swollen joint count, C-reactive protein (CRP) level, IgM rheumatoid factor (IgM-RF) positivity, and the presence of anti–cyclic citrullinated peptide (anti-CCP) antibodies (2,3). Prediction scores vary from 0 to 14 and correspond to the percent chance of developing RA at 1 year (3). Van der Helm-van Mil et al examined 570 patients with UA and found that, at cutoff levels of ≤6 and ≥8, the negative predictive values (NPVs) and positive predictive values (PPVs) were 91% and 84%, respectively (3), which indicates a score of ≥8 for initiating treatment and a score of ≤6 for withholding treatment.

The above prediction rule indicates that clinical manifestation is still the gold standard in detecting synovitis, which is also mentioned by the European Standing Committee for International Clinical Studies Including Therapeutics (ESCISIT) (4); however, the expert committee of the European League Against Rheumatism defines the importance of imaging methods such as magnetic resonance imaging (MRI) and ultrasonography as being more sensitive than clinical examination or plain radiography for the detection of early joint damage in early arthritis (4). For instance, plain radiography does not detect synovitis, early bone erosion, or bone edema, whereas MRI is able to do so (5). Given the utility of the detection of early joint damage by MRI, our investigation has focused on how to identify patients with early arthritis likely to progress to RA by not using MRI of the wrists and finger joints and serologic variables (6–8). Our previous reports have shown that MRI-proven symmetric synovitis, MRI-proven bone changes (bone edema or bone erosion), and the presence of serologic autoantibodies (anti-CCP antibodies or IgM-RF) upon admission are predictive factors for early-stage RA (6). However, some of the patients examined in the previous studies already fulfilled international disease criteria for RA or osteoarthritis upon admission (6,7), which was a weak point of our previous prediction rule.

The present study is a reevaluation of our prediction rule in patients with UA. Additionally, we examined correlations with a predictive role such as that reported by the Leiden Early Arthritis Cohort.

PATIENTS AND METHODS

Patients. The Early Arthritis Clinic opened in 2001 as a part of the Unit of Translational Medicine, the Department of Immunology and Rheumatology, and the Graduate School of Biomedical Sciences at Nagasaki University. Patients were referred from an area in the western part of Japan, Nagasaki Prefecture, which has ~450,000 inhabitants. From this clinic, 129 patients with UA were included in the present study; their disease status was formally confirmed by a rheumatologist for at least 1 year. We have examined MRI results of both wrists and finger joints for all of the subjects; therefore, all of the 129 patients with UA expressed rheumatic manifestations of the wrists and finger joints at study entry. The characterization of UA upon admission was determined as previously reported (3), i.e., as arthritis that could not be classified according to ACR criteria within 2 weeks after being included in the study, when laboratory and radiographic results were available. At a prospective followup of 1 year, 75 patients were found to have progressed to RA based on the 1987 ACR criteria for RA (1).

Baseline clinical manifestations and variables included sex, age, localization of arthritis, morning stiffness score measured on a 100-mm visual analog scale, the number of tender joints, the number of swollen joints, the CRP level (measured by latex turbidimetric immunosorbent assay; Daiichi Pure Chemicals, Fukuoka, Japan), IgM-RF positivity (measured by latex-enhanced immunonephelometric assay with a cutoff value of 14 IU/ml; Dade Behring, Marburg, Germany), positive status for anti-CCP antibodies (measured by enzyme-linked immunosorbent assay [ELISA] with a cutoff value of 4.5 units/ml; DIASTAT Anti-CCP; Axis-Shield, Dundee, UK), matrix metalloproteinase 3 (measured by ELISA with cutoff values of 59.7 ng/ml for women and 121.0 ng/ml for men; Daiichi Pure Chemicals) (9), and MRI of both wrists and finger joints, as previously described (6–8). All variables were examined on the same day, as previously reported (6–8). Each patient provided a signed consent form to participate in the study, which was approved by the Institutional Review Board of Nagasaki University.

MRI of the wrists and finger joints. MRI of both wrists and finger joints were acquired using a 1.5T system (Sigma; General Electric Medical Systems, Milwaukee, WI) with an extremity coil. Coronal T1-weighted spin-echo (repetition time [TR] 450, echo time [TE] 13) and STIR (TR 3,000, TE 12, T1 160) images were also acquired. The images were evaluated for bone edema, bone erosion, and synovitis in 15 sites in each finger and wrist, including the distal radioulnar joint, the radiocarpal joint, the midcarpal joint, the first carpometacarpal joint, the second through fifth carpometacarpal joints (together), the first through fifth metacarpophalangeal joints, and the first through fifth proximal interphalangeal joints separately (a total of 30 sites in both hands), as we recently reported (6–8). The presence of synovitis, bone edema, and bone erosion was evaluated by 2 experienced radiologists (MU, ST) as described by Lassere et al (10) and Conaghan et al (11), and decisions were reached by consensus. The evaluation of MRI features has been established by several groups (10–13); however, it is a complex task. Therefore, as we previously reported (6–8), we simply determined the presence or absence of synovitis, bone edema, and bone erosion on MRI after the intravenous injection of 0.1 mmoles/kg of gadolinium-diethylenetriamine (Magnevist, Schering, Germany). Our method is qualitative rather than quantitative; however, it is sensitive enough to identify early joint damage in patients with early-stage RA (6–8).

Assessment of disease status at 1 year and statistical analysis. Our previous reports have shown the preferential expression of MRI-proven symmetric synovitis, MRI-proven bone edema, MRI-proven bone erosion, IgM-RF, and anti-CCP antibodies in patients with early-stage RA (6–8). Logistic regression analysis of the previous study identified subjects with positive values for 2 or 3 of the 3 objective measures (anti-CCP antibodies and/or IgM-RF, MRI-proven symmetric synovitis, and MRI-proven bone

Table 1. Baseline characteristics of 129 patients with undifferentiated arthritis*

	RA progression (n = 75)	No RA progression (n = 54)	*P*†
Age, median (range) years	53 (25–80)	52 (16–79)	NS
Sex, male:female (% female)	17:58 (77.3)	12:42 (77.8)	NS
Duration of symptoms at baseline, median (range) months	3 (0.5–15)	3 (0.5–24)	NS
Morning stiffness, median (range) minutes	60 (0–960)	15 (0–960)	< 0.0005
Tender joint count, median (range)	7 (0–39)	4.5 (0–27)	< 0.05
Swollen joint count, median (range)	3 (0–26)	0 (0–24)	< 0.0001
DAS28 tender joint count, median (range)	6 (0–28)	4 (0–24)	< 0.005
DAS28 swollen joint count, median (range)	3 (0–23)	0 (0–22)	< 0.0001
HAQ score, mean ± SD	7.3 ± 4.3	4.8 ± 3.8	< 0.005
Patients' pain on a 100-mm VAS, mean ± SD	52.6 ± 27.1	52.4 ± 32.0	NS
Patients' global on a 100-mm VAS, mean ± SD	52.5 ± 25.4	56.4 ± 29.5	NS
1987 ACR criteria for RA‡			
Morning stiffness for 1 hour	41 (54.7)	15 (27.8)	< 0.005
Arthritis in ≥3 joints	42 (56.0)	10 (18.5)	< 0.00001
Arthritis of the wrists and finger joints	56 (74.7)	22 (40.7)	0.0001
Symmetric arthritis	41 (54.7)	11 (20.4)	< 0.0001
IgM-RF positivity	39 (52.0)	16 (29.6)	< 0.05
HLA–DRB1*0405 allele carriership	27 (36.0)	13 (24.1)	NS
DAS28-CRP, mean ± SD	4.31 ± 1.22	3.46 ± 1.33	< 0.0001
Serologic variables			
Anti-CCP antibody positivity	43 (57.3)	4 (7.4)	< 0.0001
IgM-RF and/or anti-CCP antibody positivity	50 (66.7)	18 (33.3)	< 0.0005
MMP-3 positivity	27 (36.0)	8 (14.8)	< 0.01
MMP-3 level, median (range) ng/ml	50.2 (0–1,250)	34.8 (10.66–419.6)	< 0.005
CRP positivity	51 (68.0)	16 (29.6)	< 0.0001
CRP level, median (range) mg/dl	0.50 (0.01–18.4)	0.10 (0–8.36)	< 0.0001
MRI features			
Synovitis positivity	68 (90.7)	30 (55.6)	< 0.0001
Symmetric synovitis positivity	56 (74.7)	22 (40.7)	< 0.005
Bone edema positivity	31 (41.3)	5 (9.3)	< 0.0001
Bone erosion positivity	22 (29.3)	5 (9.3)	< 0.0001
Bone edema and/or erosion positivity	36 (48.0)	9 (16.7)	< 0.0001

* Values are the number (percentage) unless otherwise indicated. RA = rheumatoid arthritis; NS = no significant difference; DAS28 = Disease Activity Score in 28 joints; HAQ = Health Assessment Questionnaire; VAS = visual analog scale; ACR = American College of Rheumatology; IgM-RF = IgM rheumatoid factor; CRP = C-reactive protein; anti-CCP = anti–cyclic citrullinated peptide; MMP-3 = matrix metalloproteinase 3; MRI = magnetic resonance imaging.
† Indicates the difference between RA progression and no RA progression.
‡ We did not refer to the duration of the components in the 1987 ACR criteria for RA: morning stiffness for 1 hour, arthritis in ≥3 joints, arthritis of the wrists and finger joints, and symmetric synovitis.

edema and/or bone erosion); the patients were classified as having early-stage RA with 82.5% sensitivity and 84.8% specificity (6). However, our previous study is somewhat inaccurate because some of the subjects were already classified as having early-stage RA or rheumatic diseases other than RA upon admission (6). Therefore, in the present study, all of the selected subjects were classified as having UA upon admission, having been evaluated by the same objective measures in comparison with the prediction rule by the Leiden Early Arthritis Cohort (3). For all tests (chi-square test, Mann-Whitney U test, and Spearman's rank correlation), P values less than 0.05 were considered significant.

RESULTS

Evaluation of the Leiden Early Arthritis Cohort prediction rule in 129 UA patients. We collected the demographic clinical manifestations, serologic data, and MRI features of 129 patients with UA upon admission (Table 1). As expected, these arthritis conditions were condensed in UA that progressed to RA, as compared with UA that did not progress to RA. Although the choice of therapies for the patients was based on the decision of each physician, the difference between clinical manifestations at baseline may reflect on the therapies within the first year. Therefore, the percentage of patients receiving disease-modifying antirheumatic drugs (DMARDs) and glucocorticoids was much higher in 75 patients with UA that progressed to RA than in 54 patients with UA that did not progress to RA. In regard to DMARDs, 63 (84.0%) of 75 patients with UA that progressed to RA were treated with DMARDs, including 39 patients with sulfasalazine, 13 patients with methotrexate, 2 patients with infliximab, and 1 patient with adalimumab, whereas only 3 patients (5.6%) received DMARDs among 54 patients with UA that did not progress to RA ($P < 0.0001$). In regard to glucocorticoids, 45 (60.8%) of 75 patients with UA that progressed to RA were

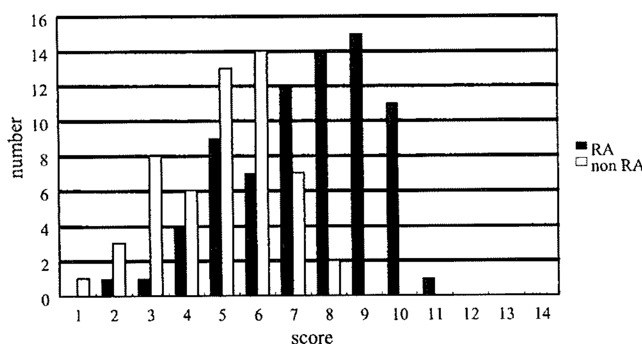

Figure 1. Scoring of 129 patients with undifferentiated arthritis by the Leiden Early Arthritis Cohort prediction rule; 75 had progressed to rheumatoid arthritis (RA) at 1 year. The distribution of the scores at baseline is shown, calculated according to the Leiden Early Arthritis Cohort prediction rule. Scores were rounded to the nearest number encoding in 0.5 or 0.0 (i.e., scores ≤0.5 are in category 0, scores >0.5 to 1.5 are in category 1, etc.), as described previously (3). RA = progression to RA group (n = 75); non RA = no progression to RA group (n = 54).

