

Fig. 4. (A) Life table analysis for the development of diabetes following administration of PC61 alone. Closed circles, PC61(+)PBS ($n = 9$); open circles, PC61(-)PBS ($n = 24$). $*P < 0.005$. (B) Life table analysis for the development of diabetes following pre-treatment of PC61 before the combination therapy with B:9-23 peptide + poly I:C. Closed squares, PC61(+)B:9-23 + poly I:C ($n = 8$); open squares, PC61(-)B:9-23 + poly I:C ($n = 16$). $*P < 0.005$.

and poly I:C, but only mild to moderate infiltration was seen in those treated with PC61 alone 2.5 weeks after PC61 injection (6 weeks of age) (Fig. 2B). Neither thyroid nor salivary glands were intact in all the mice.

These results indicate that B:9-23 peptide and poly I:C promote infiltration of both autoimmune pathogenic T cells and Foxp3⁺ Tregs into the islets, and that the latter keeps the former in check, preventing development of diabetes, and following depletion of the latter with PC61 antibody diabetogenesis is remarkably accelerated.

Discussion

It has been extremely difficult to precisely define environmental determinants of type 1 diabetes of man despite early evidence that viral infections may contribute. We believe that a part of this difficulty may be dependent upon complex interplay between activation of innate and adaptive immunity, which may result in either protection or disease induction in genetically susceptible hosts. For instance, the effects of poly I:C on anti-islet autoimmunity vary in different mouse models of type 1 diabetes. Thus, our previous studies showed that poly I:C enhances anti-insulin autoimmune reaction in BALB/c mice immunized with B:9-23 peptide [8], whereas our present study (Fig. 1A) demonstrates that poly I:C does not reverse inhibitory effect of B:9-23 peptide on development of diabetes in NOD mice. Although the exact reason(s) for this difference are at present unclear, a recent study shows that the effect of poly I:C is dose-related; high dose poly I:C accelerates but low dose prevents diabetes in BioBreeding rats [17].

It was unexpected that the prevention of diabetes of NOD mice by combined poly I:C and B:9-23 peptide administration was accompanied by enhanced insulinitis (Fig. 1B). Previous studies suggest that progression from insulinitis to overt diabetes is regulated by Tregs in NOD

mice. NOD mice only develop diabetes at 3 or 4 months of age, however, insulinitis occurs long before the clinical onset of diabetes [18]. Disease transfer into irradiated recipients by spleen cells from diabetic NOD mice is prevented by CD4⁺ T cells from young pre-diabetic NOD mice were co-transferred with diabetogenic T cells [19]. Recent data have indicated that this protective effect is confined to a CD4⁺CD25⁺CD62L⁺ Treg subset [20,21].

Our immunofluorescence study indeed showed an elevated proportion of CD4⁺Foxp3⁺ T cells in the islets (but not in the PLN) from mice treated with poly I:C and B:9-23 peptide (Fig. 3). The functional significance of Tregs was verified by our depletion study, in which antibody (PC61)-mediated Treg depletion accelerated development of diabetes in untreated mice and remarkably in those treated with poly I:C and B:9-23 peptide (Fig. 4). Extremely acute onset of diabetes was observed in Treg-depleted, poly I:C and B:9-23 peptide-treated mice (Fig. 4B). Thus, PC61 antibody sensitive cells (presumably Tregs) play a role not only in suppression of spontaneous development of diabetes in untreated NOD mice, but also in mice treated with poly I:C and B:9-23 peptide. Our data indicate that combined treatment with poly I:C and insulin peptide B:9-23, recruits both pathogenic T cells and predominantly Tregs into the islets; the latter keeps the former in check, thereby suppressing progression from insulinitis to diabetes. However, Treg-depletion in this situation reveals the enhanced action of the pathogenic T cells, by allowing the pathogenic T cells to infiltrate into the islet rapidly, thereby resulting in massive insulinitis and acute onset diabetes.

In NOD mice, administration of insulin or insulin-derived peptides can be used to prevent diabetes [22–24] but similar approaches have failed in a clinical setting [25], possibly because of inadequate induction and accumulation of antigen-specific Tregs which can suppress the autoimmune pathogenic T cells over the long disease

course. Many therapeutic interventions with antigen-specific Tregs will be attempted to treat autoimmunity [26,27]. Our results indicate that treatment of NOD mice with insulin B:9-23 peptide, a potential primary autoantigenic epitope, and poly I:C expands the pathogenic T cells as well as Tregs and recruits them into the islets. There appear to be a fine balance between the pathogenic T cells and Tregs resulting in enhancing insulinitis but suppressing progression from insulinitis to overt diabetes in NOD mice. Thus, to obtain disease inhibition by an antigenic peptide vaccination, an efficient therapy which could both induce antigen-specific regulatory cell populations and purge pathogenic T cells should be considered.

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Altered B:9–23 Insulin, When Administered Intranasally with Cholera Toxin Adjuvant, Suppresses the Expression of Insulin Autoantibodies and Prevents Diabetes¹

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Insulin peptide B:9–23 is a major autoantigen in type 1 diabetes that contains two distinct CD4 epitopes (B:9–16 and B:13–23). One of the two epitopes, B:13–23, overlaps with a CTL epitope (B:15–23). In this study, we report that the elimination of the CTL epitope from the B:9–23 peptide by amino acid substitution (with alanine) at positions B:16 and 19 (A16,19 altered peptide ligand) or truncation of the C-terminal amino acids from the peptide (B:9–21), neither of which stimulated the proliferation of insulin B:15–23 reactive CD8 T cells, provided significant intranasally induced suppression of diabetes when coadministered with a potent mucosal adjuvant cholera toxin (CT). Intranasal treatment with A16,19 resulted in the elimination of spontaneous insulin autoantibodies, significant inhibition of insulinitis and remission from hyperglycemia, and prevented the progression to diabetes. Intranasal administration of native B:9–23/CT or B:11–23/CT resulted in a significant enhancement of insulin autoantibody expression and severity of insulinitis and failed to prevent diabetes. Our present study indicates that elimination of the CTL epitope from the B:9–23 peptide was critically important for mucosally induced diabetes prevention. The A16,19 altered peptide ligand, but not other native insulin peptides, suppresses insulin autoantibodies associated with protection from and remission of diabetes. *The Journal of Immunology*, 2007, 179: 2082–2088.

Peptide immunotherapy targeting autoreactive T cells is a potential strategy for the treatment of autoimmune diseases where an autoantigen has been identified. However, it has been suggested that mucosal administration of an autoantigenic peptide may act as a double-edged sword with the potential to induce not only protective but also pathogenic immunity, especially if a CTL epitope is present within the peptide. Martinez and coworkers suggested that the induction of CTL immunity needs to be avoided for efficient and safe diabetes prevention in mucosal peptide immunotherapy with an autoantigen, which can be accomplished by disabling the integral CTL epitopes (1). Insulin is a major target in type 1 diabetes, and the insulin B chain peptide

with amino acids 9–23 (B:9–23)⁴ has been suggested as a primary antigenic epitope in the pathogenesis of type 1 diabetes in NOD mice (2, 3). The B:9–23 peptide is capable of preventing diabetes in NOD mice when given s.c. (4) or as an altered peptide ligand (APL) of insulin. The peptide contains two distinct CD4 epitopes (B:9–16 and B:13–23), and B:13–23 overlaps with a CTL epitope (B:15–23) (5, 6). Insulin B:15–23 binds the K^d MHC class I molecule, with the B chain tyrosine at position 16 (binding in p2) providing the primary anchor and the glycine at position 23 (p9) providing the secondary anchor. Substitution of the tyrosine residue at position 16 with alanine or truncation from the C-terminal of B:9–23, i.e., B:9–21/22 peptide, abrogates binding of the peptide to the K^d molecule (7). An APL of B:9–23 that contains an alanine substitution at positions B:16 and B:19 (A16,19 APL) has been shown to prevent spontaneous diabetes in NOD mice when administered s.c. (8). In this study, we test the hypothesis that replacing the CTL epitope of B:9–23 with B:9–21 or A16,19 APL is superior to the native B:9–23 peptide in preventing diabetes.

We have previously demonstrated that s.c. administration of ~50 μg of B:9–23 peptide without the use of IFA induces high levels of insulin autoantibodies (IAA) in normal BALB/c mice that specifically react with intact insulin and are not absorbed by the peptide (9). However, our preliminary study showed that intranasal administration of native B:9–23 peptide (10–80 μg) failed to induce IAA in BALB/c mice. Based on our previous study that cholera toxin (CT) was able to strongly augment Th2-type humoral immunity when intranasally coadministered with a vaccine Ag

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⁴ Abbreviations used in this paper: B:9–23, insulin B chain peptide with aa 9–23; APL, altered peptide ligand; A16,19 APL, APL with alanine substitutions at positions 16 and 19; CT, cholera toxin; IAA, insulin autoantibody; TT, tetanus toxin.

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Table I. Summary of the amino acid sequences of the insulin derived peptides studied and known epitopes for NOD islet derived T cell clones

Peptide	Amino Acid Sequence	Reference
B:9-23	S-H-L-V-E-A-L-Y-L-V-C-G-E-R-G	
B:9-21	S-H-L-V-E-A-L-Y-L-V-C-G-E	
B:11-23	L-V-E-A-L-Y-L-V-C-G-E-R-G	
A16,19 APL	S-H-L-V-E-A-L-A-L-V-A-G-E-R-G	
Known epitopes		
For CD4 T cell clone	S-H-L-V-E-A-L-Y	5
	E-A-L-Y-L-V-C-G-E-R-G	
For CD8 T cell clone	L-Y*-L-V-C-G-E-R-G*	6, 7

Asterisks denote residues for binding to K^d.

(10), we used CT (2 μ g) as a mucosal adjuvant with the peptide and successfully induced high levels of IAA in the BALB/c mice. In this study, we evaluated the effects of disabling the CTL epitope within the B:9-23 peptide (i.e., B:9-23 derived truncated peptide and A16,19 APL) in mucosal immunization regimens on IAA induction and the development of insulinitis and diabetes in NOD mice. Our results indicate that elimination of the CTL epitope from the insulin peptide B:9-23 conferred efficient suppression of insulinitis and diabetes in NOD mice when the peptide was intranasally coadministered with CT. In addition, prevention of diabetes by A16,19 APL/CT was associated with the elimination of IAA expression and significant inhibition of insulinitis, while treatment with the native peptide that contains the CTL epitope enhanced IAA expression without providing any protection from the disease.

