Table II. *Summary of peptide/CT vaccination*

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

the Victor^2 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 ^{125}I-labeled insulin Ag (Amersham Biosciences) at 20,000 cpm was incubated with 5 μl 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 μl 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 (Δcpm) between wells with and without cold insulin and expressed as an index defined as follows: (sample Δcpm − human negative control Δcpm)/(human positive control Δcpm − human negative control Δcpm). 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 TCRC$\alpha^{-/-}$ 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 μCi) 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.

^{51}Cr-release cytotoxicity assay

P815 cells (1×10^6) were incubated with 0.1 μCi of ^{51}Cr-labeled sodium chromate in 100 μl for 1 h, washed, and resuspended at 10^4 cells per 50 μl. 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 ^{51}Cr-release in a gamma counter. Specific lysis was determined as follows: ((cytotoxic release − minimum release)/(maximum − minimum)) × 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 insulitis, and the levels of insulitis 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. Insulitis 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 μg of the insulin B:9–23 peptide failed to induce anti-B:9–23-specific Abs (Fig. 1*A*). 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

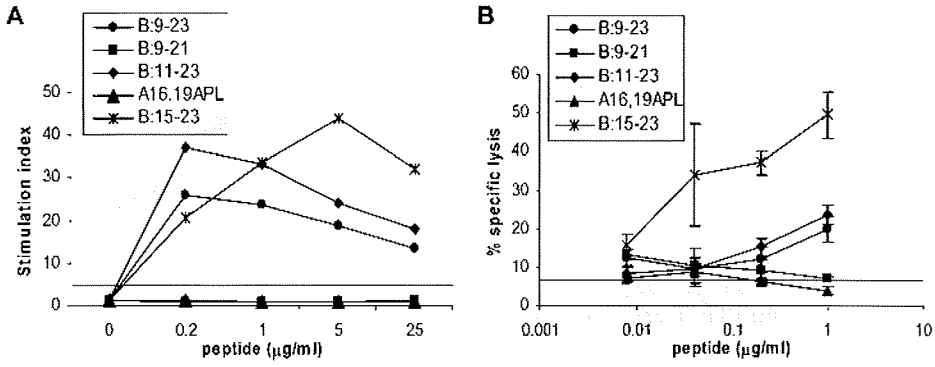

FIGURE 2. Functional responses of insulin-reactive CD8 T cells to peptides. *A*, Proliferation of insulin-reactive CD8 T cells to increasing concentrations of insulin B chain-derived peptides. The results are shown as a stimulation index (proliferation with peptide/proliferation without peptide). Background counts without peptide were 6786 + 657 cpm. *B*, ^{51}Cr-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.

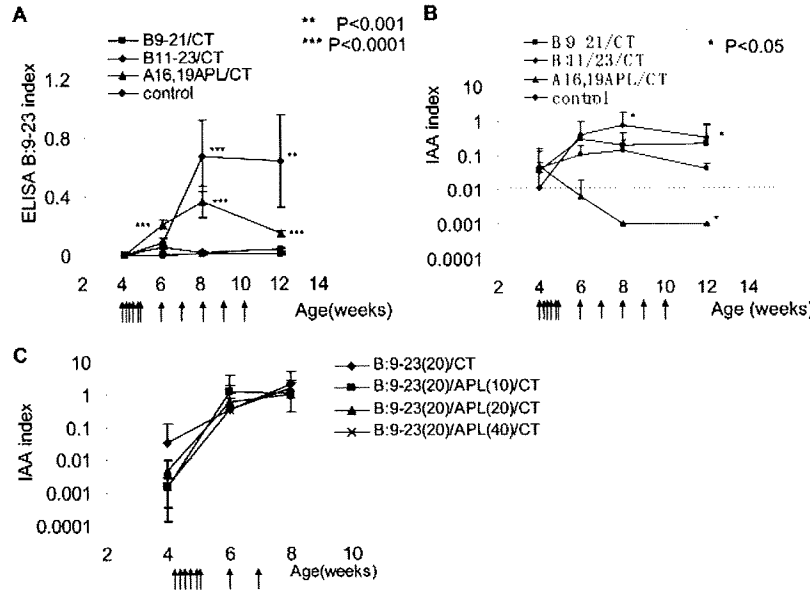

FIGURE 3. *A* and *B*, Anti-B:9–23 peptide Ab expression (*A*) and IAA expression (*B*) following intranasal administration of truncated B:9–23 and A16,19 APL together with CT in NOD mice. ■, B:9–21/CT (n = 10); ♦, B:11–23/CT (n = 9); ▲, A16,19 APL/CT (n = 8); ●, control (CT/TT and PBS) (n = 14 and 15, 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 ^{51}Cr-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*).

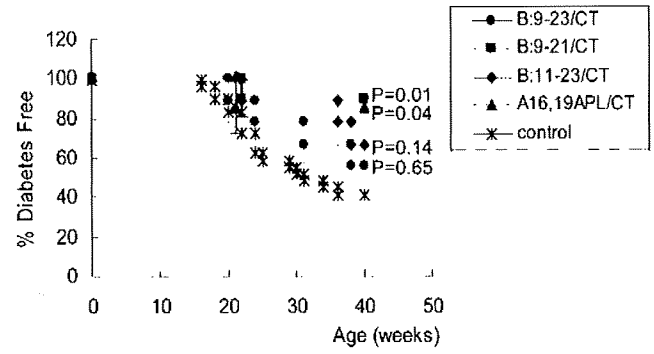

FIGURE 4. Life table analysis for the development of diabetes following intranasal administration of native B:9–23, truncated B:9–23, and A16,19 APL together with CT in NOD mice. Both intranasal-A16,19 APL/CT and intranasal-B:9–21/CT significantly suppressed the development of diabetes.

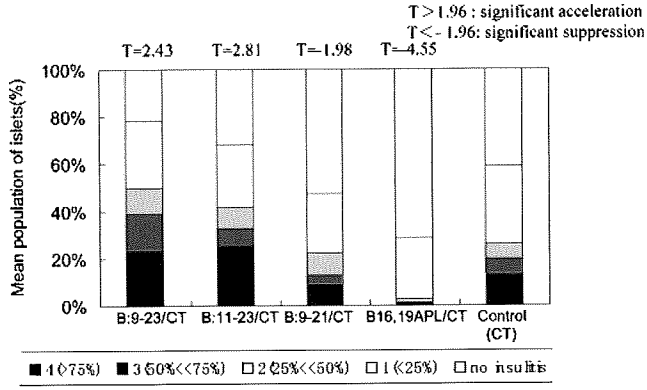

FIGURE 5. Insulitis levels at 12 wk determined by Ridit analysis. A level of $T > 1.96$ was regarded as significant increase. A level of $T < -1.96$ was regarded as significant suppression. Insulitis levels were significantly increased in the B:9–23/CT ($T = 2.43$) and B:11–23/CT ($T = 2.81$) compared 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 insulitis 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 insulitis 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 insulitis ($T = -1.98$ and -4.55, respectively), and all islets of mice immunized with the A16,19 APL/CT had no insulitis or only minimal peri-insulitis (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