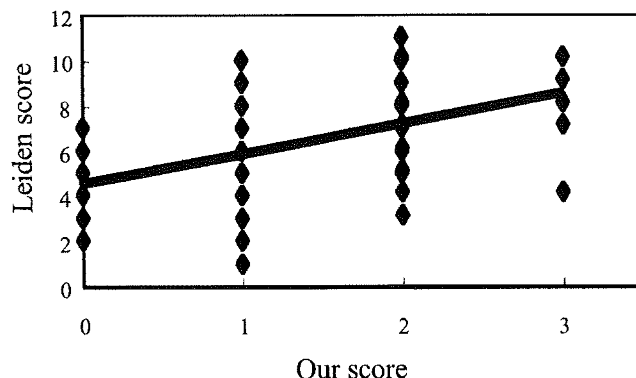

Figure 2. A positive correlation between the Leiden Early Arthritis Cohort prediction score and our prediction score. The statistical association was calculated by Spearman's rank correlation and a strong correlation was found between the 2 scores (R = 0.635, $P < 0.0001$).

treated with glucocorticoids, whereas 10 patients (18.5%) received glucocorticoids among 54 patients with UA that did not progress to RA ($P < 0.0001$). The diagnoses of 54 patients with UA that did not progress to RA at 1 year include Sjögren's syndrome (n = 11), osteoarthritis (n = 9), chronic hepatitis (n = 6), scleroderma (n = 3), palindromic rheumatism (n = 3), systemic lupus erythematosus (n = 2), fibromyalgia syndrome (n = 2), adult-onset Still's disease (n = 1), myofasciitis of unknown etiology (n = 1), polymyalgia rheumatica (n = 1), remitting seronegative symmetrical synovitis with pitting edema (n = 1), pseudogout (n = 1), and UA (n = 13). The Leiden Early Arthritis Cohort prediction scores were also calculated. Figure 1 shows the distribution of prediction scores, with a mean high score in patients with UA that progressed to RA of 8 versus 5 in patients with UA that did not progress to RA ($P < 0.0001$). According to cutoff levels of ≤6 and ≥8 in the Leiden Early Arthritis Cohort prediction rule, the NPV and PPV were 67.2% and 95.3%, respectively, in the present study population.

Evaluation of the prediction rule by serologic variables and MRI in comparison with the Leiden Early Arthritis Cohort prediction score. We evaluated the prediction rule by serologic variables and MRI in patients with UA, according to our previous report (6–8) as described above. The statistics demonstrate that the PPV was 79.7%, the NPV was 63.0%, the specificity was 75.9%, the sensitivity was 68.0%, and the accuracy was 71.3%. With respect to UA patients whose Leiden Early Arthritis Cohort prediction score was ≥8 (n = 43; PPV of 95.3% among the 41 of these 43 patients who progressed to RA by the Leiden Early Arthritis Cohort prediction score), our prediction rule was able to classify the progression to RA equally well (38 [88.4%] of 43 patients, not significantly different versus the Leiden Early Arthritis Cohort prediction score). In addition, with respect to UA patients whose Leiden Early Arthritis Cohort prediction score was ≤6 (n = 67; NPV of 67.2% among the 45 patients who did not progress to RA by the Leiden Early Arthritis Cohort prediction score), the present prediction rule predicted that 52 of 67 patients would not progress to RA (77.6%; not significantly equal to the Leiden Early Arthritis Cohort prediction rule). Fifteen of 67 patients whose Leiden Early Arthritis Cohort prediction score was ≤6 at baseline were classified as having RA by our score, and in fact, 7 patients progressed to RA at 1 year (PPV in this population is 46.7%). Accordingly, a positive correlation between the Leiden Early Arthritis Cohort prediction score and our score was clearly determined (R = 0.635, $P < 0.0001$) (Figure 2). Furthermore, the 3 critical objective characteristics were preferentially found among UA patients whose Leiden Early Arthritis Cohort prediction score was ≥8 as compared with those whose score was ≤6 (Table 2). Anti-CCP antibodies and MRI-proven bone edema were the most specifically distributed in UA patients with a score of ≥8 (Table 2).

Table 2. Distribution of MRI-proven symmetric synovitis, MRI-proven bone edema, anti-CCP antibodies, and IgM-RF in 129 patients with UA according to the Leiden Early Arthritis Cohort prediction score*

	Leiden Early Arthritis score		
	Score ≤6 (n = 67)	Score ≥8 (n = 43)	*P*
Symmetric synovitis	44.8	81.4	0.0001
Bone edema	10.4	48.8	< 0.0001
Bone erosion	13.4	30.2	0.032
Anti-CCP antibodies	6.0	86.0	< 0.0001
IgM-RF	26.9	67.4	< 0.0001
CRP level	38.8	69.8	0.0015
MMP-3	16.4	37.2	0.013
Progression to RA	32.8	95.3	< 0.0001

* Values are the percentage. Compared with patients with undifferentiated arthritis (UA) who scored ≤6, MRI-proven symmetric synovitis, MRI-proven bone edema, anti-CCP antibodies, and IgM-RF were densely distributed in the UA patients who scored ≥8. See Table 1 for definitions.

Table 3. Qualification of each variable at baseline for the prediction of progression to rheumatoid arthritis from undifferentiated arthritis*

	Sensitivity, %	Specificity, %	OR	*P*	95% CI	PPV, %	NPV, %	LR positive	LR negative	Accuracy, %
Serologic variables										
IgM-RF	52.0	70.4	2.57	< 0.05	1.53–4.34	70.9	51.4	1.76	0.682	59.7
Anti-CCP antibodies	57.3	92.6	16.8†	< 0.0001†	7.63–36.99	91.5†	61.0	7.74	0.461	72.1
MMP-3	36.0	85.2	3.23	< 0.01	1.73–6.03	77.1	48.9	2.43	0.751	56.6
MRI findings										
Symmetric synovitis	74.7	59.3	4.07	< 0.005	2.52–7.30	71.8	62.7	1.84	0.427	68.2
Bone edema	41.3	90.7	6.90†	< 0.0001†	3.34–14.29	86.1†	52.7	4.44	0.647	62.0
Bone erosion	29.3	90.7	4.07	< 0.0001	1.94–8.52	81.5	48.0	3.18	0.779	55.0

* OR = odds ratio; 95% CI = 95% confidence interval; PPV = positive predictive value; NPV = negative predictive value; LR = likelihood ratio; see Table 1 for additional definitions.
† Most significant in serologic variables or MRI findings.

Procedure for the improvement of PPV for the prediction of RA development by our objective measures. The present data showed a 79.7% PPV for the prediction of RA development using our objective measures, which is not sufficient evidence to recommend that physicians start administering DMARDs if the patients do not yet fulfill the established classification criteria. According to *P* values and odds ratios of independent predictive variables for the development of RA, anti-CCP antibodies as a serologic variable and bone edema on MRI were found to be the most specific (Table 3). Therefore, if UA patients tested positive for anti-CCP antibodies and showed MRI-proven bone edema, they will progress to RA within 1 year; the PPV in such cases was 100% (Table 4). In the case of the score in Table 4, we examined MRI-proven bone edema of the symptomatic hand instead of both hands. As shown in Table 4, the PPV in such cases was still 100%, whereas the sensitivity of detection was low as compared with both hands (16 patients by the symptomatic hand versus 22 patients by both hands, 27.3% reduction by the symptomatic hand).

DISCUSSION

In clinical practice, patients presenting with early arthritis frequently have an undifferentiated disease that may progress to polyarthritis by fulfilling the ACR criteria for RA, or they may have a more benign disease course. The ACR criteria have been criticized for their low discriminative ability in patients presenting with recent-onset arthritis (14–16). Therefore, a new set of criteria that applies to early UA and that identifies patients with UA who will progress to RA is needed, since a recent study strongly suggests that treatment is effective in the early phase of arthritis, before the disease is established (4).

To our knowledge, the present study is the first validation report of Japanese patients with UA using the Leiden Early Arthritis Cohort prediction rule. The Leiden Early Arthritis Cohort prediction rule is clinically useful, especially to identify patients who will progress to RA, i.e., those whose prediction score is ≥8. However, a difference was found in the low NPV of the Leiden score in our study population as compared with the original report (91% NPV in the original report from The Netherlands) (3). This could be due to the fact that the present study population may have included more RA patients with low disease activity whose disease developed from UA compared with the original study population in The Netherlands. In addition, the rate of progression to RA in this cohort is high as compared with previous observations, including the Leiden Early Arthritis Cohort (3,17). The Leiden Early Arthritis Cohort has identified that the presence of arthritis

Table 4. An achievement of 100% PPV for the development of RA from UA by a combination of anti-CCP antibodies and MRI-proven bone edema*

No. patients	Variables at baseline: Anti-CCP antibodies	Bone edema by both hands	Bone edema by the symptomatic hand	RA progression (n = 75)	No RA progression (n = 54)
22	Positive	Positive		22 (100)	0 (0.0)
25	Positive	Negative		21 (84.0)	4 (16.0)
14	Negative	Positive		9 (64.3)	5 (35.7)
68	Negative	Negative		23 (33.8)	45 (66.2)
16	Positive		Positive	16 (100)	0 (0.0)
31	Positive		Negative	27 (87.1)	4 (12.9)
10	Negative		Positive	5 (50.0)	5 (50.0)
72	Negative		Negative	27 (37.5)	45 (62.5)

* Values are the number (percentage). PPV = positive predictive value; RA = rheumatoid arthritis; UA = undifferentiated arthritis; anti-CCP = anti–cyclic citrullinated peptide; MRI = magnetic resonance imaging.

in the wrists and finger joints, as well as in the upper extremities at study entry, is an advantage in the progression of RA (3). All of the subjects in the present study expressed rheumatic manifestations of the wrists and finger joints; therefore, they could already be selected as being biased to the progression of RA. This discrepancy may cause a difference in the prediction efficacy of the Leiden Early Arthritis Cohort prediction rule toward the 2 cohort populations. A prospective clinical analysis of the present study population, including a radiographic joint damage study, is necessary to answer this question.

