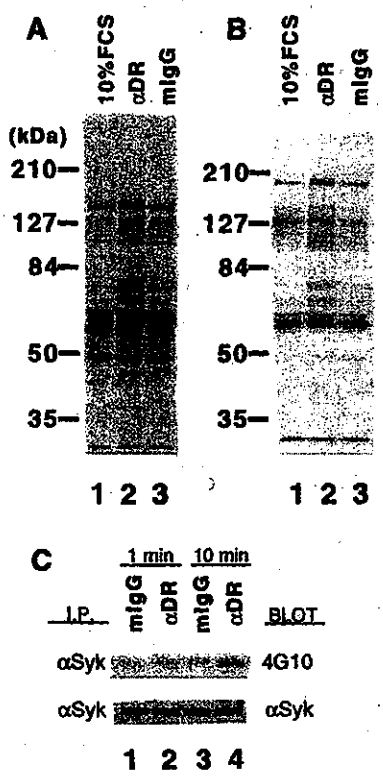


**Figure 7.** Cross-linking DR up-regulates protein-tyrosine phosphorylation of Syk. The peripheral B cells (*A*) and LD2B B cells (*B* and *C*) were incubated with biotinylated anti-DR mAb, L243 or biotinylated mouse IgG followed by avidin for 10 min (*A* and *B*) or for 1 and 10 min (*C*), then cells were lysed. Cell lysates were either immunoprecipitated by anti-Syk Ab (*C*) or directly (*A* and *B*) resolved by 9 % SDS-PAGE, and then transferred to nitrocellulose membranes. Protein-tyrosine phosphorylation was detected by using anti-phosphotyrosine mAb (4G10) and ECL.

130 kDa were reproducibly hyper-phosphorylated. Likewise, cross-linking of DR molecules on LD2B induced tyrosine-phosphorylation of 65 and 70 kDa proteins (Fig. 7B, *lane* 2 vs 1, 3). Furthermore, immunoprecipitation by anti-Syk Ab followed by blotting with anti-phosphotyrosine mAb 4G10, exhibited anti-DR-induced tyrosine phosphorylation of Syk molecules expressed in LD2B cells (Fig. 7C).

To further confirm that Syk is activated directly via HLA-DR, Syk kinase activity was determined by in vitro kinase assay, using Syk molecules immunoprecipitated with anti-Syk Ab, and MBP as a substrate. Because a large number of B cells are required for immunoprecipitation followed by in vitro Syk kinase assay, we used a B lymphoblastoid cell line LD2B homozygous for DRB1*1501, which secretes IgM in the absence of specific stimuli. LD2B was selected among many B cell lines because (*a*) anti-DR-induced phosphorylation pattern of LD2B was similar to that of peripheral B cells, including the phosphorylation of 70-kDa protein (Fig. 7); and (*b*) LD2B B cells expressed IgM heavy chain genes. Enhancement of IgM production from LD2B B cells after cross-linking DR molecules was only marginal, probably because LD2B cells constitutively showed a 50- to 80-fold higher IgM secretion than did peripheral B cells, on a single cell basis (data not shown). As shown in Fig. 8A, however, cross-linking of DR molecules by biotinylated anti-DR mAb plus avidin, induced marked phosphorylation of MBP (*lane* 3), whereas incubation of LD2B with biotinylated mouse IgG + avidin, only marginally induced phosphorylation of MBP (*lane* 2 vs 1). Because MBP is not a substrate specific for Syk kinase, and it might be that MBP was phosphorylated by certain kinases co-precipitating with Syk, we also asked if the effect of Syk on MBP would be inhibited competitively by HS1p388-402 peptide, a substrate specific for Syk. In vitro Syk kinase assay with MBP (2 μg/sample; 0.11 nmol/sample) was done in the presence of either a 250-fold molar excess of the HS1 peptide (27.5 nmol/sample) or an irrelevant peptide carrying two tyrosine residues (EIKYNGEEYLIL; 27.5 nmol/sample). Indeed, MBP phosphorylation was inhibited by the HS1 peptide, but not by the irrelevant peptide (*lanes* 4 and 5).

It is also important to note that Syk molecules are associated with FcγR and are activated by cross-linking of the receptor (24,25). It is therefore conceivable that the increment in Syk kinase activity we observed

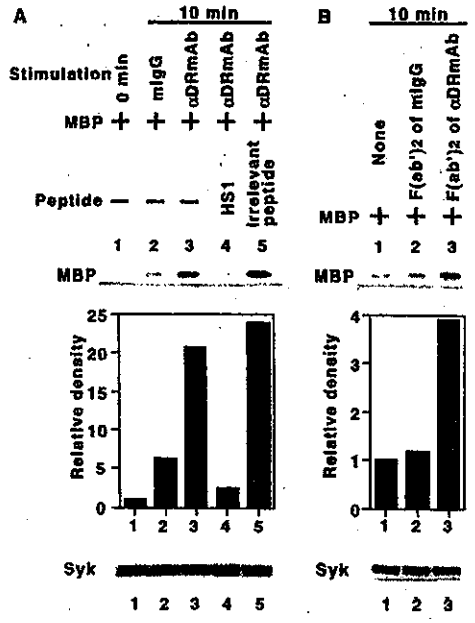


**Figure 8.** Cross-linking DR up-regulates Syk kinase activity. LD2B B cells were incubated with biotinylated anti-DR mAb L243 or biotinylated mouse IgG followed by avidin for 10 min, then cells were lysed. Lysates were immunoprecipitated with anti-Syk Ab. (*A*) An aliquot of immunoprecipitated proteins were immunoblotted with anti-Syk Ab. Residual Syk proteins on agarose beads were used for in vitro immune complex kinase assay, using MBP as a substrate. The HS1 peptide or an irrelevant peptide was added to the assay. (*B*) LD2B B cells were incubated with biotinylated F(ab')2 of anti-DR mAb L243, or biotinylated F(ab')2 of control mouse IgG followed by avidin for 10 min. Cells were lysed, immunoprecipitated, and subjected to in vitro Syk kinase assay. MBP phosphorylation levels were quantified using a bio-imaging analyzer (BAS2000, Fuji Film, Tokyo), and represented by relative values compared with those of 0 min (*A*) or 10 min (*B*) (unstimulated cells).

may be due to cross-reaction of mouse Ig with human FcγR expressed on B cells. To exclude this possibility, we prepared a biotinylated F(ab')2 fragment of anti-DR mAb L243 or that of control mouse Ig. As shown in Fig. 8B, cross-linking of F(ab')2 fragment of anti-DR mAb L243 induced phosphorylation of MBP (*lane* 3), whereas F(ab')2 fragment of mouse Ig induced little phosphorylation of MBP (*lane* 2), compared with a control (avidin only; *lane* 1). This indicates that Syk phosphorylation is induced by cross-linking DR but not FcγR. These differences in phosphorylation patterns were not due to the enhanced recovery of these kinases, as evidenced by the presence of an equal amount of Syk protein molecule in each sample (Fig. 8A and 8B). These data are consistent with results obtained using the Syk inhibitor piceatannol on IgM production, thereby collectively indicating that HLA-DR molecules on B cells not only present antigenic peptides to T cells, but also up-regulate IgM production, in association with Syk activation and without the involvement of Src kinases.

## Kinetics of proliferation induced by cross-linking HLA-DR molecules on CD4+ T cells

We investigated kinetics of T-cell proliferation when HLA-DR molecules on CD4+ T cell were cross-linked by solid-phase mAbs (Fig. 9). Cross-linking by anti-HLA-DR mAb L243 stimulated T cells to proliferate while control Ab did not do so. The proliferative response reached maximum at 24 hr, as did the response induced by anti-CD3 mAb. T cells incubated with biotinylated anti-DR + avidin proliferated as well (data not shown). However, T cells stimulated by biotinylated anti-DR alone did not do so (data not shown). Thus not only binding of anti-DR mAb molecules but also their cross-linking is necessary for T cells to proliferate (25).