Materials and Methods

Mice and antigens

Female NOD mice were purchased from Clea Japan and maintained in the Laboratory Animal Center for Biomedical Research at Nagasaki University (Nagasaki, Japan) under specific pathogen-free condition. All animal experiments described in this study were approved by the institutional animal experimentation committee and were conducted in accordance with the committee's guidelines for animal experimentation.

Mouse proinsulin II B chain-derived peptides B:9-23, B:9-21, B:11-23, A16,19 APL (Table I), and tetanus toxin (TT) peptide:830-843 (QYIKANSKFIGITE) were chemically synthesized and purified by HPLC to >95% homogeneity (purchased from Sigma-Aldrich). Insulin-related peptides and the TT peptide were dissolved in sterile saline and adjusted to a neutral pH at a concentration of 4 mg/ml. CT was pur-

chased from List Biological Laboratories and dissolved in sterile saline at a concentration of 400 μ g/ml until use.

Intranasal administration of synthetic peptides to mice

For diabetes prevention studies, mice were intranasally immunized starting at 4 wk of age (days 1-5 and 8 and then weekly until 10 wk of age) with 20 μ g of peptide alone or together with 2 μ g of CT. For remission studies, NOD mice at age 16-24 wk were treated with intranasal peptide administration once they were confirmed to have blood glucose levels between 200 and 249 mg/dl. The first day of intranasal treatment was counted as day 1 for this immunization regimen. Ten micrograms of the peptide was administered on days 1-5 and then twice a week for 40 days. Mice were anesthetized with ether, and 10 μ l of a mixture containing the peptide and the adjuvant was slowly introduced into each nostril with a micropipette.

Measurement of anti-peptide-specific Abs and IAA

Mice were bled and serum were obtained at 4, 6, 8, and 12 wk of age and stored at -20°C until the Ab assay was done. Abs against insulin peptide B:9-23 and A16,19 APL were measured by ELISA as previously described (11). Briefly, high binding Costar EIA/RIA 96-well microplates (catalog no. 3369; Corning) were coated overnight at room temperature with 1 μ g of B:9-23 peptide dissolved in 100 μ l of PBS per well. Wells were thoroughly washed three times with PBS and blocked with PBS containing 2% BSA and 0.01% sodium azide for a minimum of 2 h at room temperature. Wells were washed and 100 μ l of 1/500 buffer-diluted serum samples were incubated for 2 h at room temperature. Wells were washed and biotin-conjugated rat anti-mouse IgG1 mAb (catalog no. 553441; BD Pharmingen) diluted 1/5000 in PBS was added to each well (100 μ l/well) and incubated for 30 min. Five microliters of europium-labeled streptavidin (Wallac) was diluted in 10 ml and 100 μ l was added to each well for 15 min. Wells were washed and 200 μ l of enhancement solution (Wallac) was added per well and gently shaken for 10 min. Plates were analyzed by using

FIGURE 1. Anti-B:9-23 peptide Ab expression (A), IAA expression (B), and life table analysis for the development of diabetes (C) following intranasal administration of B:9-23 either alone or together with CT in NOD mice are depicted. Peptides were intranasally administered starting at 4 wk of age (days 1-5 and 8, and then weekly until 10 wk of age). □, B:9-23 alone ($n = 10$); ■, B:9-23/CT ($n = 10$); △, TT/CT ($n = 10$); ×, PBS, ($n = 10$); ***, $p < 0.0001$ vs PBS.

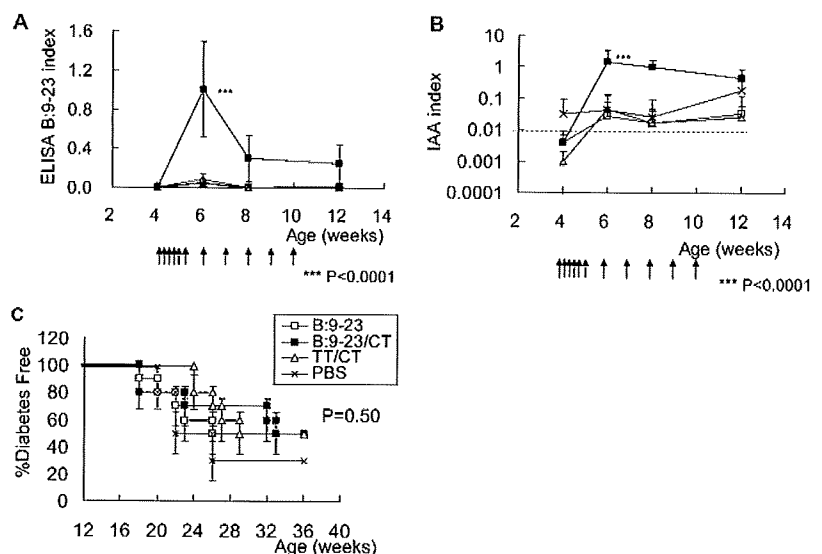


Table II. Summary of peptide/CT vaccination

Peptide	CD8 Reactivity	IAA	Insulinitis	Diabetes
B:9-23/CT	↑↑↑	↑↑↑	↑↑↑	→
B:9-21/CT	→	→	↓	↓↓↓
B:11-23/CT	↑↑↑	↑↑↑	↑↑↑	↓↓↓
A16,19 APL/CT	→	↓↓↓	↓↓↓	↓↓↓

the Victor³ V 1420 multilabel counter (Wallac). Specific Ab levels were expressed as the index defined as follows: (sample cpm - negative control cpm)/(positive control cpm - negative control cpm).

The levels of IAA in serum were evaluated prospectively at 4, 6, 8, and 12 wk of age by using a 96-well filtration plate micro-IAA assay as previously described (12). The ¹²⁵I-labeled insulin Ag (Amersham Biosciences) at 20,000 cpm was incubated with 5 μ l of serum with and without cold human insulin, respectively, for 3 days at 4°C in buffer A (20 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl, 1% BSA, 0.15% Tween 20, and 0.1% sodium azide). Fifty microliters of 50% protein A with 8% protein G-Sepharose (Amersham Biosciences) was added to the serum/insulin mixture solution in a MultiScreen NOB 96-well filtration plate (Corning) that was precoated with buffer A. The plate was shaken for 45 min at 4°C followed by two cycles of four washes with cold buffer B (identical to the buffer A except for 0.1% BSA). After washing the plates, 40 μ l of scintillation liquid (Microscint-20; Packard Instrument) was added to each well and radioactivity was measured by using a TopCount scintillation counter (96-well plate beta counter; Packard). The result was calculated based on the differences in counts per minute (Δ cpm) between wells with and without cold insulin and expressed as an index defined as follows: (sample Δ cpm - human negative control Δ cpm)/(human positive control Δ cpm - human negative control Δ cpm). The index value of 0.01 was chosen as the cutoff limit of the normal serum level of IAA in a nondiabetic mouse strain.

Monitoring diabetes by blood glucose levels

Blood glucose levels were monitored by using Glutest-Ace (Sanwa Kagaku Kenkyusho) every other week starting at 12 wk of age for diabetes prevention studies. Mice with blood glucose levels >250 mg/dl for two consecutive measurements were considered diabetic. For remission studies, blood glucose levels were monitored twice a week for 40 days and mice having blood glucose levels >600 mg/dl or >400 mg/dl for four consecutive measurements were considered to have reached the study endpoint.

Insulin-reactive CD8 T Cells

Cloned CD8 T cells, derived from the islets of young NOD mice and designated G9C8, recognize the peptide insulin B:15-23 complex (6). A TCR transgenic mouse was generated from the TCR of the G9C8 clone on the NOD background (designated G9 transgenic mouse) and crossed to TCR $\alpha^{-/-}$ mice to ensure that all of the TCR transgenic cells are monoclonal and express the transgenic receptor. These mice are designated G9C $\alpha^{-/-}$ NOD. The mice express CD8 T cells that respond to insulin and insulin peptide in an identical manner to that of the original clone (our

unpublished observations). CD8 T cells were purified from G9C $\alpha^{-/-}$ NOD splenocytes by using positive selection beads (Miltenyi Biotec) with >95% purity for proliferation and cytotoxic assays.

Proliferation assays

Purified insulin-reactive CD8 T cells (10^5) were incubated with 10^5 irradiated NOD spleen cells together with different concentrations of peptide, in triplicate, in a 96-well round-bottom plate for 72 h. Tritiated (0.5 μ Cl) thymidine was added for a further 14-h incubation. The plates were harvested and thymidine uptake was measured in counts per minute using a beta plate counter.

⁵¹Cr-release cytotoxicity assay

P815 cells (1×10^6) were incubated with 0.1 μ Ci of ⁵¹Cr-labeled sodium chromate in 100 μ l for 1 h, washed, and resuspended at 10^4 cells per 50 μ l. The cells were incubated, in triplicate, at 25°C with different peptide concentrations in round-bottom 96-well plates for 1 h. Purified insulin-reactive CD8 T cells were added to the plate at an E:T ratio of 10:1. The cell mixture was incubated for a further 16 h at 37°C. Fifty microliters of supernatant was assayed for ⁵¹Cr-release in a gamma counter. Specific lysis was determined as follows: ((cytotoxic release - minimum release)/(maximum - minimum)) \times 100.

Histology

Pancreatic sections of mice immunized with insulin-related peptides were histologically analyzed at 12 wk of age by fixing tissues in 10% formalin and staining the paraffin-embedded samples with H&E. A minimum of 20 islets from each mouse were microscopically observed by two different observers for the presence of insulinitis, and the levels of insulinitis were scored according to the following criteria: 0, no lymphocyte infiltration; 1, islets with lymphocyte infiltration in <25% of the area; 2, 25-50% of the islet infiltrated; 3, 50-75% the islet infiltrated; 4, >75% infiltrated or small retracted islets.