There is a significant difference between our score and that established by the Leiden Early Arthritis Cohort, with respect to not only the prediction rule but also to the selection of the variables. The Leiden Early Arthritis Cohort adopted a cutoff value of ≥8 for the PPV and ≤6 for the NPV for the prediction, whereas our score can draw a threshold of only one line of prediction. The Leiden Early Arthritis Cohort variables stress clinical manifestations; however, our scoring system gives weight only to serologic autoantibodies and early joint damage as verified by MRI. The NPV of the 2 prediction rules was similar (63.0% versus 67.2%), although the PPV was superior in the Leiden score (79.7% versus 95.3%). Nevertheless, our prediction rule identified 52 patients of 65 predicted upon admission, whereas the Leiden score identified only 41 patients of 43 predicted. In an attempt to improve the PPV, we demonstrated that the combination of anti-CCP antibodies with bone edema gave a 100% PPV in 22 patients (Table 4). Considering the significant correlation between the 2 rules, our prediction rule is considered to be equally valuable to predict the development of RA in patients with UA.

The ESCISIT states that clinical examination is still the gold standard in detecting synovial inflammation; however, the expert committee is aware of the importance of MRI in greater sensitivity for detection (4). In the case of the patients who progressed to RA that were identified by our prediction rule rather than by the Leiden score, MRI helped identify patients with UA who were not able to be identified by clinical manifestation. The result that our rule can predict the progression of RA whose Leiden Early Arthritis Cohort prediction score was ≤6 at baseline may reflect this notion. Our present data give clear evidence of MRI that is sensitive as well as clinically valuable for patients with early arthritis. Based on a combination of serologic anti-CCP antibodies, we suggest that a UA patient whose score is ≥2 should receive DMARDs early, especially if they both show MRI-proven bone edema and are anti-CCP positive. We also tried to simplify the method by using MRI of the symptomatic hand instead of both hands, in the case of seeking MRI-proven bone edema. In this case, detection sensitivity decreased by 27.3%, whereas the PPV was still 100%. This would show practical advantages for clinical use if a single-hand MRI is as good as both hands; however, additional studies by other groups are necessary.

It remains to be determined whether the present rule is also effective in predicting radiographic joint destruction. It is likely to be effective, since bone change in MRI as well as serologic autoantibodies are predictors for subsequent radiographic progression (10,11,16,17). The present prediction rule revealed that patients with early-stage RA with both MRI-proven bone edema and anti-CCP antibodies upon admission progressed with a high frequency to erosive disease (Tamai M et al: unpublished observations). However, a prospective analysis of the present study remains to be carried out in order to precisely answer these questions.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Eguchi had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.
Study conception and design. Tamai, Kawakami, Uetani, Aoyagi, Eguchi.
Acquisition of data. Tamai, Kawakami, Uetani, Takao, Arima, Iwamoto, Fujikawa, Aramaki, Kawashiri, Ichinose, Kamachi, Nakamura, Origuchi, Ida, Eguchi.
Analysis and interpretation of data. Tamai, Kawakami, Aoyagi.

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High Serum Cartilage Oligomeric Matrix Protein Determines the Subset of Patients with Early-Stage Rheumatoid Arthritis with High Serum C-Reactive Protein, Matrix Metalloproteinase-3, and MRI-Proven Bone Erosion

KEITA FUJIKAWA, ATSUSHI KAWAKAMI, MAMI TAMAI, MASATAKA UETANI, SHOICHIRO TAKAO, KAZUHIKO ARIMA, NAOKI IWAMOTO, TOSHIYUKI ARAMAKI, SHINYA KAWASHIRI, KUNIHIRO ICHINOSE, MAKOTO KAMACHI, HIDEKI NAKAMURA, TOMOKI ORIGUCHI, HIROAKI IDA, KIYOSHI AOYAGI, and KATSUMI EGUCHI

ABSTRACT. ***Objective.*** To identify the significance of serum cartilage oligomeric matrix protein (COMP), a marker of cartilage turnover, in patients with early-stage rheumatoid arthritis (RA) in relation to other serologic variables and magnetic resonance imaging (MRI) features.

Methods. Ninety-eight patients with early-stage RA, whose disease duration from onset was less than 2 years, were enrolled. The objective measures at baseline were Disease Activity Score (DAS28), serum C-reactive protein (CRP), serum matrix metalloproteinase-3 (MMP-3), serum antibodies against cyclic citrullinated peptide (anti-CCP), and MRI features of both wrist and finger joints. The MRI features included the number of sites scored positive for synovitis, bone edema, and bone erosion.

Results. Serum COMP concentration was not different among groups identified with low, moderate, and high DAS28-CRP values. However, COMP values were statistically high in subjects positive for bone erosions on MRI compared with the subjects who were negative for bone erosions. A positive correlation of COMP with CRP and with MMP-3 values was also identified.

Conclusion. Elevation of COMP may reflect joint damage that is dependent on the synovial inflammatory process in early-stage RA. (First Release May 15 2009; J Rheumatol 2009;36:1126–9; doi:10.3899/ jrheum.080926)

Key Indexing Terms:
EARLY-STAGE RHEUMATOID ARTHRITIS
CARTILAGE OLIGOMERIC MATRIX PROTEIN
C-REACTIVE PROTEIN
MATRIX METALLOPROTEINASE-3
BONE EROSION
MAGNETIC RESONANCE IMAGING
ANTI-CYCLIC CITRULLINATED PEPTIDE ANTIBODIES

Synovitis in the context of rheumatoid arthritis (RA) leads to pathologic changes in adjacent structures, such as the articular cartilage, the cortical bone surfaces, and the underlying bone marrow, changes that have recently been verified by comparison of magnetic resonance images (MRI) on the day before surgery and the tissue specimens at joint replacement surgery (metacarpophalangeal or proximal interphalangeal joints) in patients with established RA[1]. These findings were obtained in patients with late-stage RA. A similar process should be occurring in early-stage RA, but it is very difficult to prove, since joint replacement surgery is performed infrequently in cases of early-stage RA. In contrast, qualifying pathologic features in the rheumatoid inflammatory process by serologic variables and MRI is recommend-

From the Unit of Translational Medicine, Department of Immunology and Rheumatology; Department of Radiology and Radiation Research; and Department of Public Health, Graduate School of Biomedical Sciences, Nagasaki University School of Health Sciences, Nagasaki, Japan.

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K. Fujikawa MD, PhD; A. Kawakami, MD, PhD; M. Tamai, MD, PhD, Unit of Translational Medicine, Department of Immunology and Rheumatology; M. Uetani, MD, PhD; S. Takao, MD, PhD, Department of Radiology and Radiation Research; K. Arima, MD, PhD; N. Iwamoto, MD; T. Aramaki, MD, PhD; S. Kawashiri, MD; K. Ichinose, MD, PhD; M. Kamachi, MD, PhD; H. Nakamura, MD, PhD, Unit of Translational Medicine, Department of Immunology and Rheumatology; T. Origuchi, MD, PhD, Department of Public Health, Graduate School of Biomedical Sciences; H. Ida, MD, PhD, Unit of Translational Medicine, Department of Immunology and Rheumatology; K. Aoyagi, MD, PhD, Department of Public Health, Graduate School of Biomedical Sciences; K. Eguchi, MD, PhD, Unit of Translational Medicine, Department of Immunology and Rheumatology, Nagasaki University School of Health Sciences.

Address reprint requests to Prof. K. Eguchi, Unit of Translational Medicine, Department of Immunology and Rheumatology, Graduate School of Biomedical Sciences, Nagasaki University, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan. E-mail: eguchi@net.nagasaki-u.ac.jp

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ed in early-stage patients rather than late-stage patients, since there may be fewer secondary disease process-independent phenomena in early-stage patients. We recently reported that MRI-proven synovitis, bone edema, and bone erosion in the wrist and finger joints in early-stage RA reflect systemic inflammatory indices of serum C-reactive protein (CRP) and serum matrix metalloproteinase-3 (MMP-3)[2].

An interesting serum biomarker is cartilage oligomeric matrix protein (COMP), which is thought to be increased in the serum early in the course of RA as a sign of cartilage involvement[3]. However, it is difficult to identify early cartilage involvement by radiography, even in MRI. Thus, the estimation of early cartilage involvement in early-stage RA using established variables, including COMP, would be valuable for prognostication. Our study is the first report to qualify serum COMP values in patients with early-stage RA, in conjunction with other inflammatory indices, as well as MRI detection of early joint damage.

MATERIALS AND METHODS

Ninety-eight patients with early stage RA were enrolled from the Early Arthritis Clinic at the Unit of Translational Medicine, Department of Immunology and Rheumatology (First Department of Internal Medicine), Graduate School of Biomedical Sciences, Nagasaki University. They gave their informed consent to the protocol, which was approved by the Institutional Review Board of Nagasaki University.

The mean disease duration from onset of symptoms to entry was 4.9 months. Disease duration for each of the 98 patients was < 2 years, similar to other recent reports[3,4]. Baseline characteristics of the 98 patients are described in Table 1. All patients fulfilled the 1987 criteria of the American College of Rheumatology for RA[5]. Since serum COMP is reported to be high in patients with osteoarthritis (OA) of large joints[6,7], we excluded the cases complicated with OA of hip or knee joint, classified according to the established criteria[8,9].

The following variables were examined at entry. Serologic tests included COMP (COMP ELISA®, AnaMar Medical AB, Göteborg, Sweden), CRP (Eiken Chemical Co. Ltd., Tokyo, Japan), MMP-3 (Daiichi Pure Chemicals, Fukuoka, Japan), and anti-CCP antibodies (Diastat Anti-CCP, Axis-Shield, Dundee, UK; cutoff value 4.5 U/ml). Clinical disease activity was qualified by the Disease Activity Score (DAS28-CRP; high disease activity > 4.1, low disease activity < 2.7, remission < 2.3)[10]. MR images of both wrists and finger joints (1.5 Tesla Sigma device; GE Medical Systems, Milwaukee, WI, USA) were evaluated for bone edema, bone erosion, and synovitis in 15 sites in each finger and wrist, i.e., the distal radioulnar joint, radiocarpal joint, mid-carpal joint, first carpometacarpal joint, second–fifth carpometacarpal joint (together), and first–fifth metacarpophalangeal joints (proximal interphalangeal joints) separately (a total of 30 sites in both hands), as we reported[2,11,12]. The MR images were interpreted independently by 2 board certified radiologists experienced in musculoskeletal imaging (MU and ST), who were blinded to the clinical status of the patients. Both radiologists read each image according to the definition, as described[13,14], and disagreements were resolved by consensus. The degree of MRI features was evaluated as we recently published: synovitis; the number of sites scored as positive for MRI synovitis, bone edema; number of bones scored positive for bone edema, bone erosion; and number of bones scored positive for MRI bone erosion[2].

Serum COMP concentration, in general, is reported to be elevated with age[3], as we found in the 98 patients with early-stage RA we examined ($r = 0.39$, $p < 0.001$). Thus, a partial correlation coefficient adjusting for age was calculated. Since MMP-3 was not normally distributed, we conducted logarithmic transformation. Differences between groups were examined using the age-adjusted mean. A test for trend was performed using the general linear modeling method. A p value < 0.05 denoted the presence of a statistically significant difference.

Table 1. Baseline characteristics of the 98 patients with early-stage RA.