## T cell clonal anergy induced by a soluble form of antigenic peptide, anti-CD3 mAb and anti-HLA-DR mAb

SF36.16 cells cultured with the soluble form of antigenic peptide for 7 days resulted in low responsiveness to irradiated PBMC in the presence of the indicated concentration of peptide (Fig. 10A). Thus, when these cells are re-stimulated with 1 nM antigenic peptide and irradiated autologous PBMC, there maybe only marginal proliferation, whereas SF36.16 cells cultured for 7 days with irradiated autologous PBMC prepulsed with 5 μM peptide did exhibit marked proliferative responses when re-stimulated with 1 nM peptide and irradiated

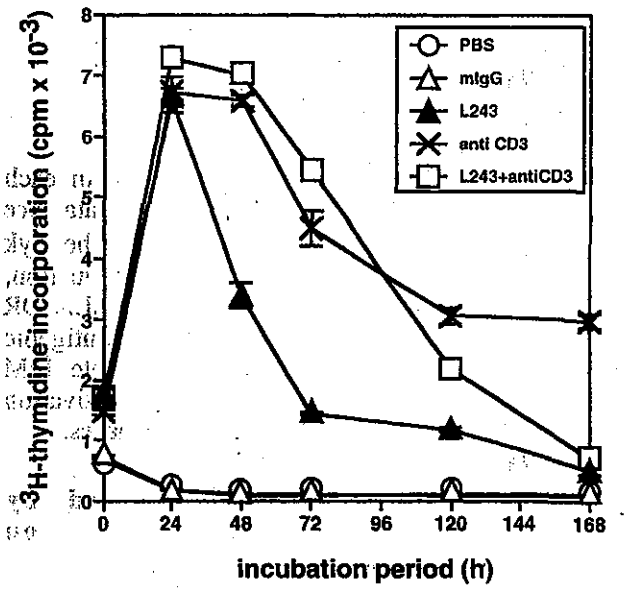


**Figure 9.** Kinetics of proliferation induced by cross-linking HLA-DR molecules on $CD4^+$ T cells. Kinetics of T-cell proliferation was investigated when HLA-DR or CD3 molecules on $CD4^+$ T cell were cross-linked by solid-phase mAbs (open circle, PBS; open triangle, control mIgG; closed triangle, anti-HLA-DR mAb L243; cross, anti-CD3 mAb OKT3; open square, L243 + OKT3). After indicated incubation periods (0-168 hr), T cells were cultured in the presence of 1 μCi/well [$^3$H]thymidine during the final 16-h period, and the incorporated radioactivity was measured by scintillation counting. All data are expressed as the mean value of duplicate determinations ± standard error. The experiment shown is representative of two independent experiments.

autologous PBMC (Fig. 10A).

SF36.16 cells cultured in the presence of solid-phase anti-CD3 mAb OKT3 for 7 days also showed low responsiveness (Fig. 10B). Interestingly, SF36.16 cells cultured in the presence of solid-phase anti-HLA-DR mAb L243 for 7 days also showed a low response (Fig. 10C). Furthermore, when exogenous IL-2 at 200 U/ml was added to re-stimulation the culture, the responsiveness was restored ($p$ = 0.0041), although human recombinant IL-2 only marginally exhibited an enhancing effect in control cultures ($p$ = 0.25; Fig. 10D). All these observations indicate that L243-stimulated T cells were rendered anergic. It is unlikely that solid-phase L243 blocked antigen presentation to T cells in the re-stimulation culture, because levels of responsiveness were the same, between L243-stimulated and control cultures, in the presence of IL-2 ($p$ = 0.11; Fig. 10D).

## Expression of CDK inhibitors $p21^{Cip1}$ and $p27^{Kip1}$ in anergic $CD4^+$ T cells

As shown in Fig. 11, T cells with clonal anergy induced by stimulation with the soluble form of an antigenic peptide, expressed higher level of CDK inhibitor $p27^{Kip1}$ on day7, than did those cultured with irradiated autologous PBMC prepulsed with 5 μM agonistic peptide. Likewise, anergic T cells induced by cross-linking its CD3 or HLA-DR, expressed higher level of CDK inhibitor $p27^{Kip1}$ . In contrast, both expressed the same levels of CDK inhibitor $p21^{Cip1}$ (Fig. 11), $p15^{INK4b}$ and $p16^{INK4a}$ (data not shown). Neither T cells stimulated with control IgG (Fig. 9) nor those stimulated with peptide-unpulsed PBMC showed proliferative responses. Therefore, T cells under such stimuli died on day 7, which did not enable us to test protein expression.

### *Cytokine production from fibroblasts by stimulus via HLA-II molecules*

Since we did not observe proliferative responses of T cells when fibroblasts were used as APC, we then considered the possibility that HLA-DR molecules on fibroblasts may act as receptor molecules (27). The cytokine production from PDL was first evaluated by determining cytokine levels in culture supernatants, when fibroblasts were stimulated via HLA-DR molecules by anti-HLA-DR mAbs. The isotype-matched immunogloblin was used as a control. As shown in Figure 12, stimulated PDL produced larger amounts of IL-8, IL-6, MCP-1 and RANTES compared with control cells.

We then examined whether cytokine production from fibroblasts actually occurred when HLA-DR molecules on fibroblasts are ligated by their natural ligands. To address this issue, peptide pulsed fibroblasts were co-cultured with emetine-treated T cells. Since

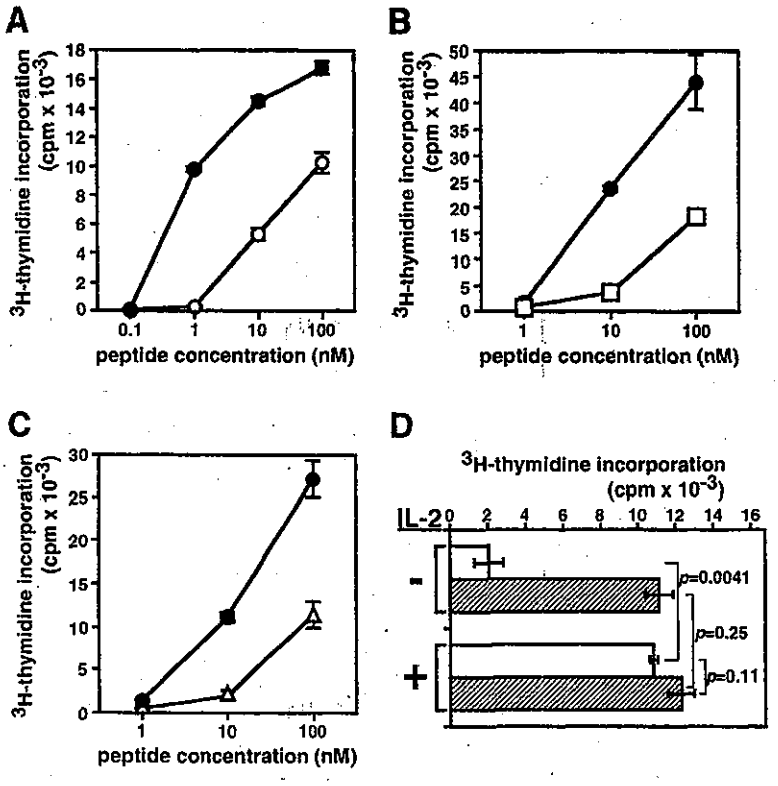


**Figure 10.** T cell clonal anergy induced by a soluble form of the antigenic peptide (A), anti-CD3 mAb (B) and anti-HLA-DR mAb (C, D). SF36.16 T cells were primarily stimulated with irradiated autologous PBMC prepulsed with the wild-type peptide (5 μM BCGap84-100 for 5 h at 37 °C; closed circle or hatched bar), soluble wild-type peptide (1 μM BCGap84-100; open circle), plastic immobilized anti-CD3 (OKT3; open square) or plastic immobilized anti-DR (L243; open triangle or open bar). Seven days after initiation of culture, T cells in these cultures were re-stimulated (secondary stimulation) with various concentrations of wild-type BCGap84-100 plus irradiated PBMC (A, B, C), or with 10 nM wild-type BCGap84-100 plus irradiated PBMC, in the presence or absence of 200 U/ml of exogeneous human recombinant IL-2 (D). The T cells were cultured on a 96-well plate for 72 h, and subjected to [$^3$H]thymidine incorporation assay. All data are expressed as the mean value of duplicate determinations ± standard error. Data are representative of two independent experiments. (D) Proliferative response of T cells to exogeneous IL-2. Primary stimulation, secondary stimulation and [$^3$H]thymidine incorporation assay were done as described above. Results are expressed as the means ± SD of duplicate measurement. Statistical significance was analyzed using Student's *t* test.