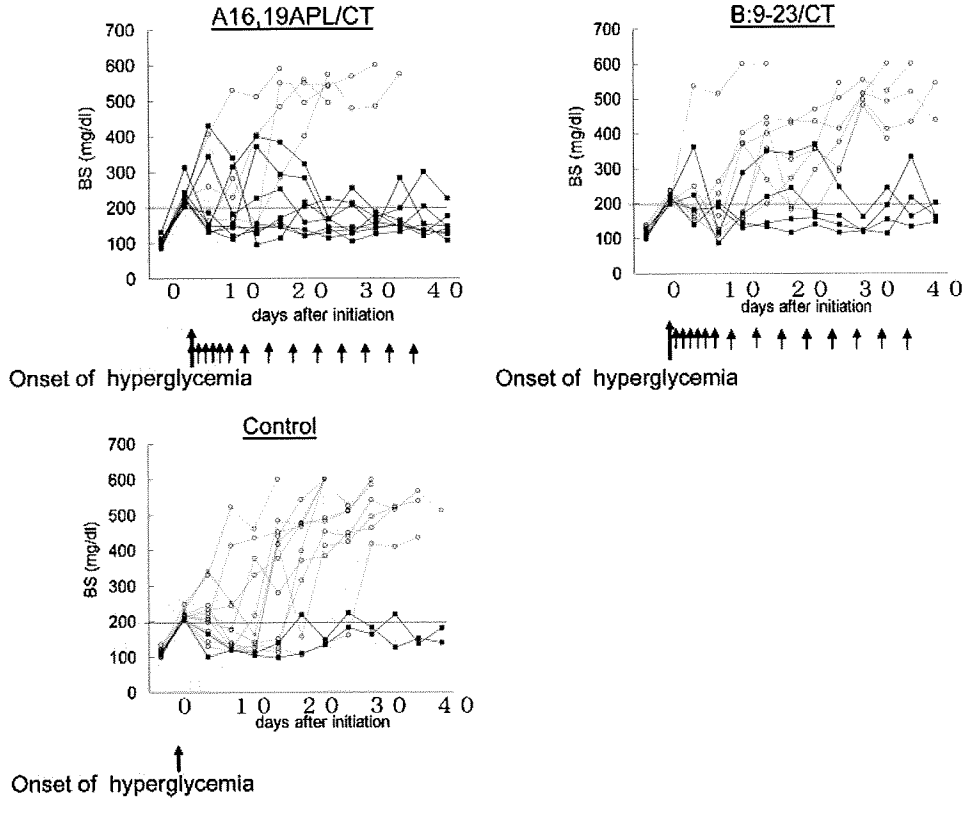

FIGURE 6. Blood glucose levels in the NOD mice intranasally treated with A16,19 APL/CT (*top left*), B:9–23/CT (*top right*), and control (*bottom left*) starting on the onset of diabetes with glucose levels between 200 mg/dl and 249 mg/dl. The peptide was intranasally immunized on days 1–5 and then 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. 3*B*). 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 insulitis 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 insulitis, 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 insulitis (Figs. 4 and 5) and without any effect on the spontaneous expression of IAA (Fig. 3*B*).

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 previously 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 insulitis, similar to the native peptide, intranasal immunization with this peptide combined with CT completely eliminated IAA expression and strongly suppressed the development of insulitis. The inhibition of insulitis 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.

Acknowledgment

We thank Marcella Li at Barbara Davis Center for Childhood Diabetes (Denver, CO) for her technical assistance.

Disclosures

The authors have no financial conflict of interest.

References

1. Martinez, N. R., P. Augstein, A. K. Moustakas, G. K. Papadopoulos, S. Gregori, L. Adorini, D. C. Jackson, and L. C. Harrison. 2003. Disabling an integral CTL epitope allows suppression of autoimmune diabetes by intranasal proinsulin peptide. *J. Clin. Invest.* 111: 1365–1371.
2. Moriyama, H., N. Abiru, J. Paronen, K. Sikora, E. Liu, D. Miao, D. Devendra, J. Beilke, R. Gianani, R. G. Gill, and G. S. Eisenbarth. 2003. Evidence for a primary islet autoantigen (preproinsulin 1) for insulitis and diabetes in the nonobese diabetic mouse. *Proc. Natl. Acad. Sci. USA* 100: 10376–10381.
3. Nakayama, M., N. Abiru, H. Moriyama, N. Babaya, E. Liu, D. Miao, L. Yu, D. R. Wegmann, J. C. Hutton, J. F. Elliott, and G. S. Eisenbarth. 2005. Prime role for an insulin epitope in the development of type 1 diabetes in NOD mice. *Nature* 435: 220–223.
4. Daniel, D., and D. R. Wegmann. 1996. Protection of nonobese diabetic mice from diabetes by intranasal or subcutaneous administration of insulin peptide B-(9-23). *Proc. Natl. Acad. Sci. USA* 93: 956–960.
5. Abiru, N., D. Wegmann, E. Kawasaki, P. Gottlieb, E. Simone, and G. S. Eisenbarth. 2000. Dual overlapping peptides recognized by insulin peptide B:9-23 reactive T cell receptor AV13S3 T cell clones of the NOD mouse. *J. Autoimmun.* 14: 231–237.
6. Wong, F. S., J. Karttunen, C. Dumont, L. Wen, I. Visintin, I. M. Pilip, N. Shastri, E. G. Pamer, and C. A. Janeway, Jr. 1999. Identification of an MHC class I-restricted autoantigen in type 1 diabetes by screening an organ-specific cDNA library. *Nat. Med.* 5: 1026–1031.
7. Wong, F. S., A. K. Moustakas, L. Wen, G. K. Papadopoulos, and C. A. Janeway, Jr. 2002. Analysis of structure and function relationships of an autoantigenic peptide of insulin bound to H-2K(d) that stimulates CD8 T cells in insulin-dependent diabetes mellitus. *Proc. Natl. Acad. Sci. USA* 99: 5551–5556.
8. Alleva, D. G., A. Gaur, L. Jin, D. Wegmann, P. A. Gottlieb, A. Pahuja, E. B. Johnson, T. Motheral, A. Putnam, P. D. Crowe, et al. 2002. Immunological characterization and therapeutic activity of an altered-peptide ligand, NBI-6024,

