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IMMUNOLOGY ORIGINAL ARTICLE

Critical role of *M. tuberculosis* for dendritic cell maturation to induce collagen-induced arthritis in H-2b background of C57BL/6 mice

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Summary

Collagen-induced arthritis (CIA) can be induced even in CIA-resistant H-2^b background of C57BL/6 mice when these mice are immunized with type II collagen (CII) emulsified in complete Freund's adjuvant (CFA) containing high, but not low, dose of *Mycobacterium tuberculosis*. Here, we investigated the pathogenesis of CIA in C57BL/6 mice induced by the immunizing protocol. We examined expressions of cytokines, costimulatory molecules and major histocompatibility complex (MHC) class II in draining lymph nodes (DLN) in CIA-induced C57BL/6 mice by quantitative reverse transcription–polymerase chain reaction. We also examined an effect of *M. tuberculosis* on the expression of these molecules on dendritic cells (DC) *in vitro* by flow cytometry. We finally examined an effect of *M. tuberculosis* in CFA used for immunization with CII antigen on priming of $CD4^+$ helper T cells specific to CII in DLN of CIA-induced C57BL/6 mice. The expression of interferon-γ (IFN-γ), Interleukin-12p40 (IL-12p40), costimulatory molecules CD40, CD80 and CD86 and MHC class II were up-regulated in DLN of CIA-induced C57BL/6 mice. Expressions of these costimulatory molecules were also up-regulated on DC after stimulation with high, but not low, dose of *M. tuberculosis in vitro*. Furthermore, priming of $CD4^+$ helper T cells specific to CII antigen in DLN required immunization with CII using CFA containing high, but not low, dose of *M. tuberculosis*. These results suggested that high dose of *M. tuberculosis* were required for maturation of DC enough to prime $CD4^+$ helper T cells specific to CII antigen in DLN of H-2^b background of C57BL/6 mice.

Keywords: collagen-induced arthritis (CIA); C57BL/6 mice; *M. tuberculosis*; dendritic cell, costimulatory molecules

Introduction

Rheumatoid arthritis (RA) is an autoimmune disease characterized by chronic inflammation and synovial infiltration of immune cells. Collagen-induced arthritis (CIA) is a mouse model for RA in humans.[1] The histopathology of this arthritis is characterized by a proliferative synovitis that erodes the adjacent cartilage, ultimately producing articular injury and ankylosis, like the arthritis in human RA. Therefore, CIA is a useful model of human RA for analysis of pathogenesis of inflammatory joint diseases.[2–4]

CIA is purportedly restricted to mice bearing the major histocompatibility complex (MHC) class II H-2^q or H-2^r, but not H-2^b, haplotypes.[5,6] Therefore, C57BL/6 mice have been thought to be resistant to CIA because of their H-2^b background. However, it should be useful if we could analyse C57BL/6 mice with CIA, because there are a number of C57BL/6 mice deficient in certain genes, which might be involved in the pathogenesis of RA. Recently, Campbell and colleague demonstrated that C57BL/6 mice could be susceptible to CIA using a particular immunization protocol with complete Freund's adjuvant (CFA) containing high dose of *Mycobacterium tuberculosis*.[7] However, it is unclear how a high dose of *M. tuberculosis* in CFA leads to the induction of CIA in C57BL/6 mice.

In the present study, we investigated the pathogenesis of CIA in C57BL/6 mice and found that high, but not

low, dose *M. tuberculosis* in CFA is required for activation and/or maturation of dendritic cells (DC) to prime type II collagen (C-II) reactive T cells in draining lymph nodes (DLN) of C57BL/6 mice.

Materials and methods

Animals and induction of CIA

C57BL/6 mice (6–10-week-old, female) were purchased from the Japan Charles River Breeding Laboratories and maintained in the animal facility under specific pathogen-free conditions at the University of Tsukuba. CIA was induced as previously described.[7] In brief, complete Freund's adjuvant (CFA) was prepared by grinding 2 mg or 10 mg heat-killed *M. tuberculosis* (H37Ra; Difco Laboratories, Detroit, MI) in 2 ml incomplete Freund's adjuvant (IFA; Sigma Chemical Co., St. Louis, MO). An emulsion was formed by dissolving 2 mg/ml chick collagen type II (CII; Sigma Chemical Co.) overnight at 4° in 10 mM acetic acid, followed by mixture with an equal volume of CFA. Mice were injected intradermally (i.d.) at the base of tail with a total of 100 μl emulsion of 50 μl of CII (2 mg/ml) and 50 μl of IFA or CFA containing 1 or 5 mg/ml *M. tuberculosis*, followed by the second injection as a booster 21 days after the first injection. The day of the second immunization (booster) was designated as day 0.

Clinical assessment of CIA

The swelling of each paw was graded as follows: grade 0, no swelling; grade 1, swelling or focal redness of finger joints; grade 2, mild swelling of wrist or ankle joints; grade 3, severe swelling of the entire paw. The scores of four paws were totalled so that the maximal score per mouse was 12. Incidence of CIA was calculated by dividing the number of mice showing swelling of any paws with the number of total mice tested. Anteroposterior radiographs of the four limbs were obtained, using X-ray apparatus (muFX-1000, FUJIFILM, Tokyo) and BAS-5000 (FIJIFILM), 12 weeks after the second immunization. Paraffin sections of hind paws after decalcification with 10% ethylenediaminetetraacetic acid were stained with haematoxylin-eosin (H&E, 100×).

RNA Extraction and polymerase chain reaction (PCR) analysis

Total RNA was prepared from draining LN (DLN) cells and hind paw joints, which were removed 10 days after the second immunization. DLN cells were prepared from 10 arthritic or control non-arthritic mice that were immunized with CII in IFA. Gene expression was measured by TaqMan real-time PCR (Applied Biosystems, Foster City, CA), according to the manufacturer's protocol. Probes and primer sets for mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH), CD80, CD86, CD40, MHC II, interleukin-4 (IL-4), and IL-12p40 were purchased from Applied Biosystems, and those for interferon-γ (IFN-γ) were designed as follows; TCACC ATCCTTTTGCCAGTTCCTCCAG (probe), TCAAGTGG CATAGATGTGGAAGAA (forward primer), and TGGCT CTGCAGGATTTTCATG (reverse primer). Target gene probes were labelled with 6-carboxyfluorescein (FAM) and internal reference probes and the rodent GAPDH were labelled with VIC. PCR were performed on the ABI PRISM 7700 Sequence Detection System (Applied Biosystems) using the following conditions: 2 min at 50°, 10 min at 95°, followed by two-step PCR for 40 cycles of 95° for 15 s followed by 60° for 1 min. mRNA expression of each cytokine was normalized by those of GAPDH in each sample.

Effect of M. tuberculosis *on DC maturation* in vitro

Splenocytes prepared from naïve C57BL/6 mice were cultured in 12-well flat-bottom plates (1×10^7/well) in the presence of 2·5 or 5·0 μg/ml or absence of *M. tuberculosis* for 48 hr. Cells were then incubated with anti-FcγR (BD PharMingen, San Diego, CA) to block non-specific binding of antibody, followed by simultaneous staining with fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD80, CD86, CD40 or MHC II, and phycoerythrin (PE)-conjugated anti-mouse CD11c (BD PharMingen). Cells were analysed by flow cytometry.

Effect of M. tuberculosis *on DC maturation* in vivo

Mice were injected into the foot pads with a total of 100 μl emulsion of 50 μl of CII (2 mg/ml) and 50 μl of IFA or CFA containing 1 or 5 mg/ml *M. tuberculosis*. Five days after the immunization, DLN were removed and gene expressions were measured by TaqMan real-time PCR, as described above.

CII-specific T-cell proliferation and cytokine secretion

Five days after immunization at the base of tail with CII emulsified with IFA or CFA, $CD4^+$ T cells were purified from the draining lymph node by magnetic-antibody cell sorting (MACS, Miltenyi Biotec). The purity of $CD4^+$ T cells was more than 95%, as determined by flow cytometry. $CD4^+$ T cells were cocultured in 96-well flat-bottom microtitre plates (1×10^5/well) in the presence (50 μg/ml) or absence of the denatured chick CII for 48 hr with splenocytes (5×10^6/well) from naïve C57BL/6 mice as antigen-presenting cells (APC), which had been treated with 50 μg/ml mitomycin C (Sigma-Aldrich) at 37° for 30 min. T-cell proliferations specific to CII were measured

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by enzyme-linked immunosorbent assay (ELISA) using BrdU Kit (Roche, Mannheim, Germany). IFN-γ and IL-4 levels in the culture supernatants were determined by ELISA (Ready-SET-Go; eBioscience, San Diego, CA).

Statistical analyses

Statistical analyses were performed using the Mann–Whitney *U*-test.

Results

Induction of CIA in mice of C57BL/6 background

To investigate whether CIA can be induced in mice of C57BL/6, we immunized C57BL/6 mice with CII emulsified in IFA or CFA containing either 1 or 5 mg/ml *M. tuberculosis*. We observed that neither mice immunized with CII in IFA nor those with CFA containing 1 mg/ml of *M. tuberculosis* developed any clinical signs of arthritis. However, immunization of CII emulsified in CFA containing 5 mg/ml of *M. tuberculosis* induced CIA in more than half of C57BL/6 10 days after immunization (Fig. 1a–c). The incidence of CIA and the mean clinical score reached to the maximum around 2 weeks after immunization (Fig. 1a, b). The histopathological analyses showed mononuclear cells, synovial hyperplasia, pannus formation, cartilage destruction and bone erosion (Fig. 1d). Radiological examination at 12 weeks after immunization showed bone erosion and destruction in the affected joints (Fig. 1e). These data were consistent with a previous report that CIA can be induced in mice bearing the MHC class II haplotypes of not only H-2^q or H-2^r, but also H-2b,[7] and suggested that susceptibility to CIA may reflect immunization conditions.