emetine is a *de novo* protein synthesis inhibitor, we first treated T cells with emetine to prevent T cells from up-regulating cytokines or cell-surface molecules. As shown in Figure 13, IL-8, IL-6, MCP-1 and RANTES were indeed produced from peptide-pulsed PDL following 16 hr culture ($1x10^4$ cells/well) with emetine-treated T cells, whereas no cytokines were produced when the cells were merely pulsed with peptide. Peptide-pulsed and IFN-γ treated PDL produced larger amounts of cytokines including IL-8, IL-6, MCP-1 and RANTES

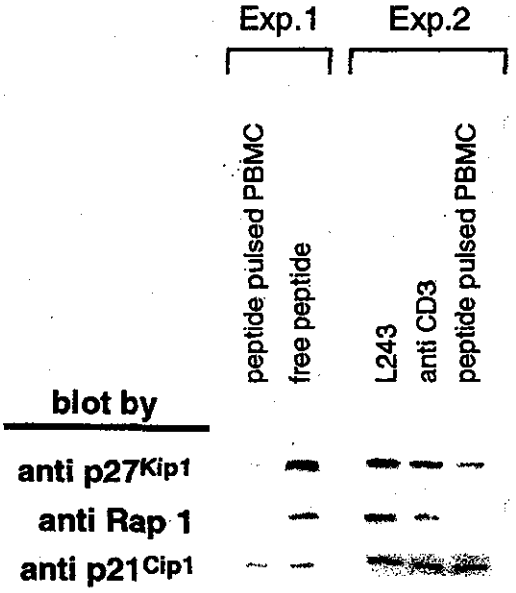


**Figure 11.** Expression of CDK inhibitors. T cell clone SF36. 16 were stimulated using a soluble form of antigenic peptide (1μM BCGap84-100), immobilized anti-CD3 (OKT3), immobilized anti-DR (L243) or irradiated autologous PBMC prepulsed with the wild-type peptide (5 μM BCGap84-100 for 5 h at 37 °C) at 37 °C. After 7 days of incubation, the T cells were lysed. Cell lysates were directly resolved on 13.5 % SDS-PAGE, transferred to nitrocellulose membranes then blotted using Abs to CDK inhibitor $p27^{Kip1}$ and CDK inhibitor $p21^{Cip1}$.

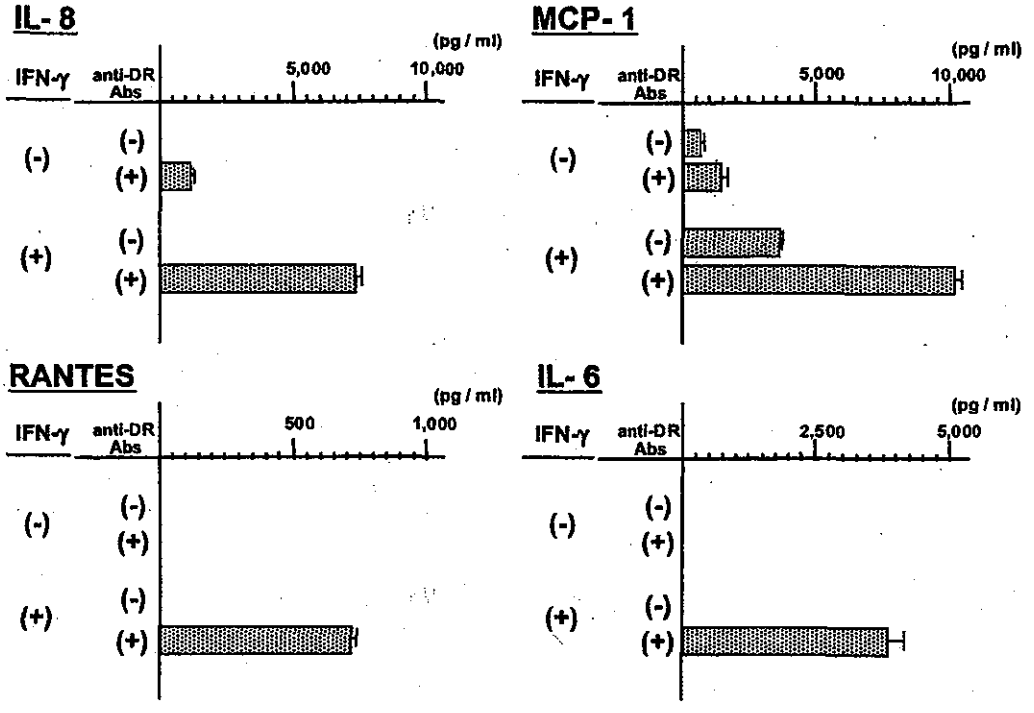


**Figure 12.** Cytokine productivity of PDL by the stimulus with anti-HLA-DR (L243) mAb. IFN-γ-treated or –untreated PDL ($1x10^4$ cells/well) were cultured with or without L243 in the presence of goat anti-mouse IgG2a Ab. The supernatants were collected following 16 hr culture of the cells, and concentrations of IL-1β, IL-6, IL-8, MCP-1 and RANTES were determined using ELISA kits as in "Materials and Methods".

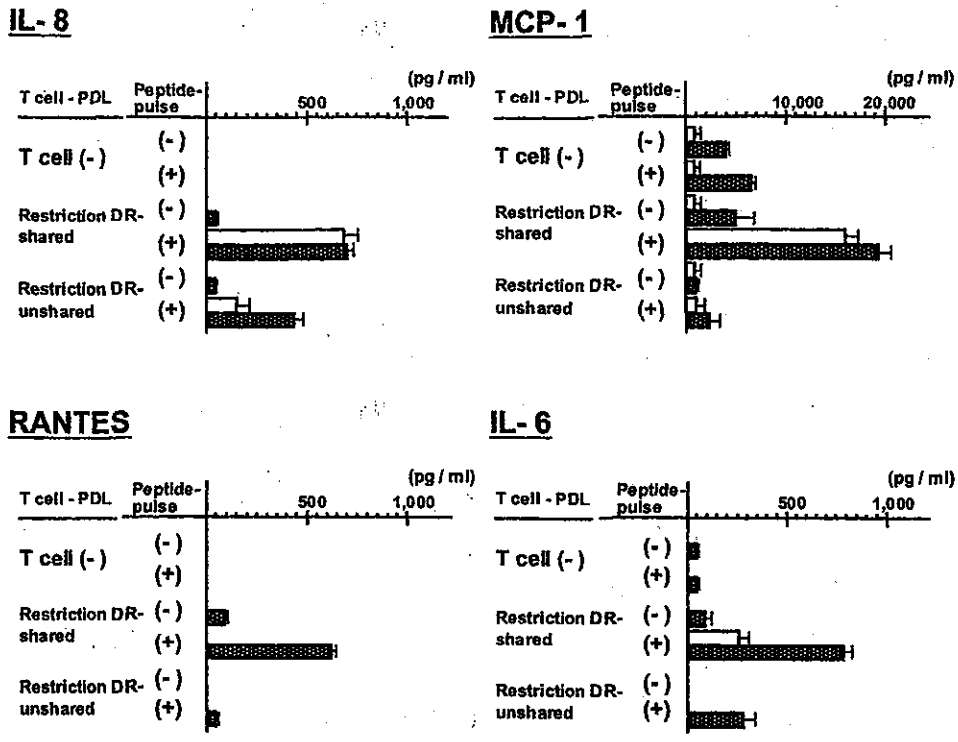


**Figure 13.** Cytokine productivity of PDL by stimulation via HLA-II molecules when PDL were co-cultured with emetine-treated T cells. PDL ($1x10^4$ cells/well) was pulsed with or without antigenic peptide in the serum free medium for 24 hr following 72 hr culture with or without IFN-γ before the cells were cultured with emetine-treated T cells. The supernatants were collected following 16 hr culture of the cells, and cytokine concentrations were determined. Shaded and open bars indicate the results of IFN-γ-treated and –untreated PDL, respectively.

compared with non-treated and non-pulsed PDL. Moreover, PDL expressing HLA-DRB1*1501 molecules produced the large amounts of IL-8, IL-6, MCP-1 and RANTES when emetine-treated T cells (restricted by DRB1*1501) was co-cultured with PDL. MCP-1 and RANTES were produced only when peptide-pulsed PDL was cultured with restriction DR-shared T cells, whereas IL-6 and IL-8 were produced even when restriction DR-unshared T cells were co-cultured. Although RANTES and IL-6 productivity was relatively higher in IFN-γ treated, peptide-pulsed PDL cultured with DR-restricted T cells, no significant difference was found in IL-8 and MCP-1 productivity between them.