based on the immunodominant type 1 diabetes autoantigen insulin B-chain (9-23) peptide. *Diabetes* 51: 2126–2134.
9. Abiru, N., A. K. Maniatis, L. Yu, D. Miao, H. Moriyama, D. Wegmann, and G. S. Eisenbarth. 2001. Peptide and MHC-specific breaking of humoral tolerance to native insulin with the B:9-23 peptide in diabetes-prone and normal mice. *Diabetes* 50: 1274–1281.
10. Arakawa, T., T. Tsuboi, A. Kishimoto, J. Sattabongkot, N. Suwanabun, T. Rungruang, Y. Matsumoto, N. Tsuji, H. Hisaeda, A. Stowers, et al. 2003. Serum antibodies induced by intranasal immunization of mice with *Plasmodium vivax* Pvs25 co-administered with cholera toxin completely block parasite transmission to mosquitoes. *Vaccine* 21: 3143–3148.
11. Liu, E., H. Moriyama, N. Abiru, D. Miao, L. Yu, R. M. Taylor, F. D. Finkelman, and G. S. Eisenbarth. 2002. Anti-peptide autoantibodies and fatal anaphylaxis in NOD mice in response to insulin self-peptides B:9-23 and B:13-23. *J. Clin. Invest.* 110: 1021–1027.
12. Yu, L., D. T. Robles, N. Abiru, P. Kaur, M. Rewers, K. Kelemen, and G. S. Eisenbarth. 2000. Early expression of anti-insulin autoantibodies of man and the NOD mouse: evidence for early determination of subsequent diabetes. *Proc. Natl. Acad. Sci. USA* 97: 1701–1706.
13. Abiru, N., L. Yu, D. Miao, A. K. Maniatis, E. Liu, H. Moriyama, and G. S. Eisenbarth. 2001. Transient insulin autoantibody expression independent of development of diabetes: comparison of NOD and NOR strains. *J. Autoimmun.* 17: 1–6.
14. Liu, E., N. Abiru, H. Moriyama, D. Miao, and G. S. Eisenbarth. 2002. Induction of insulin autoantibodies and protection from diabetes with subcutaneous insulin B:9-23 peptide without adjuvant. *Ann. NY Acad. Sci.* 958: 224–227.
15. Chen, W., I. Bergerot, J. F. Elliott, L. C. Harrison, N. Abiru, G. S. Eisenbarth, and T. L. Delovitch. 2001. Evidence that a peptide spanning the B-C junction of proinsulin is an early autoantigen epitope in the pathogenesis of type 1 diabetes. *J. Immunol.* 167: 4926–4935.
16. Kent, S. C., Y. Chen, L. Bregoli, S. M. Clemmings, N. S. Kenyon, C. Ricordi, B. J. Hering, and D. A. Hafler. 2005. Expanded T cells from pancreatic lymph nodes of type 1 diabetic subjects recognize an insulin epitope. *Nature* 435: 224–228.
17. Alleva, D. G., P. D. Crowe, L. Jin, W. W. Kwok, N. Ling, M. Gottschalk, P. J. Conlon, P. A. Gottlieb, A. L. Putnam, and A. Gaur. 2001. A disease-associated cellular immune response in type 1 diabetics to an immunodominant epitope of insulin. *J Clin. Invest.* 107: 173–180.
18. Daniel, D., R. G. Gill, N. Schloot, and D. Wegmann. 1995. Epitope specificity, cytokine production profile and diabetogenic activity of insulin-specific T cell clones isolated from NOD mice. *Eur. J. Immunol.* 25: 1056–1062.
19. Wegmann, D. R., M. Norbury-Glaser, and D. Daniel. 1994. Insulin-specific T cells are a predominant component of islet infiltrates in pre-diabetic NOD mice. *Eur. J. Immunol.* 24: 1853–1857.
20. Metzler, B., and D. C. Wraith. 1993. Inhibition of experimental autoimmune encephalomyelitis by inhalation but not oral administration of the encephalitogenic peptide: influence of MHC binding affinity. *Int. Immunol.* 5: 1159–1165.
21. von Herrath, M. G., and L. C. Harrison. 2003. Antigen-induced regulatory T cells in autoimmunity. *Nat. Rev. Immunol.* 3: 223–232.
22. De Magistris, M. T., J. Alexander, M. Coggeshall, A. Altman, F. C. Gaeta, H. M. Grey, and A. Sette. 1992. Antigen analog-major histocompatibility complexes act as antagonists of the T cell receptor. *Cell* 68: 625–634.
23. Basu, D., C. B. Williams, and P. M. Allen. 1998. In vivo antagonism of a T cell response by an endogenously expressed ligand. *Proc. Natl. Acad. Sci. USA* 95: 14332–14336.
24. Sloan-Lancaster, J., B. D. Evavold, and P. M. Allen. 1993. Induction of T-cell anergy by altered T-cell-receptor ligand on live antigen-presenting cells. *Nature* 363: 156–159.
25. Tsitoura, D. C., W. Holter, A. Cerwenka, C. M. Gelder, and J. R. Lamb. 1996. Induction of anergy in human T helper 0 cells by stimulation with altered T cell antigen receptor ligands. *J. Immunol.* 156: 2801–2808.
26. Nicholson, L. B., J. M. Greer, R. A. Sobel, M. B. Lees, and V. K. Kuchroo. 1995. An altered peptide ligand mediates immune deviation and prevents autoimmune encephalomyelitis. *Immunity* 3: 397–405.
27. Saito, M., S. Otake, M. Ohmura, M. Hirasawa, K. Takada, J. Mega, I. Takahashi, H. Kiyono, J. R. McGhee, Y. Takeda, and M. Yamamoto. 2001. Protective immunity to *Streptococcus* mutans induced by nasal vaccination with surface protein antigen and mutant cholera toxin adjuvant. *J. Infect. Dis.* 183: 823–826.
28. Yamamoto, M., J. R. McGhee, Y. Hagiwara, S. Otake, and H. Kiyono. 2001. Genetically manipulated bacterial toxin as a new generation mucosal adjuvant. *Scand. J. Immunol.* 53: 211–217.

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

MAMI TAMAI, ATSUSHI KAWAKAMI, MASATAKA UETANI, SHOICHIRO TAKAO, FUMIKO TANAKA, KEITA FUJIKAWA, TOSHIYUKI ARAMAKI, HIDEKI NAKAMURA, NOZOMI IWANAGA, YASUMORI IZUMI, KAZUHIKO ARIMA, KOUICHIRO ARATAKE, MAKOTO KAMACHI, MINGGUO HUANG, TOMOKI ORIGUCHI, HIROAKI IDA, KIYOSHI AOYAGI, and KATSUMI EGUCHI

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 carriership, 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 | MAGNETIC RESONANCE IMAGING
BONE EDEMA | SEROLOGIC VARIABLES | 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].

From the First Department of Internal Medicine; Department of Radiology and Radiation Research; Department of Public Health, Graduate School of Biomedical Sciences; and Nagasaki University School of Health Sciences, Nagasaki University, Nagasaki, Japan.

Supported by a grant from The Ministry of Health, Labour and Welfare, Japan.

M. Tamai, MD, PhD; A. Kawakami, MD, PhD, First Department of Internal Medicine; M. Uetani, MD, PhD; S. Takao, MD, Department of Radiology and Radiation Research; F. Tanaka, MD, PhD; K. Fujikawa, MD; T. Aramaki, MD; H. Nakamura, MD, PhD; N. Iwanaga, MD, PhD; Y. Izumi, MD, PhD; K. Arima, MD, PhD; K. Aratake, MD, PhD; M. Kamachi, MD, PhD; M. Huang, MD, PhD, First Department of Internal Medicine; T. Origuchi, MD, PhD, Nagasaki University School of Health Sciences; H. Ida, MD, PhD, First Department of Internal Medicine; K. Aoyagi, MD, PhD, Department of Public Health, Graduate School of Biomedical Sciences; K. Eguchi, MD, PhD, First Department of Internal Medicine.

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

Accepted for publication June 29, 2007.

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 report[7]. Diagnosis was made based on 1987 American College of Rheumatology (ACR) criteria for RA[8]. Thirty-six patients were already classifed 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 described[3-6], and disagreements were resolved by consensus. Degree of MRI features was evaluated per our recent report: synovitis; number of sites scored positive for MRI

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, respectively[5].

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.

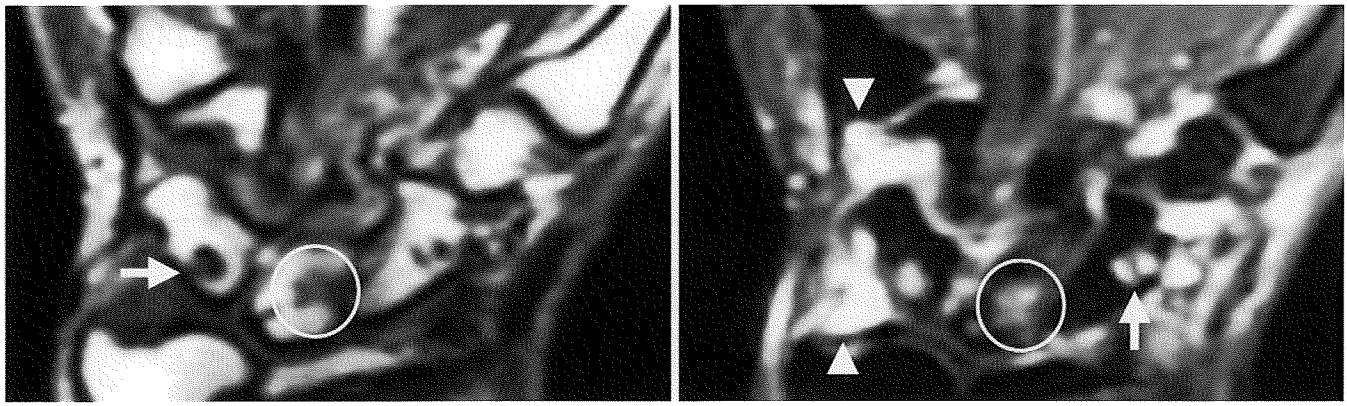

Figure 1. Representative MR images of patients with early-stage RA. T1-weighted spin-echo images show bone edema (circle), bone erosion (arrow), and synovitis (arrowheads). Right panel: gadolinium-diethylenetriamine-enhanced image.