Age, yrs (range)	53 (16–80)
No. female/male	77/21
Duration of disease, mo (range)	3 (1.5–24)
Tender joint count, n (range)	6 (0–28)
Swollen joint count, n (range)	3 (0–24)
Global health, 100 mm VAS (range)	50.5 (0–100)
DAS28-CRP (range)	4.3 (2.1–8.2)
MRI	
Synovitis positivity, %	90
n (range)	11.5 (0–30)
Bone edema positivity, %	47
n (range)	0 (0–15) mean 2.1
Bone erosion positivity, %	33
n (range)	0 (0–11) mean 1.3
Serologic markers	
COMP, U/l (range)	10.3 (0.6–24.9)
CRP positivity, %	66
Titer, mg/dl (range)	0.52 (0.02–12.7)
Anti-CCP antibody positivity, %	66
Titer U/ml (range)	23 (0.2–2115.3)
MMP-3 positivity, %	42
Titer, ng/ml (range)	66.6 (12.5–1160)

VAS: visual analog scale, COMP: cartilage oligomeric matrix protein, MRI: magnetic resonance imaging, CRP: C-reactive protein, CCP: citric citrullinated peptide, MMP: matrix metalloproteinase.

RESULTS

Table 1 shows the baseline characteristics of the 98 patients. Briefly, the median DAS28-CRP at entry was 4.3. Forty-seven percent of subjects were positive for MRI-proven bone edema, and 33% were positive for bone erosions. The median serum COMP at entry was 10.3 U/ml, median titer of anti-CCP antibodies was 20.3 U/ml, and seropositivity to anti-CCP antibodies was found in 66% of subjects. For MMP-3, the median serum concentration was 66.6 ng/ml, and seropositivity for MMP-3, denoted as in the higher than normal range, was 42%. As described[3], a positive correlation was found between serum COMP value and age in the 98 patients ($r = 0.39$, $p < 0.001$, Spearman's rank correlation test), and thus a partial correlation coefficient adjusting for age was calculated in the following data, as described above.

A partial correlation coefficient adjusting for age showed positive correlation of COMP with CRP ($r = 0.21$, $p = 0.035$) as well as MMP-3 values ($r = 0.20$, $p = 0.046$). Although the difference was not statistically significant, a weak association was determined between the number of MRI-proven bone erosions and the serum COMP ($r = 0.19$, $p = 0.06$). Therefore, we divided the 98 patients into 2 groups according to the MRI-proven bone erosions, and examined the distribution of serum COMP. Figure 1 shows that the age-adjusted mean concentration of serum COMP

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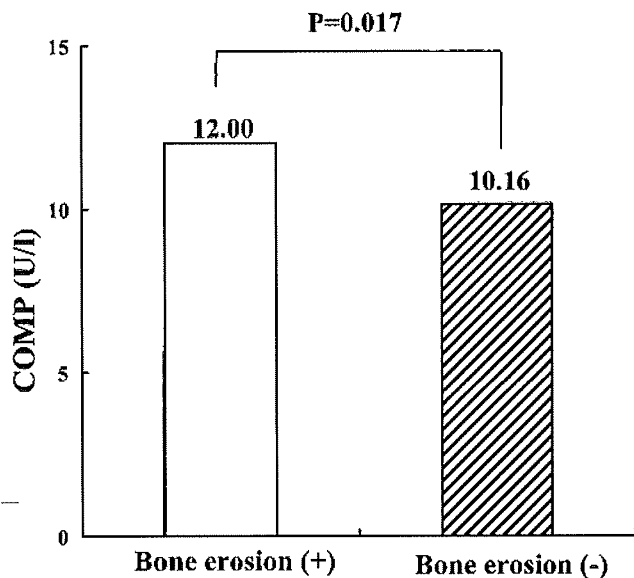

Figure 1. The age-adjusted mean serum COMP concentration was high in subjects with MRI-proven bone erosions (n = 32) compared to those without bone erosions (n = 66).

was statistically significantly high in the subjects who were positive for bone erosions on MRI, compared with subjects who were negative for erosions. As suspected, the mean values of CRP and MMP-3 were also high in the subjects who were positive for bone erosions on MRI (data not shown). We also examined the distribution of serum COMP by DAS28-CRP, and observed no differences among subjects who had low, moderate, and high DAS28-CRP scores (Figure 2). A test for trend showed no association between serum COMP and DAS28-CRP (p = 0.31). In addition, the

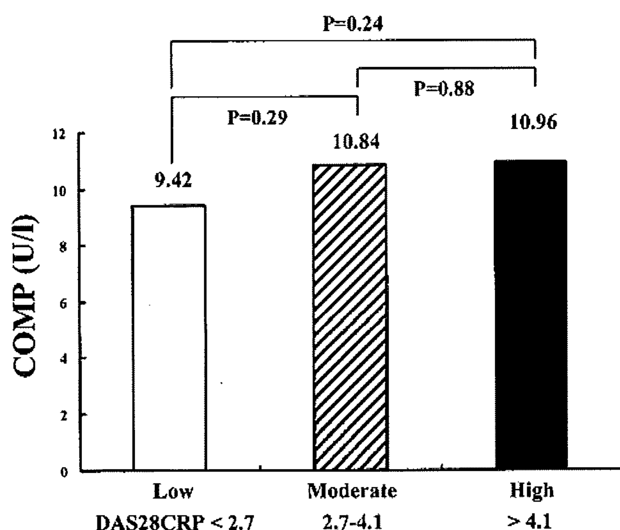

Figure 2. Serum COMP values of 98 patients with early-stage RA were not different among those with low, moderate, and high scores for DAS28-CRP. P value between the groups was calculated by age-adjusted mean. P value was nonsignificant.

age-adjusted mean concentration of serum COMP was not statistically different between subjects with different anti-CCP antibody status (mean COMP concentration was 10.54 U/l in subjects who were anti-CCP antibody-positive; and 11.26 U/l in subjects who were anti-CCP antibody-negative; p = 0.36).

DISCUSSION

COMP is a marker of articular cartilage damage, originally described as the determinant of radiographic progression in OA of the large joints, such as hip OA and knee OA[6,7,15]. Recently, serum COMP was evaluated in patients with RA, and was found to be preferentially elevated not in late-stage RA but in early-stage RA[3]. In addition, high serum COMP at baseline in early-stage RA indicates future radiographic progression[3]. The serum COMP concentration at baseline may also reflect the therapeutic efficacy of radiography in patients with active RA: low COMP at baseline predicted a better radiographic outcome in clinical trials of adalimumab treatment in RA[16]. We have investigated the role of serologic variables as well as MRI features of the wrist and fingers in early-stage RA[2,11,12]; in the present study we focused on the serum COMP value. MR images of the small joints in the wrist and fingers were closely examined, and thus patients with early-stage RA with clinically definite large-joint OA (hip and knee OA) were excluded, since a large amount of COMP could be produced from the affected articular cartilage, which might weaken the significance of the RA-related COMP value.

We initially expected COMP values to correlate with bone damage-prone markers, such as MMP-3, and MRI-proven bone edema and erosion. Raw data also showed an association of COMP with the number of MRI-proven bone erosions (r = 0.33, p = 0.02); however, age-adjusted data identified statistically significant association of COMP with only CRP and MMP-3. Since rheumatoid bone and cartilage damage are suggested to be driven by the neighboring synovial tissues[1], it was recognized that the inflammatory index provided by CRP and MMP-3 correlates with the cartilage turnover marker COMP. In addition, the findings that COMP concentrations were higher in the subjects with MRI-proven bone erosions than in those without erosions may support the speculation that rheumatoid synovial inflammation promotes articular cartilage turnover *in situ* in early-stage RA. Our findings that CRP and MMP-3 levels were high in the subjects with MRI-proven bone erosions also support this. Our recent study showed that MRI-proven bone edema, in early-stage RA, was significantly correlated with inflammatory indexes and DAS[2], but in the present study we did not see a relationship between the serum COMP value and MRI-proven bone edema. These observations may indicate that COMP reflects another aspect of the disease process of RA, which should be confirmed by our continuing prospective study.

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This is the first report to investigate the significance of the relation of serum COMP value with MRI detection of early joint damage in early-stage RA. Serum COMP values at entry correlated with CRP and MMP-3 values, and MRI-proven bone erosion also predicted high serum COMP concentrations in study subjects.

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Proinflammatory Cytokines Synergistically Enhance the Production of Chemokine Ligand 20 (CCL20) from Rheumatoid Fibroblast-like Synovial Cells *in vitro* and Serum CCL20 Is Reduced *in vivo* by Biologic Disease-modifying Antirheumatic Drugs

SHIN-YA KAWASHIRI, ATSUSHI KAWAKAMI, NAOKI IWAMOTO, KEITA FUJIKAWA, TOSHIYUKI ARAMAKI, MAMI TAMAI, KAZUHIKO ARIMA, MAKOTO KAMACHI, SATOSHI YAMASAKI, HIDEKI NAKAMURA, TOSHIYUKI TSURUMOTO, MASAFUMI KONO, HIROYUKI SHINDO, HIROAKI IDA, TOMOKI ORIGUCHI, and KATSUMI EGUCHI

ABSTRACT. ***Objective.*** Chemokine ligand 20 (CCL20) is a selective ligand for chemokine receptor 6 (CCR6). We investigated, both *in vitro* and *in vivo*, whether CCL20 is critically involved in the disease process of rheumatoid arthritis (RA).

Methods. *In vitro* study investigated the effect of proinflammatory cytokines and biologic disease-modifying antirheumatic drugs (DMARD) on the production of CCL20 by rheumatoid fibroblast-like synovial cells (FLS). The *in vivo* role of CCL20 was studied by screening for serum CCL20 concentration in patients with RA during the therapeutic course of biologic DMARD, i.e., infliximab, etanercept, and tocilizumab.

Results. Spontaneous CCL20 production from rheumatoid FLS was minimal; however, its production was significantly stimulated by interleukin 1ß (IL-1ß), tumor necrosis factor-α (TNF-α), or IL-17. IL-1ß was the most potent for stimulating the production of CCL20. CCL20 production was synergistically augmented by a combination of IL-1ß, TNF-α, and IL-17. In contrast, interferon-γ suppressed IL-1ß-induced CCL20 production. IL-6, in combination with soluble IL-6 receptor (sIL-6R), did not modulate CCL20 production, whereas IL-1ß-induced, TNF-α-induced, and IL-17-induced production were increased by IL-6. These production levels were clearly suppressed by biologic DMARD *in vitro*. Serum CCL20 was significantly higher in RA than in control subjects, and was clearly decreased by the treatment with infliximab, etanercept, and tocilizumab.

Conclusion. Proinflammatory cytokines modulate the production of CCL20 from FLS. Our data suggest that therapeutic efficacy of biologic DMARD may result from the inhibition of CCL20 production in rheumatoid synovium. (First Release Oct 1 2009; J Rheumatol 2009;36:2397–402; doi:10.3899/jrheum.090132)

Key Indexing Terms:
BIOLOGIC DISEASE-MODIFYING ANTIRHEUMATIC DRUGS
CHEMOKINE LIGAND 20
FIBROBLAST-LIKE SYNOVIAL CELLS
PROINFLAMMATORY CYTOKINES
RHEUMATOID ARTHRITIS

Chemokine ligand 20 (CCL20) is a selective ligand for chemokine receptor 6 (CCR6)[1]. The expression pattern of CCR6 has been revealed in recent investigations; interleukin 17 (IL-17)-producing helper T cells (TH17 cells) highly express CCR6 and also synthesize CCL20[2-4]. TH17 cells are supposed to accumulate in the rheumatoid synovi-

From the Unit of Translational Medicine, Department of Immunology and Rheumatology; Department of Orthopedic Surgery, Graduate School of Biomedical Sciences, Nagasaki University; and Department of Orthopedic Surgery, Japanese Red Cross Nagasaki Genbaku Hospital, Nagasaki, Japan.