## DISCUSSION

The observation that IFN-γ / IL-4 produced by T cells are associated with HLA restriction molecules even in freshly isolated short-term T-cell clonal responses to crude protein antigens or randomized peptide antigens, is evidence that the phenomenon is not limited to three T-cell clones used in this study. Thus, although not being complete as shown in Fig. 3, HLA class II subregions may determine T cell differentiation patterns or IFN-γ / IL-4, probably through monocyte responses. However, one might speculate that DR-peptide complex deliver strongest avidity between TCR, leading to Th1-prone responses (28,29). However, absence of DR-restricted T cell clones

with low IFN-γ / IL-4 rules out this possibility, because low-affinity DR-binding peptides should exist, which should activate Th2-prone responses, if the phenomenon is attributed to avidity alone. Indeed, it is likely that such a phenomenon is attributed to high IL-12 production through DR signaling (Table 1).

PKC and Syk are associated (30) and involved in signaling through MHC molecules. We observed a slight enhancement of monokine production by PKC inhibitor GF109203X and Syk inhibitor piceatannol. The precise mechanisms are yet to be determined, but one might speculate that they negatively regulate monokine production induced by ligating class II HLA.

Because transmembrane and intracellular domains are markedly different among α and β chains of HLA-DR, -DQ and -DP, and MHC molecules have no immunoreceptor tyrosine-based activation motif, it is reasonable to speculate that HLA-DR, -DQ or -DP molecules are associated with distinct signal transduction molecules. Differential endosomal trafficking / recycling (31), differential signaling in monocyte subsets (32), including contaminated dendritic cells, and even differential localization in membrane microdomains would also need to be considered. Study is currently underway, to address these questions, using various monocytic cell lines and mass mapping techniques.

IgM contributes to early defense against microbial infections (33). When B cells are exposed to non-self antigens, such as those of microbial origin, B cells bearing surface IgM specific for the antigen are capable of concentrating the antigen and present it effectively to T cells. We found that cross-linking DR molecules up-regulates not only secretory-type but also membrane-type μ chains, which may indicate that cross-linking DR molecules leads to more effective antigen presentation. It is also important to note that CD40-generated signals arrest B-cell terminal differentiation to produce Igs (34). Although DR-mediated signals appear to up-regulate IgM production in the absence of CD40-CD154 interaction, further investigation is needed to determine whether or not the generation of signals via CD40 under physiological T-B interactions interferes with IgM production induced by DR-mediated signals. In this study, ligation of DR molecules not only with specific Abs (either solid-phase Abs or soluble Abs), but also with HLA-peptide-TCR interaction, induced IgM production, suggesting that signals via DR alone are capable of inducing up-regulation of IgM, which may also occur in physiological T-B interactions. In this relation, DR-mismatched transplantation should be one of rare cases, in which massive T-B interaction via DR occurs *in vivo*. Indeed, when DNA typing of HLA-DR was unavailable, graft-vs-host disease was frequent, and such patients reportedly had deposition of IgM at the dermo-epidermal junction. (35).

BCR-Ag-complex is internalized to supply T-cell epitopes, and subsequent DR-peptide-TCR interaction results in class switching, which eventually leads to decreased IgM production (36). Indeed, our experimental system did not allow BCR to interact with protein antigens, and T cells were treated with emetine (thereby bearing no class switch pressure). Such a system might have up-regulated IgM to be readily detected. However, because the disappearance of surface IgM at antigen presentation (before class switching) is incomplete, one might speculate that signaling through DR supplies new IgM molecules, for a short and critical period of time for T-B interaction before class switching is initiated. Other factors should also be considered, because even with thymus-independent antigens, IgM production from B cells can be induced (37).

In case of the anergy induced by soluble antigenic peptide, signals should be transmitted to T cells not only via TCR but also via class II HLA because T cells express both TCR and HLA-DR. Although anergy induced by the lack of co-stimulation was rescued by signaling via CD28, anergy induced by soluble antigenic peptide was not rescued (38), which is also the case in our present study (data not shown). It is therefore likely that molecular mechanisms differ between anergy induced by the lack of co-stimulation and that induced by anti-DR, even though the behavior of CDK inhibitors are apparently the same.

Fibroblasts are known to participate in the immune system because of their expression of several immunoregulatory molecules on their cell surfaces, as well as many cytokine species. It was reported that fibroblasts produce IL-8 by the stimulus with several inflammatory cytokines (IL-1β and TNF-α; 39,40) and LPS (41,42). A previous study demonstrated that the engagement of MHC class II molecules by the superantigen including SEA and *Mycoplasma arthritidis*-derived superantigen induced the RANTES, MCP-1 and IL-8 mRNA expression in synovial fibroblasts (43). Herein, we demonstrated that PDL produced large amounts of chemokines when cultured with emetine-treated T cells, under restriction DR-shared conditions. Thus, signaling via HLA-II molecules into fibroblasts is not only induced by the engagement of MHC class II molecules with superantigens, but also by making the DR-peptide-TCR complex.

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# Monocytes of distinct clinical types of leprosy are differentially activated by cross-linking class II HLA molecules to secrete IL-12

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Ohyama H, Kato K, Takeuchi K, Soga Y, Uemura Y, Nishimura F, Matsushita S. Monocytes of distinct clinical types of leprosy are differentially activated by cross-linking class II HLA molecules to secrete IL-12. APMIS 2004;112:271–4.

Leprosy is characterized by a wide spectrum of clinical features depending on the individual differences in Th1-type immunity. The objective of this study was to evaluate whether monocyte activation by stimulus via class II HLA molecules would be correlated with the differences in cellular immune responses among diverse clinical forms of leprosy. IL-1β and IL-12 productivity in monocyte preparations obtained from PBMCs was estimated in patients with lepromatous- and tuberculoid-type leprosy. We found that monocytes from lepromatous patients produced significantly higher (about 4-fold higher) amounts of IL-12 as compared to in patients with tuberculoid type of leprosy when class II HLA molecules were cross-linked with anti-HLA class II antibodies, whereas almost equal amounts of IL-1β were produced from each monocyte preparation by stimulus via class II HLA molecules regardless of the clinical form of leprosy. These results suggest that monocyte activation differs between lepromatous and tuberculoid patients in terms of IL-12 secretion, which might be related to individual differences in the cellular immune responses according to the clinical type of leprosy.

Key words: Class II HLA; IL-12; leprosy patients; cellular immune response.

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Leprosy, a chronic disease caused by infection with *Mycobacterium leprae*, shows a wide spectrum of clinical features (1). Tuberculoid type of leprosy is at one end and lepromatous leprosy at the other end of the spectrum. Tuberculoid patients show a high level of cell-mediated immunity (CMI) responses against *M. leprae*, which results in resistance to infection, whereas lepromatous patients show poor CMI responses against the pathogen and progressive form of the disease. This clinical spectrum of leprosy is explained by the differences in responses to *M. leprae* among individuals. The differences of T-cell subsets accumulating in leprosy lesions may account for the diversity of protective patterns to *M. leprae*. In the case of *M. leprae* infection, the clinical type of the disease depends on individual differences in Th1-type immunity. One of the most important cytokines produced by Th1, IFN-γ, promotes activation of macrophages, thus leading the host immunity toward cellular immunity to these bacteria. IL-12, which is produced by antigen-presenting cells (APCs), is

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well-known as a powerful inducer of IFN-γ production from Th1 cells. In leprosy patients, IL-12 productivity is likely to influence the cellular immune responsiveness in patients with lepromatous and tuberculoid type of the disease (2, 3). Toll-like receptor 2 (TLR2) is thought to be a key molecule in inducing the IL-12 production from monocytes in the response against mycobacterial pathogens (4), and the polymorphism of the TLR2 gene is likely to affect the low productivities of IL-12 in the lepromatous type of leprosy patients (5).

Meanwhile, recent studies have suggested that class II HLA (HLA-II) molecules not only act as antigen-presenting molecules, but also as receptor molecules to transduce signals into APCs, resulting in the production of several cytokines, including IL-1β and IL-12 (6). From this point of view, it is likely that the difference in IL-12 production induced by the stimulus via HLA-II molecules will also be a possible factor implicated in determination of the clinical type of leprosy.