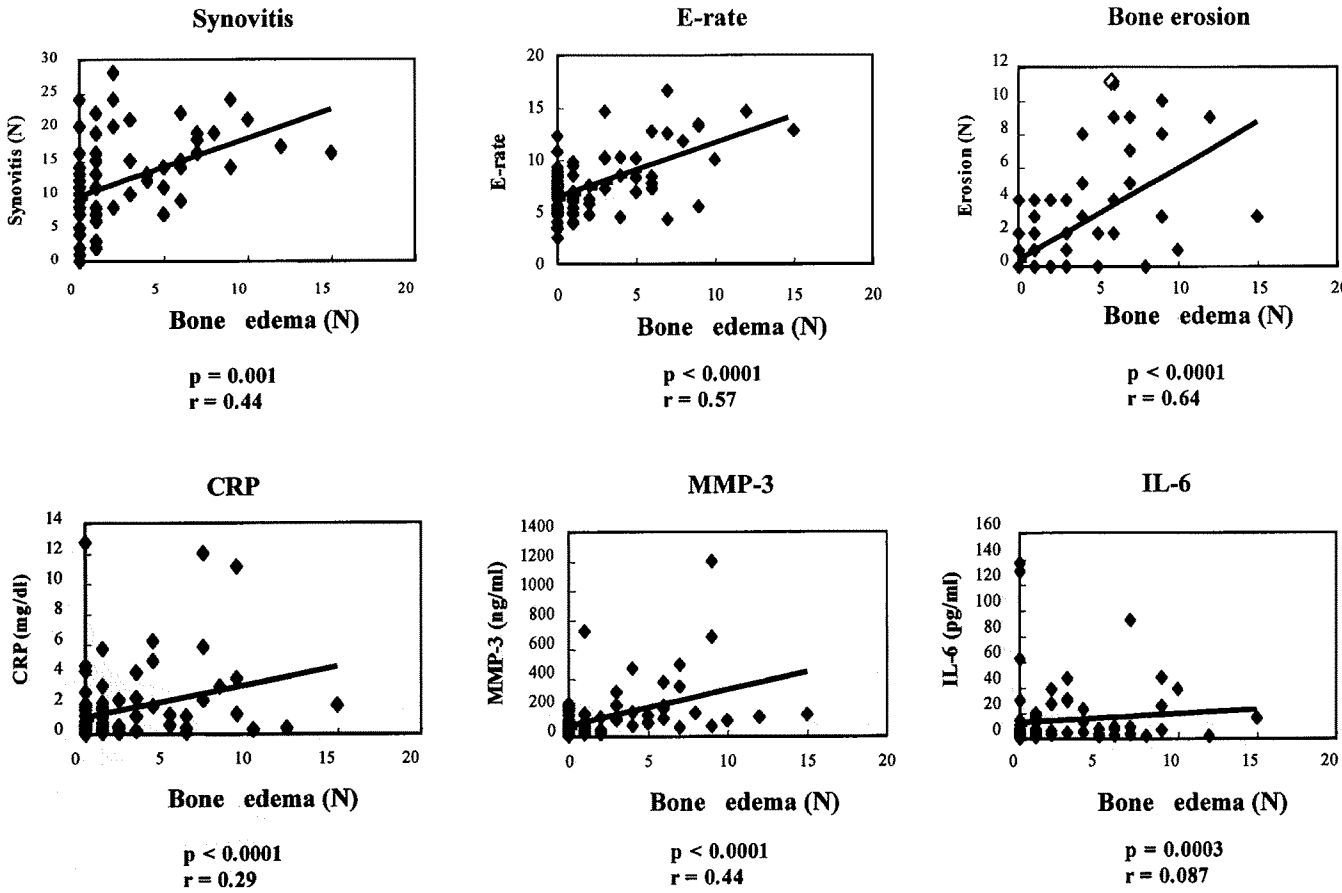

Figure 2. Positive correlation between the number of bones scored as positive for bone edema and the number of sites scored for MRI synovitis and MRI bone erosion, enhancement rate (E-rate), serum concentrations of CRP, MMP-3, and IL-6.

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 ($p = 0.00026$, $rs = 0.398$).

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 patients[9]. 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 disease[7]. Considering bone edema is a forerunner of bone erosion[10], 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 microenviroment 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.

ACKNOWLEDGMENT

We thank Dr. Masako Furuyama, Dr. Akinari Mizokami, Dr. Toshiaki Tsukada, Dr. Munetoshi Nakashima, Dr. Yojiro Kawabe, and Dr. Masanobu Mine for referring patients. We also thank Maiko Kubo, Nobuko Fukuda, and Junko Matsushita for their excellent technical assistance.

REFERENCES

1. Lassere M, McQueen F, Ostergaard M, et al. OMERACT rheumatoid arthritis magnetic resonance imaging studies. Exercise 3: An international multicenter reliability study using the RA-MRI score. J Rheumatol 2003;30:1366-75.
2. Conaghan P, Lassere M, Ostergaard M, et al. OMERACT rheumatoid arthritis magnetic resonance imaging studies. Exercise 4: An international multicenter longitudinal study using the RA-MRI score. J Rheumatol 2003;30:1376-9.
3. McQueen FM, Beneton N, Perry D, et al. Bone edema scored on magnetic resonance imaging scans of the hands and feet six years later in patients with rheumatoid arthritis. Arthritis Rheum 2003;48:1814-27.
4. Conaghan PG, O'Connor P, McGonagle D, Astin P, et al. Elucidation of the relationship between synovitis and bone damage: A randomized magnetic resonance imaging study of individual joints in patients with early rheumatoid arthritis. Arthritis Rheum 2003;48:64-71.
5. Tamai M, Kawakami A, Uetani M, et al. The presence of anti-cyclic citrullinated peptide antibody is associated with MRI detection of bone marrow oedema in early-stage rheumatoid arthritis. Ann Rheum Dis 2006;65:133-4.
6. Tamai M, Kawakami A, Uetani M, et al. Early prediction of rheumatoid arthritis by serologic variables and magnetic resonance imaging of the wrists and finger joints: Results from prospective clinical examination. Ann Rheum Dis 2006;65:134-5.

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.

7. van Gaalen FA, van Aken J, Huizinga TW, et al. Association between HLA class II genes and autoantibodies to cyclic citrullinated peptides (CCPs) influences the severity of rheumatoid arthritis. Arthritis Rheum 2004;50:2113-21.
8. Arnett EC, Edworthy SM, Bloch DA, et al. The American Rheumatology Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 1988;31:315-24.
9. Shibue T, Tsuchiya N, Komata T, et al. Tumor necrosis factor alpha 5'-flanking region, tumor necrosis factor receptor II, and HLA-DRB1 polymorphisms in Japanese patients with rheumatoid arthritis. Arthritis Rheum 2000;43:753-7.
10. McQueen FM. Magnetic resonance imaging in early inflammatory arthritis: what is its role? Rheumatology Oxford 2000;39:700-6.

ARTHRITIS & RHEUMATISM
Vol. 58, No. 7, July 2008, pp 1940–1946
DOI 10.1002/art.23494

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

Shu Kobayashi,[1] Katsunori Ikari,[2] Hirotaka Kaneko,[2] Yuta Kochi,[3] Kazuhiko Yamamoto,[4] Kenichi Shimane,[4] Yusuke Nakamura,[5] Yoshiaki Toyama,[6] Takeshi Mochizuki,[2] So Tsukahara,[2] Yasushi Kawaguchi,[2] Chihiro Terai,[2] Masako Hara,[2] Taisuke Tomatsu,[2] Hisashi Yamanaka,[2] Takahiko Horiuchi,[7] Kayoko Tao,[8] Koji Yasutomo,[8] Daisuke Hamada,[8] Natsuo Yasui,[8] Hiroshi Inoue,[8] Mitsuo Itakura,[8] Hiroshi Okamoto,[2] Naoyuki Kamatani,[2] and Shigeki Momohara[2]

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.**

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.**

Dr. Ikari's work was supported by a Japan Orthopaedics and Traumatology Foundation grant, a Takeda Science Foundation grant, and a Japanese Ministry of Education, Culture, Sports, Science, and Technology grant-in-aid for scientific research. Drs. Kochi and Yamamoto's work was supported by a Japanese Ministry of Health, Labor, and Welfare grant. Dr. Yasui's work was supported by a Japan Rheumatism Foundation grant. Drs. Inoue and Itakura's work was supported by a Japanese Ministry of Education, Culture, Sports, Science, and Technology Knowledge Cluster Initiative grant. The Institute of Rheumatology Rheumatoid Arthritis cohort studies were supported by 36 pharmaceutical companies.