S. Kawashiri, MD; A. Kawakami, MD, PhD; N. Iwamoto, MD; K. Fujikawa, MD, PhD; T. Aramaki, MD, PhD; M. Tamai, PhD; K. Arima, MD, PhD; M. Kamachi, MD, PhD; S. Yamasaki, MD, PhD; H. Nakamura, MD, PhD, Unit of Translational Medicine, Department of Immunology and Rheumatology; T. Tsurumoto, MD, PhD, Department of Orthopedic Surgery, Graduate School of Biomedical Sciences, Nagasaki University; M. Kono, MD, PhD, Department of Orthopedic Surgery, Japanese Red Cross Nagasaki Genbaku Hospital; H. Shindo, MD, PhD, Department of Orthopedic Surgery, Graduate School of Biomedical Sciences; H. Ida, MD, PhD; T. Origuchi, MD, PhD; K. Eguchi, MD, PhD, Unit of Translational Medicine, Department of Immunology and Rheumatology, Graduate School of Biomedical Sciences, Nagasaki University.

Address correspondence to Prof. K. Eguchi, Unit of Translational Medicine, Department of Immunology and Rheumatology, Graduate School of Biomedical Sciences, Nagasaki University, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan. E-mail: eguchi@net.nagasaki-u.ac.jp

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um[5-7], and the protein concentrations of CCL20 in both peripheral blood and synovial fluid are high in patients with rheumatoid arthritis (RA)[2,8-11]. These data suggest an interaction of CCL20 with CCR6 in TH17 cells that may perpetuate the chronic inflammatory process of RA. Previous studies have found an inducible effect of proinflammatory cytokines on CCL20 synthesis from fibroblast-like synovial cells (FLS)[8-10]. Since the blockage of the cytokine network by biologic disease-modifying antirheumatic drugs (DMARD) including infliximab, etanercept, and tocilizumab shows remarkable therapeutic efficacy for patients with RA[12-14], the proinflammatory cytokine-biologic DMARD interaction in CCL20 is supposed to be present, although few reports are available.

We showed in our study that FLS-derived CCL20 production is synergistically induced by proinflammatory cytokines, but is inhibited by biologic DMARD and interferon-γ (IFN-γ), which may be reflected in the decrease of serum CCL20 during treatment with infliximab, etanercept, and tocilizumab.

MATERIALS AND METHODS

RA patient samples for the isolation of FLS. Rheumatoid FLS for use in *in vitro* experiments was obtained from 14 patients with RA in the Department of Orthopedic Surgery, Graduate School of Biomedical Sciences, Nagasaki University and Japanese Red Cross Nagasaki Genbaku Hospital at the time of orthopedic surgery. They gave their informed consent to the protocol that was approved by the Institutional Review Board of Nagasaki University and Japanese Red Cross Nagasaki Genbaku Hospital. All patients fulfilled the 1987 criteria of the American College of Rheumatology (ACR)[15] for RA.

Detection of CCL20 in the culture supernatants of FLS in vitro. Rheumatoid FLS were isolated from 14 patients with RA. FLS were grown in 24-well plates (Becton Dickinson, Franklin Lakes, NJ, USA) at 100,000 cells/ml for 48 h at 37°C in a 5% CO_2 atmosphere in RPMI-1640 medium (Wako, Osaka, Japan) supplemented with 10% FBS/1% penicillin/streptomycin (Gibco-BRL, Grand Island, NY, USA). Proinflammatory cytokines of recombinant human IL-1ß (range 1–1000 pg/ml), IL-17 (range 1–1000 ng/ml), IL-6 (100 ng/ml), soluble IL-6 receptor (IL-6R; 100 ng/ml; R&D Systems, Abingdon, UK), and tumor necrosis factor-α (TNF-α; range 1–1000 ng/ml; Upstate Biochemical Co., Lake Placid, NY, USA) were added into the culture either singly or in a variety of combinations for 48 h. The protein concentration of CCL20 was examined using a commercial ELISA detection kit (Quantikine, R&D Systems). In some experiments, FLS were cultured in the presence of proinflammatory cytokines with biologic DMARD [infliximab (Centocor, Malvern, PA, USA), 500 μg/ml; etanercept (Wyeth, Madison, NJ, USA), 500 μg/ml; tocilizumab (Chugai, Tokyo, Japan), 500 μg/ml], and the CCL20 concentration was examined. The effect of IFN-γ (Shionogi, Osaka, Japan), an inhibitory cytokine for TH17, on CCL20 production was also studied. The numbers of individual FLS for each experiment are 14 in Figure 1 and 12 in Figure 2, Figure 3, and Table 1.

Measurement of serum CCL20 concentration in patients with RA during treatment with biologic DMARD. Serum CCL20 concentration in 14 patients with RA (12 women, 2 men) during treatment with biologic DMARD (infliximab, 5 patients; etanercept, 4 patients; and tocilizumab, 5 patients) and 13 healthy controls (11 women, 2 men) were studied using the same ELISA kit. Age, disease duration, and Disease Activity Score 28-erythrocyte sedimentation rate (DAS28-ESR) of the 14 RA patients at entry were 52.6 ± 13.0 years, 7.0 ± 9.4 years, and 6.0 ± 1.2 (high disease activity at the entry), respectively. Age of healthy controls was 36.5 ± 9.5 years; thus, they were statistically younger than patients with RA. We examined correlation of age and serum CCL20 concentration among 13 healthy controls by Spearman's rank correlation, and did not find any association (data not shown). Sex distribution was similar in healthy controls to patients with RA. Therefore, the samples from 13 healthy subjects were employed as control as comparison to patients with RA. CCL20 concentration of 14 patients with RA was serially examined at 3 to 6 months after the treatment with biologic DMARD, which achieved more than the moderate response determined by the EULAR response criteria[16] at the second measurement of CCL20.

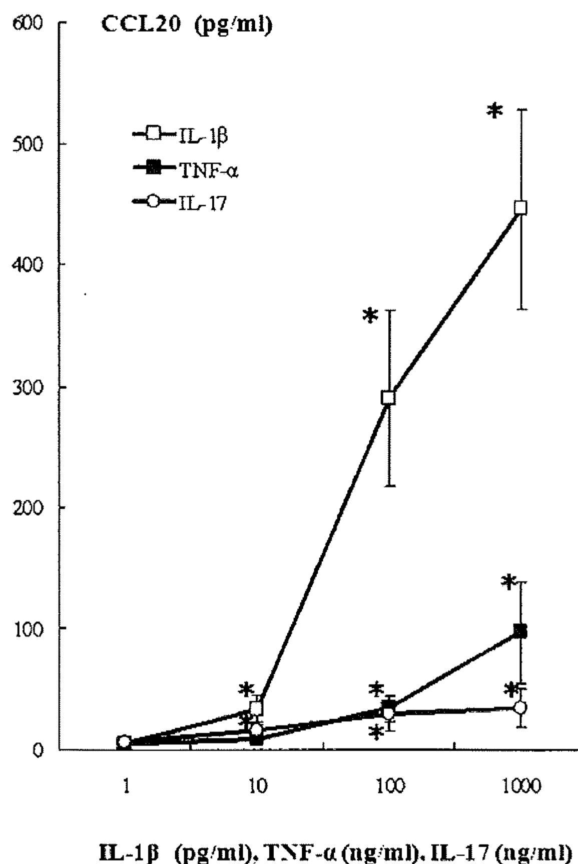

Figure 1. Dose-dependent increase of CCL20 production from cultured fibroblast-like synovial cells (FLS) by interleukin 1ß (IL-ß, tumor necrosis factor-α (TNF-α), and IL-17. Rheumatoid FLS were cultured for 48 h in the presence of 1–1000 pg/ml IL-ß, 1–1000 ng/ml TNF-α, or IL-17, and the CCL20 concentration in the supernatants was examined as described in Materials and Methods. IL-ß, TNF-α, and IL-17, especially IL-1ß, clearly induced CCL20 production from FLS. Results are expressed as means ± standard deviation from 14 independent experiments. *p < 0.01, vs 1 ng/ml of each cytokine.

Statistical analyses. Within-group comparisons were made using Mann-Whitney's U-test and Wilcoxon's signed-rank test. The overall significance level for statistical analysis was 5% (2-sided). P values less than 0.05 were considered statistically significant.

RESULTS

Proinflammatory cytokines synergistically stimulate the production of CCL20 from FLS, while TNF inhibitors suppress production. Spontaneous production of CCL20 from

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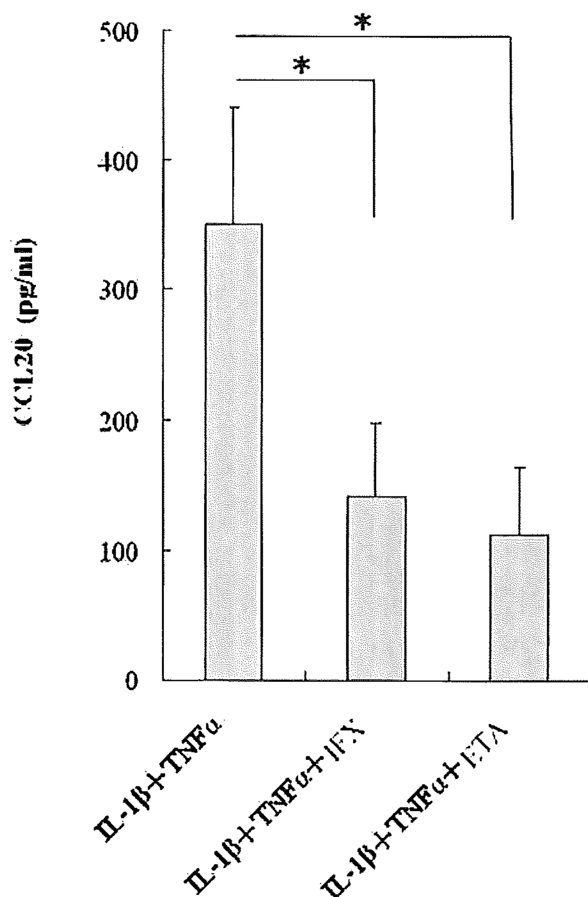

Figure 2. Suppression of CCL20 production from FLS stimulated with IL-1ß and TNF-α by infliximab (IFX) or etanercept (ETA). Rheumatoid FLS were cultured for 48 h in the presence of 100 pg/ml IL-ß and 50 ng/ml TNF-α in the presence or absence of either infliximab or etanercept (500 μg/ml). CCL20 concentration in the supernatants was examined as described in Materials and Methods. Results are expressed as means ± standard deviation from 12 independent experiments. *p < 0.01 vs absence of infliximab or etanercept.