Elevated T helper 1 (Th1) responses in draining lymphnodes and arthritis joints of CIA-induced C57BL/6 mice

It has been suggested that RA is mediated by IFN-γ-producing Th1 cells, which stimulate inflammatory responses.[8,9] It is also reported that Th1 responses are predominant in DLN of CIA-induced mice of DBA/1 background at the time of onset of arthritis.[10] To examine whether Th1 cells are predominant also in CIA-induced C57BL/6 mice, DLN cells were isolated from mice with CIA induced by CII in CFA containing 5 mg/ml of *M. tuberculosis* or control mice that were immunized with CII in IFA 10 days after the second immunization and were examined for cytokine production by quantitative reverse transcription (RT)–PCR. As demonstrated in Fig. 2(a), while IL-4 mRNA levels were

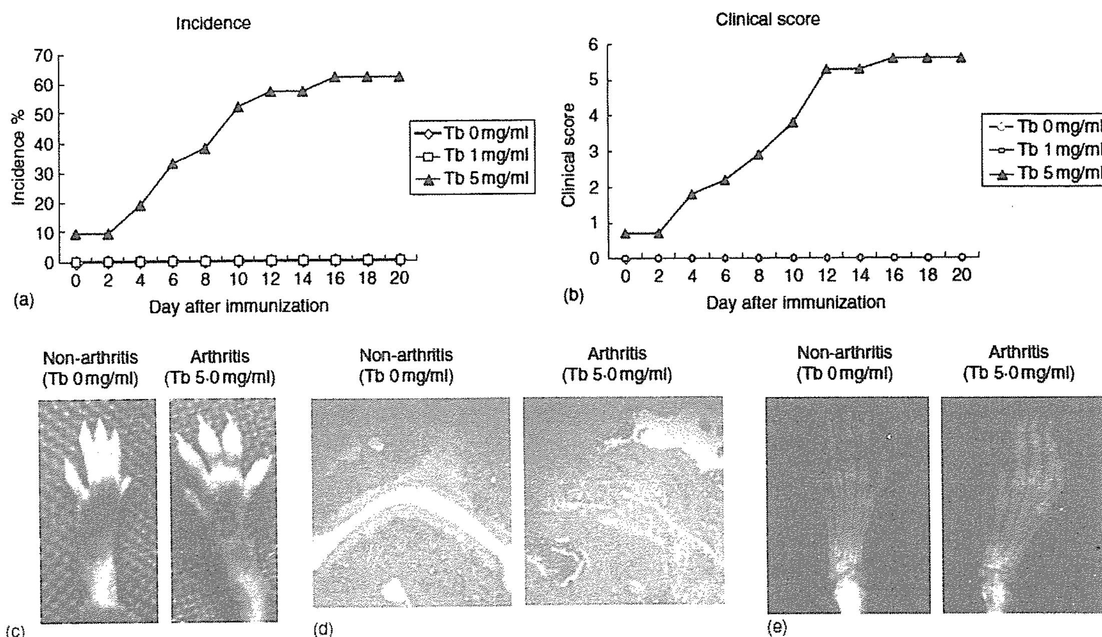

Figure 1. Induction of CIA in C57BL/6 mice. C57BL/6 mice were injected i.d. twice at day −21 and 0 with 100 μg CII emulsified in either IFA (Tb 0 mg/ml) (◇), or CFA containing *M. tuberculosis* at a concentration of 1 or 5 mg/ml (□ and ▲, respectively) ($n = 21$ in each group). Incidences of CIA and clinical scores were determined, as described in Materials and methods. Data are shown as cumulative incidence (a) and mean clinical score (b) of each group. A hind paw swelling in CIA-induced mouse 10 days after the second immunization and a control non-arthritis paw are shown (c). Representative histological (d) and radiological (e) findings on an affected joint of a CIA-induced C57BL/6 mouse (12 days (d) and 12 weeks (e)) after the second immunization and control joints are also shown (H&E, original magnification ×100).

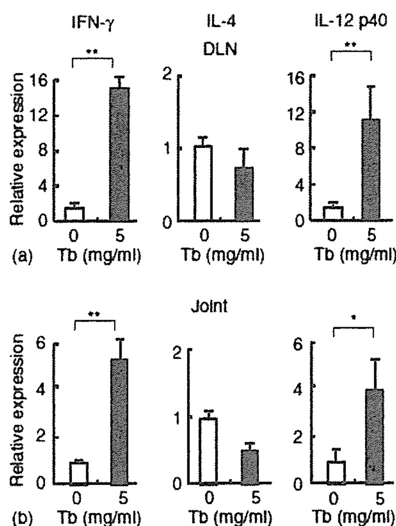

Figure 2. Expression of cytokines in DLN and affected joints of CIA-induced C57BL/6 mice. mRNA was extracted from DLN (a) and affected joints (b) of CIA-induced C57BL/6 mice 10 days after the second immunization and expression of cytokines indicated were analysed by quantitative RT–PCR analysis. Expressions of these cytokines in corresponding DLN and joints of mice (immunized with CII in IFA) were also shown as controls. Relative expressions of IFN-γ and IL-12 in DLN and joints from CIA-induced C57BL/6 mice significantly increased compared with those of control non-arthritic mice (*; $P < 0{\cdot}05$, **; $P < 0{\cdot}01$) ($n = 10$). Data are the means ± SEM of the relative expression of each gene.

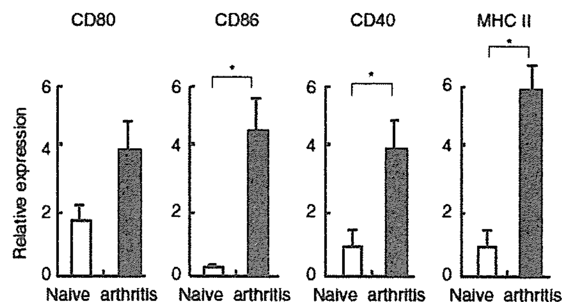

Figure 3. Expression of costimulatory molecules and MHC II in DLN of CIA-induced C57BL/6 mice mRNA was extracted from DLN of CIA-induced C57BL/6 mice 10 days after the second immunization and expression of costimulatory molecules indicated were analysed by quantitative RT–PCR analysis. Expressions of these molecules in corresponding DLN of control naive mice were also shown as controls. Relative expressions of CD86, CD40 and MHC II in DLN from CIA-induced C57BL/6 mice significantly increased compared with those of control mice (*$P < 0{\cdot}05$) ($n = 10$). Data are the mean ± SEM of the relative expression of each gene.

not different between arthritis and control non-arthritis mice, IFN-γ mRNA levels were significantly higher in arthritis than control mice ($P < 0{\cdot}01$). We also observed the similar results in the synovial tissues of arthritis joints of CIA-induced C57BL/6 mice (Fig. 2b). These results indicated that Th1 responses were predominant during the arthritic phase in both DLN and arthritis joints of CIA-induced C57BL/6 mice.

Up-regulation of IL-12 and costimulatory molecules in DLN of CIA-induced C57BL/6 mice

Because IL-12 is essentially required for Th1 development and activation[11,12] we examined whether IL-12p40 mRNA was expressed in DLN and arthritis joints of CIA-induced C57BL/6 mice by quantitative RT–PCR. As demonstrated in Fig. 2, expression of IL-12p40 mRNA in DLN of CIA-induced C57BL/6 mice was significantly higher than that of control non-arthritis mice ($P < 0{\cdot}01$). Because IL-12 is produced from APC such as DC and macrophages, these results suggested that APC were activated in DLN of CIA-induced C57BL/6 mice. We therefore further examined whether expression of costimulatory molecules were also up-regulated on these APC. As demonstrated in Fig. 3, CD40, CD80, CD86 and MHC II were expressed in DLN of CIA-induced C57BL/6 mice significantly higher than those of control naïve mice ($P < 0{\cdot}05$) 10 days after the second immunization, indicating that APC were activated and/or matured in DLN of CIA-induced C57BL/6 mice. Taken together, these results suggested a hypothesis that antigen presentation by mature APC differentiated naïve helper T cells into collagen-specific Th1 cells in DLN of CIA-induced C57BL/6 mice.

M. tuberculosis up-regulated costimulatory molecules expression on DC

We have shown that CIA can be induced in C57BL/6 mice when these mice are immunized with CII emulsified with CFA containing high, rather than low, dose of *M. tuberculosis*. However, it has not been determined how the immunization conditions induce CIA in these mice. To address this issue, we examined whether high, but not low, concentrations of *M. tuberculosis* could induce maturation of DC *in vitro*. Splenocytes from naïve C57BL/6 mice were cultured in the absence or presence of heat-killed *M. tuberculosis* for 48 hr and then simultaneously stained with anti-CD11c and either anti-CD80, anti-CD86 or anti-CD40. Analysis by flow cytometry demonstrated that expression of these costimulatory molecules were up-regulated on $CD11c^+$ cells in a dose of *M. tuberculosis*-dependent manner (Fig. 4), suggesting that maturation of DC by high dose of *M. tuberculosis* may be critical for induction of CIA in C57BL/6 mice.

