lower tibia (arrow).

reaction in this model. Together with the reports on CIA and aggrecan-induced arthritis,^{1,3} immunodominant epitopes may be needed to produce severe destructive arthritis. Further, the cellular responses to each of the fragments C2F1, C2F2, and C2F3 were comparable with that to C2. This indicates that C2F1, C2F2, and C2F3 each carry immunodominant epitope(s), and thus immunodominant epitopes would be different from the arthritogenic epitopes.

On the third point, immunisation of C1 alone or C2 alone led to an immune reaction to both C1 and C2. As the generated anti-C1 antibodies did not react with C2 and vice versa (data not shown), the immune reactions to C1 and C2 were not cross reactive. Thus we should consider the involvement of a third molecule that intervenes between C1 and C2. An important possible candidate might be a mouse CILP molecule. Even though the protein has not been identified, its reference sequence supported by expressed sequence tags evidence was presented recently (XP-146393, GI: 20864514). According to the reference sequence, the putative mouse CILP molecule has about 50% amino acid sequence identity with the human CILP molecule (data not shown). Therefore,

Figure 8 NTPPHase activity in the sera of mice immunised with CILP proteins. Mice were immunised with the mixture of C1/C2 with FCA followed four times by a booster immunisation over a period of 70 days. Control mice received βGal and FCA with the same protocol. Error bars show the mean (SD). NTPPHase-specific activities were decreased in the sera of the mice significantly compared with non-treated mice or mice repeatedly injected with βGal alone ($p < 0.05$).

immunisation of C2 would generate autoimmunity to putative mouse CILP, and the observed anti-C1 immune reaction would be part of the immune reaction to the whole molecule of mouse CILP. This scenario can be applied to an explanation for the fourth finding as follows.

We found calcification of the tendocalcaneus and irregularity of the bone cortex after repeated immunisation of CILP. The COOH terminus of the CILP molecule was very similar (90%) to a porcine NTPPHase.⁹ The NTPPHase generates inorganic pyrophosphate (PPi) by hydrolysing extracellular nucleoside triphosphate (ATP), and PPi is a major inhibitor in calcification, such as soft tissue calcification and bone mineralisation.¹⁸ In fact, deficiency of PC-1, one of the PPi-generating NTPPHases, causes ankylosis in mice^{23–26} and infantile arterial calcification with periarticular calcification.²⁷ We may thus speculate that the autoimmunity to the putative mouse CILP molecule, which might spread to other NTPPHases, reduced the NTPPHase activity. In addition, possibly the autoantibody against CILP/NTPPH indirectly affects the PPi metabolism by, for example, a negative feedback mechanism at the levels either of extracellular ATP accumulation or the NTPPHase, or by affecting the function of other pyrophosphate related proteins such as ank.²⁸ However, because the NTPPHase activity of CILP is still controversial,²⁹ the mechanism should be further investigated. For example, it would be helpful to detect the functional activity of anti-CILP antibody or sera in directly blocking the NTPPHase activity.

In conclusion, we demonstrated that autoimmunity to CILP causes mild polyarthritis to develop, and when the antigen stimulation is repeated, ectopic calcification on tendons and abnormal mineralisation of the bone cortex are induced in the immunised mice. The overall clinical features of the arthropathy may be determined by a balance between the antigenic potential and the functional loss of the sensitised cartilage related antigens.

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