[1]Shu Kobayashi, MD: Tokyo Women's Medical University, and Keio University, Tokyo, Japan; [2]Katsunori Ikari, MD, PhD, Hirotaka Kaneko, MSc, Takeshi Mochizuki, MD, So Tsukahara, MD, PhD, Yasushi Kawaguchi, MD, PhD, Chihiro Terai, MD, PhD, Masako Hara, MD, PhD, Taisuke Tomatsu, MD, PhD, Hisashi Yamanaka, MD, PhD, Hiroshi Okamoto, MD, PhD, Naoyuki Kamatani, MD, PhD, Shigeki Momohara, MD, PhD: Tokyo Women's Medical University, Tokyo, Japan; [3]Yuta Kochi, MD, PhD: Institute of Physical and Chemical Research, Yokohama, Japan; [4]Kazuhiko Yamamoto, MD, PhD, Kenichi Shimane, MD: Institute of Physical and Chemical Research, Yokohama, and University of Tokyo, Tokyo, Japan; [5]Yusuke Nakamura, MD, PhD: University of Tokyo, Tokyo, Japan; [6]Yoshiaki Toyama, MD, PhD: Keio University, Tokyo, Japan; [7]Takahiko Horiuchi, MD, PhD: Kyushu University Graduate School of Medical Sciences, Fukuoka, Japan; [8]Kayoko Tao, MD, PhD, Koji Yasutomo, MD, PhD, Daisuke Hamada, MD, PhD, Natsuo Yasui, MD, PhD, Hiroshi Inoue, MD, PhD, Mitsuo Itakura, MD, PhD: University of Tokushima, Tokushima, Japan.

Address correspondence and reprint requests to Katsunori Ikari, MD, PhD, Institute of Rheumatology, Tokyo Women's Medical University, 10-22 Kawada, Shinjuku, Tokyo 162-0054, Japan. E-mail: kikari@ior.twmu.ac.jp.

Submitted for publication December 14, 2007; accepted in revised form March 5, 2008.

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 association 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)	*P*	OR (95% CI)	*P*	OR (95% CI)	*P*
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)	*P*	OR (95% CI)	*P*	OR (95% CI)	*P*
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.

ACKNOWLEDGMENTS

We thank all of the DNA donors for making this study possible. We are grateful to Dr. Noriko Iikuni for assistance in preparation of the manuscript, and to Dr. Akihiko Miyatake (Miyatake Asthma Clinic, Osaka, Japan), the members of the Rotary Club of Osaka-Midosuji District 2660 Rotary International in Japan, and the staff of the BioBank Japan Project for supporting the study and collecting the clinical samples (RIKEN case–control series). We also thank Ms Kazue Tsugawa and Ms Aya Sato (University of Tokushima) for technical assistance, and Drs. Yoichiro Takata, Shunji Nakano (University of Tokushima) and Fumio Shinomiya (Mima Hospital, Tokushima, Japan) for aiding with patient recruitment (Tokushima case–control series). We are grateful to Dr. Atsuo Taniguchi, Mr. Eisuke Inoue, and other members of the Institute of Rheumatology, Tokyo Women's Medical University, for their work with the IORRA cohort.

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.
Statistical analysis. Kobayashi, Ikari.

REFERENCES

1. Amos CI, Chen WV, Lee A, Li W, Kern M, Lundsten R, et al. High-density SNP analysis of 642 Caucasian families with rheumatoid arthritis identifies two new linkage regions on 11p12 and 2q33. Genes Immun 2006;7:277–86.
2. Remmers EF, Plenge RM, Lee AT, Graham RR, Hom G, Behrens TW, et al. STAT4 and the risk of rheumatoid arthritis and systemic lupus erythematosus. N Engl J Med 2007;357:977–86.
3. Lee HS, Remmers EF, Le JM, Kastner DL, Bae SC, Gregersen PK. Association of STAT4 with rheumatoid arthritis in the Korean population. Mol Med 2007;13:455–60.
4. Nishikomori R, Usui T, Wu CY, Morinobu A, O'Shea JJ, Strober W. Activated STAT4 has an essential role in Th1 differentiation and proliferation that is independent of its role in the maintenance of IL-12Rβ2 chain expression and signaling. J Immunol 2002;169: 4388–98.
5. Mathur AN, Chang HC, Zisoulis DG, Stritesky GL, Yu Q, O'Malley JT, et al. Stat3 and Stat4 direct development of IL-17-secreting Th cells. J Immunol 2007;178:4901–7.
6. Oliver JE, Worthington J, Silman AJ. Genetic epidemiology of rheumatoid arthritis. Curr Opin Rheumatol 2006;18:141–6.
7. Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 1988;31:315–24.
8. Tan EM, Cohen AS, Fries JF, Masi AT, McShane DJ, Rothfield NF, et al. The 1982 revised criteria for the classification of systemic lupus erythematosus. Arthritis Rheum 1982;25:1271–7.
9. Iwamoto T, Ikari K, Inoue E, Toyama Y, Hara M, Yamanaka H, et al. Failure to confirm association between PDCD1 polymorphisms and rheumatoid arthritis in a Japanese population. J Hum Genet 2007;52:557–60.
10. Prevoo ML, van 't Hof MA, Kuper HH, van Leeuwen MA, van de Putte LB, van Riel PL. Modified disease activity scores that include twenty-eight–joint counts: development and validation in a prospective longitudinal study of patients with rheumatoid arthritis. Arthritis Rheum 1995;38:44–8.
11. Matsuda Y, Singh G, Yamanaka H, Tanaka E, Urano W, Taniguchi A, et al. Validation of a Japanese version of the Stanford Health Assessment Questionnaire in 3,763 patients with rheumatoid arthritis. Arthritis Rheum 2003;49:784–8.
12. Van der Heijde D. How to read radiographs according to the Sharp/van der Heijde method [corrected and republished in J Rheumatol 2000;27:261–3]. J Rheumatol 1999;26:743–5.
13. Nakamura Y. The BioBank Japan Project. Clin Adv Hematol Oncol 2007;5:696–7.
14. Takata Y, Hamada D, Miyatake K, Nakano S, Shinomiya F, Scafe CR, et al. Genetic association between the PRKCH gene encoding protein kinase Cη isozyme and rheumatoid arthritis in the Japanese population. Arthritis Rheum 2007;56:30–42.
15. Tao K, Fujii M, Tsukumo S, Maekawa Y, Kishihara K, Kimoto Y, et al. Genetic variations of Toll-like receptor 9 predispose to systemic lupus erythematosus in Japanese population. Ann Rheum Dis 2007;66:905–9.
16. Gregersen PK, Lee HS, Batliwalla F, Begovich AB. PTPN22: setting thresholds for autoimmunity. Semin Immunol 2006;18: 214–23.
17. Ikari K, Momohara S, Inoue E, Tomatsu T, Hara M, Yamanaka H, et al. Haplotype analysis revealed no association between the PTPN22 gene and RA in a Japanese population. Rheumatology (Oxford) 2006;45:1345–8.
18. Iwamoto T, Ikari K, Nakamura T, Kuwahara M, Toyama Y, Tomatsu T, et al. Association between PADI4 and rheumatoid arthritis: a meta-analysis. Rheumatology (Oxford) 2006;45:804–7.
19. Ueda H, Howson JM, Esposito L, Heward J, Snook H, Chamberlain G, et al. Association of the T-cell regulatory gene CTLA4 with susceptibility to autoimmune disease. Nature 2003;423:506–11.
20. Tsukahara S, Shinozaki M, Ikari K, Mochizuki T, Inoue E, Tomatsu T, et al. Effect of matrix metalloproteinase-3 functional SNP on serum matrix metalloproteinase-3 level and outcome measures in Japanese RA patients. Rheumatology (Oxford) 2008; 47:41–4.