To further examine whether DC maturation is dependent on the dose of *M. tuberculosis in vivo*, mice were injected into their foot pads with CII plus IFA or CFA containing 1 or 5 mg/ml of *M. tuberculosis* and the

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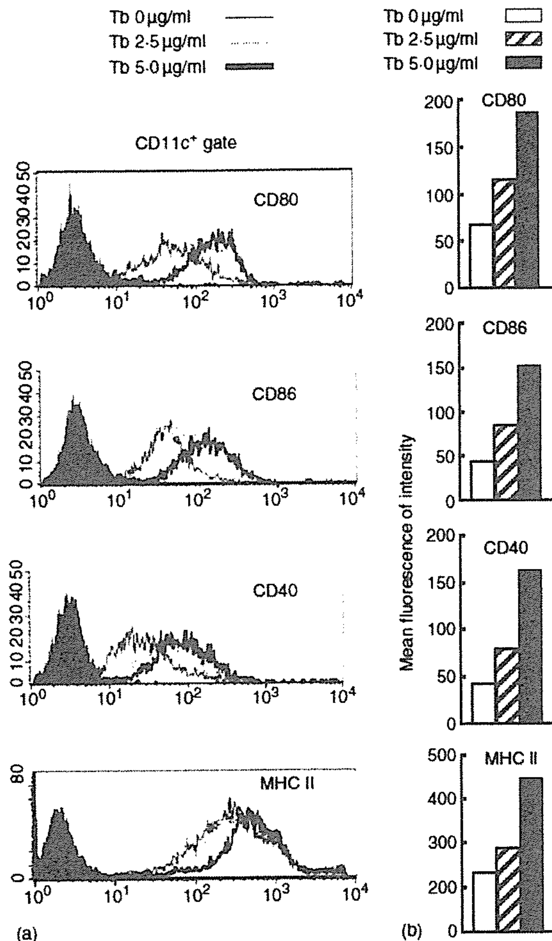

Figure 4. Maturation of DC induced by *M. tuberculosis in vitro*. Splenocytes prepared from naïve C57BL/6 mice were cultured and stimulated or not with 2·5 or 5·0 µg/ml heat-killed *M. tuberculosis* for 48 hr. Cells were then simultaneously stained with PE-conjugated CD11c and FITC-conjugated monoclonal antibodies against CD80, CD86, CD40 (open histograms) or an isotype control (shaded histograms). Expressions of the costimulatory molecules and MHC II on CD11c⁺ DC were analysed (a) and mean fluorescence intensities were determined (b) by flow cytometry. Data are representative of four independent experiments.

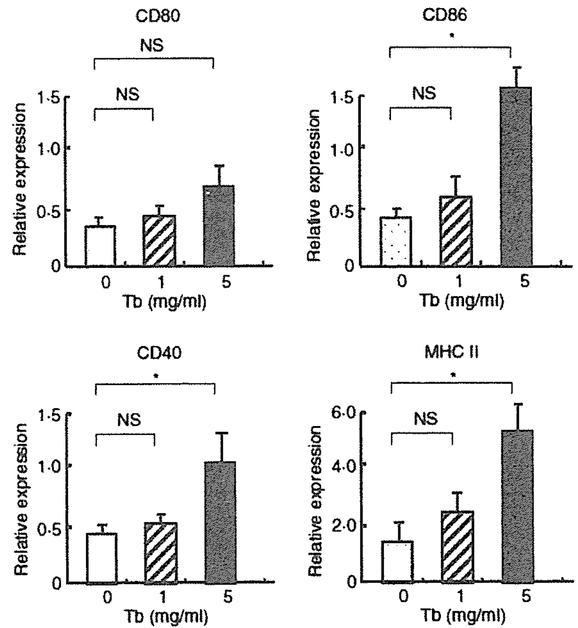

Figure 5. Maturation of DC induced by M. tuberculosis *in vivo*. Mice were injected into their foot pads with CII in IFA (Tb; 0 mg/ml) or CFA containing 1 or 5 mg/ml of *M. tuberculosis*. Five days after the immunization, total RNA was prepared from DLN cells and the expression of each gene was measured by quantitative RT–PCR analysis. Relative expressions of CD86, CD40 and MHC II in DLN from mice immunized with CII with 5 mg/ml of *M. tuberculosis* were significantly increased compared with those of the other groups ($^*P < 0{\cdot}05$) ($n = 6$). Data are the mean ± SEM of the relative expression of each gene.

mRNA expressions of costimulatory molecules and MHC II were determined in DLN (Fig. 5). Consistent with the results of *in vitro* studies, the mRNA levels of these molecules in DLN of mice that injected with CII plus CFA containing high does (5 mg/ml) of *M. tuberculosis* were significantly higher than those injected with CII plus IFA or CFA containing low does (1 mg/ml) of *M. tuberculosis*.

CII-specific Th1 responses by high, but not low, dose of *M. tuberculosis*

To examine whether immunization of C57BL/6 mice with CII emulsified with CFA containing high, but not low, dose of *M. tuberculosis* induces Th1 cells specific to CII in DLN, CD4⁺ T cells were sorted from DLN of immunized mice and stimulated with denatured CII for 48 hr in the presence of splenocytes that were used as APC. As shown in Fig. 6(a), CD4⁺ T cells from mice immunized using a high dose of *M. tuberculosis* significantly proliferated in response to CII. By contrast, we observed significantly less proliferation of CD4⁺ T cells from mice immunized using no or a low dose of *M. tuberculosis*. Similarly, IFN-γ production was detected in the culture of CD4⁺ T cells from mice immunized using a high dose of *M. tuberculosis* significantly more than those from mice immunized using no or a low dose of *M. tuberculosis* (Fig. 6b). These results suggest that high dose of *M. tuberculosis* is required for priming of CII-specific Th1 cells *in vivo*.

Discussion

The majority of autoimmune diseases are associated with the expression of specific MHC class II.[13,14] The susceptibility of CIA has been considered to be restricted by H-2^q and H-2^r haplotypes.[6] However, the present study

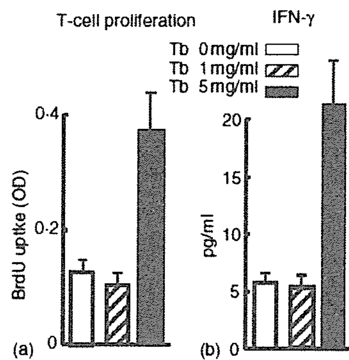

Figure 6. Collagen specific T cells were recruited to draining lymph nodes in Tb 5 mice. Naïve C57BL/6 mice were immunized with CII antigen emulsified in IFA or CFA containing *M. tuberculosis* at a final concentration of 1 or 5 mg/ml. Five days after the immunization, $CD4^+$ T cells were purified from DLN and stimulated with denatured chick CII antigen (50 μg/ml) for 48 hr in the presence of mitomycin C-treated splenocytes from naïve C57BL/6 mice as APC. All cultures were pulsed with BrdU for the last 16 hr and $CD4^+$ T-cell proliferation was determined by ELISA. IFN-γ production from $CD4^+$ T cells was also analysed in the culture supernatants by ELISA. Data are the means ± SEM and are representative of three independent experiments.

indicated that MHC class II molecule I-Ab could also present the C-II peptide antigen enough to prime antigen-specific $CD4^+$ helper T cells and induce CIA.

In the present study, we have shown that expressions of IFN-γ, IL-12p40, costimulatory molecules CD40, CD80 and CD86 and MHC II were up-regulated in DLN of CIA-induced C57BL/6 mice by immunization with CII emulsified in CFA containing high dose of *M. tuberculosis*. Because Toll-like receptor 2 expressed on DC recognizes *M. tuberculosis* and induces up-regulation of costimulatory molecule expression[15] and IL-12 secretion,[16,17] immunization with CII using high, but not low, dose of *M. tuberculosis* might induce maturation of DC in DLN; in these DLN DC secreted IL-12 and induced differentiation of CII-specific naïve helper T cells into IFN-γ-producing Th1 cells. In fact, we have demonstrated that *M. tuberculosis* induced maturation of DC *in vitro* in a dose-dependent manner. Furthermore, we have shown that priming of $CD4^+$ helper T cells specific to CII antigen in DLN required immunization with CII using CFA containing high, but not low, doses of *M. tuberculosis in vivo*. Taken together, these results suggested that high doses of *M. tuberculosis* were required for maturation of DC enough to prime $CD4^+$ helper T cells specific to CII antigen in DLN of $H\text{-}2^b$ background of C57BL/6 mice.

Th1/Th2 cytokine imbalance has been implicated in the pathogenesis of CIA in mice.[10,18] In the present study, we demonstrated the predominant Th1 phenotype in DLN of C57BL/6 mice with CIA. High level of IFN-γ production in response to CII has also been reported in DBA/1 mice, a susceptible strain of CIA, suggesting a critical role of Th1 responses in the pathogenesis of CIA in both DBA/1 and C57BL/6 mice.[10,19] However, C57BL/6 mice deficient in IFN-γ or IFN-γ receptor genes demonstrated increased incidence and severity of CIA.[20,21] A protective role of IFN-γ has been demonstrated in a number of distinct organ-specific autoimmune diseases, including EAE,[22] thyroiditis[23] and uveitis.[24] A number of models have been proposed for the down-regulatory effects of IFN-γ in autoimmunity, including the induction of inducible nitric oxide (NO) synthetase,[25] which leads to an increase in NO-mediated apoptosis of activated T cells.[26] Together, these studies suggested that IFN-γ may have dual roles in pathogenesis of CIA.

Th1 cells are differentiated from $CD4^+$ naïve T cells, in which IL-12 plays a critical role.[27] In the present study, we observed that the expression of IL-12p40 mRNA in DLN and joint tissues of CIA-induced C57BL/6 mice was significantly higher than that of control mice. Consistent with our results, a previous report showed that *in vivo* injection of IL-12 into mice accelerated incidence and severity of CIA.[28] Moreover, mice deficient in the IL-12p40 gene, a subunit of the IL-12 heterodimer, showed a significant reduction in both incidence and severity of CIA.[29] In contrast, mice deficient in the IL-12p35 gene, another subunit of the IL-12 heterodimer, showed exacerbated CIA.[30] Recent evidence indicated that IL-12p40 is a subunit of not only IL-12 but also IL-23 heterodimer[31] suggesting that IL-23, rather than IL-12, may play an important role in CIA development. Because microbial Toll-like receptor ligands stimulate APC, such as macrophages and DC, to produce IL-12 and IL-23, immunization with C II in CFA containing a high dose of *M. tuberculosis* induced IL-12 and/or IL-23 in C57BL/6 mice, which might regulate the development of CIA.