In this study, we measured IL-12 production from APCs by stimulus via HLA-II molecules in humans with leprosy, in order to find the differences in CMI activities in vitro between the patients with lepromatous and tuberculoid types of leprosy.

## MATERIALS AND METHODS

*Study population*

Ten leprosy patients, including 5 LL patients and 5 TT patients, and 7 healthy individuals were enrolled in this study. Patients were clinically diagnosed as having each type of leprosy based on the description of Ridley & Jopling (1) and with reference to their results in the Mitsuda test and their sequelae.

*Cell preparation*

Mononuclear cells were prepared by the Ficoll-paque (Pharmacia Biotech, Uppsala, Sweden) gradient solution method. Peripheral blood mononuclear cells (PBMC) were suspended in RPMI 1640 medium supplemented with 10% heat-inactivated pooled human sera (HS) and placed on HS-pretreated plastic dishes (Falcon, Becton Dickinson, Lincoln Park, NJ). These dishes were incubated for 2 h at 37°C. After the dish-nonadherent cells were harvested and extensive washes, the adherent cells were removed by washing with ice-cold PBS containing 0.04% EDTA and by scraping them with a rubber policeman. This fraction was served as a monocyte fraction. More than 90% of the adherent cell fraction was CD14-positive.

*Inducing the monokine production from monocytes*

Mouse anti-HLA-DR (L243: Leinco Technologies Inc., Ballwin, MO), anti-DQ (1a3: Leinco Technologies Inc.) monoclonal antibodies (mAbs) and isotype-matched control antibodies (mouse IgG2a) were coated onto 96-well flat-bottomed culture plates for a day at 10 μg/ml PBS. After extensive washing of the plates with PBS, monocytes were added at $2\times10^4$ cells/well, and incubated for 16 h at 37 °C in a $CO_2$ incubator (7).

*Quantitation of cytokines*

Culture supernatants of monocytes stimulated via HLA-II molecules were collected. The hIL-1β and hIL-12 (p40 & p70) ELISA kits (Endogen Inc. Woburn, MA) were used for the quantitation of cytokines in the supernatants. The detection limit of both these cytokines is 5 pg/ml.

## RESULTS AND DISCUSSION

We first estimated IL-1β and IL-12 productivity in monocyte preparations obtained from PBMCs of lepromatous, tuberculoid, and healthy Japanese subjects. Monocytes from lepromatous patients produced significantly higher amounts of IL-12 as compared with those from patients with tuberculoid type leprosy (Mann-Whitney *U* test; $p<0.05$), whereas almost equal amounts of IL-1β were produced from each monocyte preparation by stimulus via HLA-II molecules regardless of the clinical form of leprosy. In particular, monocyte preparations from three tuberculoid patients produced extremely low amounts of IL-12. A small amount of IL-β and IL-12 was detected when monocytes were cultured in a dish coated with isotype-matched antibodies as control. There were no differences between DR-induced and DQ-induced cytokine productivity from monocytes.

In this regard, the present findings are not in agreement with those of some previous studies showing that IL-12 mRNA is more highly expressed in tuberculoid lesions as compared to lepromatous lesions (2, 3). According to the results of these previous studies, tuberculoid patients are supposed to allow *M. leprae* infection although they have the ability to produce a suf-

TABLE 1. *IL-1β and IL-12 production from monocytes by signaling via HLA-II molecules*

| Clinical type | IL-1β (pg/ml) | IL-12 (pg/ml) | IFN-γ/IL-12 |
|---|---|---|---|
| L-lep | 322±107 | 402±299 † | 2.93±2.89 ‡ |
| T-lep | 264±138 | 110±119 † | 28.03±8.71 ‡ |
| HC | 418±340 | 304±225 | N.D |

The amounts of cytokines are given as the mean value of duplicate cultures with L243, after subtraction of the mean value obtained from cultures with control antibodies in each subject. L-lep, T-lep and HC represent patients with lepromatous type of leprosy, tuberculoid type of leprosy, and healthy controls, respectively.
†; Mann-Whitney *U* test, $p<0.05$.
‡; Mann-Whitney *U* test, $p<0.01$.

ficient volume of IL-12 to induce CMI responses. Moreover, *M. leprae* has the longest doubling time of all known bacteria and has massive gene decay, including the genes coding virulence determinants (8). From this viewpoint, we believe that tuberculoid patients are also susceptible to *M. leprae.* Thus, we speculate that the low productivities of IL-12 by stimulus via HLA-II molecules in tuberculoid patients might have an influence on the host defense function in early stage *M. leprae* infection.

We previously reported the lymphokine productivity of T-cell lines established from PBMCs in response to Major Membrane Protein II (MMPII), one of the outer membrane protein species derived from *M. leprae* (9). In such studies, however, it was impossible to classify leprosy patients based on IFN-γ productivity in T-cell lines. We thus considered that the balance between IL-12 from monocytes produced by stimulus via HLA class II molecules, and IFN-γ from Th cells might explain individual differences in CMI activities of clinical types of leprosy. The IFN-γ/IL-12 ratio in each subject was calculated and revealed that the ratio of lepromatous patients was significantly lower than that of tuberculoid patients ($p<0.01$). These findings indicate that insufficient IFN-γ production was induced by IL-12 in lepromatous patients, and this might reflect the abnormality of cellular immune responses observed in the lepromatous type of leprosy.

We speculate that this phenomenon might reflect the abnormality in cellular immune responses seen in the lepromatous type of leprosy. In this study, however, we did not evaluate the cytokine productivity from monocytes using antigen-specific stimuli. Further studies are needed to clarify whether the difference of IFN-γ/IL-12 ratio between lepromatous and tuberculoid patients depends upon the antigen-specific immune responses.

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JOURNAL OF MEDICAL PRIMATOLOGY

# Detection of CD3ε polymorphism in cynomolgus monkeys by a method based on RFLP

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Abstract: We previously reported that peripheral lymphocytes from about 12% of cynomolgus monkeys lacked reactivity with anti-rhesus monkey CD3 monoclonal antibody (FN18). The nucleotide sequence analysis of the genes encoding CD3 component proteins revealed that a single amino acid substitutions found in the CD3ε chain determined the phenotype. In this study, we attempted to develop a method based on the restriction fragment length polymorphism (RFLP) and apply it for determination of the genotypes of individual monkeys. Comparison of the phenotype determined by fluorescence-activated cell sorter analysis with the genotype determined by RFLP analysis revealed that the FN18 -positive trait was dominant over the FN18-negative trait. It was also revealed that allele frequency was significantly different among macaques depending on the geographical region where their ancestors were derived from.

Key words: allele – CD3 – cynomolgus – FN18 – polymorphism

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## Introduction

Cynomolgus monkeys (*Macaca fascicularis*) are important experimental animals for biomedical research and understanding immunobiology of these animals is essential for interpretation of experimental data. The FN18 monoclonal antibody (mAb), which was raised against CD3 molecules of rhesus monkey (*Macaca mulatta*), is also able to be used for identification of T cells of cynomolgus monkey; however, it was shown that T cells from some cynomolgus monkeys and rhesus monkeys did not react with FN18 mAb [1–3, 7, 8].

The nucleotide sequence analysis of cDNAs coding for CD3 components showed that CD3ε chain from FN18 non-reactive cynomolgus monkeys had two common amino acid substitutions at positions 67 and 72 [8]. We have further shown that the amino acid at position 67 played a key role in determining the FN18 responsiveness by *in vitro* experiments using several mutated CD3ε genes [7].

In this study, we attempted to establish a method for genotyping individual monkeys based on the polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) method and used the method to determine the allele frequencies among cynomolgus monkeys derived from different geographical regions.

## Materials and Methods

### Animals

All the cynomolgus monkeys studied here were raised and reared in the Tsukuba Primate Center for Medical Science, NIID. Both genders were involved and the ages of the monkeys were between 2 and 16 years. This study was conducted in accordance with the Guide for Animal Experiments Performed at the National Institute of Infectious Disease.