Statistics analysis

Group differences were analyzed with the Tukey honestly significant difference (HSD) test and differences between Kaplan-Meier survival curves were estimated by the long rank test, with the use of Dr. SPSS II for Windows software (SPSS). Values of $p < 0.05$ were considered statistically significant. Insulinitis levels were analyzed by Ridit analysis, and levels of $t > 1.96$ or < -1.96 were considered statistically significant.

Results

CT functions as an effective mucosal adjuvant for intranasally administered insulin B:9-23 peptide

Intranasal administration of 10 μ g of the insulin B:9-23 peptide failed to induce anti-B:9-23-specific Abs (Fig. 1A). However, when the peptide was coadministered with a small amount of CT the Ab responses were significantly augmented, particularly at 6 wk of age ($p < 0.0001$ vs PBS group). The small amount of CT did not induce any obvious toxic effects. Mice given TT peptide

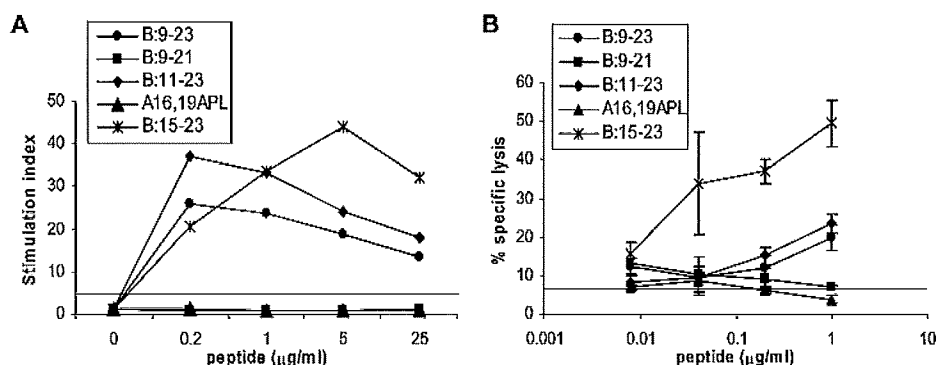
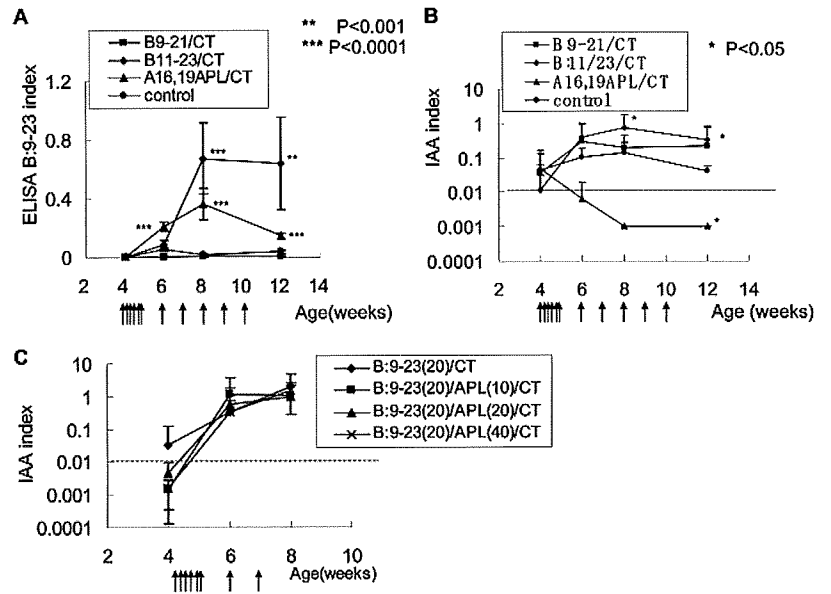


FIGURE 2. Functional responses of insulin-reactive CD8 T cells to peptides. *A*, Proliferation of insulin-reactive CD8 T cells to increasing concentrations of insulin B chain-derived peptides. The results are shown as a stimulation index (proliferation with peptide/proliferation without peptide). Background counts without peptide were 6786 ± 657 cpm. *B*, ⁵¹Cr-release cytotoxicity assay showing lysis of P815 targets by the insulin-reactive CD8 T cells with increasing concentrations of peptides. The horizontal line shown is the cytotoxicity in the absence of peptide. ●, B:9-23; ■, B:9-21; ◆, B:11-23; ▲, A16,19 APL; ×, B:15-23.

FIGURE 3. *A* and *B*, Anti-B:9–23 peptide Ab expression (*A*) and IAA expression (*B*) following intranasal administration of truncated B:9–23 and A16,19 APL together with CT in NOD mice. ■, B:9–21/CT (*n* = 10); ◆, B:11–23/CT (*n* = 9); ▲, A16,19 APL/CT (*n* = 8); ●, control (CT/TT and PBS) (*n* = 14 and 15, respectively); *, *p* < 0.05; **, *p* < 0.001; ***, *p* < 0.0001 significant vs control. *C*, IAA expression following intranasal administration of 20 μg of B:9–23 peptide/CT supplemented with different doses (0–40 μg) of A16,19 APL in NOD mice. ◆, B:9–23 peptide/CT alone (*n* = 10); ■, B:9–23/CT with 10 μg of APL/CT (*n* = 10); ▲, B:9–23/CT with 20 μg of APL/CT (*n* = 10); ×, B:9–23/CT with 40 μg of APL/CT (*n* = 10).



with CT had no effect on B:9–23 peptide-specific responses, confirming that the response was insulin peptide-specific and that CT critically influenced the level of the specific immune response.

We next examined the effects of intranasal immunization with insulin peptides on IAA. IAA can be spontaneously expressed in NOD mice as early as 4 wk of age (13). Intranasal administration of B:9–23/CT, but not B:9–23 alone or TT/CT, significantly enhanced IAA expression at 6 wk of age (Fig. 1*B* and Table II) as compared with a group given PBS (*p* < 0.0001). Based on previously reported findings that the s.c. administration of B:9–23 peptide alone induced high levels of IAA and effectively prevented diabetes in the NOD mice (14), we expected that intranasal immunization with B:9–23/CT would also prevent the development of diabetes. However, in contrast to our expectation, intranasal administration of B:9–23/CT completely failed to prevent diabetes (Fig. 1*C* and Table II). As expected, the B:9–23 peptide alone or TT/CT had no effect on the development of diabetes.

CTL epitope elimination

As described in the Introduction, the failure of disease prevention by intranasal B:9–23/CT immunization could be partly due to the induction of peptide-specific CTL immunity by the CD8 insulin B:15–23 T cell epitope. Thus, we decided to evaluate the effects of CTL epitope elimination from the B:9–23 peptide by amino acid substitutions at the B:16 and 19 positions (i.e., A16,19 APL) or by truncation of the C-terminal amino acids (i.e., B:9–21 peptide) on nasally induced Ab responses and disease prevention. To confirm whether the substitution and truncation of a CTL epitope disabled B:15–23 specific CTL immunity, proliferation and ⁵¹Cr-release cytotoxicity assays of cloned insulin B:15–23 reactive CD8 T cells were performed. A comparison was made of insulin B:9–23 derived peptides with the B:15–23 peptide. As we expected, neither the A16,19 APL nor the B:9–21 peptide stimulated the proliferation of insulin-reactive CD8 T cells, while all other peptides containing the CTL epitope stimulated the CD8 T cells (Fig. 2*A*). In the proliferation, the larger peptides containing the B:15–23 epitope (B:9–23 and B:11–23) can be further processed to generate the epitope that can stimulate the CD8 T cells. In addition, as expected no cytotoxicity was stimulated by the A16,19 APL and the B:9–21 peptide. However unlike the proliferation assay, there was relatively low cytotoxicity toward targets coated with B:9–23

and B:11–23 (Fig. 2*B*). In the cytotoxicity assay, which is set up for peptides that will bind well into the K^d groove, the larger peptides are unlikely to bind appropriately to the MHC and, therefore, the stimulation of cytotoxicity using these peptides was limited.

We evaluated both Abs to the peptide B:9–23 (Fig. 3*A*) and Abs to insulin (Fig. 3*B*). Although intranasal administration of A16,19 APL/CT or B:11–23/CT induced significant levels of anti-B:9–23 Abs as compared with control, B:9–21/CT was almost completely unable to induce anti-B:9–23 Abs (Fig. 3*A* and Table II). Analyses of serum IAA revealed that significantly higher IAA was induced at 8 and 12 wk of age in mice given the B:11–23/CT group (*p* < 0.05), but not in the B:9–21/CT-immunized mice as compared with spontaneously expressed levels of IAA in the control mice. Interestingly, mice intranasally treated with A16,19 APL/CT never expressed IAA at 6, 8, and 12 wk of age except for one mouse that was IAA positive at 4 wk of age before peptide immunization (Fig. 3*B* and Table II). To test whether A16,19 APL modulated the autoimmune response to native B:9–23 peptide, we coadministered A16,19 APL and native peptide. The addition of different doses of A16,19 APL did not suppress the IAA induced by the 20-μg dose of B:9–23 peptide/CT, indicating that A16,19 APL/CT suppressed spontaneous, but not induced, autoantibody formation (Fig. 3*C*).

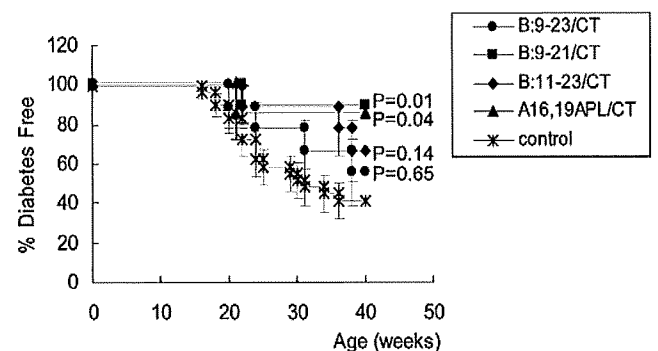


FIGURE 4. Life table analysis for the development of diabetes following intranasal administration of native B:9–23, truncated B:9–23, and A16,19 APL together with CT in NOD mice. Both intranasal-A16,19 APL/CT and intranasal-B:9–21/CT significantly suppressed the development of diabetes.