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on timely referral should also include variables as discussed above. This is also suggested in gender studies in other health domains [9]. Multivariate analyses in large prospective studies will allow firm conclusions to be reached.

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Characterisation of Th1/Th2 type, glucose-6-phosphate isomerase reactive T cells in the generation of rheumatoid arthritis

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Rheumatoid arthritis (RA) is a chronic inflammatory disorder characterised by an unknown inflammatory process in multiple joints. The K/BxN T cell receptor transgenic mouse model is a striking model of inflammatory arthritis characterised by arthritic manifestations similar to those of RA.[1] Matsumoto *et al* reported that arthritis could be provoked by linked T and B cell recognition of a ubiquitously expressed self antigen glucose-6-phosphate isomerase (GPI).[2] Recently, immunisation with recombinant human GPI was reported to induce T cell dependent arthritis in DBA/1 mice,[3] supporting the notion that GPI reactive T cells have a crucial role in the induction of arthritis.

In our previous study we reported the presence of high titres of anti-GPI antibodies (Abs) in some patients with RA, although a few control subjects were also positive.[4] To examine the role of GPI-specific T cells in patients with RA, we investigated the spontaneous Th1/Th2 response to GPI in patients with RA, systemic lupus erythematosus (SLE), and in healthy subjects with anti-GPI Abs.

To select anti-GPI Ab positive patients, an enzyme linked immunosorbent assay (ELISA) was performed using two different sources of GPI: a recombinant human GPI (huGPI), and a rabbit muscle GPI (raGPI; Sigma Chemical Co, St Louis, MO, USA), which have been described in detail previously.[4] Fifteen anti-GPI Ab positive patients with RA (from 185 with RA), four patients with SLE (from 135 with SLE), and four healthy subjects (from 145 controls) were studied (table 1). To analyse the possible relationship between HLA-DRB1 and anti-GPI Ab positivity, HLA-DRB1 alleles were screened. As shown in Table 1, 10 (67%) patients with RA and anti-GPI Abs shared the HLA-DRB1*0405 allele, which is one of the genes for susceptibility to RA in Japanese people, and five (33%) patients were DRB1*0901. In a recent report, the DRB1*0405 and *0901 alleles showed the most significant associations with RA in Korean people.[5] However, none of the four patients with SLE or four control subjects positive for anti-GPI Abs retained these alleles, suggesting a strong linkage between anti-GPI positive patients with RA with anti-GPI Abs and HLA DRB1*0405 and *0901 alleles (table 1).

To investigate the pathogenic relevance of GPI reactive T cells in subjects with anti-GPI Abs, a magnetic activated cell sorting cytokine secretion assay was performed using peripheral blood mononuclear cells plus GPI (in the presence of 10 μg purified human GPI protein digested by thrombin or 13.5 ng thrombin as a control). As a positive control, we used staphylococcal enterotoxin B (1 μg/ml). Cells (2×10^6) were harvested Ab-Ab directed against CD45 and either interferon (IFN) γ or interleukin (IL) 4 conjugates, and stained with phycoerythrin (PE)-conjugated anti-IFNγ or anti-IL4. Cells were magnetically labelled by anti-PE Ab microbeads, and were analysed on a FACSCalibur flow cytometer (Becton Dickinson). IFNγ secreting T cells were detected in seven (47%) patients with RA (RA3, 6, 7, 9, 10, 11, 15). IFNγ may be produced by GPI reactive T cells (table 1). IL4 secreting T cells were detected in four (27%) patients (RA1, 3, 7, 10), although they were less frequent than IFNγ+ T cells. Three patients (RA3, 7, 10) had both IFNγ and IL4 secreting T cells. In contrast, only one healthy subject (control 2) showed weak response to GPI (IFNγ and IL4). Interestingly, all seven patients with RA bearing GPI reactive IFNγ+ T cells shared either DRB1*0405 or *0901 (table 1). Our results demonstrated that GPI-specific Th1 and Th2-type cells (especially

Table 1 Anti-GPI Abs and DRB1 genotype in patients with RA, SLE, and in healthy subjects

Subject	huGPI	raGPI	DRB1 genotype	IFNγ+ T cells	IL4+ T cells
RA1	1.32	2087	0405 0901	4	9
RA2	2.73	3.02	0409 0803	0	0
RA3	1.33	1.15	0405 1501	**190**	**12**
RA4	2.43	2.55	0101 0803	0	0
RA5	1.79	3.14	0405 0401	6	3
RA6	1.65	2.67	0802 0901	**15**	3
RA7	1.88	1.15	1402 0901	**55**	**10**
RA8	2.60	3.47	0405 0901	0	0
RA9	2.46	1.70	1502 0405	**8**	1
RA10	1.93	2.65	0405 0803	**16**	**24**
RA11	1.72	0.95	0405 0803	**49**	3
RA12	1.40	1.61	0405 1502	7	0
RA13	1.49	0.94	0405 0803	0	0
RA14	1.39	0.99	0405 1502	0	0
RA15	2.48	3.32	0901 0901	**20**	2
SLE1	3.68	1.86	0803 1404	0	0
SLE2	1.32	1.38	0427 0427	0	0
SLE3	1.91	2.41	0101 0428	2	0
SLE4	2.89	2.97	0803 0803	0	0
Control1	3.75	3.54	1501 1403	2	0
Control2	3.05	3.19	1302 0803	**9**	**4**
Control3	2.19	3.28	1501 0803	3	0
Control4	2.51	2.59	1329 1406	1	1

The cut off optical density was calculated from an ELISA of 145 healthy subjects, the mean value + two standard deviation was 1.32 to human recombinant GPI, and 0.94 to rabbit native GPI. Double positive populations were considered anti-GPI Ab positive. For MACS cytokine secretion assay, positive cell numbers were determined after subtracting control cells, either reacted on thrombin or spontaneously secreting cytokines, from GPI reactive IFNγ+ and IL4+ T cells. The cut off cell numbers were calculated from the reaction of four patients with SLE and four control subjects who were all anti-GPI Ab positive. The mean value + two standard deviation was 7.6 to IFNγ, and 3.6 to IL4. Bold numbers indicate a positive reaction to GPI.
Abs, antibodies; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; IFN, interferon; IL, interleukin.

Th1-type cells) were frequently detected in patients with RA with anti-GPI Abs, suggesting that these cytokines may be associated with the production of arthritogenic Abs, especially when associated with HLA-DRB1*0405 or *0901.

In conclusion, our findings suggest that GPI reactive IFNγ+/IL4+ T cells may have a crucial role in the generation of arthritis in HLA-DRB1*0405 or *0901 positive patients with RA and anti-GPI Abs.

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ORIGINAL ARTICLE

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Significance of antiprothrombin antibodies in patients with systemic lupus erythematosus: clinical evaluation of the antiprothrombin assay and the antiphosphatidylserine/prothrombin assay, and comparison with other antiphospholipid antibody assays

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Abstract Antibodies against prothrombin are detected by enzyme immunoassays (EIA) in sera of patients with antiphospholipid syndrome (APS). However, there are two methods for antiprothrombin EIA; one that uses high binding plates (aPT-A), and another that utilizes phosphatidylserine bound plates (aPS/PT). We aimed to evaluate and compare aPT-A and aPS/PT in a clinical setting. We performed EIA for anti-PT, anti-PS/PT, IgG, and IgM anticardiolipin antibodies (aCL), and IgG β2-glycoprotein I-dependent aCL (aβ2GPI/CL) with serum samples from 139 systemic lupus erythematosus (SLE) patients (16 with history of at least one thrombotic episode) and 148 controls. We observed that: (1) although titers of anti-PT and anti-PS/PT were significantly related with each other ($P < 0.0001$, $\rho = 0.548$), titer of anti-PT and anti-PS/PT differed greatly in some samples; (2) odds ratio and 95% confidence interval for each assay was 3.556 (1.221–10.355) for aPT-A, 4.591 (1.555–15.560) for aPS/PT, 4.204 (1.250–14.148) for IgG aCL, 1.809 (0.354–9.232) for IgM aCL, and 7.246 (2.391–21.966) for aβ2GPI/CL. We conclude that, while all EIA performed in this study except IgM aCL are of potential value in assessing the risk of thrombosis, aPS/PT and aβ2GPI/CL seemed to be highly valuable in clinical practice, and that autoantibodies detected by anti-PT and anti-PS/PT are not completely identical.

Key words Antiphospholipid syndrome · Antiprothrombin antibody · Enzyme immunoassay · Systemic lupus erythematosus (SLE)

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Introduction

Antiphospholipid antibodies (aPL) are a group of heterogeneous autoantibodies against a variety of phospholipid binding proteins. Detection of aPL by radioimmunoassay was first reported by Harris et al.,[1] and detection of anticardiolipin antibody (aCL) by enzyme immunoassay (EIA) was reported by Koike et al. in 1984.[2] The development of these relatively simple methods enabled researchers to perform a number of clinical studies, and the clinical entity antiphospholipid syndrome (APS), proposed by Hughes et al., was established during the 1980s. This syndrome is characterized by the presence of aPL and occurrence of thrombotic episodes and/or intrauterine fetal deaths.