ARTHRITIS & RHEUMATISM
Vol. 60, No. 8, August 2009, pp 2294–2303
DOI 10.1002/art.24687

Gamma/Delta T Cells Are the Predominant Source of Interleukin-17 in Affected Joints in Collagen-Induced Arthritis, but Not in Rheumatoid Arthritis

Yoshinaga Ito,[1] Takashi Usui,[1] Shio Kobayashi,[1] Mikiko Iguchi-Hashimoto,[1] Hiromu Ito,[1] Hiroyuki Yoshitomi,[1] Takashi Nakamura,[1] Masakazu Shimizu,[1] Daisuke Kawabata,[1] Naoichiro Yukawa,[1] Motomu Hashimoto,[2] Noriko Sakaguchi,[3] Shimon Sakaguchi,[2] Hajime Yoshifuji,[1] Takaki Nojima,[1] Koichiro Ohmura,[1] Takao Fujii,[1] and Tsuneyo Mimori[1]

Objective. **Although interleukin-17 (IL-17)–producing γ/δ T cells were reported to play pathogenic roles in collagen-induced arthritis (CIA), their characteristics remain unknown. The aim of this study was to clarify whether γ/δ T cells or CD4+ T cells are the predominant IL-17–producing cells, and to determine what stimulates γ/δ T cells to secret IL-17 in mice with CIA. The involvement of IL-17–producing γ/δ T cells in SKG mice with autoimmune arthritis and patients with rheumatoid arthritis (RA) was also investigated.**

Methods. **IL-17–producing cells in the affected joints of mice with CIA were counted by intracellular cytokine staining during 6 distinct disease phases, and these cells were stimulated with various combinations of cytokines or specific antigens to determine the signaling requirements. Similar studies were performed using SKG mice with arthritis and patients with RA.**

Results. **Gamma/delta T cells were the predominant population in IL-17–producing cells in the swollen joints of mice with CIA, and the absolute numbers of these cells increased in parallel with disease activity. IL-17–producing γ/δ T cells expressed CC chemokine receptor 6, were maintained by IL-23 but not by type II collagen in vitro, and were induced antigen independently in vivo. Furthermore, IL-17 production by γ/δ T cells was induced by IL-1β plus IL-23 independently of T cell receptor. In contrast to what was observed in mice with CIA, IL-17–producing γ/δ T cells were nearly absent in the affected joints of SKG mice and patients with RA, and Th1 cells were predominant in the joints of patients with RA.**

Conclusion. **Gamma/delta T cells were antigen independently stimulated by inflammation at affected joints and produced enhanced amounts of IL-17 to exacerbate arthritis in mice with CIA but not in SKG mice with arthritis or patients with RA.**

Rheumatoid arthritis (RA) is a chronic autoimmune disease that results in the destruction of cartilage and bone in joints. Collagen-induced arthritis (CIA) is a well-established murine model of this disease and shares many features with RA (1,2). Specifically, susceptibility to both CIA and RA is associated with the specific class II major histocompatibility complex allele (3,4). In addition, autoantibodies to type II collagen have been detected in the synovial fluid of patients with RA, and these autoantibodies have an aggravating effect on CIA in mice (5–7). In addition, pathogenic contributions of CD4+ T helper cells have been reported in both CIA and RA (8,9).

Interleukin-17 (IL-17) is a cytokine secreted by T cells, natural killer (NK) cells, and neutrophils (10),

Supported by grants from the Japan Society for the Promotion of Science and the Ministry of Health, Labor, and Welfare of Japan.

[1]Yoshinaga Ito, MD, Takashi Usui, MD, PhD, Shio Kobayashi, Mikiko Iguchi-Hashimoto, MD, Hiromu Ito, MD, PhD, Hiroyuki Yoshitomi, MD, PhD, Takashi Nakamura, MD, PhD, Masakazu Shimizu, PhD, Daisuke Kawabata, MD, PhD, Naoichiro Yukawa, MD, Hajime Yoshifuji, MD, PhD, Takaki Nojima, MD, PhD, Koichiro Ohmura, MD, PhD, Takao Fujii, MD, PhD, Tsuneyo Mimori, MD, PhD: Kyoto University, Kyoto, Japan; [2]Motomu Hashimoto, MD, Shimon Sakaguchi, MD, PhD: Kyoto University, Kyoto, and Osaka University, Osaka, Japan; [3]Noriko Sakaguchi, MD: Osaka University, Osaka, Japan.

Address correspondence and reprint requests to Takashi Usui, MD, PhD, Center for Innovation in Immunoregulative Technology and Therapeutics, Graduate School of Medicine, Kyoto University, Building B, Konoe-cho, Yoshida, Sakyo-ku Kyoto 606-8501, Japan. E-mail: takausui@kuhp.kyoto-u.ac.jp.

Submitted for publication November 17, 2008; accepted in revised form April 18, 2009.

and it induces IL-6, IL-8, chemokine, and metalloproteinase production by target cells (11). Central pathogenic roles of IL-17 in CIA have been reported recently. For example, systemic or local IL-17 gene transfer aggravated CIA, whereas administration of an IL-17–blocking antibody ameliorated CIA even after the onset of arthritis (12,13), and IL-17–deficient mice also showed reduced severity of CIA (14). Furthermore, IL-23–deficient mice, which show an impaired Th17 response, do not exhibit CIA, because IL-23 is an essential factor for the maintenance of Th17 cells (15).

Although Roark et al recently reported the infiltration of IL-17–producing γ/δ T cells together with IL-17–producing CD4+ T (Th17) cells in inflamed joints of mice with CIA (16), the precise predominance, distribution, kinetics, cytokine-production requirements, and characteristics of these cells, especially in the context of IL-17–producing γ/δ T versus Th17 cells, remain unclear. Elucidation of these factors will be critical in terms of understanding the pathogenesis of CIA, finding novel therapeutic targets associated with IL-17, and determining the optimal timing and site for therapeutic intervention in CIA.

In the current study, we performed spatiotemporal analysis of IL-17–producing cells in CIA and demonstrated that γ/δ T cells are the predominant source of IL-17 in swollen joints of mice with CIA. IL-17–producing γ/δ T cells were maintained by IL-23 but not by type II collagen in vitro. Furthermore, IL-17 production by γ/δ T cells was efficiently stimulated by inflammatory cytokines independently of T cell receptor (TCR). Contrary to the results observed in mice with CIA, IL-17–producing γ/δ T cells could not be detected in the affected joints of patients with RA.

MATERIALS AND METHODS

Animals. DBA1/J mice and BALB/c mice were purchased from Charles River (Wilmington, MA). Eight-week-old male mice were used for induction of CIA, and 2-week-old mice were used to analyze thymocytes. The procedures for the induction of arthritis in SKG mice were described previously (17). Mice were maintained in our animal facility under specific pathogen–free conditions, and all animal procedures were approved by the Ethics Committee of Kyoto University.

Induction of CIA. Immunization-grade bovine type II collagen was purchased from Chondrex (Redmond, WA) and reconstituted at 2 mg/ml in 0.05*M* acetic acid and then emulsified with an equal volume of Freund's complete adjuvant (CFA) containing 4 mg/ml of heat-killed *Mycobacterium tuberculosis* (Arthrogen-CIA; Chondrex). In order to examine the immune process at the immunized site, CIA was initiated by subcutaneous injection with 100 μl of emulsified type II collagen into the left footpad rather than the tail base. This altered method of immunization did not result in skewed disease kinetics, severity, or cytokine profiles of cells in swollen joints (data not shown). A booster immunization was not given. Each joint was designated as follows: immunized joint = left hind paw that received immunization; swollen joint = a fore paw in which arthritis developed; nonswollen joint = right hind paw that was not immunized and in which arthritis did not develop macroscopically (Figure 1A). SKG and BALB/c mice were also immunized with CFA plus type II collagen into the left hind paw to analyze locally infiltrated cells 10 days later. In some experiments, control mice were treated with type II collagen emulsified in Freund's incomplete adjuvant (IFA; Difco, Detroit, MI) or 0.05*M* acetic acid emulsified in IFA or phosphate buffered saline (PBS) alone.