### RFLP analysis

PolyA mRNA extracted from peripheral blood mononuclear cells (PBMCs) of FN18-reactive

cynomolgus monkeys using the Quickprep micro mRNA purification kit (Amersham, Uppsala, Sweden) was converted into cDNA using high fidelity RNA PCR kit (Takara, Shiga, Japan). Genomic DNA was extracted form PBMCs using the GeneTLE solution (Takara). PCR of CD3ε chain was performed in 20 μl reaction mixture containing ε-sense primer (5′-CTC CAT CTC TGG AAC CAC AGT A-3′) and ε-anti-sense primer (5′-CAG GTA GAG ATG ATG GCT CG-3′), 0.6–0.8 μg of genomic DNA or cDNA and 0.6 U of *ExTaq* polymerase (Takara). The size of PCR products was expected to be 207 bp. The reaction mixtures were heated at 95°C for 5 minutes, and then 40 cycles of amplification consisting of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 30 s was carried out followed by an additional extension at 72°C for 7 minutes. About 0.3–0.5 μg of amplified DNA were digested with 2.5 U of *Mbo*II at 37°C for 2 hour followed by agarose gel electrophoresis using 4% gel. As similar results were obtained using both cDNA and genomic DNA as templates for PCR, most part of this study was conducted using the genomic DNA as template.

### Fluorescence-activated cell sorter (FACS) analysis

The PBMCs were isolated from fresh blood by standard Ficoll-Hypaque gradient centrifugation method. PBMCs were washed with phosphate-buffered saline (PBS) and resuspended in RPMI 1640 containing 100 U/ml, 0.1 mg/ml streptomycin, and 10% foetal calf serum at a concentration of $10^6$ cells/ml. PBMCs ($10^5$) were incubated at 4°C for 1 hour with FN18 mAb (Biosource, Camlio, CA, USA), and washed twice with PBS containing 1% bovine serum albumin. After fixation with 1% paraformaldehyde at 4°C for 30 minutes, FACS analysis (FACS Caliber; Becton Dickinson, Cockeysville, MD, USA) was performed.

## Results

### Differentiation of genotype by the PCR–RFLP

As the nucleotide at position 200 of the FN18+ and FN18– cDNA clone was A and G, respectively, and the flanking sequence, GAAGA, gave rise to a recognition site by restriction enzyme *Mbo*II (Fig. 1), it was likely that the nucleotide difference could readily be differentiated by *Mbo*II digestion. As expected, the PCR amplicons derived from cloned FN18+ DNA was digested by *Mbo*II yielding two smaller bands, but that from FN18– clone was resistant to the digestion with *Mbo*II (Fig. 2A). We therefore attempted to apply this technique to determine the genotype of individual monkeys. There should be three distinct electrophoreic patterns of *Mbo*II-digested fragments. The PCR amplicons from homozygotes bearing A/A at position 200 should be resistant to *Mbo*II digestion, whereas those bearing G/G must be cut into two fragments. The PCR fragments from heterozygotes, however, would give rise to three fragments of 207, 113 and 94 bp, if properly digested. As incomplete digestion may be misleading, the PCR amplicons were subjected to complete digestion. In order to accomplish complete digestion, we used sufficient amount (2.5 U) of the enzyme to digest 0.3–0.5 μg of a short DNA fragment containing only one cutting site. Furthermore, the PCR amplicons from presumed heterozygotes were subjected to the nucleotide sequencing to assure that appearance of three fragments were due indeed to the heterozygocity. The results showed that both A and G were present at position 200 (data not shown), indicating that the presence of a 207-bp fragment after *Mbo*II digestion was because of the absence of *Mbo*II recognition site but not of incomplete digestion. These results indicate that RFLP analysis with *Mbo*II could be used as a powerful tool to determine the genotype of macaques.

### Inheritance of the polymorphism

To analyse how this polymorphism is inherited, a family consisted of three parents (one sire and two dams) and four offspring were selected and subjected to the PCR–RFLP analysis. They were bred and raised in the Tsukuba Primate Center for Medical Science, NIID. By FACS analysis it was shown that the sire (3028) was FN18 negative while the two dams (3032 and 1159) were positive

```
                                  67          72
          61  H N G K N K E D S G D R L F L P E F S E  80
FN18 +/+ 181  CACAATGGTA AAAACAAAGA AGATTCTGGG GATCGGCTGT TTCTGCCGGA ATTTTCAGAA 240
FN18 -/- 181  ---------- ---------G ---------- ----AA---- ---------- ---------- 240
          61  - - - - - - G - - - - Q - - - - - - - - -  80
```

*Fig. 1.* Nucleotide sequence of CD3ε. The nucleotide and the deduced amino acid sequences around polymorphic region were shown. Dots indicated identical nucleotide or amino acids. Recognition and cleavage site of *Mbo*II are indicated by underline and arrowhead, respectively.

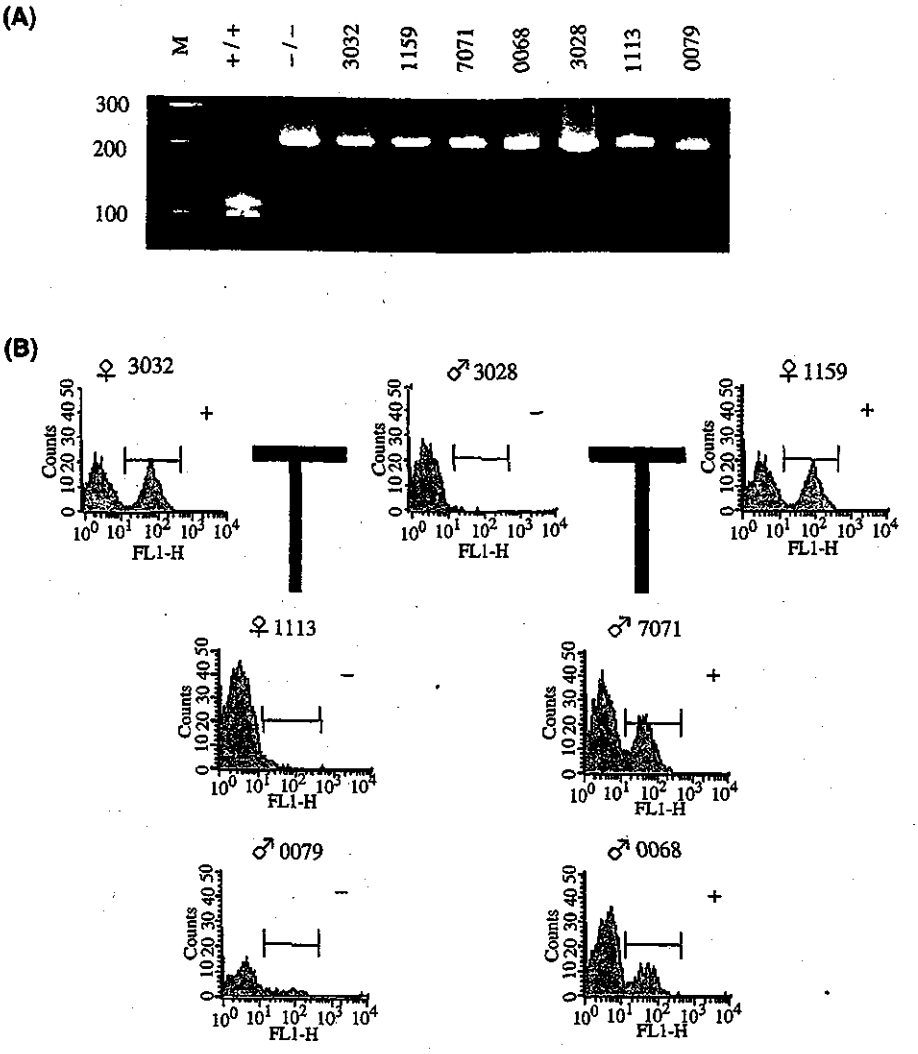


*Fig. 2.* The family pedigree demonstrating the inheritance of CD3ε genotypes and phenotypes. (A). The PCR products (207 bp) amplified from the genomic DNA of PBMCs were digested with *Mbo*II. The PCR products from cloned FN18 +/+ or −/− were also included as a positive or negative control. (B) The phenotypes regarding the reactivity with FN18 mAb were determined by FACS analysis. 1-H (*x*-axis): the fluorescence intensity of FN18 mAb.