Methods currently used for detecting aPL can be divided to two categories: lupus anticoagulant (LAC) tests and EIA. Enzyme immunoassays are frequently used for detection of aPL, due to their relative simplicity and reliability. A standardized aCL EIA, described by Harris et al.,[3] is widely used for detection of aPL and the diagnosis of APS. However, during the last decade it has become evident that anticardiolipin antibodies are in fact autoantibodies against phospholipid binding proteins. The first such protein described is β2-glycoprotein I (β2GPI). It is suggested that β2GPI undergo a conformational change upon binding to negatively charged phospholipids, which in turn causes exposure of neoepitopes that are detected by aCL. Alternatively, the density of β2GPI may be important for recognition by aCL, and appropriate β2GPI density is achieved by binding to negatively charged phospholipids. Enzyme immunoassay for detection of β2GPI dependent aCL is reported to be of value in clinical practice[4–6] and is included in the proposed Sapporo criteria for classification of APS.[7] Enzyme immunoassays using β2GPI directly coated on oxidized plates (high binding plates) are also reported to be useful.[8–12]

Prothrombin is another important autoantigen recognized by aPL. Shortly after the description of β2GPI as the aCL "cofactor,"[13–15] Bevers et al.[16] reported that the IgG with LAC activity recognizes a complex of phospholipids

and prothrombin. Thereafter, a number of studies with regard to the relationship between thrombotic events and presence of antiprothrombin antibodies as measured by EIA have been reported, with conflicting conclusions.[17] Interestingly, two methods for antiprothrombin antibody detection have been suggested, similar to the case for anti-β2GPI detection. One is an assay that utilizes prothrombin coated directly onto high binding plates (in this paper referred to as aPT-A), and the other is an assay that detects antibodies bound to prothrombin on phosphatidylserine-coated plates (in this paper referred to as aPS/PT). The large differences in the results among the reports that studied the relevance of antiprothrombin EIA may partly be due to the methods of the EIA employed in individual studies. However, very few studies have directly compared the aPT-A and the aPS/PT assays.[18,19]

In this study, we aimed to compare these two antiprothrombin EIA, along with other aPL EIA (IgG and IgM aCL, IgG aβ2GPI/CL) and LAC, for their values in assessing the risk of thrombosis in patients with systemic lupus erythematosus (SLE). In particular, for all EIA, we measured the values of each antibody in our own healthy controls and have set the cutoff points anew to enable comparison of these assays from a same viewpoint.

Material and methods

Subjects and samples

Serum and plasma samples were taken from 139 SLE patients followed at University of Tsukuba Hospital, Tsukuba, Ibaraki, Japan. All SLE patients fulfilled the 1987 ACR criteria[20,21] for the classification of SLE. Among these patients, 16 had one or more documented episodes of thrombosis (11 with arterial thromboses, 8 with venous thromboses, 3 with both arterial and venous thromboses). Mean follow-up period at the time of blood sampling was 9.95 ± 8.23 (mean ± SD) years; 9.00 ± 9.49 years for patients with history of thromboses, and 10.07 ± 8.09 years for patients without history of thromboses. Warfarin was started in 11 patients after diagnosis of thrombosis, and in a mean follow-up of 2.64 ± 2.87 years, no additional thrombotic episodes were noticed. Samples from 148 healthy volunteers, with no apparent history of thrombosis or autoimmune diseases, served as controls. Written informed consent was obtained from all subjects of this study, and the study was approved by the ethics committee of the University of Tsukuba.

Antiphospholipid antibody assays

Antiprothrombin antibodies measurement using high binding plates

Nunc Maxisorp plates (Nalge Nunc, Rochester, NY) were used. One hundred microliters per well (μl/well) of human prothrombin (Haematologica Technologies, Essex Junction, VT, USA) in carbonate buffer, pH 9.6, at a concentration of 10 μg/ml was coated onto wells at room temperature overnight. After blocking by 1% bovine serum albumin (BSA) in Tris-buffered saline including 5 mM calcium (TBS), 100 μl/well of sample sera diluted 1:200 in TBS with 1% BSA and 0.5% Tween-20 (TBS/Tw) were added to the wells, and the plates were incubated for 2 h at room temperature. After washing with TBS/Tw, alkaline phosphatase labeled goat antihuman IgG antibody (American Qualex, SanClemente, CA, USA) diluted 1:2500 in TBS/Tw were added to the wells, and the plates were incubated for 1 h at room temperature. After further washing, substrate was added and the optical density was measured at 405 nM by an autoreader. A standard curve was always generated using a standard serum, the antibody concentration in which designated as 100 units/ml.

Anti-phosphatidylserine/prothrombin antibodies

For the measurement of aPS/PT, a commercially available EIA kit (Medical and Biological Laboratories, Nagoya, Japan)[22] was used. The kit detects aPS/PT in the presence of calcium and follows, in principle, the method described by Atsumi et al.[18] The wells of the plates are coated with phosphatidylserine/human prothrombin by the supplier. Sample sera were diluted at 1:100 with the supplied dilution buffer, and 100 μl of each sample were added to the wells. The plates were incubated for 1 h at room temperature, and bound antibodies were detected by a peroxidase labeled goat anti-human IgG antibody.

Anticardiolipin antibodies

Anticardiolipin antibodies were measured according to the methods described by Harris et al.,[3] using a Mesacup cardiolipin kit (Medical and Biological Laboratories) and a Mesacup cardiolipin IgM kit (Medical and Biological Laboratories).

Anticardiolipin/β2-glycoprotein I antibodies

Serum aβ2GPI/CL were measured using anticardiolipin/β2-glycoprotein I antibody enzyme-linked immunosorbent assay (ELISA) kit (Yamasa Shoyu, Chosi, Japan). The kit is supplied with plates coated with cardiolipin. The wells were first incubated with sample dilution buffer with or without β2GPI. Sample sera were diluted at 1:101 with the supplied dilution buffer, and 50 μl /well of each sample was added to both the aβ2GPI added and non-added wells. Plates were incubated for 30 min at room temperature. Bound antibodies were detected by a peroxidase labeled anti-human IgG antibody. Samples were considered positive for aβ2GPI/CL when the values obtained from the β2GPI added wells exceeded the cutoff point, and were above the values obtained from β2GPI non-added wells.

Determination of cutoff levels for enzyme immunoassays

Although the cutoff levels for commercially available EIA tests are determined by the suppliers, the definition of a

cutoff level differs among assays, and different control populations are used for the determination of each cutoff level in an assay. Therefore, to compare various assays from a same viewpoint, the cutoff levels for all EIA were determined anew. For all assays, the cutoff levels were set at mean + 2SD of our control samples.

Lupus anticoagulant assay

Lupus anticoagulant assay was performed using an LA test kit (Gradipore, North Ryde, Australia), which is based on a simplified dilute Russel viper venom time described by Exner et al.[23] Ratios equal to or larger than 1.3 were considered positive for LA.

Statistical analyses

Relationships between the levels of EIA were compared by Spearman's rank correlation test. Differences in titer of any aPL measured by EIA between SLE patients with history of thromboses and patients without such history were compared by the Mann–Whitney *U*-test. Positivity for any aPL assay and having histories of thromboses were compared by Fisher's exact test. *P* values equal to or less than 0.05 were considered as statistically significant.

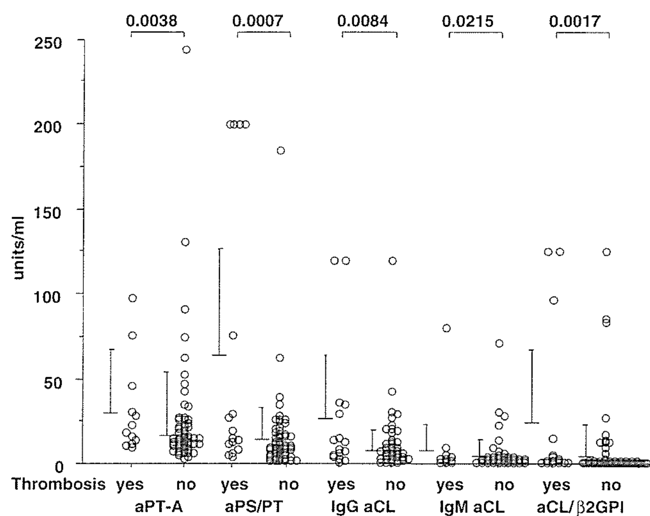

Fig. 1. Values of antiphospholipid antibodies as measured by enzyme immunoassays in patients with systemic lupus erythematosus. One hundred and thirty-nine patients with systemic lupus erythematosus were divided into two groups (patients with or without history of thrombosis) and were applied to various antiphospholipid antibody enzyme immunoassays. Values are in arbitrary units determined independently for each enzyme immunoassay. Numbers above indicate *P* values calculated by Mann–Whitney *U*-test *aPT-A*, antiprothrombin antibody measured using high binding plates; *aPS/PT*, antiphosphatidylserine/prothrombin antibody; *aCL*, anticardiolipin antibody; *aβ2GPI/CL*, β2 glycoprotein I-dependent anticardiolipin antibody

Results

Cutoff levels of enzyme immunoassays

The cutoff levels of all five EIA performed in this study were determined anew from the same 148 sample sera. The cutoff levels for aPT-A, aPS/PT, aβ2GPI/CL, IgG aCL, and IgM aCL assays were 17.95, 17.83, 0.57, 15.43, and 5.69, respectively. Values above these cutoff levels were considered positive for a given assay.

Titer of various antiphospholipid antibodies in SLE patients with or without history of thrombotic episodes

The levels of each EIA were compared between patients with history of thrombosis and those without thrombosis. In all assays, patients with history of thrombosis had significantly higher values compared to those without such history (Fig. 1). The differences observed between patients with or without thrombotic episodes seemed especially large in aPS/PT, although it is difficult to compare the assays in this way since the "unit" in each assay was defined independently among each other.

Results of antiprothrombin antibodies detected using high binding plates and those of antiphosphatidylserine/ prothromnbin antibodies are significantly correlated with each other

It has been suggested that the values obtained by aPT-A and aPS/PT assays do not necessarily correlate with each other.[18] We compared the values of aPT-A and aPS/PT among sera from SLE patients. These values were significantly correlated with each other ($\rho = 0.514$, $P < 0.0001$ by Spearman's rank correlation), compared to relationships among other aPL such as between aPT-A and aβ2GPI/CL (Fig. 2 and results not shown). However, some sera had high value for only one or the other of those assays.

Positivity of antiphosphatidylserine/prothrombin antibody and/or antiprothrombin antibody is correlated with having histories of thrombotic episodes

Positivity for aPS/PT was significantly related with having history of thrombosis (Tables 1 and 2). aPT-A positivity was also significantly related with history of thrombosis.