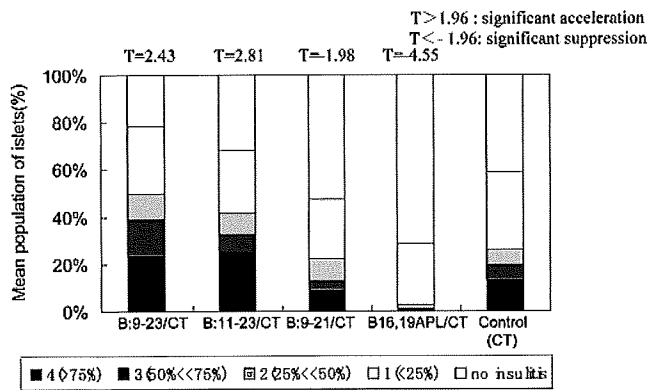


FIGURE 5. Insulinitis levels at 12 wk determined by Riddit analysis. A level of $T > 1.96$ was regarded as significant increase. A level of $T < -1.96$ was regarded as significant suppression. Insulinitis levels were significantly increased in the B:9-23/CT ($T = 2.43$) and B:11-23/CT ($T = 2.81$) compared with the CT alone in contrast to being suppressed slightly in the B:9-21/CT ($T = -1.98$) and strongly in the A16,19 APL/CT ($T = -4.55$).

Elimination of CTL epitope critically influenced diabetes prevention efficacy

As expected, intranasal administration of A16,19 APL/CT, or B:9-21/CT strongly suppressed the development of diabetes as compared with the control group ($p < 0.05$) (Fig. 4 and Table II). The disease prevention efficacy attained by intranasal B:11-23/CT immunization was comparable with that achieved by the A16,19 APL/CT- and B:9-21/CT-immunized groups until 34 wk of age; however, the B:11-23/CT group started to develop the disease at 40 wk of age. We also found that the blood glucose levels in most of the A16,19 APL/CT immunized mice were < 100 mg/dl until 40 wk of age, whereas animals of the other groups showed blood glucose levels higher than 100 mg/dl, suggesting that the A16,19

APL was able to more effectively suppress subclinical β cell damage compared with the C-terminally truncated form of the peptide (data not shown).

Prevention of diabetes was associated with the degree of insulinitis inhibition

Histological analysis of pancreatic islets of mice intranasally immunized with insulin-derived peptides revealed that B:9-23/CT and B:11-23/CT accelerated the development of insulinitis as compared with the control group ($T = 2.43$ and 2.81 , respectively) (Fig. 5 and Table II). In contrast, we found that administration of B:9-21/CT or A16,19 APL/CT significantly suppressed the development of insulinitis ($T = -1.98$ and -4.55 , respectively), and all islets of mice immunized with the A16,19 APL/CT had no insulinitis or only minimal peri-insulinitis (Fig. 5 and Table II).

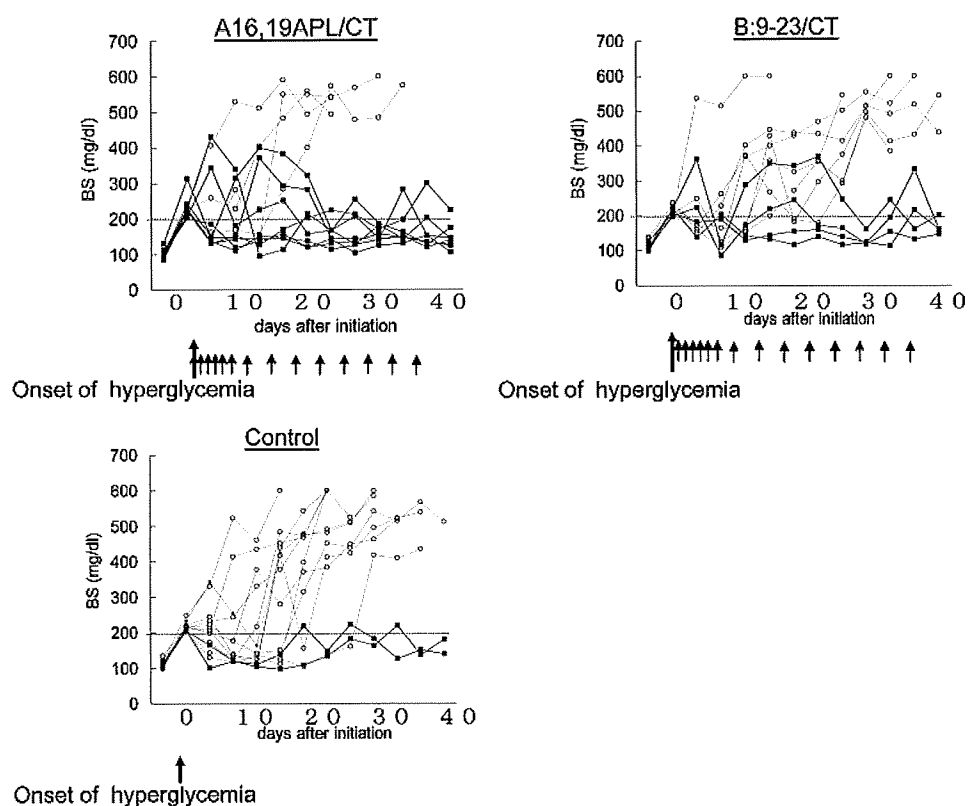
Reversal of hyperglycemia and prevention of progression to diabetes was more often achieved by intranasal administration of the A16,19 APL than the native B:9-23 peptide

Finally, we evaluated the efficacy of insulin-derived peptides to reverse diabetes after the onset of hyperglycemia (Fig. 6). We found that 85% of the control NOD mice that became hyperglycemic (glucose levels reaching 200–249 mg/dl) eventually developed the endpoint of sustained severe hyperglycemia (> 400 mg/dl) within 40 days of initial hyperglycemia. In contrast, intranasal immunization with A16,19 APL/CT resulted in remission from hyperglycemia in 65% of mice, more often than the control mice given PBS ($p < 0.05$). Native B:9-23 peptide/CT-treated mice showed a 36% remission.

Discussion

Multiple studies indicate that insulin/proinsulin is a key target in the pathogenesis of type 1 diabetes (2, 15). Several proinsulin-derived peptides, including insulin A and B chain peptides and a B-C junction peptide, have been reported to be recognized by T

FIGURE 6. Blood glucose levels in the NOD mice intranasally treated with A16,19 APL/CT (top left), B:9-23/CT (top right), and control (bottom left) starting on the onset of diabetes with glucose levels between 200 mg/dl and 249 mg/dl. The peptide was intranasally immunized on days 1–5 and then twice a week until 40 days. Blood glucose levels were monitored twice a week until 40 days and we stopped the experiment at the endpoint when blood glucose levels were > 600 mg/dl or 400 mg/dl four times in continuity. The mice that developed endpoint are shown as open circles (○) and others are shown as filled squares (■).



cells from humans at risk for type 1 diabetes (16, 17) and from NOD mice (15, 18). B:9–23-specific autoreactive T cell clones are found in islet infiltrates (19) and adoptively transfer diabetes into NOD SCID mice (18). The B:9–23 peptide protects NOD mice from diabetes when given subcutaneously (4, 14). With this “vaccine” NOD mice express significantly higher and long-lasting IAA compared with the levels of spontaneous IAA expression (9). Using insulin gene knockouts we have recently reported that the insulin peptide B:9–23 might be an essential target of immune destruction in the NOD mouse (3).

Mucosally induced immune regulation has been harnessed to prevent experimental autoimmune diseases, and therapeutic responses have been reported after nasal, but not oral, administration of Ags (20, 21). In this study, we found that intranasal immunization with native B:9–23 peptide in combination with CT induced significant levels of IAA without protecting against the development of diabetes in NOD mice (Fig. 1). Martinez and coworkers have reported that intranasal administration of proinsulin B24–C36 induces regulatory CD4⁺ T cells that, however, could not inhibit the development of spontaneous diabetes in the NOD mice (1). They found that B25–C34 was a CTL epitope and that disabling it (truncation at its C terminus) inhibited diabetes (1). In this study, we have altered the B:15–23 CTL K^d epitope, previously identified (6), by truncation at the B:9–23 C terminus or amino acid substitution (alanine for tyrosine) at position B:16. Intranasal immunization with B:11–23/CT that retains the B:15–23 CD8 epitope, but not with B:9–21/CT, induced significant levels of IAA (Fig. 3B). This is consistent with our previous finding that one of the identified minimum epitopes, B:13–23, but not the B:9–16 epitope, induces IAA expression in normal BALB/c mice (9). Interestingly, intranasal B:11–23/CT as well as B:9–23/CT accelerated the development of insulinitis but did not accelerate or inhibit diabetes development (Fig. 4). Immunization of young NOD mice with islet autoantigens such as the insulin B chain peptide prevents autoimmune diabetes (4); however, it can accelerate the development of insulinitis, especially when peptides are coadministered with polyinosinic-polycytidylic acid in NOD mice (K. Fukushima, N. Abiru, and M. Kobayashi, unpublished observations). The observations made in this study and by others suggest that the B:9–23-derived peptide containing a functional CTL epitope can expand both regulatory and pathogenic T cells. In contrast to the native peptide, intranasal B:9–21/CT immunization provided significant prevention of spontaneous diabetes development with a suppressive effect on insulinitis (Figs. 4 and 5) and without any effect on the spontaneous expression of IAA (Fig. 3B).