Preparation of mononuclear cells from joints. To prepare cells from the joints, the previously described technique (3) was used. Although a previous report confirmed that contamination of bone marrow cells had not occurred using this procedure (18), we compared the absolute counts of γ/δ T cells and CD19+ cells collected by this procedure and collected from the remaining tissues of the normal joints of naive DBA1/J mice. Cells in the remaining tissues were collected by mincing the remaining tissues, including bone marrow. The cells were stained with biotinylated anti-CD19 monoclonal antibody (mAb) (1D3; BD Biosciences, San Jose, CA) or anti-γ/δ TCR mAb (UC7-13D5), detected with streptavidin–allophycocyanin, and analyzed using fluorescence-activated cell sorting. Human synovial tissue or synovial fluid was obtained from patients with RA who were undergoing joint replacement surgery or subcutaneous puncture of the knee joints. Synovial tissue was dissected into small pieces with scissors, and lymphocytes were collected by density-gradient centrifugation with Ficoll-Paque PLUS (GE Healthcare, Amersham, UK). All human procedures were approved by the Ethics Committee of Kyoto University and were performed after receiving informed consent.

Intracellular cytokine staining. Cell stimulation with phorbol myristate acetate (PMA) and ionomycin and intracellular cytokine staining were performed as described previously (16). When IL-17 production requirements were analyzed, 2×10^4 cells/well in a 96-well plate were stimulated with 10 μg/ml of plate-bound anti-γ/δ TCR (UC7-13D5), 2 μg/ml of soluble anti-CD28 (37. 51; BD Biosciences), 5 ng/ml of recombinant mouse IL-23 (1887-ML-010; R&D Systems, Minneapolis, MN), 50 ng/ml of recombinant mouse IL-1β (094-04681; Wako, Osaka, Japan), or 50 ng/ml of recombinant human transforming growth factor β1 (TGFβ1) (240-B; R&D Systems) for 24 hours, in the presence of 15 μ*M* monensin for the last 4 hours. Other stimulants were not included in the analysis of IL-17 production requirements.

To analyze surface antigens, the following antibodies were used: fluorescein isothiocyanate (FITC)–labeled anti-CD8 (53-6.7), FITC-conjugated anti-CD3e (145-2C11), FITC-conjugated anti-mouse CC chemokine receptor 6 (CCR6) (140706; R&D Systems), peridinin chlorophyll protein complex–labeled anti-CD4 (L3T4; BD Biosciences), biotinylated anti-γ/δ TCR (UC7-13D5), and biotinylated anti-CD49b (DX5) mAb detected using streptavidin–allophycocyanin or streptavidin–Cy-Chrome (BD Biosciences). Cytokines were detected using FITC- or allophycocyanin-labeled anti–

interferon-γ (IFNγ) (XMG1.2), phycoerythrin (PE)–labeled anti–IL-17 mAb (TC11-18H10; BD Biosciences), or an isotype control. When human synoviocytes were analyzed, the cells were stained using FITC-conjugated anti-human IL-17A (eBio64DEC17), allophycocyanin-conjugated anti-human IFNγ (4S. B3), Cy-Chrome–conjugated anti-human CD4 (PM-30158X; BD Biosciences), and PE-conjugated anti-human γ/δ TCR mAb (B1.1). Unless specified otherwise, all antibodies were purchased from eBioscience (San Diego, CA).

Flow cytometry analysis. The absolute numbers of cytokine-producing cells were analyzed using a FACSCalibur flow cytometer (BD Biosciences). Lymphocytes were gated based on their forward and side scatter. The cytokine-positive subsets were determined by a comparison with isotype control staining. By applying cells from a whole joint, the absolute numbers of cytokine-positive cells in each joint were counted, and the data were analyzed using CellQuest software (BD Biosciences).

Sorting of γ/δ T cells. To analyze the IL-17 production requirements, cells were collected from peripheral lymph nodes of naive DBA1/J mice or from the draining lymph nodes (DLNs) of the swollen joints of mice with CIA. Cells were stained with FITC-conjugated anti-mouse γ/δ TCR mAb (UC7-13D5) and anti-FITC microbeads, and then γ/δ T cells were prepared by positive selection using an MS column (Miltenyi Biotec, Bergisch Gladbach, Germany).

Cell culture in the presence of IL-23 or type II collagen. Cells were prepared from the DLNs of swollen joints of mice with CIA. Then, 5×10^5 cells/well were cultured in 200 μl of RPMI 1640 complete medium in the presence or absence of 1 ng/ml of IL-23. For type II collagen, cells were cultured in the presence or absence of 15 μg/ml of type II collagen. After 7 days, the cells were stimulated with PMA and ionomycin for 4 hours. IL-17–producing cells were detected by intracellular cytokine staining. The ratios of the numbers of IL-17–producing cells in the presence of IL-23 or type II collagen to those in medium alone were calculated.

Analysis of the γ/δ TCR repertoire of IL-17–producing γ/δ T cells (CCR6+ γ/δ T cells). Cells from the DLNs of swollen joints were stained with FITC-conjugated anti-mouse CCR6 mAb (140706; R&D Systems) and anti-FITC microbeads, and then CCR6+ cells were prepared by positive selection using an MS column (Miltenyi Biotec). The purity of CCR6+ cells among γ/δ T cells was >99%. RNA isolation, complementary DNA synthesis, and TCR repertoire analysis with polymerase chain reaction (PCR) were performed as described previously (19,20) with the same PCR primer sets.

Adoptive transfer experiments with CCR6+ γ/δ T cells. Cells from the DLNs of swollen joints of mice with CIA were prepared. To enrich CCR6+ γ/δ T cells, single-cell suspensions were depleted of CD4+, CD8a+, CD45R+, CD49b+, CD11b+, and Ter-119+ cells by negative selection with a biotin antibody cocktail and antibiotin microbeads of a CD4+ T Cell Isolation Kit, CD4+ microbeads, and an LS column (Miltenyi Biotec). The remaining γ/δ TCR–positive–enriched cells were stained with FITC-conjugated anti-mouse CCR6 mAb (140706; R&D Systems) and anti-FITC microbeads, and the CCR6+ γ/δ T cells were prepared by positive selection using an MS column (Miltenyi Biotec). Control naive CD4+ T cells were purified using the CD4+CD62L+ T Cell Isolation Kit II (Miltenyi Biotec) in accordance with the manufacturer's instructions. Using a Hamilton microsyringe (Osaka Chemical, Osaka, Japan), 60,000 CCR6+ γ/δ T cells or naive CD4+ T cells in 10 μl of PBS, or PBS alone, were injected around each wrist or ankle of naive mice or mice immunized with type II collagen plus CFA 2 weeks previously (n = 79). Arthritis in each joint was examined every 3–4 days according to the scoring system described previously (21).