(Fig. 2B). Similarly two offspring (1113 and 0079) were negative whereas the others (7071 and 0068) were positive. As the FACS profile of 0079 was rather ambiguous, we stained PBMC of this monkey with an mAb directed to monomorphic epitope of CD3ε (SP34). It was shown that SP34 positive cells did not react with FN18 confirming that 0079 was FN18 negative. The PCR–RFLP analysis using cDNA as templates showed that three monkeys (3028, 1113 and 0079) were homozygous (−/−) while the other monkeys (3032, 1159, 7071 and 0068) were heterozygous (+/−) (Fig. 2A). This finding indicates that these genes were co-dominantly expressed on RNA level. As there is no antibody available that would react with the protein expressed from the FN18 −/− genotype, the FN18-positive phenotype appeared to be inherited according to the Mendelian rules, and dominant over FN18.

### Allele frequency of monkeys from different geographical areas

We then applied the RFLP technique to determine the allele frequency of this particular single nucleotide polymorphism among cynomolgus monkeys whose ancestors were introduced from three different countries – Malaysia, Indonesia and Philippines. As shown in Table 1, the frequency of three genotypes, FN18 +/+, FN18 +/− and FN18 −/− in total, was 0.576, 0.339 and 0.085, respectively. It was noted, however, that the frequency of FN18 −/− was significantly higher (0.208) in the monkeys derived from Philippines

Table 1. The frequency of CD3ε genotype in cynomolgus monkeys

| | Country | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Malaysia | | Indonesia | | Philippine | | Total | |
| Genotype | Frequency | n | Frequency | n | Frequency | n | Frequency | n |
| FN18 +/+ | 0.808 | 38 | 0.675 | 52 | 0.226 | 12 | 0.576 | 102 |
| FN18 +/− | 0.149 | 7 | 0.299 | 23 | 0.566 | 30 | 0.339 | 60 |
| FN18 −/− | 0.043 | 2 | 0.026 | 2 | 0.208 | 11 | 0.085 | 15 |
| Total | 1.000 | 47 | 1.000 | 77 | 1.000 | 53 | 1.000 | 177 |

than in those from Malaysia (0.043) and Indonesia (0.026).

## Discussion

In this study, we established a simple method for the detection of CD3 polymorphism, and applied the method to analyse the mode of inheritance of the CD3ε polymorphism. We also determined allele frequency among monkeys originated from different countries. We found that the frequency of FN18 −/− genotype was higher in the Philippine population. This might be caused by bottleneck effect as mating was carried out among monkeys of the same origin. Another possibility was that FN18-negative gene had diffused widely into Philippine population as FN18-negative phenotype might be advantageous in reproduction or adaptation, in particular environment of Philippines.

There are several reports suggesting that the polymorphism found in human CD3 might be related to type I diabetes [4–6], but controversial results are also reported. It would be interesting to see whether there are any differences in biological properties between macaques of Philippine and other places. It also seems important to look at whether there are linkages between this polymorphism and certain diseases in cynomolgus monkeys.

In conclusion, we established a simple method to identify the polymorphism of CD3ε by genotyping using RFLP. The RFLP analysis of a large number of monkeys demonstrated that the frequency of the genotype of the CD3ε differed among cynomolgus monkeys of different origin of country.

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ORIGINAL PAPER

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# Detection of 14 alleles derived from the MHC class I *A* locus in cynomolgus monkeys

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**Abstract** A basic understanding of the major histocompatibility complex (MHC) class I, which, together with T-cell receptors, is a key player in antigen recognition by cytotoxic T lymphocytes, is necessary to study the cellular immune response to intracellular pathogens. The MHC has hardly been reported in cynomolgus monkeys (*Macaca facicularis*), although cynomolgus monkeys have been frequently used as the surrogate animal model. We attempted to determine the nucleotide sequences of the MHC class I *A* locus of cynomolgus monkeys (*Mafa-A*) and eventually 34 independent sequences of *Mafa-A* were obtained from 29 cynomolgus monkeys. These 34 sequences were classified into 14 *Mafa-A* alleles according to the results of phylogenetic analyses using the neighbor-joining method. One to three *Mafa-A* alleles were obtained from a single animal. We also tried to establish a multiplex PCR-SSP method for convenient typing of *Mafa-A* alleles. cDNA from a family of cynomolgus monkeys, which is composed of four sirs and four dams, were examined by multiplex PCR-SSP. The result of multiplex PCR-SSP showed that an individual cynomolgus monkey had two or three *Mafa-A* alleles, suggesting that the *A* locus of cynomolgus monkeys might be duplicated.

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**Keywords** Cynomolgus · Major histocompatibility complex · *Macaca facicularis* · Allele · PCR-SSP

## Introduction

The major histocompatibility complex (MHC) class I consists of heavy chain, $\beta_2$-microglobulin ($\beta_2$m), and antigen peptide (Hennecke et al. 2001). Human cells are known to express three highly polymorphic MHC heavy chains (*HLA-A*, *-B*, and *-C*) and three conserved MHC heavy chains (*HLA-E*, *-F*, and *-G*). *HLA-A*, *-B*, and *-C* present antigen peptides to cytotoxic T lymphocytes (CTL) and the CTL are then activated (Flynn et al. 1992; Hou et al. 1992; York and Rock 1996). These classical molecules, especially *HLA-C*, also provide both stimulatory and inhibitory signals to natural killer (NK) cells through killer cell immunoglobulin-like receptors (KIR) (Valiante et al. 1997).

The gene encoding the class I heavy chain is composed of eight exons. Exon 1 encodes the signal peptide, exons 2–4 specify the extracellular domains $\alpha_1$–$\alpha_3$, exon 5 codes for the transmembrane domain, and exons 6–8 code for the cytoplasmic domain. The $\alpha_1$ and $\alpha_2$ domains are the most polymorphic, while the $\alpha_3$ domain contributes to the $\beta_2$m association (Hebert et al. 2001) and interaction with the CD8 molecule (Salter et al. 1990).

The rhesus MHC has been extensively studied among non-human primates because rhesus monkeys are most frequently used as the surrogate animal model (Allen et al. 2001; Horton et al. 2001; Mothe et al. 2002) for HIV infection in human. Rhesus MHC (*Mamu*) class I *A* (Boyson et al. 1996b; Miller et al.1991; Urvater et al. 2000a; Voss and Letvin 1996; Watanabe et al. 1994), *B* (Boyson et al. 1996b; Voss and Letvin 1996; Yasutomi et al. 1995), *E* (Boyson et al. 1995), *F* (Otting and Bontrop 1993), *G* (Boyson et al. 1996a), *AG* (Slukvin et al. 1999), and *I* (Urvater et al. 2000b) have already been reported. Rhesus monkeys were shown to carry at least one *A* and two *B* loci, because three *Mamu-A* and five *Mamu-B* alleles have been identified in a single animal (Boyson et

al. 1996b). *HLA-C* homologues have been identified in the common chimpanzee, bonobo, gorilla, and orangutan (Adams et al. 1999, 2000; Cooper et al. 1998; de Groot et al. 2000; Lawlor et al. 1990, 1991), while no evidence of an *HLA-C* homologue was observed in old and new world monkeys (Adams and Parham 2001). Although SIV infection in cynomolgus monkeys is also used as the animal model for human HIV infection (McClure et al. 1990; Putkonen et al. 1992), there are few reports about cynomolgus MHC (*Mafa*) except for class II loci (Gaur and Nepom 1996; Kriener et al. 2000; Otting et al. 1992), class I *E* (Alvarez et al. 1997; Boyson et al. 1995), and *I* loci (Urvater et al. 2000b).

In this study, we have determined the nucleotide sequences of the genes coding for the cynomolgus MHC class I *A* molecules and found 14 *Mafa-A* alleles. In addition, we established a convenient method to detect the *Mafa-A* alleles.

## Materials and methods

### Animals

All the cynomolgus monkeys were raised and reared in the Tsukuba Primate Center for Medical Science, National Institute of Infectious Diseases. Both genders were involved and the cynomolgus monkeys were between 2 and 23 years old. This study was conducted in accordance with the Guide for Animal Experiments Performed at the National Institute of Infectious Diseases.