Positivity of β2GPI-dependent aCL and/or lupus anticoagulant is significantly correlated with having histories of thrombotic events

When the cutoff level of aβ2GPI/CL was set at 3.5 units as recommended by the supplier, 17 were positive, among whom 5 had history of thrombotic episodes ($P = 0.0282$ by Fisher's exact test). When the cutoff level was adjusted using data from our own healthy controls, a more significant relationship was observed (Tables 1 and 2). The OD values equivalent to 0.6 units were around 0.060–0.070 in the presence of β2GPI, and around 0.015–0.040 in the absence of β2GPI (not shown). Being positive for LAC was also significantly associated with history of thrombosis.

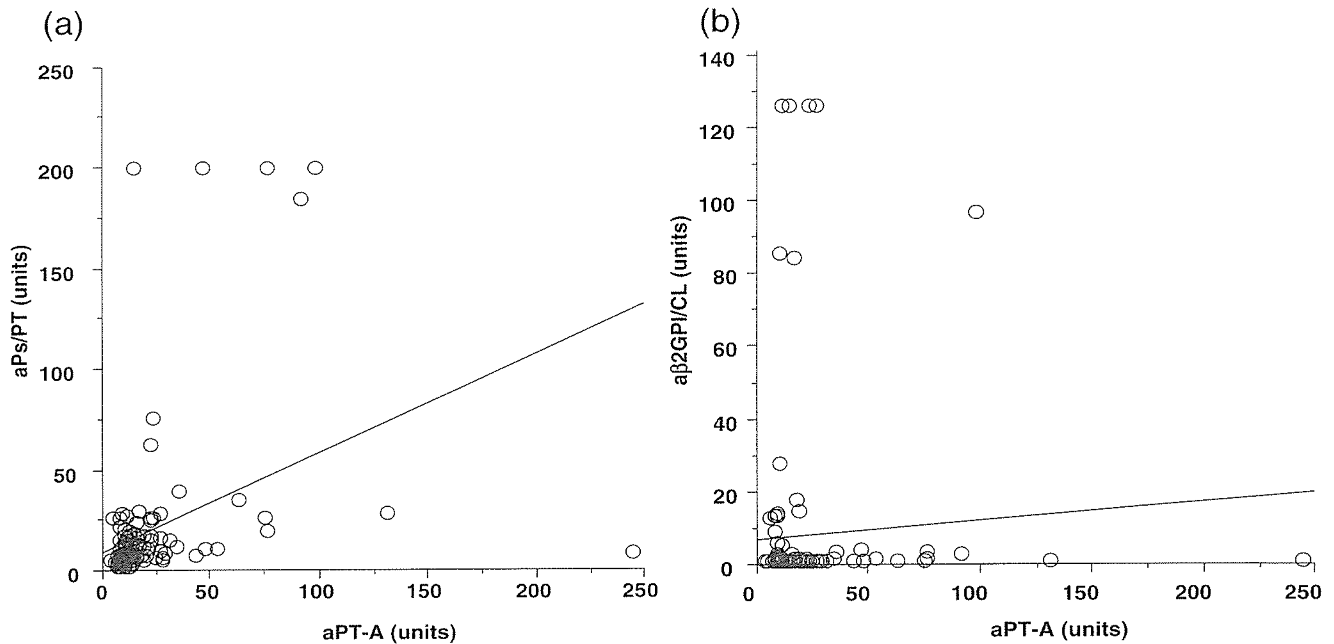

Fig. 2a,b. Relationship between values of antiprothrombin antibody and antiphosphatidylserine/prothrombin antibody in sera of patients with systemic lupus erythematosus. The values of antiprothrombin antibody and antiphosphatidylserine/prothrombin antibody in patients with systemic lupus erythematosus were compared. For comparison, the values of antiprothrombin antibody and β2-glycoprotein I-dependent anticardiolipin antibody were also compared. **a** Antiprothrombin antibody and antiphosphatidylserine/prothrombin antibody. $\rho = 0.514$, $P < 0.0001$ by Spearman's rank correlation. **b** Antiprothrombin antibody and β2 glycoprotein I-dependent anticardiolipin antibody. $\rho = 0.086$, $P = 0.3103$ by Spearman's rank correlation. *aPT-A*, antiprothrombin antibody measured using high binding plates; *aPS/PT*, antiphosphatidylserine/prothrombin antibody; *aβ2GPI/CL*, β2 glycoprotein I-dependent anticardiolipin antibody

Table 1. Relationship between positivity of antiphospholipid assays and history of thrombosis

		Thrombosis		Total	Odds ratio	95% CI	*P* value
		Yes	No				
aPT-A	Positive	8	27	35	3.556	1.221–10.355	0.0278
	Negative	8	96	104			
aPS/PT	Positive	8	22	30	4.591	1.555–15.560	0.0072
	Negative	8	101	109			
aβ2GPI/CL	Positive	10	23	33	7.246	2.391–21.966	0.0005
	Negative	6	100	106			
IgG aCL	Positive	5	12	17	4.204	1.250–14.148	0.0282
	Negative	11	111	122			
IgM aCL	Positive	2	9	11	1.809	0.354–9.232	0.6158
	Negative	14	114	128			
LAC	Positive	7	9	16	9.852	2.972–32.657	0.0004
	Negative	9	114	123			

aPT-A, antiprothrombin antibody measured using high binding plates; aPS/PT, antiphosphatidylserine/prothrombin antibody; aβ2GPI/CL, β2-glycoprotein I dependent anticardiolipin antibody; aCL, anticardiolipin antibody; LAC, lupus anticoagulant

Relationships between conventional aCL assays, lupus anticoagulant assay, and history of thrombosis

The relationship between positivity of IgG aCL and history of thrombosis was statistically significant when the cutoff value was set at mean + 2SD of our control samples (Table 1). When the cutoff level was set at 10 units, originally set by the manufacturer, among the 31 patients positive for IgG aCL, 8 had history of one or more thrombosis episodes, yielding a *P* value of 0.0091 as calculated by Fisher's exact test. No association between IgM aCL positivity and history of thrombosis was seen, regardless of whether the cutoff point was set at the values set by the manufacturer (1 of 7 positive patients with history of thrombosis, $P = 0.5837$ by Fisher's exact test), or that set by ourselves (Table 1).

Positivity of antiphospholipid antibodies among SLE patients with history of thrombosis

The 16 SLE patients with history of thrombosis were assessed for their aPL positivity (Table 3). Thirteen patients were positive for at least one of the antiphospholipid antibody assays performed. While a combination of IgG aCL and LAC enabled us to judge 8 patients as positive for aPL, a combination of aPS/PT and aβ2GPI/CL indicated 10 as positive for aPL.

Table 2. Sensitivity, specificity, and positive predictive values of antiphospholipid assays for history of thromboses in patients with systemic lupus erythematosus

	Sensitivity	Specificity	Positive predictive value
aPT-A	0.500	0.775	0.229
aPS/PT	0.500	0.821	0.267
aβ2GPI/CL	0.625	0.813	0.303
IgG aCL	0.313	0.902	0.294
IgM aCL	0.125	0.927	0.182
LAC	0.438	0.927	0.438

aPT-A, antiprothrombin antibody measured using high binding plates; aPS/PT, antiphosphatidylserine/prothrombin antibody; aβ2GPI/CL, β2-glycoprotein I dependent anticardiolipin antibody; aCL, anticardiolipin antibody; LAC, lupus anticoagulant

Positivity of antiphospholipid antibodies among SLE patients with history of pregnancy

Among 69 SLE patients who had history of at least one pregnancy, only 3 had a history of recurrent fetal losses. Among patients with history of at least one pregnancy, the number of patients positive for a given antibody and with history of fetal loss, the number of patients of patients positive for the antibody, P values calculated by Fisher's exact test were 2/16, 0.1321 for aPT-A, 1/17, >0.9999 for aPS/PT, 0/5, >0.9999 for IgG aCL, 0/4, >0.9999 for IgM aCL, 2/13, 0.0888 for aβ2GPI/CL, and 1/6, 0.2421 for LAC, respectively. For aβ2GPI/CL, when the cutoff value was set at 3.5 units, as determined by the supplier, the numbers became 2/

Table 3. Positivity of antiphospholipid assays in patients with history of thrombosis

Patient	Thrombosis	aPT-A	aPS/PT	aβ2GPI/CL	IgG aCL	IgM aCL	LAC
1	A	–	–	–	–	–	–
2	A	–	–	–	–	–	–
3	A	+	+	+	–	–	–
4	V	+	–	+	–	–	+
5	V	–	–	–	–	–	+
6	A,V	+	+	+	–	–	+
7	A	+	–	–	+	–	–
8	A	+	+	+	+	–	+
9	A	+	+	+	+	–	+
10	A	–	–	–	–	–	–
11	V	+	–	–	–	–	–
12	V	–	+	+	+	+	+
13	A,V	–	+	+	–	+	–
14	A	–	–	+	–	–	–
15	V	–	+	+	–	–	–
16	A,V	+	+	+	+	–	+

A, with history of arterial thromboses; V, with history of venous thromboses; aPT-A, antiprothrombin antibody measured using high binding plates; aPS/PT, antiphosphatidylserine/prothrombin antibody; aβ2GPI/CL, β2-glycoprotein I-dependent anticardiolipin antibody; aCL, anticardiolipin antibody; LAC, lupus anticoagulant