APLs derived from T cell-reactive self-Ags have been shown to function as protective therapeutic agents in animal models of autoimmunity. The mechanisms by which APLs may modulate immune response include antagonism (22, 23), anergy (24, 25), and immune deviation (26). Many of the analogues to date were agonistic peptides rather than antagonistic ones. B16 tyrosine is a critical amino acid for peptides that activate both NOD islet-derived CD4⁺ and CD8⁺ T cell clones. We previously reported that alteration of the tyrosine residue to alanine at this position abrogated the proliferative responses of anti-B:9–23 CD4⁺ T cell clones (5). The substitution of tyrosine with alanine at the B:16 position results in the failure of the B:15–23 peptide to bind to the K^d molecule (7). Female NOD mice with a single amino acid alteration at residue 16 (from tyrosine to alanine) in the insulin B chain are dramatically protected from diabetes (3). Alleva and coworkers reported that the B:9–23-altered peptide ligand that contains alanine substitutions at residues 16 and 19, A16,19 APL (namely NBI-6024), substantially delays the onset and reduces the incidence of diabetes when given s.c. without adjuvant (8). We pre-

viously found that the induction of insulin autoantibodies is specific for the B:9–23 peptide among proinsulin-derived peptides. The Abs induced by the B:9–23 peptide react with intact insulin but are not absorbed with the peptide itself, suggesting that the B:9–23 peptide is not a B cell epitope in intact insulin and that spontaneously occurring anti-insulin B cells are activated with the help of activated T cells specific to the B:9–23 peptide (9). In our study, intranasal administration of A16,19 APL coadministered with a potent mucosal immune potentiator efficiently prevented diabetes and induced Abs reacting not only with A16,19 APL but also with the native B:9–23 peptide. Contrary to our expectation that the nasally administered A16,19 APL/CT would be associated with the enhancement of IAA expression and the acceleration of insulinitis, similar to the native peptide, intranasal immunization with this peptide combined with CT completely eliminated IAA expression and strongly suppressed the development of insulinitis. The inhibition of insulinitis with A16,19 APL/CT should not be considered as an antagonist of the native insulin peptide binding to the K^d molecule, because A16,19 APL by itself is unable to bind MHC class I (7).

The presence of small amounts of CT was critically important for the induction of the Abs and disease prevention. Alternative adjuvants that might be safer for human use, such as a nontoxic mutant of CT or a related heat-labile toxin from enterotoxigenic *Escherichia coli* have been developed and their efficacy as mucosal adjuvants has been demonstrated (27, 28).

These studies in NOD mice demonstrate unique (native B:9–23 vs A16,19 APL) differential immunologic effects (induction vs suppression of IAA) and enhanced protection by combining the APL with specific adjuvant. Such a potent combined therapy we believe provides initial proof of concept that, with an appropriate adjuvant and a peptide, potent disease and autoantibody suppression can be achieved.

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Disclosures

The authors have no financial conflict of interest.

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Bone Edema Determined by Magnetic Resonance Imaging Reflects Severe Disease Status in Patients with Early-Stage Rheumatoid Arthritis

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ABSTRACT. *Objective.* To determine the significance of bone edema, detected by magnetic resonance imaging (MRI), in early-stage rheumatoid arthritis (RA).

Methods. We simultaneously examined serologic variables, MRI of wrist sites and finger joints of both hands, clinical disease activity score (DAS), and HLA-DR typing at entry in 80 patients with early-stage RA.

Results. The number of bones scored as positive for bone edema correlated with the number of sites scored as positive for MRI synovitis and MRI bone erosion, rate of enhancement (E-rate), and serum C-reactive protein (CRP), matrix metalloproteinase 3 (MMP-3), and interleukin 6 (IL-6). Findings for MRI synovitis and MRI bone erosion, E-rate, CRP, MMP-3, IL-6, seropositivity, and titer of anti-cyclic citrullinated peptide antibody (anti-CCP antibody), DAS28-CRP and HLA-DRB1*0405 allele carrier-ship, were significantly higher in the positive versus the negative bone edema group.

Conclusion. Bone edema based on our scoring system may reflect severe disease status in patients with early-stage RA. However, its clinical value at entry in prognostication of RA should be examined through prospective clinical followup studies. (First Release Oct 1 2007; J Rheumatol 2007;34:2154–7)

Key Indexing Terms:

EARLY-STAGE RHEUMATOID ARTHRITIS
BONE EDEMA

SEROLOGIC VARIABLES

MAGNETIC RESONANCE IMAGING
HLA-DRB1*0405 ALLELE

Evaluation of magnetic resonance imaging (MRI) in rheumatoid arthritis (RA) has been analyzed using the OMERACT 5 RA-MRI scoring system (RAMRIS)^{1,2} and by others^{3,4}; however, the scoring process is complicated. We evaluated MRI features by imaging only wrist sites and finger joints in early-stage RA using different qualification parameters^{5,6}.

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MATERIALS AND METHODS

Enrolled patients were from the Early Arthritis Clinic, First Department of Internal Medicine, Graduate School of Biomedical Sciences, Nagasaki University. The study consists of 80 patients with early-stage RA, who gave their informed consent to the protocol that was approved by the Institutional Review Board of Nagasaki University. Median disease duration from onset of symptoms to entry was 3 months. Disease duration in all 80 patients was < 2 years, similar to a recent report⁷. Diagnosis was made based on 1987 American College of Rheumatology (ACR) criteria for RA⁸. Thirty-six patients were already classified as RA at entry, while the remaining 44, who were classified as undifferentiated arthritis at entry, developed to RA later. Median modified Genant-Sharp score at entry by plain radiography was 0.26.

The following variables were examined at entry. Serologic tests included matrix metalloproteinase-3 (MMP-3), anti-cyclic citrullinated peptide (anti-CCP), and interleukin 6 (IL-6). Clinical disease activity was qualified by DAS28 (Disease Activity Score 28-C-reactive protein (DAS28-CRP)). High resolution analysis of HLA-DRB1 genotyping was performed, as described^{7,9}, by polymerase chain reaction. MR images of both wrists and finger joints (1.5 T system, Sigma, 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., distal radioulnar joint, radiocarpal joint, mid-carpal joint, first carpometacarpal joint, 2nd-5th carpometacarpal joint (together), 1st-5th metacarpophalangeal joints (proximal interphalangeal) separately (total 30 sites in both hands) as reported^{5,6}. MR images were interpreted independently by 2 board-certified radiologists experienced in musculoskeletal imaging (MU and ST) who were blinded to patients' clinical status. Both radiologists read each image according to the definition as described³⁻⁶, and disagreements were resolved by consensus. Degree of MRI features was evaluated per our recent report: synovitis; number of sites scored positive for MRI

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synovitis and E-rate, bone edema; number of bones scored positive for bone edema, bone erosion; number of bones scored positive for MRI bone erosion, respectively⁵.

Differences between groups were examined using the Mann-Whitney U test and chi-square test. A correlation between the 2 variables was calculated by Spearman's rank correlation. A p value < 0.05 denoted a statistically significant difference.

RESULTS

Representative MR images are shown in Figure 1. The number of bones scored as positive for bone edema clearly correlated with the number of sites scored as positive for MRI synovitis and MRI bone erosion, mean E-rate from 30 sites, bone erosion number, CRP, MMP-3, and IL-6 as shown in Figure 2.

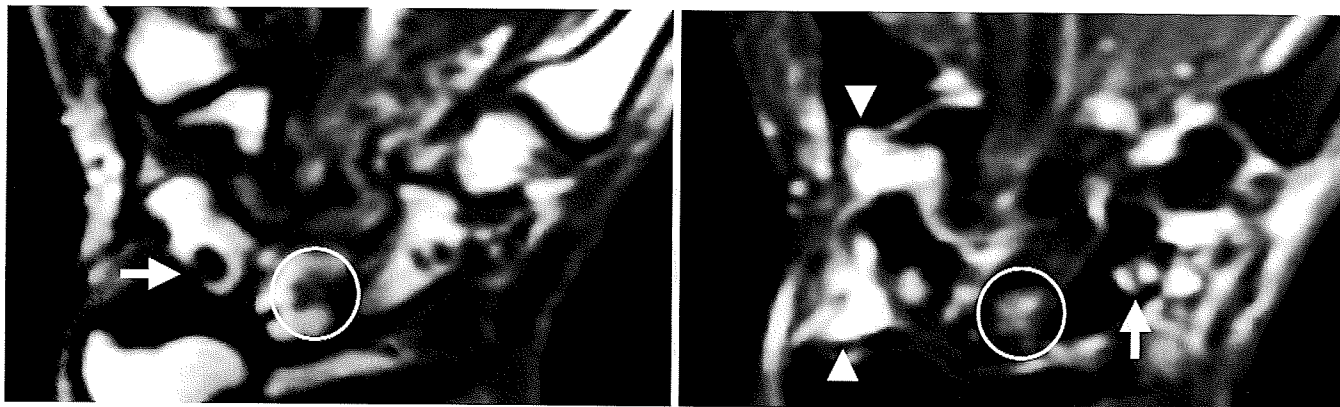


Figure 1. Representative MR images of patients with early-stage RA. T1-weighted spin-echo images show bone edema (circle), bone erosion (arrow), and synovitis (arrowheads). Right panel: gadolinium-diethylenetriamine-enhanced image.