FLS was very slight (4.5 ± 5.1 pg/ml, n = 14). We next verified an inducible effect of proinflammatory cytokines in CCL20 production. As reported[8-10], CCL20 production was induced by IL-1ß, TNF-α, or IL-17 in a dose-dependent fashion (Figure 1). The most prominent effect was induced by IL-1ß (Figure 1), and a synergy of these cytokines with each other was demonstrated since the CCL20 value induced by their combination was higher than the sum of each value (Table 1). TH1 cytokine IFN-γ inhibits the development of TH17 cells[17], and accordingly, IFN-γ (100 U/ml) was found to significantly inhibit IL-1ß (100 pg/ml)-induced CCL20 production (58.5% reduction by IFN-γ, Table 1). FLS were used for all experiments. However, all experiments cannot be performed at the same time, and a generation of FLS passage may be different among the experiments. Thus, the baseline CCL20 productions from FLS in the presence of cytokines are different in each figure and table.

We also examined whether proinflammatory cytokine-mediated CCL20 production from FLS is suppressed by TNF inhibitors. These experiments were performed by means of the incubation of FLS stimulated by IL-1ß and TNF-α in the presence of infliximab (500 μg/ml) or etanercept (500 μg/ml). As shown in Figure 2, both infliximab and etanercept *in vitro* clearly suppressed the production of CCL20 by FLS (59.4% reduction by infliximab and 67.9% reduction by etanercept).

IL-6 signal alone does not stimulate CCL20 production, but increases the production of CCL20 stimulated by proinflammatory cytokines, an increase abrogated by tocilizumab. The role of IL-6 in the production of CCL20 was investigated. Based on the previous finding that FLS do not express membrane-bound IL-6R[18,19], these experiments were performed in the presence of soluble IL-6R. As shown in Figure 3, the combination of IL-6 with soluble IL-6R did not increase CCL20 production; however, CCL20 production from FLS stimulated by IL-1ß, IL-1ß+TNF-α, and IL-1ß+IL-17 was clearly increased in the presence of the IL-6 signal. The increase of CCL20 production by IL-6 with soluble IL-6R was abrogated by the anti-IL-6R monoclonal antibody, tocilizumab (500 μg/ml; Figure 3).

Decrease of serum CCL20 concentration in patients with RA by biologic DMARD. Before the treatment, serum CCL20 was significantly higher in patients with RA than in healthy controls (Table 2). Serum CCL20 concentration did not correlate with DAS28-ESR at baseline, probably due to all their disease activity being similarly classified as high disease activity. Biologic DMARD treatment for 3 to 6 months markedly decreased serum CCL20 concentration (Table 2).

DISCUSSION

Recent investigations have revealed that TH17 cells, a T helper subset, specifically produce IL-17, which is critically involved in the disease process of RA[6,7,20]. Prominent TH17 cells are supposed to infiltrate the rheumatoid synovial tissues[5]; thus, a certain RA-specific microenvironment that facilitates the migration of TH17 cells is developed in affected tissues. Since TH17 cells express CCR6[2-4], *in situ* condensation of CCL20, a selective ligand of CCR6, is supposed to perpetuate the rheumatoid synovial inflammation. Therefore, the first half of our study focused on the regulation of CCL20 production from FLS.

The confirmatory results were obtained that IL-1ß, TNF-α, and IL-17 synergistically stimulate the production of CCL20. Cellular subsets observed in the rheumatoid synovial tissues, including TH17 cells, have been shown to produce these proinflammatory cytokines[6,20,21], presumably resulting in the augmentation of CCL20 production from neighboring FLS. Infliximab directly neutralizes TNF-α, and etanercept inhibits the TNF-α-eliciting signal at the TNF receptor. Thus, the result shown in Figure 2 is reasonable, and our data represent the first definitive result to be published.

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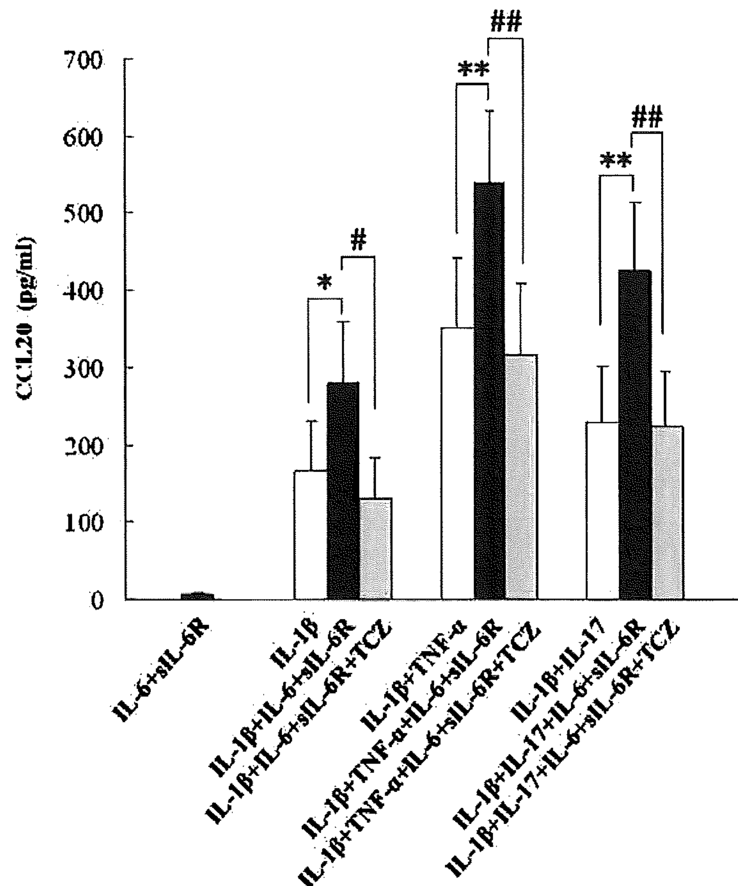

Figure 3. IL-6 combined with soluble IL-6 receptor (sIL-6R) does not induce CCL20 production, whereas it stimulates CCL20 production in the presence of IL-1ß, TNF-α, or IL-17; production is abolished by tocilizumab (TCZ). Rheumatoid FLS were cultured for 48 h in the presence of IL-6 (100 ng/ml) and sIL-6R (100 ng/ml) (1st column); IL-1ß (100 pg/ml), IL-1ß (100 pg/ml) + IL-6 (100 ng/ml), and sIL-6R (100 ng/ml), each in the presence or absence of 500 μg/ml tocilizumab (2nd column); IL-1ß (100 pg/ml) + TNF-α (50 ng/ml), IL-1ß (100 pg/ml) + TNF-α (50 ng/ml) + IL-6 (100 ng/ml), and sIL-6R (100 ng/ml), each in the presence or absence of 500 μg/ml tocilizumab (3rd column); and IL-1ß (100 pg/ml) + IL-17 (50 ng/ml), IL-1ß (100 pg/ml) + IL-17 (50 ng/ml) + IL-6 (100 ng/ml), and sIL-6R (100 ng/ml), each in the presence or absence of 500 μg/ml tocilizumab (4th column). CCL20 concentration in the supernatants was examined as described in Materials and Methods. Results are expressed as means ± standard deviation from 12 independent experiments. IL-6 with sIL-6R did not induce CCL20 production (1st column), whereas it stimulated production in the presence of IL-1ß (2nd column), IL-1ß + TNF-α (3rd column), and IL-1ß + IL-17 (4th column). *p < 0.05, **p < 0.01 vs absence of IL-6+sIL-6R. These increases were abolished by tocilizumab. $^{\#}$p < 0.05, $^{\#\#}$p < 0.01 vs absence of tocilizumab.

This article has identified a novel role for the IL-6 signal in CCL20 production from FLS. Our data suggest that the IL-6-eliciting signal amplifies the production of CCL20 by IL-1ß, TNF-α, and IL-17, the signals being transmitted through nuclear factor-κB (NF-κB)[22]. Janus kinase and signal transducer and activator of transcription (Jak-STAT), but not NF-κB, is dominantly triggered by IL-6[23], and thus unique phenomena may be induced in the presence of IL-6 with IL-1ß, TNF-α, or IL-17, as represented by our data. Further investigations are necessary to clarify the molecular interactions in FLS stimulated by these cytokines with regard to CCL20 production. It remains unclear whether the amount of biologic DMARD used in *in vitro* experiments can be achieved in the synovial tissues of RA treated by infliximab, etanercept, or tocilizumab. We can determine the serum concentration of infliximab or etanercept in the treated patients to be low compared with the concentrations used in the present *in vitro* study[24,25]. Concentration of biologic DMARD in synovial tissue or synovial fluid of the treated RA patients is not reported; however, the concentrations used in our experiment might be higher. Therefore, CCL20 from cell populations other than FLS are also supposed to

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Table 1. Synergistic effects of IL-1ß, TNF-α, IL-17 on CCL20 production by rheumatoid fibroblast-like synovial cells.

Cytokines	Concentrations of CCL20
IL-1ß, 100 pg/ml	166.5 ± 222.8
TNF-α (100 ng/ml)	34.2 ± 40.0
IL-17 (100 ng/ml)	29.4 ± 54.1
IL-1ß + TNF-α (50 ng/ml)	349.8 ± 313.4*
IL-ß+IL-17 (50 ng/ml)	225.8 ± 225.8*
IL-ß+IFN-γ (100 U/ml)	69.1 ± 103.3*

Differences in CCL20 production from FLS stimulated by IL-1ß, IL-1ß+TNF-α, or IL-1ß+IL-17 or IL-1ß+IFN-γ were analyzed using nonparametric paired Wilcoxon test. * $p < 0.01$, significantly different from CCL20 production by IL-1ß stimulation alone.

Table 2. High serum CCL20 concentration is decreased by treatment with biologic DMARD. Data are mean ± standard deviation.

Samples	Concentrations of CCL20
Healthy controls, n = 13	6.6 ± 6.6
RA patients at baseline	49.7 ± 37.5*
RA patients treated with biologics	19.5 ± 13.6**

Differences in concentrations of serum CCL20 between each group were analyzed using nonparametric paired Wilcoxon test. RA: n = 14 (infliximab 5, etanercept 4, tocilizumab 5). * $p < 0.001$, significantly different from controls. ** p , 0.01, significantly different from RA patients at baseline.

involve *in vivo* change of serum CCL20 during the treatment with biologic DMARD in patients with RA. Additionally, our study identified that IFN-γ inhibits CCL20 production from FLS in one effect of the suppression of TH17 function in humans. Indeed, the TH17-dependent arthritis model is inhibited by the introduction of IFN-γ[7].

As far as we know, only a few clinical values of CCL20 during treatment with biologic DMARD in patients with RA can be found in the literature[11]. We have shown here that serum CCL20 declined in patients with RA who responded well to biologic DMARD. The decrease of CCL20 was found in all 3 treatment arms, namely infliximab, etanercept, and tocilizimab, with no statistical difference between the TNF inhibitors (infliximab and etanercept) and tocilizumab (data not shown). Both the TNF inhibitors and tocilizumab inhibited the production of CCL20 from FLS *in vitro*, and probably *in vivo*. TNF-α and IL-6 cooperatively act on the effector cell population to stimulate CCL20 production. Thus, serum CCL20 concentration in patients with RA was clearly downregulated by both of the TNF inhibitors and tocilizumab in a similar fashion.

The CCL20-mediated TH17 cell activation process is supposed to play a central role in the disease process of RA. We do not provide direct evidence of FLS-derived CCL20 interaction with TH17 cells in our study. Development of TH17 cells requires several cytokines, including IL-1, TNF-α, and IL-6, whereas IFN-γ suppresses the process[17,26-28]. CCL20 production from FLS is clearly modulated by IL-1ß, TNF-α, IL-6, and IFN-γ, indicating that proinflammatory cytokine-mediated regulation of CCL20 in FLS may be involved in the TH17 cell-dependent disease process of RA. Further studies are necessary to identify the *in vivo* or *ex vivo* role of FLS-derived CCL20 for the accumulation of TH17 cells in rheumatoid synovial tissues. The CCL20-mediated TH17 cell activation process is supposed to play a central role in the disease process of RA.