Summary of thrombotic history: 1. Cerebral infarction (CI), occurring before diagnosis of systemic lupus erythematosus (SLE). No additional events with low-dose aspirin. 2. Left atrial thrombosis, found at the time of diagnosis of SLE. No recurrence with warfarin plus low-dose aspirin. 3. CI, occurring 21 years after diagnosis of SLE, while on low-dose aspirin. No further events after addition of warfarin. 4. Deep vein thrombosis (DVT) and pulmonary embolism (PE), occurring 3 years after diagnosis of SLE, while on low-dose aspirin. No further events after addition of warfarin. 5. PE found 2 years after diagnosis of SLE, while without any anticoagulation. Dipyridamole was added. Warfarin added 10 years after diagnosis of SLE to delay the progression of pulmonary hypertension. 6. Old lung infarction noticed 22 years after diagnosis of SLE, while under low-dose aspirin. No further episodes. 7. CI occurred 15 years before occurrence of SLE. 8. DVT occurred in the first year after diagnosis of SLE while under low-dose aspirin. Warfarin was added and no further episodes noticed. 9. PE, found 17 years after diagnosis of SLE while drug free. No episodes after addition of warfarin. 10. CI, occurring before diagnosis of SLE. 11. DVT, occurring 4 years after diagnosis of SLE. No recurrence after addition of warfarin. 12. DVT, found at the time of diagnosis of SLE. No recurrence after addition of warfarin and low-dose aspirin. 13. DVT, PE, both found at the time of diagnosis of SLE. No recurrence after addition of warfarin and low-dose aspirin. 14. Myocardial infarction occurring 24 years after diagnosis of SLE. CI found by further examination. No recurrence after low-dose aspirin and ticlopidine hydrochloride. 15. DVT, occurring 5 years after diagnosis of SLE while under no anticoagulation. No recurrence after addition of warfarin and low-dose aspirin. 16. CI, PE, both found at the time of diagnosis of SLE. No recurrence after addition of warfarin

8 and 0.0337. Other assays did not yield significant *P* values. However, the number of patients with history of recurrent fetal losses was too small to allow us to draw a reliable conclusion.

Discussion

Previous studies suggested that aPT-A and aPS/PT assays detect significantly different populations of autoantibodies and the results of the two assays may not correlate with each other. We applied the two assays to the same samples consisting of SLE patients and healthy individuals to assess the differences between the two assays. Results show that both assays, aPS/PT in particular, are of potential value in assessing the risk of thrombosis in SLE patients.

Very recently, Bertolaccini et al.[19] also compared the results of aPT-A and aPS/PT in their cohort of 212 SLE patients. In their study they also found a significant relationship between the results of aPT-A and aPT/PS. Similar to our study, they also noted the presence of a number of patients with discrepant results. Thus, along with the results of our study, it is conceivable that aPT-A and aPT/PS recognize an overlapping, but not identical, population of autoantibodies. In their study, Bertolaccini et al. found a slightly stronger relationship between positivity of IgG aPS/PS and a history of thromboses than IgG aPT-A positivity and a history of thromboses. However, they have not documented how the aPS/PT-positive and aPT-A-positive patients overlap, when the presence of thrombotic history of thrombosis was put into consideration. In our study, between 8 aPT-A-positive SLE patients with a history of thrombosis and 8 aPS/PT-positive patients with a history of thrombosis, only 5 overlapped. These results could imply that performing both aPT-A and aPS/PT assays, if possible, or developing a new method that would enable detection of both populations of autoantibodies would be desirable for more sensitive detection of clinically relevant antiprothrombin antibodies. The reason why aPT-A and aPS/PT assays recognize overlapping, but not identical, populations of autoantibodies is not clear, but a most reasonable explanation would be that the nature of conformational changes introduced to prothrombin is different between when prothrombin is bound to high binding plates and when it is bound to phosphatidylserine bound plates. It is possible that the conformational changes introduced when bound to phosphatidylserine-coated plates is more similar to the changes introduced in vivo, and this may account for the higher clinical relevance of aPS/PT assay than that of aPT-A assay observed in our study.

Assay for aβ2GPI/CL has become increasingly popular in the clinical management of SLE in Japan. The assay has been approved by the Ministry of Science Health and Welfare of Japan as a tool for diagnosis of APS. As seen in Table 1, positivity for this antibody was strongly correlated with histories of thrombosis, suggesting its value in a clinical setting. However, the cutoff value of aβ2GPI/CL in this study was set at 0.57 units, different from 3.5 units recommended by the manufacturer of the assay kit. In addition, the OD values at 0.6 units were around 0.060 in the assay we used. Thus, while setting the cutoff value at this level does give us a better *P* value, implying the validity of the assay, refinement of the assay would be necessary to more accurately measure low-titer aβ2GPI/CL. By standardizing the methodology and appropriately setting the cutoff levels, aβ2GPI/CL could become a more widely used measure for the diagnosis of APS. Efforts to standardize this assay are necessary. Previously, we have suggested[24] that aβ2GPI/CL assays may be suitable to be included in the 1987 ACR criteria,[20,21] which currently includes only LAC and conventional aCL assays as means of aPL detection. Results presented in this study imply that aβ2GPI/CL EIA has similar or superior value for the detection of aPL as IgG aCL. We believe that inclusion of aβ2GPI/CL in the ACR criteria for SLE may be beneficial.

Currently, the Sapporo criteria, proposed in 1999, are widely used for the diagnosis of APS.[7] In the Sapporo criteria, for the detection of aPL, aCL, aβ2GPI/aCL, and LAC assays are recommended. However, studies reported thereafter have suggested the values of antiprothrombin assays in the diagnosis of APS. We wished to determine the values of aPT-A and aPS/PT in a clinical setting and in particular, wanted to determine whether routine measurement of these antibodies would aid in the diagnosis of APS. For this purpose, we decided to set the cutoff level of each EIA anew, to evaluate each assay from the same viewpoint. Among our 16 SLE patients with history of thrombosis, 8 were judged positive for aPL by the combination of IgG aCL and LAC. Addition of aβ2GPI/aCL raised the number of patients positive for aPL to 12, while addition of either aPT-A or aPS/PT raised the number to 11. A combination of aβ2GPI/aCL and aPS/PT judged 10 patients as positive for aPL, and a combination of aPT-A, aPS/PT, and aβ2GPI/aCL judged 12 as aPL-positive (Table 3). In our SLE patients, among the patients with history of thrombosis, all but one of those positive for LAC were positive for at least either aPS/PT or aβ2GPI/aCL. These results are quite reasonable since a large part, if not the majority, of LAC activity is β2GPI or prothrombin dependent. Refinement of these assays may show that these assays should have a position in the diagnostic criteria of APS, and that they have the potential to substitute LAC assays in the future. Although the clinical significance of LAC assays is well established, carefully collected and preserved plasma needs to be used, the methods are tedious and are still not completely standardized.

By directly comparing aPS/PT and aPT-A assays along with other aPL assays, using the same samples and the same criteria to set cutoff levels, our current study indicated that aPS/PT and aβ2GPI/aCL assays have more clinical relevance than the aPT-A assay. However, aPT-A assay seemed to be detecting a spectrum of autoantibodies not detected by aPS/PT, and we believe that it is premature to dismiss the assay as clinically meaningless.

In conclusion, we have shown the heterogeneity of autoantibodies detected by aPT-A or aPS/PT assays, but have also shown that these EIAs may be valuable in the

diagnosis of APS. Although interlaboratory studies and standardization of the assays are necessary, we believe that these assays may have the potential to be included in the future diagnostic criteria for APS and SLE.

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ORIGINAL ARTICLE

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Altered peptide ligands control type II collagen-reactive T cells from rheumatoid arthritis patients

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Abstract We previously reported that peripheral blood mononuclear cells from HLA-DRB1*0101 Japanese patients with rheumatoid arthritis (RA) were highly reactive to 256–271 peptide of type II collagen (CII). In this report, we tried to regulate the CII reactivity of T cells from RA patients with HLA-DRB1*0101 by altered peptide ligand (APL), which is a single amino acid substitution of the T-cell epitope on CII 256–271 peptide. Antagonistic activity of 21 APLs was assessed using three different T-cell lines. Results showed that 262 (G→A) APL of CII 256–271 exhibited antagonistic activity in all T-cell lines and it was suggested that the application of CII APL might be a new therapeutic strategy in the regulation of RA.

Key words Altered peptide ligand (APL) · Antagonist · Rheumatoid arthritis (RA) · T cells · Type II collagen

Introduction

T-cell activation depends on the ability of the T-cell receptor (TCR) to recognize 8–20 amino acid peptides that are bound to major histocompatibility complex (MHC) molecules. The way TCR recognizes peptide is flexible. If the amino acid residue of peptide ligands for TCR is substituted for a different amino acid and can still bind to MHC molecules (altered peptide ligands; APLs), these APLs could regulate the activation of T cells. Several studies have shown that APL had a potential to induce differential cytokine secretion, anergy, and antagonism of the response to the wild-type antigens.[1–3] Therefore, it is possible to use APL as a therapeutic agent against T-cell-mediated diseases such as autoimmune diseases and allergic disorders.

Rheumatoid arthritis (RA) is generally considered to be a T-cell-mediated autoimmune disease. Type II collagen (CII), a molecule abundant in articular cartilage, is an attractive candidate as a target antigen (autoantigen) responsible for pathogenicity of RA. We previously reported that peripheral blood mononuclear cells (PBMCs) from RA patients with HLA-DRB1*0101 haplotype, which is one of the major alleles in Japanese RA patients, reacted to CII 256–271 peptide and this CII fragment was suggested to be a major T-cell epitope in RA patients with this haplotype.[4] In the present study, we established three different CII256–271-specific T-cell lines (E01, H01, H07) from two HLA-DRB1*0101-positive RA patients and tried to regulate CII reactive T cells by inducing TCR antagonism using 21 different APLs.

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Patients and methods

Patients

Two RA patients, who met the 1987 classification criteria of the American College of Rheumatology,[5] were selected after confirmation that their PBMCs included CII 256–271 peptide-reactive T cells. Their HLA-DRB1 haplotypes were DRB1*0101/*0405 and DRB1*0101/*0901. They agreed to participate in the present study and written informed consent was obtained from these subjects before collection of blood samples. The study design was approved by the Ethical Committee of the University of Tsukuba.