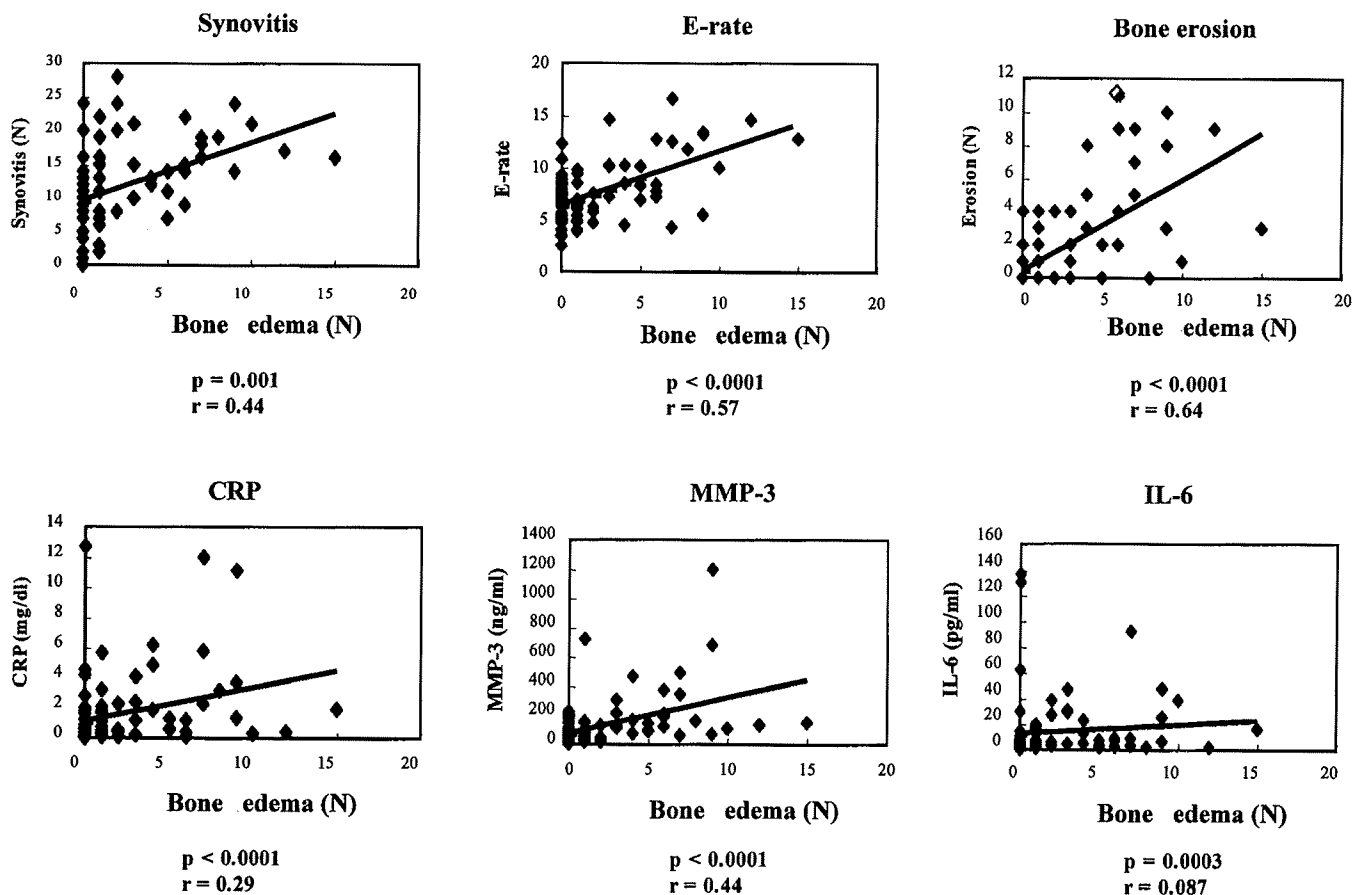


Figure 2. Positive correlation between the number of bones scored as positive for bone edema and the number of sites scored for MRI synovitis and MRI bone erosion, enhancement rate (E-rate), serum concentrations of CRP, MMP-3, and IL-6.

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Patients with early-stage RA were divided into 2 groups: with or without bone edema. MRI evidence of synovial inflammation and bone erosion was more remarkable in bone edema-positive patients. Seropositivity and titer for anti-CCP antibody, CRP, MMP-3, IL-6, and DAS28-CRP were also higher in the bone edema-positive group (Table 1). Positive correlation between DAS28-CRP and the number of bones scored as positive for bone edema was also identified by Spearman's rank correlation test ($p = 0.00026$, $r_s = 0.398$).

In 36 of the 80 (45%) patients with early-stage RA, we confirmed at least one HLA-DRB1*0405 allele, the most frequent shared epitope in Japanese RA patients⁹. This allele was more predominantly distributed in the bone edema-positive group versus the -negative group (Table 1). Our data also showed that significantly more patients who were positive for anti-CCP antibody and HLA-DRB1*0405 allele ($N = 26$) had a positive score for bone edema (77%) compared to patients who were negative for both these markers ($N = 16$), in whom bone edema was scored in only 25% ($p = 0.0013$).

DISCUSSION

Trying to solve the complexity of standard MRI scoring methods, we semiquantitatively evaluated MRI features. The present data suggest that our score, especially bone edema score, classified the disease status of early-stage RA.

Employing prospective analysis, van Gaalen, *et al* recently showed that patients with early-stage RA at entry who are positive for both anti-CCP antibody and HLA-DRB1 shared epitope developed severe erosive disease⁷. Considering bone edema is a forerunner of bone erosion¹⁰, our findings that bone edema-positive early-stage RA patients are preferentially distributed in the subgroup positive for anti-CCP antibody and HLA-DRB1*0405 allele support the findings of van Gaalen, *et al* as well as the prognostic value of bone edema.

Our results suggest that bone edema in patients with early-

stage RA develops through an inflammatory synovial microenvironment with specific HLA-antigen interaction, and our evaluation method for MRI may be an alternative for the standard methods. However, since a therapeutic regimen was already administered in some patients at entry to the study, its clinical value should be verified through prospective followup studies.

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Table 1. Clinical features in early-stage RA with or without bone edema. All the variables were highly significant in the bone edema-positive group.

Variable	Bone Edema, n = 45	No Bone Edema, n = 35	p
CRP, mg/ml*	2.08 ± 2.62	1.03 ± 2.33	< 0.0001
MMP-3, ng/ml*	178.2 ± 225.4	71.4 ± 61.9	0.002
No. of sites scored as positive for MRI synovitis*	13.9 ± 5.9	8.6 ± 5.8	0.0002
Mean E-rate from 30 sites*	8.34 ± 3.26	6.56 ± 2.10	0.03
Bone erosion % [†]	60.0	25.7	0.02
n*	2.71 ± 3.31	0.37 ± 0.81	0.02
IL-6, pg/ml	14.7 ± 17.2	13.1 ± 32.1	0.0003
Anti-CCP antibody %	77.8	52.3	0.02
U/ml*	218.2 ± 399.2	82.4 ± 182.2	0.01
DAS28-CRP	4.54 ± 1.02	3.88 ± 1.18	0.008
HLA-DRB1*0405, %	57.8	28.6	0.009

* Mann-Whitney U test. [†] Chi-square test, as described in text.

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Association of *STAT4* With Susceptibility to Rheumatoid Arthritis and Systemic Lupus Erythematosus in the Japanese Population

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Objective. *STAT4* encodes a transcriptional factor that transmits signals induced by several key cytokines, and it might be a key molecule in the development of autoimmune diseases. Recently, a *STAT4* haplotype was reported to be associated with rheumatoid arthritis

(RA) and systemic lupus erythematosus (SLE) in Caucasian populations. This was replicated in a Korean RA population. Interestingly, the degree of risk of RA susceptibility with the *STAT4* haplotype was similar in the Caucasian and Korean populations. The present study was undertaken to investigate the effect of *STAT4* on susceptibility to RA and SLE in the Japanese.

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Methods. We performed an association study using 3 independent Japanese RA case-control populations (total 3,567 cases and 2,199 controls) and 3 independent Japanese SLE populations (total 591 cases). All samples were genotyped using the TaqMan fluorogenic 5' nuclease assay for single-nucleotide polymorphism (SNP) rs7574865, which tags the susceptibility haplotype. The association of the SNP with disease susceptibility in each case-control study was calculated using Fisher's exact test, and the results were combined, using the Mantel-Haenszel method, to obtain combined odds ratios (ORs).

Results. We observed a significant association of the *STAT4* polymorphism with susceptibility to both RA and SLE. The combined ORs for RA and SLE, respectively, were 1.27 ($P = 8.4 \times 10^{-9}$) and 1.61 ($P = 2.1 \times 10^{-11}$) for allele frequency distribution; these ORs were quite similar to those previously observed in the Caucasian population.

Conclusion. We conclude that *STAT4* is associated with RA and SLE in the Japanese. Our results indicate that *STAT4* is a common genetic risk factor for autoimmune diseases, with similar strength across major racial groups.

Rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) are chronic inflammatory autoimmune diseases characterized by pathologic infiltration of lymphocytes in target organs. Although the pathogenesis of these diseases remains unclear, dysregulated lymphocyte activation via the breakdown of self tolerance is believed to be implicated in their pathogenesis, and multiple genetic and environmental factors are important in the development of these diseases.

Recently, Amos et al conducted a genome-wide linkage scan using >5,700 single-nucleotide polymorphisms (SNPs) in 642 Caucasian families with affected sibling pairs; they found the best evidence of linkage at chromosomes 2q33 (1). Following the linkage analysis, Remmers et al performed a large case-control study of 13 selected candidate genes within the linkage region and found an association between a common haplotype located in the third intron of *STAT4* and susceptibility to RA and SLE (2). The association was replicated in several independent Caucasian RA and SLE populations, and also in a Korean RA population (2,3).

STAT4 encodes signal transducer and activator of transcription 4, the STAT protein family member that is uniquely activated by interleukin-12 (IL-12) through its receptor, which has an essential downstream role in Th1 cell differentiation and proliferation (4). In addition, it has been reported that STAT-4 is necessary for the development of Th17 cells (IL-17-producing CD4+ T cells) (5). Since Th1 cells and Th17 cells play an important role in chronic inflammatory disorders and since STAT-4 is considered to be a key molecule in both the Th1 and Th17 lineages, STAT-4 may play a crucial role in the development of autoimmune diseases such as RA and SLE.

Genetic association between HLA-DRB1 and RA susceptibility has been well established, and several other risk genes for RA outside the HLA region have been identified. However, while DRB1 has been repeatedly shown to be an RA risk locus in Caucasian and Asian populations, the other reported RA risk genes, such as *PTPN22*, *PADI4*, and *FCRL3*, have been difficult to replicate in other ethnic populations aside from the original populations first reported (6). These conflicting results suggest that the genetic background of the disease may vary among ethnic groups.

Interestingly, the degree of risk of RA susceptibility observed with the *STAT4* haplotype was found to be similar in the Caucasian and Korean populations (2,3). This finding indicates that the risk haplotype for RA susceptibility might be common across major racial groups. In the present study, we investigated the associ-

ation of *STAT4* with RA susceptibility using large series of Japanese RA cohorts. We also tried to evaluate whether the gene is associated with RA outcome measures in a Japanese RA cohort. In addition, we tested the association between the gene and susceptibility to SLE in the Japanese population. This study is the first to investigate the association of *STAT4* with SLE in an Asian population.

PATIENTS AND METHODS

Subjects and disease criteria. All patients with RA fulfilled the American College of Rheumatology (ACR; formerly, the American Rheumatism Association) 1987 revised criteria for the disease (7). All patients with SLE met the ACR 1982 revised criteria for the disease (8).