Our study gives a possible explanation for why CCL20 is an exacerbating factor in patients with RA. The monitoring of serum CCL20 concentration may reflect the disease activity of RA.

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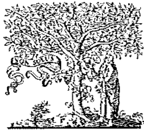
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Combined insulin B:9-23 self-peptide and polyinosinic–polycytidylic acid accelerate insulitis but inhibit development of diabetes by increasing the proportion of $CD4^+Foxp3^+$ regulatory T cells in the islets in non-obese diabetic mice

Keiko Fukushima [a,b], Norio Abiru [a,*], Yuji Nagayama [c], Masakazu Kobayashi [a,g], Tsuyoshi Satoh [a], Mami Nakahara [d], Eiji Kawasaki [b], Hironori Yamasaki [a,e], Satoshi Ueha [f], Koji Matsushima [f], Edwin Liu [g], Katsumi Eguchi [a]

[a] *Department of Endocrinology and Metabolism, Unit of Translational Medicine, Graduate School of Biomedical Science, Nagasaki University, Nagasaki, Japan*
[b] *Department of Metabolism/Diabetes and Clinical Nutrition, Nagasaki University Hospital of Medicine and Dentistry, Nagasaki, Japan*
[c] *Department of Medical Gene Technology, Nagasaki University, Nagasaki, Japan*
[d] *Department of Clinical Pharmacology, Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki, Japan*
[e] *Health Center, Nagasaki University, Nagasaki, Japan*
[f] *Department of Molecular Preventive Medicine, Graduate School of Medicine, University of Tokyo, Tokyo, Japan*
[g] *Barbara Davis Center for Childhood Diabetes, UCHSC, Denver, CO, USA*

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Abstract

Insulin peptide B:9-23 is a major autoantigen in type 1 diabetes. Combined treatment with B:9-23 peptide and polyinosinic–polycytidylic acid (poly I:C), but neither alone, induce insulitis in normal BALB/c mice. In contrast, the combined treatment accelerated insulitis, but prevented diabetes in NOD mice. Our immunofluorescence study with anti-CD4/anti-Foxp3 revealed that the proportion of Foxp3 positive $CD4^+CD25^+$ regulatory T cells (Tregs) was elevated in the islets of NOD mice treated with B:9-23 peptide and poly I:C, as compared to non-treated mice. Depletion of Tregs by anti-CD25 antibody hastened spontaneous development of diabetes in non-treated NOD mice, and abolished the protective effect of the combined treatment and conversely accelerated the onset of diabetes in the treated mice. These results indicate that poly I:C combined with B:9-23 peptide promotes infiltration of both pathogenic T cells and predominantly Tregs into the islets, thereby inhibiting progression from insulitis to overt diabetes in NOD mice.
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Keywords: Insulin; Type 1 diabetes; Insulitis; Non-obese diabetic mouse; $CD4^+$ T cell; Peptide; Polyinosinic–polycytidylic acid; Regulatory T cell

Type 1 diabetes mellitus is an autoimmune disease that develops when tolerance mechanism(s) fail to control immune responses to islet-specific autoantigens, including insulin, glutamic acid decarboxylase, and heat-shock protein [1]. Insulin is an important islet autoantigen in non-obese diabetic (NOD) mice and patients with type 1 diabetes [1]. In NOD mice, insulin-autoreactive $CD4^+$ and $CD8^+$ T cells infiltrate islets of mice, and clones of these T cells can transfer diabetes to young recipients [2,3]. The B chain peptide, B:9-23, has been suggested to be a primary autoantigenic epitope in the pathogenesis of type 1 diabetes in NOD mice [4,5]. However, of interest, immunization of NOD mice with exogenous B:9-23 peptide prevents diabetes [6].

* Corresponding author. Fax: +81 95 849 7270.
E-mail address: abirun@nagasaki-u.ac.jp (N. Abiru).

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In contrast to NOD mice, we have recently found that immunization of non-diabetes prone BALB/c mice with B:9-23 peptide readily induces insulin autoantibodies, but not insulitis or diabetes [7], and that simultaneous administration of B:9-23 peptide and polyinosinic–polycytidylic acid (poly I:C, a Toll-like receptor 3 ligand), but not poly I:C alone, induces insulitis. In addition, we have also developed an experimental autoimmune diabetes model in which B:9-23 peptide induces diabetes in transgenic BALB/c mice expressing the costimulatory molecule B7.1 in their islets [8]. Disease induction is accelerated with simultaneous administration of poly I:C in this model. These data indicate that poly I:C appears to promote anti-insulin autoimmunity in BALB/c mice immunized with B:9-23 peptide.

Since the previous report has demonstrated that poly I:C treatment confers significant protection against diabetes in NOD mice [9], we are left with the question of whether combination treatment of NOD mice with B:9-23 peptide and poly I:C would promote or prevent diabetes. We here first showed that treatment with B:9-23 peptide and poly I:C unexpectedly (or surprisingly) accelerated insulitis, but prevented diabetes in young NOD mice. We first thought that TGF-β might mediate diabetes protection despite acceleration of insulitis, because it has been reported that the TGF-β producing T cell clone which was found to react to insulin B:9-23 peptide protects NOD mice from disease induced by adoptive transfer of diabetogenic spleen cells [10]. We therefore injected TGF-β monoclonal antibody (2g7, a generous gift from Dr. Sylvaine You, INSERM U580 Hopital NECKER, Paris) to NOD mice after B:9-23 peptide immunization [11]. These studies however showed that TGF-β neutralization did not influence the disease inhibition by B:9-23 peptide and poly I:C in NOD mice (our unpublished data).

Another possibility is naturally arising $CD4^+CD25^+$ regulatory T cells (Tregs). They develop in the thymus and in the periphery, and actively maintain immunological self-tolerance. Tregs have been shown to be essential for regulation of several autoimmune diseases, including type 1 diabetes [12]. The transcriptional factor Foxp3 has been identified to be essential for development and function of Tregs [13]. Insulin-specific Tregs induced by *in vivo* immunization and *in vitro* restimulation with B:9-23 peptide efficiently prevent diabetes induced by adoptive transfer of diabetogenic T cells [14]. In patients with type 1 diabetes, it has been reported that regulatory T cell markers such as Foxp3 mRNA is upregulated in peripheral blood mononuclear cells stimulated with insulin *in vitro* [15]. In this study, we evaluated whether Tregs play a role in the aforementioned action of combined B9-23 peptide and poly I:C.

Materials and methods

Mice. Female NOD mice, 3–4 weeks of age, were purchased from Clea Japan (Tokyo, Japan). All mice were kept under specific pathogen-free conditions at the Laboratory Animal Center for Biomedical Research of Nagasaki University, and were housed in an air conditioned room with a 12-h light-darkness cycle. Animal care and all experimental procedures were performed in accordance with the Guideline for Animal Experimentation of Nagasaki University with approval of the Institutional Animal Care and Use Committee.

Injection of B:9-23 peptide, poly I:C or anti-CD25 antibody. Mouse proinsulin II B chain-derived peptide B:9-23 (SHLVEALYLVCGERG), synthesized and HPLC-purified to greater than 95% homogeneity, was purchased from SIGMA Genosys (Hokkaido, Japan). Polyinosinic–polycytidylic acid sodium salt (poly I:C) was from Sigma–Aldrich KK (Tokyo, Japan). Anti-CD25 antibody was purified from ascites of mice intraperitoneally (ip) injected with hybridoma PC61 (a generous gift from Dr. K. Yui at Nagasaki University) using a HiTrap™ protein G HP column (Amersham, Piscataway, NJ).

B:9-23 peptide (100 μg/mouse) in incomplete Freund's adjuvant (IFA) was subcutaneously injected into the scruff of NOD mice at 4 weeks of age (day 1). Poly I:C (7.5 μg/g body weight) was ip administered on days 1–5 and 8–12. PC61 was ip injected at the indicated time point.

Monitoring diabetes by blood glucose levels. The blood glucose levels of mice were monitored every other week with a Glutest-Ace meter (Sanwa Kagaku, Nagoya, Japan) starting at 12 weeks of age to determine the development of diabetes following B:9-23 peptide and/or poly I:C injection without PC61. In the study with PC61, the blood glucose levels were monitored twice a week starting at 4.5 weeks of age. Mice with blood glucose levels above 250 mg/dl for two consecutive measurements were considered diabetic.

Histology. Pancreata were obtained at 8 weeks of age after the administration of B:9-23 peptide and/or poly I:C. Pancreata, thyroid tissues, and salivary glands were obtained at 6 weeks of age after the administration of PC61 with or without B:9-23 peptide and poly I:C. Each section of the tissues was histologically analyzed by fixing in 10% formalin and staining with hematoxylin and eosin. A minimum of 20 islets from each mouse were microscopically observed by two different observers for the presence of insulitis, and the levels of insulitis were scored according to the following criteria; 0, no lymphocyte infiltration; 1, islets with lymphocyte infiltration in less than 25% of their area; 2, 25–50% of the islet area infiltrated; 3, 50–75% of the islet area infiltrated; 4, more than 75% infiltrated or small retracted islets.

Immunohistochemistry. The primary antibodies used were rabbit anti-mouse Foxp3 Ab [16] (the final concentration of 2.5 μg/ml) and FITC hamster anti-mouse CD4 (H129.19) (BD Biosciences Pharmingen, San Diego, CA) (1:250 dilution). The secondary antibodies were Alexa Fluor 555 goat anti-rabbit IgG (0.5 μg/ml) and Alexa Fluor 488 goat anti-hamster IgG (BD Biosciences Pharmingen) (1:250 dilution).

Pancreata and pancreatic draining lymph nodes (PLNs) (for four mice in each group) were embedded in Tissue-Tek OCT compound (Miles, Elkhart, IN), frozen in liquid nitrogen, and cut by a cryostat into 6- to 7-μm-thick sections. The sections were fixed with cold acetone for 10 min at 4 °C and use for immunofluorescence. We used an already established double-immunofluorescence staining protocol for FoxP3 and CD4 [16]. After blocking non-specific reactions and endogenous biotin activity using Blocking One (Nacalai Tesque, Kyoto, Japan), the samples were incubated with each primary Abs for 1 h at room temperature and subsequently with secondary Abs for 1 h at room temperature, and then fixed for 10 min at 4 °C in PBS containing 4% paraformaldehyde. All sections were analyzed with a confocal laser scan microscope LSM5Pascal (Carl Zeiss, Germany). The number of $Foxp3^+CD4^+$ cells was counted in four non-consecutive microscopic fields within the T cell areas of islets and PLN.

Statistical analysis. Group differences were analyzed with the Turkey HSD test, and differences between Kaplan–Meier survival curves were estimated by the log rank test using Dr. SPSS II for Windows software (SPSS Inc., Chicago, IL). P values less than 0.05 were considered statistically significant. Insulitis levels were analyzed by Ridit analysis, and levels of t higher than 1.96 or lower than −1.96 were considered statistically significant.