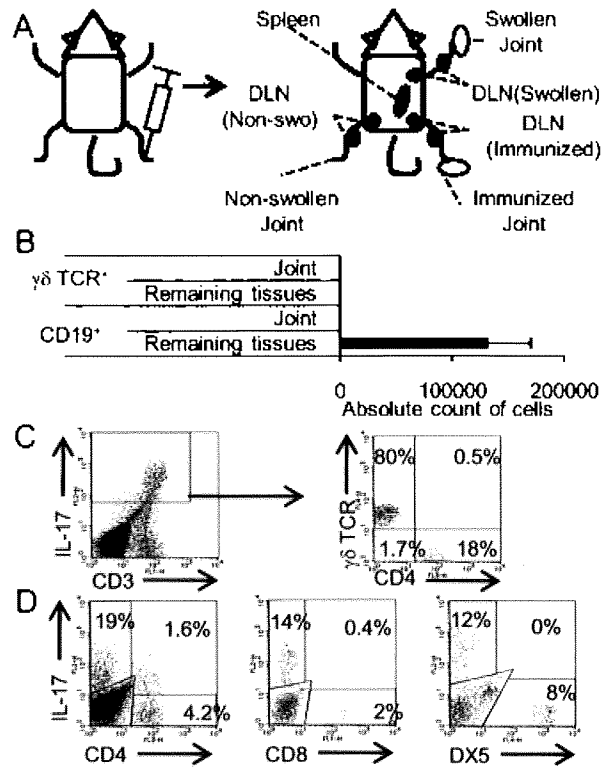

Figure 1. Predominance of interleukin-17 (IL-17)–producing γ/δ T cells in the swollen joints of mice with collagen-induced arthritis (CIA). **A,** Schematic of the analyzed joints and draining lymph nodes (DLNs) in mice with CIA. **B,** Comparison of the absolute counts of γ/δ T cells and CD19+ cells in the joints and remaining tissues of naive mice, as detected by fluorescence-activated cell sorting analysis. Values are the mean and SEM results from 3 different mice. **C** and **D,** Analysis of IL-17–producing γ/δ T cells in the swollen joints of mice. Cells were obtained from the swollen joints of mice with CIA at the peak of arthritis and stained with antibodies against CD3, CD4, CD8, DX5, and γ/δ T cell receptor (TCR). IL-17–producing cells were detected by intracellular cytokine staining. Live lymphocytes were gated based on their forward and side scatter. The percentage of cells in each region or quadrant is noted. One of 5 experiments with similar results is shown. In **C,** IL-17–producing cells were gated and plotted by their expression of CD4 and γ/δ TCR. Non-swo = nonswollen.

Patients with RA. Eleven female patients ages 37–81 years (mean ± SD 59 ± 12 years) with a diagnosis of RA based on the 1987 criteria of the American College of Rheumatology (ACR; formerly, the American Rheumatism Association) (22) were included. The duration of RA ranged from 4

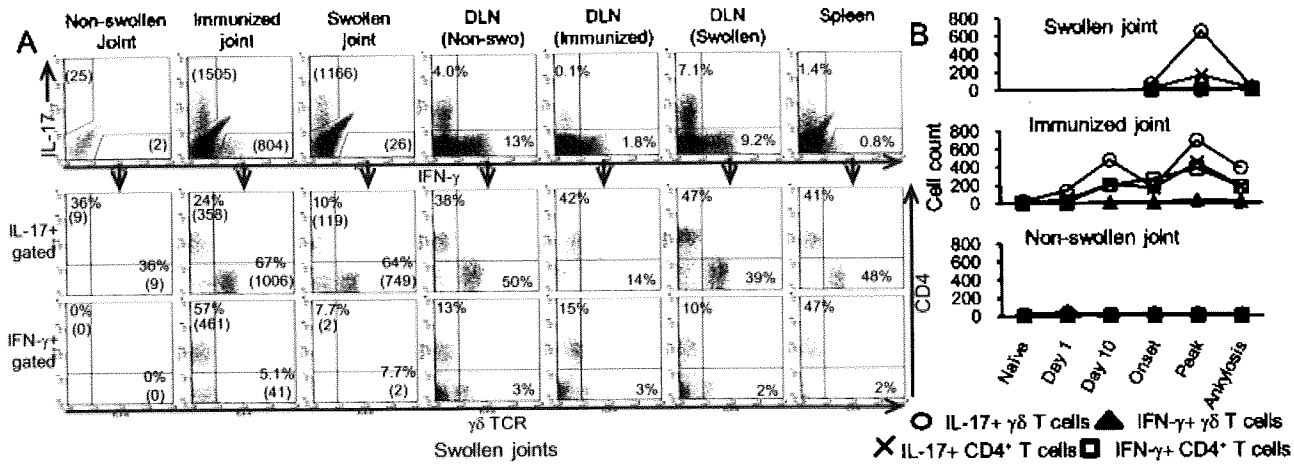

Figure 2. Distribution and kinetics of IL-17–producing γ/δ and CD4+ T cells in CIA. **A,** Cells were obtained from the joints, their DLNs, and the spleens of mice with CIA at the peak of arthritis. Viable lymphocytes were gated based on their forward and side scatter. Using intracellular cytokine staining, IL-17–producing cells and interferon-γ (IFNγ)–producing cells were detected (top row). IL-17–producing cells (second row) or IFNγ-producing cells (bottom row) were gated and plotted by their expression of γ/δ TCR and CD4. In the panels showing analysis of joints, the absolute number of IL-17–producing cells and the percentage of CD4+ cells and γ/δ TCR+ cells among them are indicated. In the panels showing analysis of DLNs and spleen, the percentage of cells in each quadrant is noted. One of 3 experiments with similar results is shown. **B,** Cells were recovered from the swollen joints, immunized joints, and nonswollen joints of mice with CIA during the 6 distinct phases of arthritis described in Materials and Methods. IL-17–producing cells and IFNγ+ cells were detected by intracellular cytokine staining, and their absolute numbers were calculated using fluorescence-activated cell sorting analysis. Values for each phase represent the mean from at least 3 different mice. In **B,** only 3 phases after the onset of arthritis are applicable for the DLNs of swollen joints. See Figure 1 for other definitions.

years to 33 years (mean ± SD 15 ± 9 years). Eight patients were receiving disease-modifying antirheumatic drugs (6 methotrexate, 2 bucillamine, and 2 sulfasalazopyridine) either as monotherapy or in combination, 9 patients were receiving prednisolone (2–10 mg/day), and 1 patient was being treated with an anti–tumor necrosis factor α biologic (etanercept). The 28-joint Disease Activity Score (23) ranged from 2.22 to 6.49 (mean ± SD 4.3 ± 1.6). The C-reactive protein level and the erythrocyte sedimentation rate ranged from 0 to 5.9 mg/dl (mean ± SD 1.9 ± 2.1) and from 9 to 83 mm/hour (mean ± SD 41.9 ± 22.5), respectively. According to the Steinbrocker criteria (24), 27% of the patients had stage III disease, and 73% had stage IV disease. According to the ACR 1991 revised criteria for the classification of global functional status in RA, 50% of the patients had stage II disease, and 50% had stage III disease (25).

Statistical analysis. All statistical analyses were performed using the Mann-Whitney U test with Microsoft Excel software (Microsoft, Redmond, WA) and Statcel2 add-in software (Hisae Yanai, Department of Mathematics, Faculty of Science, Saitama University, Japan). *P* values less than 0.05 were considered significant.

RESULTS

Predominance of IL-17–producing γ/δ T cells in swollen joints of mice with CIA. In the present study, it was first confirmed that cells from the joints were not contaminated by bone marrow cells. The number of CD19+ cells in the joints was negligible (Figure 1B). Next, IL-17–producing γ/δ T cells in the swollen joints of mice were analyzed at the peak of CIA. Interestingly, the percentage of IL-17–producing γ/δ T cells was 4.4-fold higher than that of Th17 cells (Figure 1C). Almost all of the IL-17–producing cells in swollen joints were either γ/δ T cells or CD4+ T cells, and neither CD8+ cells nor DX5+ NK cells produced IL-17 (Figures 1C and D).

Distribution and kinetics of IL-17–producing γ/δ and CD4+ T cells in CIA. To analyze the distribution and kinetics of IL-17–producing γ/δ T cells and Th17 cells in mice with CIA, intracellular cytokine staining was performed using cells obtained from the joints of mice with CIA during 6 distinct phases, as follows: before immunization (naive mice), 1 day after immunization (day 1), before onset of arthritis (day 10), onset of arthritis (day 32), peak of arthritis (day 42), and ankylosing phase of arthritis (day 70). At each phase, cells were collected from the swollen joint, an immunized joint, a nonswollen joint, the DLNs of each joint, and the spleen (Figure 1A).

In swollen joints, the absolute numbers of IL-17–producing γ/δ T cells were higher than the absolute numbers of Th17 cells, with the maximal counts ob-