DNA samples were obtained from subjects in 3 RA case-control series (Table 1). DNA from the case subjects in the Tokyo Women's Medical University (TWMU) Institute of Rheumatology RA cohort (IORRA) case-control series was obtained from the IORRA DNA collection. The IORRA is an observational RA cohort with an enrollment of nearly 5,000 Japanese RA patients, and DNA samples were collected from 1,504 of these patients (mean age 59.3 years, 84% female, 88% rheumatoid factor [RF] positive) (9). This DNA collection was also used to analyze the effect of the single-nucleotide polymorphism (SNP) (see below) on RA outcome measures. Demographic, clinical, and treatment information on IORRA patients as of the spring of 2003 was obtained from the IORRA database, which includes the Disease Activity Score in 28 joints (DAS28) (10) and the Japanese version of the Health Assessment Questionnaire (J-HAQ) (11). Radiographs of the hands and feet of the IORRA patients, obtained when the duration of disease was 5 years, were reviewed retrospectively, and radiographic joint damage was assessed by a single skilled reader, using the modified Sharp/van der Heijde score (SHS) (12). The SHS includes a count of erosions and joint space narrowing in the hands and feet and has a range of 0 (no damage) to 448 (highest damage). DNA samples from popu-

Table 1. Case-control series for the studies of rheumatoid arthritis and systemic lupus erythematosus

Series	No. of patients	No. of controls
Rheumatoid arthritis		
IORRA	1,504	752
RIKEN	1,113	940
Tokushima	950	507
Systemic lupus erythematosus		
TWMU	238	752*
RIKEN	188	940†
Tokushima/Fukuoka	165	212

* Genotype information was obtained from the controls in the Tokyo Women's Medical University (TWMU) Institute of Rheumatology Rheumatoid Arthritis cohort (IORRA) rheumatoid arthritis series.

† Genotype information was obtained from the controls in the Institute of Physical and Chemical Research (RIKEN) series.

lation controls were obtained from the Pharma SNP consortium (<http://www.jpma.or.jp/psc/index.html>).

DNA from the case subjects in the Institute of Physical and Chemical Research (RIKEN) RA case-control series (mean age 60.4 years, 82% female, 70% RF positive) was obtained from the BioBank Japan Project DNA collection. As part of the BioBank Japan Project, DNA and serum samples along with clinical data have been collected from 300,000 patients with 47 diseases, including RA (13). Sixty-six hospitals affiliated with 12 institutions are participating in the project. Population-based control subjects were recruited through the Rotary Club of Osaka-Midosuji District 2660 Rotary International in Japan.

Patients and controls in the Tokushima RA case-control series were recruited through the orthopedics clinic at University of Tokushima Hospital, its community affiliates, and the rheumatology clinic at Tokushima Kensei Hospital (Tokushima, Japan) (14). The mean age of the patients was 61.8 years, and 79% were female.

Cases with SLE were also obtained from 3 sources (Table 1). TWMU patients were recruited from Institute of Rheumatology and Aoyama Hospital, TWMU. RIKEN patients were recruited through the Specified Disease Treatment Research Program of the Japanese Ministry of Health, Labor, and Welfare. Several medical institutions nationwide are participating in the program. These 2 series included only cases; control genotype information was obtained from the RA case-control series in the IORRA and RIKEN, respectively. Patients and control subjects in the Tokushima/Fukuoka lupus case-control series were recruited from Kyushu University Hospital (Fukuoka, Japan) (15).

The ethics committee of each institution (TWMU, RIKEN, and University of Tokushima) granted approval for the study, and each individual subject signed an informed consent form after receiving a verbal explanation of the study.

SNP genotyping. A polymorphism located within intron 3 of *STAT4*, rs7574865, which tags the susceptibility haplotype, was selected for this study because it exhibited the best evidence for association in the primary study (2) and was one of the SNPs most significantly associated with RA susceptibility in the Korean replication study (3). This SNP has been considered to be in strong linkage equilibrium with a putative

functional variant. Genotyping was performed using the TaqMan fluorogenic 5' nuclease assay, according to the instructions of the manufacturer (Applied Biosystems, Tokyo, Japan). All polymerase chain reactions were performed using GeneAmp PCR System 9700 (Applied Biosystems), and end point fluorescence readings were performed with an ABI Prism 7900 HT Sequence Detection System (Applied Biosystems).

Statistical analysis. Allele frequencies of SNP rs7574865 in each case-control series were estimated by the allele counting method. Chi-square testing was used to identify significant departure from Hardy-Weinberg equilibrium.

Association of the SNP with susceptibility to RA or SLE in each study was estimated by Fisher's exact test; we compared the allelic effect of T (suspected risk allele) with G (common allele), and the genotypic effect of the homozygous genotypes with other genotypes. After assessing heterogeneity among the studies as determined based on Woolf's method, the Mantel-Haenszel test was used to evaluate combined odds ratios (ORs) and 95% confidence intervals (95% CIs), demonstrating the population-wide impact of the polymorphism on disease susceptibility.

Differences in patient characteristics among IORRA subjects with different rs7574865 genotypes were assessed by Kruskal-Wallis test or Fisher's exact test. The allelic effect of rs7574865 on the SHS was analyzed by linear regression analysis.

All statistical tests were implemented using the R software package, version 2.6.0 (<http://www.r-project.org/>).

RESULTS

On average, we achieved a genotyping success rate of 98.9%, with call rates of >98.2% for each case-control series. The genotype concordance rate was 100% as assessed by random retyping across different plates. Genotype distributions for SNP rs7574865 were in Hardy-Weinberg equilibrium in each case-control series.

Association of *STAT4* polymorphism with RA. The data summarized in Table 2 show the allele fre-

Table 2. Association of single-nucleotide polymorphism rs7574865 with RA in Japanese subjects*

Series, subjects	Genotype					Allele		GG vs. others		TT vs. others	
	GG	GT	TT	Total	MAF	OR (95% CI)	<i>P</i>	OR (95% CI)	<i>P</i>	OR (95% CI)	<i>P</i>
IORRA											
RA	588	694	199	1,481	0.37	1.29 (1.13–1.48)	1.7×10^{-4}	1.38 (1.15–1.66)	3.9×10^{-4}	1.41 (1.05–1.89)	0.020
Controls	355	316	74	745	0.31						
RIKEN											
RA	447	502	160	1,109	0.37	1.31 (1.15–1.50)	4.8×10^{-5}	1.38 (1.16–1.66)	3.0×10^{-4}	1.48 (1.12–1.96)	0.0048
Controls	453	389	96	938	0.31						
Tokushima											
RA	365	448	128	941	0.37	1.17 (0.99–1.38)	0.056	1.20 (0.96–1.50)	0.11	1.30 (0.92–1.86)	0.13
Controls	216	230	54	500	0.34						
Combined						1.27 (1.17–1.37)	8.4×10^{-9}	1.34 (1.20–1.49)	1.9×10^{-7}	1.41 (1.19–1.67)	8.5×10^{-5}

* RA = rheumatoid arthritis; MAF = minor allele frequency; OR = odds ratio; 95% CI = 95% confidence interval (see Table 1 for other definitions).

Table 3. Association of single-nucleotide polymorphism rs7574865 with SLE in Japanese subjects*

Series, subjects	Genotype					Allele		GG vs. others		TT vs. others	
	GG	GT	TT	Total	MAF	OR (95% CI)	<i>P</i>	OR (95% CI)	<i>P</i>	OR (95% CI)	<i>P</i>
TWMU											
SLE	76	103	48	227	0.44	1.73 (1.38–2.15)	8.3×10^{-7}	1.81 (1.31–2.50)	1.8×10^{-4}	2.43 (1.59–3.68)	2.9×10^{-5}
Controls	355	316	74	745	0.31						
RIKEN											
SLE	70	85	28	183	0.39	1.40 (1.10–1.77)	0.0059	1.51 (1.08–2.12)	0.015	1.58 (0.97–2.53)	0.053
Controls	453	389	96	938	0.31						
Tokushima/Fukuoka											
SLE	51	83	31	165	0.44	1.79 (1.31–2.45)	1.4×10^{-4}	2.07 (1.33–3.25)	0.0010	2.34 (1.22–4.59)	0.0059
Controls	102	91	19	212	0.30						
Combined						1.61 (1.40–1.85)	2.1×10^{-11}	1.74 (1.43–2.12)	4.9×10^{-8}	2.08 (1.59–2.72)	8.5×10^{-8}

* SLE = systemic lupus erythematosus; MAF = minor allele frequency; OR = odds ratio; 95% CI = 95% confidence interval (see Table 1 for other definitions).

quency and genotype distribution in RA patients and controls in each case–control series. We observed a significant difference in allele frequency and genotype distribution of the *STAT4* polymorphism between RA patients and controls in the IORRA and the RIKEN cohorts, while no significant difference was found in the Tokushima series. When study-specific ORs were combined using the Mantel-Haenszel method, the differences in allele frequency and genotype distributions of the SNP between patients and controls were significant (combined OR 1.27 [95% CI 1.17–1.37, $P = 8.4 \times 10^{-9}$]-1.41 [95% CI 1.19–1.67, $P = 8.5 \times 10^{-5}$]). There was no significant heterogeneity among the studies, as assessed by Woolf’s method ($P > 0.05$).

Association of *STAT4* polymorphism with SLE.

Table 3 shows the genotype distribution and minor allele frequency in the 3 SLE series. As with RA, we found differences in the allele frequency and genotype distributions of SNP rs7574865 between SLE patients and controls; these were significant in all 3 case–control series. No significant evidence of heterogeneity among the studies was identified by Woolf’s method ($P > 0.05$), and the combined OR for the polymorphism as calculated by Mantel-Haenszel testing was 1.61 (95% CI 1.40–1.85, $P = 2.1 \times 10^{-11}$). Combined ORs for the recessive trait and the dominant trait were 2.08 (95% CI 1.59–2.72) and 1.74 (95% CI 1.43–2.12), respectively.