Results

Administration of B:9-23 peptide in combination with poly I:C significantly accelerated the development of peri-insulitis but prevented development of diabetes

We first evaluated the influence of administration of exogenous B:9-23 peptide and/or poly I:C on development of insulitis and diabetes in NOD mice at 4 weeks of age. As determined by life table analysis, B:9-23 peptide alone or in combination with poly I:C significantly suppressed development of diabetes, as compared to the PBS-treated control group ($P < 0.0005$ and $P < 0.001$, respectively). Injection of poly I:C alone at 4 weeks of age slightly inhibited development of diabetes, but this inhibition was insignificant ($P = 0.1$) (Fig. 1A).

The pancreata from NOD mice at 8 weeks of age (4 weeks after the beginning of treatment) were used for histological analysis. Unexpectedly, the levels of insulitis were significantly increased by administration of B:9-23 peptide alone or poly I:C alone, compared with the control group ($T = 4.304$ or $T = 6.183$, respectively). Simultaneous administration of B:9-23 peptide and poly I:C further accelerated development of insulitis ($T = 10.77$) (Fig. 1B).

Administration of B:9-23 peptide with poly I:C increased the frequency of $CD4^+Foxp3^+$ regulatory T cells in the islets, but not in the PLNs

To further characterize insulitis enhanced by B:9-23 peptide and poly I:C, the frequency of Tregs in the islets and PLNs was determined by CD4 and Foxp3 staining as a marker of Tregs. We found that approximately 8% of $CD4^+$ T cells were Foxp3-positive both in the islets and the PLNs in 6 weeks old control mice (Figs. 2A and 3A, B). Combined treatment of B:9-23 peptide with poly I:C increased the proportion of $CD4^+Foxp3^+$ T cells in the islets up to ~17% ($P < 0.0005$) (Figs. 2A and 3A), but did not change the percentage in the PLNs (Fig. 3B). These results indicate that the combination of B:9-23 peptide and poly I:C enhanced insulitis, but concomitantly increased proportion of Tregs in T cells infiltrated into the islets.

Depletion of Tregs induced extremely rapid onset of insulitis and diabetes in mice treated with B:9-23 peptide and poly I:C

To clarify the role played by infiltrating $Foxp3^+CD4^+$ Tregs, we attempted to deplete Tregs by using PC61, a widely used means to examine the function of Tregs. In our preliminary experiment, 500 μg/mouse PC61 efficiently depleted $CD25^+$ cells (data not shown). A single administration of PC61 into mice at 4 weeks of age accelerated the spontaneous development of diabetes ($P = 0.0046$ vs. PC61(−) PBS) (Fig. 4A). Surprisingly, pre-treatment with PC61 of mice at 3.5 weeks of age abrogated the disease inhibition induced by B:9-23 peptide and poly I:C, and instead greatly accelerated the onset of diabetes. Thus, disease developed in 35% and 45% mice vs. 0% in non-depleted mice at age of 6 and 10, respectively, weeks ($P < 0.005$) (Fig. 4B).

Pre-treatment with PC61 also decreased the percentage of $Foxp3^+$ T cells among the $CD4^+$ T cells infiltrated into the islets in untreated NOD mice ($P = 0.002$ vs. PC61(−)

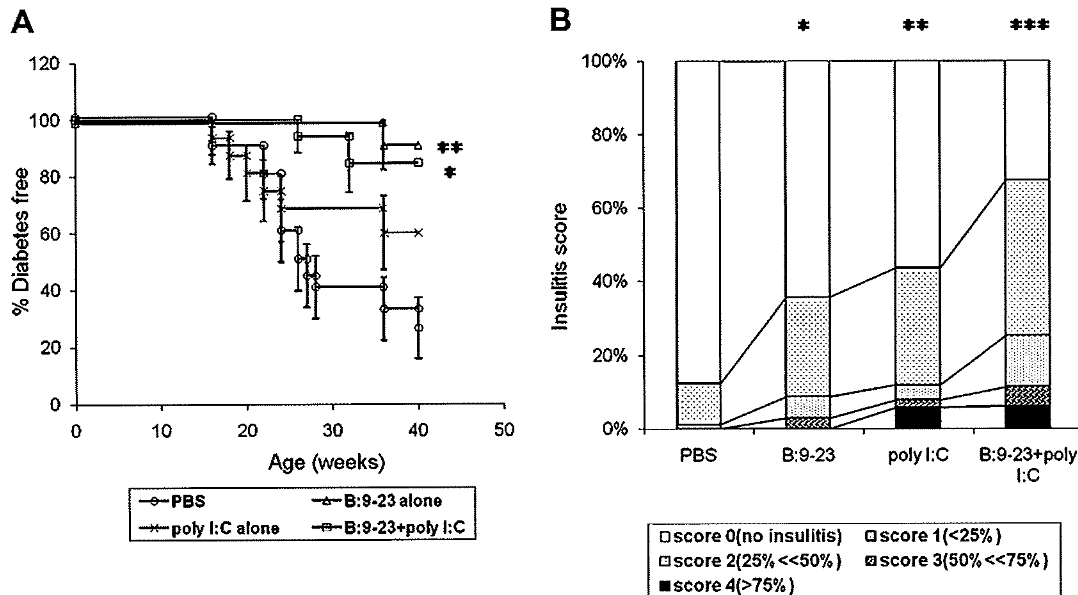

Fig. 1. (A) Life table analysis for development of diabetes following administration of B:9-23 peptide and/or poly I:C in NOD mice. Open triangles, B:9-23 peptide alone ($n = 10$); x, poly I:C alone ($n = 16$); open squares, B:9-23 peptide + poly I:C ($n = 16$); open circles, PBS ($n = 19$). $^*P < 0.001$; $^{**}P < 0.0005$. (B) Levels of insulitis at 8 weeks of age determined by Ridit analysis. A level of $T > 1.96$ was regarded as a significant increase. A level of $T < -1.96$ was regarded as a significant suppression. $^*T = 4.304$, $^{**}T = 6.183$, and $^{***}T = 10.77$.

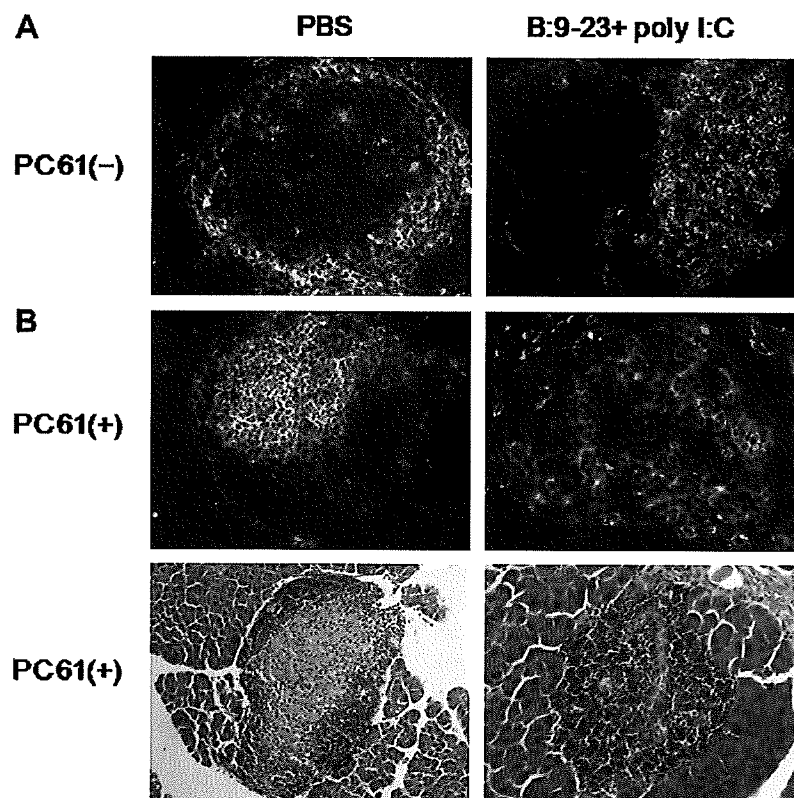

Fig. 2. (A) Immunofluorescence of the sections of pancreata from NOD mice at 6 weeks of age treated with PBS or B:9-23 + poly I:C without PC61 pretreatment stained with anti-Foxp3 Ab and anti-CD4 mAbs, followed by goat anti-rabbit IgG Alexa555 (red) and goat anti-rat IgG Alexa488 (green). Original magnification: 100×. (B) Immunofluorescence of the sections stained and H&E stained paraffin sections of the pancreas from NOD mice at 6 weeks of age treated with PC61(+) PBS or PC61(+) B:9-23+poly I:C 2 weeks before. Original magnification: 200×.

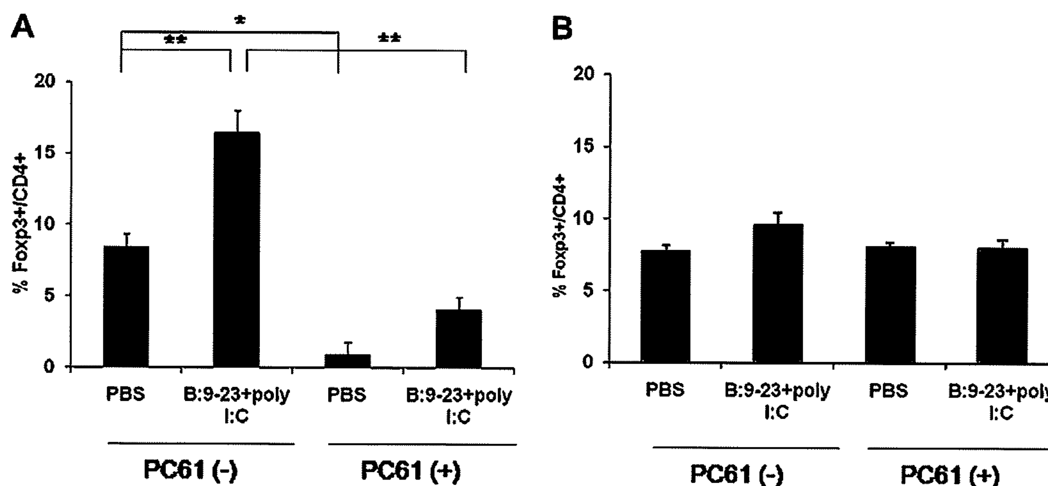

Fig. 3. The numbers of $Foxp3^+$ T cells and $CD4^+$ T cells were counted in several non-consecutive microscopic fields within the T cell area of the pancreas or pancreatic lymph nodes (PLN). The frequency of $Foxp3^+$ T cells in whole $CD4^+$ cells in the T cell area of the pancreas (A) or PLN (B) were compared within each group of treated mice, respectively. The results are presented as means ± SE. $^*P < 0.005$, $^{**}P < 0.0005$.

PBS) and in those treated with B:9-23 peptide and poly I:C ($P < 0.0005$ vs. PC61(−) B:9-23 + poly I:C) (Figs. 2B and 3A). In the PLNs, in contrast, the frequency of $CD4^+Foxp3^+$ T cells was not influenced by treatment with PC61 ($P = 0.954$ vs. PC61(−) PBS) ($P = 0.613$ vs. PC61(−) B:9-23 + poly I:C) (Fig. 3B).

Massive infiltration of lymphoid cells was observed in the islets from mice treated with PC61, B:9-23 peptide