### RT-PCR and nucleotide sequencing

Peripheral blood mononuclear cells (PBMC) were isolated from the fresh blood of 29 cynomolgus monkeys by a standard Ficoll-Hypaque gradient method. PBMC were washed twice with PBS and suspended in 5 ml of RPMI-1640 (Sigma, St. Louis, Mo.) containing 100 U/ml penicillin (*Meiji Seika Kaisha, Tokyo*, Japan), 10% FCS (GIBCO-BRL, Grand Island, N.Y.), and 5 μg/ml concanavalin A (ConA; Pharmacia, Cleveland, Ohio) at a concentration of $10^5$ cells/ml. PBMC were cultured at 37 °C for 3–4 days. Messenger RNA extracted from the cultured PBMC (2–

**Table 1** Primers used for the amplification and sequencing of MHC class I cDNAs from cynomolgus monkeys

| Primer | Sequence | Concentration (pmol/sample) | Annealing temperature (°C) |
|---|---|---|---|
| Primers used for RT-PCR | | | 60 |
| Mafa-A-s | 5′-GCAGGATCCGAATCTCCCCAGACGCGCA-3′ | 10 | |
| Mafa-A-a | 5′-GCTCTAGACCTCACAAGGCAGCTGTC-3′ | 10 | |
| Mafa-A13-s | 5′-CGAACCCTCCTCCTGG-3′ | 10 | |
| Mafa-A1013-a | 5′-CTGAGAGTAGCTCCCTCCTTTTCTAT-3′ | 10 | |
| Primers used for multiplex PCR | | | |
| Primer set 1 | | | 72 |
| IA01-s | 5′-GCAGCGGGATGGAGAGGAA-3′ | 20 | |
| IA02-s | 5′-GCTGTGGTTGTGCCTTCTGGAAAA-3′ | 10 | |
| IA03-s | 5′-ACGCTGCAGCGCGCA-3′ | 2 | |
| IA04-s | 5′-GCGGCGGATGTGGCGGAGAG-3′ | 2 | |
| IA05-s | 5′-CTGCGACCTGGGGCCG-3′ | 2 | |
| IA-a | 5′-CCTGGGCACTGTCACTGCTT-3′ | 20 | |
| Primer set 2 | | | 72 |
| IA06-s | 5′-GGGCCTGTGCGTGGAGTCCCTG-3′ | 10 | |
| IA07-s | 5′-CACACTGACCTGGCAGCGT-3′ | 10 | |
| IA08-s | 5′-CTGCGACCTGGGGCCA-3′ | 10 | |
| IA09-s | 5′-CTACAACCAGAGCGAGGCCA-3′ | 10 | |
| IA10-s | 5′-GCAGCCCCGCTTCATCT-3′ | 10 | |
| IA-a | 5′-CCTGGGCACTGTCACTGCTT-3′ | 20 | |
| Primer set 3 | | | 70 |
| IA11-s | 5′-ACACATGTGACCCATCACCCT-3′ | 5 | |
| IA12-s | 5′-GCCGGAGTATTGGGACCA-3′ | 20 | |
| IA13-s | 5′-GGCCTGCAGGAGATGGAAA-3′ | 20 | |
| IA14-s | 5′-CGGACCTGGGGGCTCAA-3′ | 15 | |
| IA-a | 5′-CCTGGGCACTGTCACTGCTT-3′ | 20 | |
| Primers used for sequencing | | | |
| T7 primer | 5′-TAATACGACTCACTATAGGG-3′ | 3 | 55 |
| SP6 primer | 5′-ATTTAGGTGACACTATAG-3′ | 3 | 55 |
| Ia698 | 5′-TAGAAGCCCAGGGCCCAGGC-3′ | 3 | 55 |
| Is437 | 5′-ATTACATCGCCCTGAACGAG-3′ | 3 | 55 |

$10\times10^6$ cells) using a Quick Prep Micro mRNA Purification kit (Pharmacia Biotech, Uppsala, Sweden) were converted into cDNA using a High Fidelity RNA PCR kit (Takara, Shiga, Japan). The amplification of *A* locus was carried out by using specific primer sets, either Mafa-As/Mafa-Aa or Mafa-A13-s/Mafa-A1013-a (Table 1). Forty cycles of amplification were carried out at 94 °C for 30 s, at 60 °C for 30 s, and at 72 °C for 30 s, followed by an additional extension at 72 °C for 7 min using GeneAmp PCR System 9700 (Applied Biosystem, Norwalk, Conn.). The PCR products were cloned into pCR4-Blunt-TOPO plasmid using Zero Blunt TOPO PCR Cloning kit (Invitrogen, Carlsbad, Calif.). The clones were sequenced with sequencing primers T7, SP6, Ia698, and Is437 (Table 1) by an ABI model 310 DNA Sequencer (Applied Biosystem, Foster City, Calif.).

## The multiplex PCR-SSP

The multiplex PCR-SSP was carried out using cDNA from cynomolgus monkeys as the template. The primers used are listed in Table 1. Primer set 1 was a mixture of IA-01s, IA-02s, IA-03s, IA-04s, IA-05s, and IA-a, primer set 2 IA-06s, IA-07s, IA-08s, IA-09s, IA-10s, and IA-a, and primer set 3 IA-11s, IA-12s, IA-13s, IA-

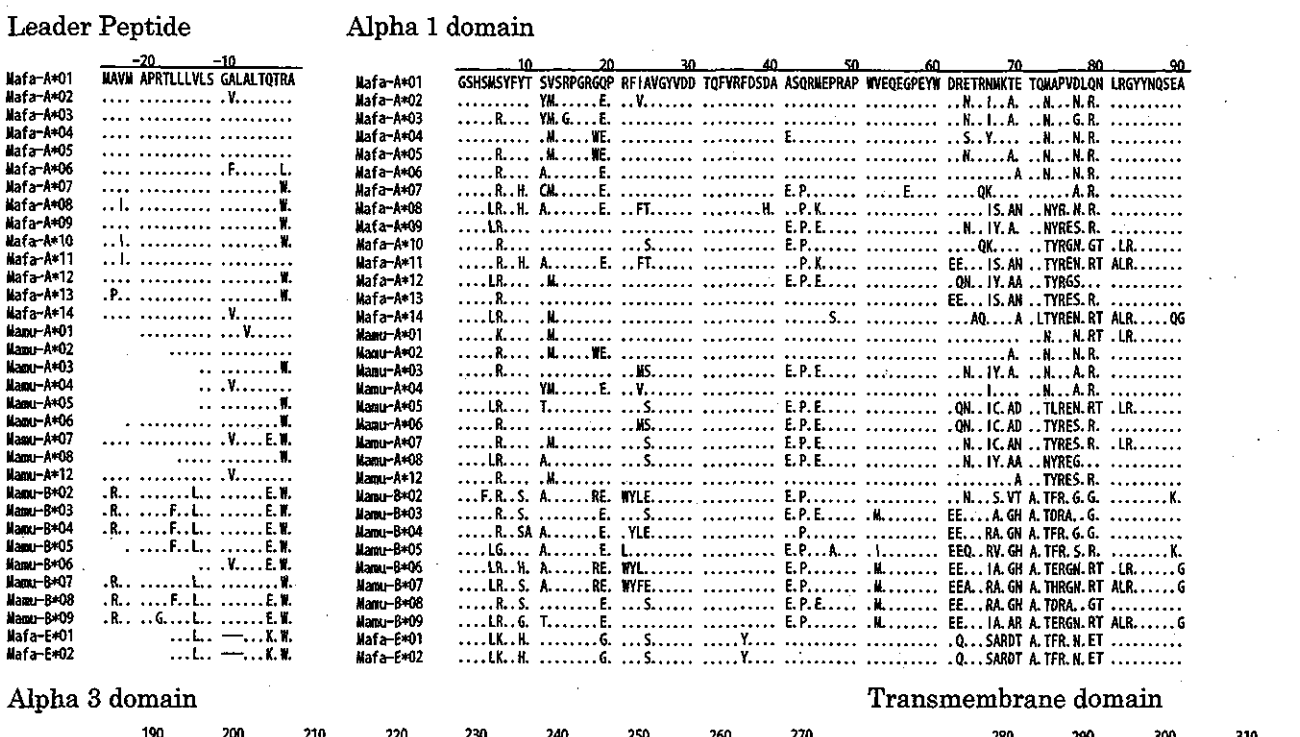


**Fig. 1** Alignment of predicted amino acid sequences of *Mafa-A* with previously reported *Mamu-A*, *Mamu-B*, and *Mafa-E* sequences. Identity to predicted amino acid sequence of *Mafa-A*01* is indicated by *dots*, whereas amino acid replacements are depicted by the conventional *one-letter code*. The deletions of amino acid are indicated by *hyphens*