Stratified analyses of clinical and laboratory variables in RA patients. Among 1,504 patients with available DNA samples, 1,335 participated in the IORRA clinical data collection in the spring of 2003, and information on their demographic, clinical, and treatment details as of that time could be obtained from the IORRA database (Table 4). Consistent with previous findings by Lee et al in a Korean population (3),

there was no significant genotypic association with age at disease onset or sex. We also found no significant differences among the genotypes in age, disease duration, family history of RA, RF status, DAS28 score, or J-HAQ score. There was a trend toward an association of risk allele with elevated levels of inflammation markers and patient’s assessment of global health, but these were not significant. Only glucocorticoid usage and glucocorticoid dosage were found to differ significantly among the genotypes, with the difference increasing in a stepwise manner according to the number of risk alleles (median dosage 0, 1, and 2.5 mg equivalent prednisolone, respectively, among patients with the GG, GT, and TT genotypes).

The SHS after 5 years of disease could be measured in 163 patients, of whom 160 were genotyped. Although a trend toward an effect of the risk allele on the SHS was observed, it was not significant ($P = 0.22$) (median score 40, 45, and 46, respectively, among patients with the GG, GT, and TT genotypes [$n = 67, 79,$ and 14, respectively]).

We did not perform a stratified analysis on anti-cyclic citrullinated peptide antibody (anti-CCP) positivity, since anti-CCP data were not available on most of the patients from the IORRA DNA collection. However, Lee and colleagues suggested that, at least among Asians, the risk of RA susceptibility associated with the *STAT4* variant may not be restricted to the anti-CCP positive disease subset (3).

DISCUSSION

This study is the first to investigate the association of a *STAT4* polymorphism with genetic susceptibility to lupus in any Asian population, and susceptibility to

Table 4. Genotypic differences in clinical or laboratory variables in RA patients*

	Total	Genotype			P†
		GG	GT	TT	
No. (%) of patients	1,335	521 (40)	610 (46)	183 (14)	
Age, years	60 (53–68)	61 (53–68)	60 (53–68)	60 (52–66)	0.14
Female, no. (%)	1,125 (84)	441 (85)	507 (83)	157 (86)	0.63
Disease duration, years	10 (5–16)	10 (5–17)	10 (5–16)	10 (5–17)	0.71
Age at RA onset, years	48 (39–57)	49 (40–57)	49 (40–57)	48 (38–55)	0.18
Family history of RA, no. (%)	415 (32)	157 (31)	187 (31)	63 (35)	0.54
RF positive, no. (%)‡	1,195 (90)	468 (90)	543 (89)	164 (90)	0.92
RF titer, IU/ml‡	116 (48–282)	115.5 (49–296)	116 (49–278)	122 (46–283)	0.97
Treatment§					
NSAID, no. (%)	980 (73)	378 (73)	446 (73)	143 (78)	0.32
DMARD, no. (%)	1,228 (92)	478 (92)	560 (92)	171 (93)	0.78
Glucocorticoid, no. (%)	719 (54)	266 (51)	325 (53)	117 (64)	0.01
Prednisolone, mg	1 (0–4.9)	0 (0–4)	1 (0–5)	2.5 (0–5)	0.01
DAS28	3.6 (2.7–4.5)	3.5 (2.7–4.4)	3.6 (2.8–4.5)	3.8 (2.8–4.6)	0.39
TJC	1 (0–3)	1 (0–3)	1 (0–3)	1 (0–3)	1.00
SJC	1 (0–3)	1 (0–3)	1 (0–3.75)	1 (0–4)	0.25
Patient's global assessment by VAS, mm	27 (10–54)	24 (9–54)	27 (11–55)	33 (14–53)	0.12
ESR, mm/hour	28 (16–48)	27 (16–46)	29 (16–48)	31 (16–54)	0.51
CRP, mg/dl	0.7 (0.2–1.6)	0.6 (0.2–1.6)	0.7 (0.2–1.6)	0.95 (0.3–1.9)	0.08
J-HAQ	0.625 (0.125–1.375)	0.625 (0.125–1.375)	0.625 (0.125–1.25)	0.625 (0.125–1.375)	0.96

* Data on some variables were missing for a small number of patients (maximum 3.1%). Data on genotype were missing for 21 patients (1.6%); therefore, values in the individual columns under Genotype are for 1,314 patients (521 for GG, 610 for GT, 183 for TT). Except where indicated otherwise, values are the median (interquartile range). RA = rheumatoid arthritis; NSAID = nonsteroidal antiinflammatory drug; DMARD = disease-modifying antirheumatic drug; DAS28 = Disease Activity Score in 28 joints; TJC = tender joint count (28 joints); SJC = swollen joint count (28 joints); VAS = visual analog scale; ESR = erythrocyte sedimentation rate; CRP = C-reactive protein; J-HAQ = Japanese version of the Health Assessment Questionnaire.

† By Kruskal-Wallis test or Fisher's exact test.

‡ The highest rheumatoid factor (RF) value measured in the cohort project during 2000–2006 for each individual was used. Cutoff for positivity = 15.0 IU/ml.

§ Biologic agents were not available in Japan at this time (spring 2003). Glucocorticoid dosage was calculated as the prednisolone equivalent dosage in milligrams.

RA in a Japanese population. Although replication studies using other ethnic populations are essential for establishing any genetic association, results are often reported as negative in the other populations. One of the reasons for this is that the degree of genetic risk differs among ethnic groups.

Concerning RA genetics, many study groups worldwide have made great efforts to newly identify susceptibility genes and to replicate findings of other groups, particularly using Caucasian or Asian populations. However, findings for most susceptibility genes identified outside the HLA region have not been replicated in the populations different from the population used in the primary study. A typical example of this is an association between *PTPN22* and susceptibility to RA. A missense SNP in *PTPN22* known as R620W was discovered as a common genetic risk factor for several autoimmune diseases including RA in a Caucasian population, and the finding has been replicated in many Caucasian RA cohorts (16). However, the risk allele is extremely rare in Asians, and attempts to validate the

association in Asian populations have been unsuccessful (17). In contrast, the association between *PADI4* and RA susceptibility is thought to be strong among Asian populations, and indeed, most replication studies in Asian populations have succeeded in validating this association. However, a meta-analysis of studies using Caucasian populations revealed the combined OR for the association to be as low as 1.1 (18), and as a result, replication studies in Caucasian populations have seldom validated the association.

Failure to replicate a genetic association in a different ethnic population from the population used in the primary study is often due to low statistical power. To avoid this problem, it is important to make the sample size as large as possible, as we did in the present multicenter Japanese case-control study. We collected 3,567 RA cases and 591 SLE cases to validate the association between *STAT4* and susceptibility to RA and lupus in the Japanese. Consistent with previous reports (2,3), we observed a significant association of the *STAT4* polymorphism with both RA and SLE susceptibility in

the Japanese. Although the risk allele frequency in control populations is slightly different between Caucasians (22%) and Japanese (30–34%), the OR shown by investigation of the allele frequency distribution of rs7574865, 1.27, is exactly the same as in the Caucasian populations. Also, the impact of the risk allele on susceptibility to SLE in the Japanese population was found to be similar to that obtained in the previous meta-analysis of studies of Caucasian populations (1.61) (2). These results suggest that the responsible functional variant, which remains unknown, is ancient in origin. Further independent studies using populations of other ethnicities would help to prove the hypothesis.

Autoimmune diseases are initiated by breakdown of self tolerance, and thus, they may share a common pathogenesis. Indeed, some RA susceptibility genes have been identified as common risk factors for clinically different autoimmune phenotypes. One of them is *PTPN22*, which has been reported as a disease susceptibility gene for type 1 diabetes, autoimmune thyroid disease, lupus, Addison's disease, and juvenile idiopathic arthritis, in addition to RA (16). *CTLA4*, one of the genes associated with lupus and RA, especially in Asians, has also been suggested to be a disease-associated gene in a variety of other autoimmune diseases (19). Both *PTPN22* and *CTLA4* negatively regulate T cell activation and maintain peripheral tolerance, and T cells play a central role in the immunopathogenesis of autoimmune diseases. STAT-4 is suggested to be a key molecule in both the Th1 and Th17 lineages, and therefore may be involved in a common pathway of pathogenesis in autoimmune diseases.

It is reasonable to speculate that a variant on *STAT4* could also affect disease activity in autoimmune diseases through dysregulation of the Th1 and Th17 pathways. Although we did not find evidence of association between *STAT4* and disease activity in RA, we did observe a trend toward an effect of the risk allele on elevated levels of inflammation markers and patient's global assessment. Both the fact that glucocorticoid usage and dosage increased significantly in a stepwise manner in parallel with the number of risk alleles and the knowledge that glucocorticoid treatment significantly reduces levels of inflammation markers suggest that the polymorphism on *STAT4* might be associated with disease activity in RA. Although a trend toward an effect of risk allele on radiographic damage in the first 5 years was observed, it was not significant, similar to findings in the Korean study (3). However, while differences in other clinical variables among the genotypes were tested using DNA from 1,335 patients, the effect

on radiographic severity was tested only in 163 patients, due to the unavailability of suitable radiographs in the others (20). As a result, the statistical power of the study of association with radiographic severity was rather limited. There were also other potential sources of artifacts that should be considered in interpretation of these preliminary data. A large prospective study, accounting for the genotypes of *STAT4*, is needed to definitively answer the question of its associations with clinical and laboratory features.

The functional variant in *STAT4* that is responsible for increased disease susceptibility remains unknown. Since the susceptibility haplotype is located within intron 3 of *STAT4*, it is considered to be responsible for splice variation or regulatory effects of STAT-4. However, it might be also possible that the putative functional variant could be responsible for a biologic effect on intragenic RNA or other factors. Studies to investigate the functional variant on the susceptibility haplotype remain to be performed.

In conclusion, using Japanese RA and SLE case-control series with large samples, we confirmed *STAT4* polymorphism as a common genetic risk factor for these autoimmune diseases. The strength of the association was found to be similar across major racial groups.

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AUTHOR CONTRIBUTIONS

Dr. Ikari 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 design. Ikari, Kochi, Inoue.

Acquisition of data. Ikari, Kochi, Yamamoto, Shimane, Nakamura, Toyama, Kawaguchi, Terai, Hara, Tomatsu, Yamanaka, Horiuchi, Tao, Yasumoto, Hamada, Yasui, Inoue, Itakura, Okamoto, Kamatani, Momohara.

Analysis and interpretation of data. Kobayashi, Ikari, Kaneko, Kochi, Mochizuki, Tsukahara, Inoue.

Manuscript preparation. Kobayashi, Ikari, Kochi, Inoue.

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