Fig. 1. Design for altered peptide ligands (*APL*). Two anchor positions on CII 256–271 peptide, CII 263 (F) and CII 264 (K), were reported and a single amino acid around these residues was substituted. Twenty-one analog peptides were designed. *A* indicates an anchor residue, which was a binding site to HLA molecule. The *dash* indicates the identical amino acid residue to that of CII 256–271 peptide

CII 256-271amino acide sequence

	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12	P13	P14	P15	P16
								A	A							
APLs	G	K	P	G	I	A	G	F	K	G	E	Q	G	P	K	G
APL1	-	-	-	-	-	S	-	-	-	-	-	-	-	-	-	-
APL2	-	-	-	-	-	D	-	-	-	-	-	-	-	-	-	-
APL3	-	-	-	-	-	-	D	-	-	-	-	-	-	-	-	-
APL4	-	-	-	-	-	-	K	-	-	-	-	-	-	-	-	-
APL5	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-
APL6	-	-	-	-	-	-	-	D	-	-	-	-	-	-	-	-
APL7	-	-	-	-	-	-	-	Q	-	-	-	-	-	-	-	-
APL8	-	-	-	-	-	-	-	E	-	-	-	-	-	-	-	-
APL9	-	-	-	-	-	-	-	S	-	-	-	-	-	-	-	-
APL10	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-
APL11	-	-	-	-	-	-	-	-	V	-	-	-	-	-	-	-
APL12	-	-	-	-	-	-	-	-	M	-	-	-	-	-	-	-
APL13	-	-	-	-	-	-	-	-	-	D	-	-	-	-	-	-
APL14	-	-	-	-	-	-	-	-	-	K	-	-	-	-	-	-
APL15	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-
APL16	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-
APL17	-	-	-	-	-	-	-	-	-	-	V	-	-	-	-	-
APL18	-	-	-	-	-	-	-	-	-	-	M	-	-	-	-	-
APL19	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-
APL20	-	-	-	-	-	-	-	-	-	-	-	V	-	-	-	-
APL21	-	-	-	-	-	-	-	-	-	-	-	M	-	-	-	-

: Negative charged
: Neutral
: Positive charged
: Hydrophobic

Peptides

It was reported that CII 263 (F) and CII 264 (K) were the dominant residues at the binding to DR1 molecule,[6] and therefore, the TCR contact site was considered to be around these residues. The amino acid sequence of APLs was designed so that a single amino acid of the TCR contact site, between CII 261 and CII 267, was changed to an amino acid that had a different charge and was of similar molecular size. Twenty-one APLs were set in this examination, and they were synthesized by Qiagen (Tokyo, Japan) including CII 256–271 wild-type peptide. Purity of each peptide was higher than 90%. The sequences of the peptides are shown in Fig. 1.

Generation of antigen-specific T-cell lines

Peripheral blood mononuclear cells from two RA patients were isolated using Ficoll-Paque (Pharmacia Biotechnology, Piscataway, NJ, USA). 2×10^6 PBMCs were suspended in RPMI-1640 containing 2mM L-glutamine (Gibco BRL, Grand Island, NY, USA), 100IU/ml penicillin/streptomycin (Gibco BRL), and 10% autologous serum, and cultured at 37°C in 5% CO_2 with 20μg/ml of CII 256–271 peptide and interleukin (IL)-2 (30U/ml, Immunace 35; Shionogi, Osaka, Japan). Cells were restimulated with the CII peptide and limiting dilution was carried out at 10, 50, or 100 cells/well in the presence of 2×10^4 feeder cells, which was the autologous B-cell line infected Epstein–Barr virus and irradiated (100Gy), and pulsed with CII peptide. Cells were restimulated at 7-day intervals, and 6 lines (E01–E06) and 10 lines (H01–H10) were established from each patient. These T-cell lines were examined for antigen specificity by proliferative response to several concentration of CII 256–271 peptide. Restriction of the DRB1 molecule was confirmed using anti-DP, DR, and DQ antibody and L-cell transfectant expressing HLA-DR 1.[3] From the results, 1 line (E01) and 2 lines (H01 and H07) were established from each individual as DRB1-restricted T-cell lines.

Measurement of TCR antagonism by APLs

Whether APL could function as an antagonist for TCR was determined as previously reported.[1] Briefly, feeder cells were pulsed with a suboptimal concentration (5μM) of CII 256–271 peptide for 2h at 37°C under 5% CO_2, washed twice, and irradiated. Feeder cells prepulsed with the CII peptide were incubated with each APL (100μM) for 12h, and thereafter, antigen-specific T cells (1×10^5) were added. After incubation for 72h, cell proliferative response was estimated. Antagonistic activity of APL was expressed as percentage of inhibition of the CII 256–271 peptide response. It was judged as positive when the percentage of inhibition was more than 80%.

Evaluation of cell proliferative response

Cell proliferative response was measured using a bromodeoxyuridine enzyme-linked immunosorbent assay (ELISA) system (Cell Proliferation ELISA kit; Roche Diagnostics, Mannheim, Germany).

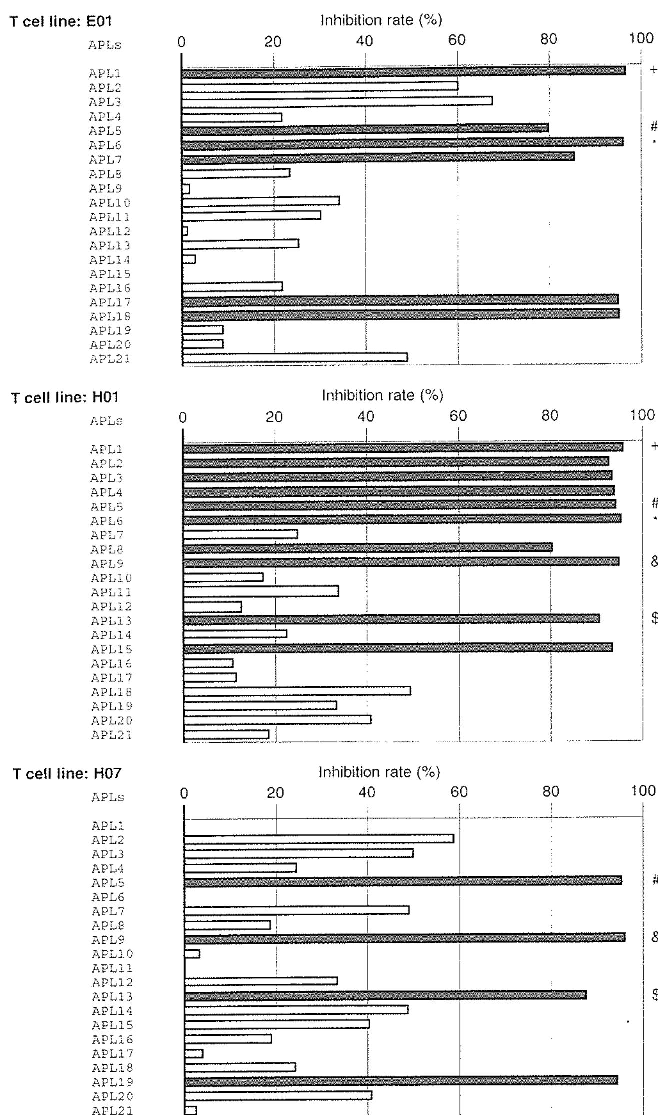

Fig. 2. Antagonistic activity of altered peptide ligands (*APL*) to T-cell lines. Three T-cell lines were established from two rheumatoid arthritis patients with DRB1*0101 and antagonistic activity of each APL was evaluated. The antagonistic activity was expressed as percentage of inhibition of the CII 256–271 peptide response. It was judged as positive when the percentage of inhibition was more than 80%. The *symbols* *, +, &, and $ designate APL inducing antagonistic activity in two T-cell lines; # designates APL inducing antagonistic activity in three T-cell lines

Results and discussion

The results demonstrated that seven APLs suppressed CII-specific T-cell response in E01 T-cell line, 10 APLs in H01, and four APLs in H07. Especially, APL5 (CII 262; G→A) inhibited the CII response in all three T-cell lines. Four other analog peptides, APL1 (CII 261; A→S), APL6 (CII 263; F→D), APL9 (CII263; F→S), and APL13 (CII 265; G→D), decreased T-cell proliferation in two T-cell lines. Although CII 263 (F) was considered an anchor residue, which is a binding site to DR1 molecule, some APLs induced T-cell suppression by antagonism when the residue was substituted. This might imply that there was another anchor residue on the peptide. Actually, CII 260 (I) was predicted as an anchor residue using the systems of prediction of MHC binding peptide (MHC Pred: http://www.jenner.ac.uk/MHCPred, RANKPEP: http://www.mifoundation.org/Tools/rankpep). CII 263 (F) might compose a TCR contact site when the peptide is bound to HLA molecule at CII 260 (I) or CII 264 (K).

To date, there have been some trials to change the T-cell response to CII. Fridkis-Hareli et al. reported that analog peptides based on CII 261–273 could block the binding of CII 261–273 peptide to DR1 molecule and suppress T-cell response to CII 261–273 peptide.[7] Myers et al. showed that CII 256–276 peptide substituted CII 263 (F→N) and CII 266 (E→D) induced lower IFNγ production and higher IL-4 and IL-10 production from splenocytes of DR1 transgenic mice, and collagen-induced arthritis was suppressed using this peptide.[8] On the other hand, we used antagonistic activity to change the T-cell response. This strategy is characterized by controlling the activation of antigen-specific T cells directly. Since RA patients do not necessarily have CII-reactive T cells, the antigen-specific therapy targeted to several autoantigens is expected to be an efficient method to control RA. This examination was probably the first trial using T cells of RA patients.

In conclusion, we observed evidence that at least four APLs of CII (256–271) were able to suppress the CII-specific T-cell proliferation. Further trials using more T-cell lines established from RA patients are required; however, these findings should shed light on a new therapy for RA in the antigen-specific fashion.

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