receiving PIs, we failed to observe a statistical difference in CD4 cell counts and viral suppression among patients with different *MDR1* 3435 SNPs (Figures 5A and 6A). Furthermore, we found that patients with the *MDR1* 1236 T/T genotype showed a greater increase in the CD4 cell counts during HAART therapy with PI at months 1 and 9 than patients with the *MDR1* 1236 C/C genotype (Figure 5C). The contribution of genetic variations in the *MDR1* gene to the patients' clinical characteristics, if any, seems very complicated and thus is difficult to evaluate in a straightforward manner.

As the steady-state intracellular concentration of NFV was about 250 times higher than that in the medium (10 μM), the uptake of NFV seems active rather than passive. However, these *in vitro* data depart from what has been observed in *in vivo* measurements of NFV in patients [19,20] presumably due to the presence of alpha(1)-acid glycoprotein to which NFV binds in plasma [21]. Furthermore, this discrepancy may also be due to the differential distribution of NFV among tissues rather than in free artificial medium. Therefore, our *in vitro* data should be considered as such, that is, *in vivo* lymphocytes may be unlikely to have this high intracellular to extracellular concentration ratio (250:1).

We observed an association of slower efflux of NFV *in vitro* with the T/T genotype at *MDR1* 3435. In fact, P-gp has been found to export PIs from lymphocytes and reduce their anti-HIV activity *in vitro*, and its low activity has been found to be associated with the T/T genotype at *MDR1* 3435 [13]. As the SNP at *MDR1* 3435 is a silent mutation, one possible explanation for this association is that the T/T genotype at *MDR1* 3435 renders *MDR1* mRNA unstable in the cell. Another possible explanation for the association is that *MDR1* 3435 SNP is in linkage disequilibrium with the SNPs at *MDR1* 1236 (exon 12) and *MDR1* 2677 (exon 21), the latter of which is a substitution mutation. This amino acid substitution from the *MDR1* 2677 SNP may be responsible for the observed difference (Figure 4) [11]. Another possible explanation is that *MDR1* 3435 SNPs are in linkage disequilibrium with a polymorphism(s) elsewhere in the genome that modifies *MDR1* expression or function [3,12].

Although an *in vitro* study showed that the velocity of NFV efflux in patients' LCLs with the *MDR1* 3435 T/T genotype was slower than that with the C/C genotype, we failed to observe a statistical difference in CD4 cell counts and viral suppression among patients with different *MDR1* 3435 genotypes (Figures 5A and 6A). Four equally possible accounts seem to explain this discrepancy. Firstly, since the C/C genotype at *MDR1* 3435 is also correlated with higher expression of P-gp in intestinal epithelial cells that adsorb PIs, the *MDR1* 3435 C/C is likely to be associated with higher absorption of PIs and higher PI concentration in plasma [12,22]. The higher plasma levels of NFV in 3435 C/C patients in one study [12] is puzzling and as yet not fully understood. Secondly, the sample size (n=21) in this study may be too small to evaluate CD4 cell counts or viral suppression in a statistical way. Thirdly, since the enrolled patients received different treatment combinations of PIs and reverse transcription inhibitors during antiretroviral therapy, the clinical evaluation was not normalized. Finally, because LCLs – immobilized B cells – but not CD4+ T cells were used in this study, the function of P-gp in a setting of HIV-1 infection may not have been accurately tested. In contrast to the *MDR1* 3435, we observed a marginal but statistically significant association of the *MDR1* 1236 SNP with the CD4 cell count increase although this SNP is a silent mutation. To our knowledge, this clinical association of *MDR1* 1236 with statistical significance is unprecedented, although its clinical significance remains to be investigated. In conclusion, a large-scale and case-controlled study would be required to test whether SNPs of *MDR1* affect the clinical course during antiretroviral therapy with PIs and the prognosis of infected patients.

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ABSTRACT 98

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Changes in prevalence and patterns of drug resistant mutations in Japan – summary of nationwide HIV-1 drug resistance surveillance study (1996 to 2003) in Japan

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BACKGROUND AND OBJECTIVE: Antiretroviral treatment situation in Japan has been similar to other Western countries, zidovudine became available in 1987 and HAART was introduced in 1997. As has been widely reported, HAART has improved the prognosis of HIV/AIDS significantly. However, treatment failure due to antiretroviral drug resistance is a critical issue that must be overcome. We started HIV drug resistance genotyping in 1996 to support the treatment, and also to understand prevalence and patterns of HIV drug resistance. Here we present a summary of our 7-year surveillance study of HIV drug resistance in Japan.

METHODS: HIV/AIDS patient blood samples sent to our laboratory from November 1996 to December 2003 were analysed. Drug resistance genotyping was performed using *in-house* protocol. Briefly, HIV-1 RNA was extracted from patient serum, and protease and reverse transcriptase fragments were amplified and sequenced. Drug resistance mutations were defined according to IAS-USA drug resistance mutation list.

RESULTS: During the study period, 5561 samples from 1156 HIV/AIDS patients were collected and analysed. The prevalence of NRTI resistance was 45% in the beginning of our study, and remained at 40–45% for throughout the study period. In contrast, the prevalence of PI resistance increased dramatically. There was no resistance case in 1996, but it increased up to 35% in 2000. Subsequently, PI resistance frequency decreased in 2001 to 2002, and reciprocally the prevalence of NNRTI increased from 5% in 2000 to 15% in 2002. The data appears to reflect the availability and trends of antiretroviral treatment in Japan. PI was approved in 1997, prescriptions increased thereafter, and the increase in PI resistance matched this progression. Subsequent changes in the prevalence of PI resistance and NNRTI resistance coincided with the availability of efavirenz, which was approved in 2000 and experienced increased use as a replacement for PIs in HAART.

CONCLUSION: Our data demonstrates significant increase of HIV drug resistance in these 7 years. Further continuation of the surveillance is necessary not only to understand epidemiological status, but also to find effective strategy to overcome the HIV drug resistance issue.

ABSTRACT 2

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Novel small-molecule compounds which inhibit strand transfer activity of HIV-1 integrase

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OBJECTIVE: Integration of the proviral DNA into the host genome is essential event in the human immunodeficiency virus type 1 (HIV-1) replication life cycle. Therefore, integrase (IN), which plays crucial role in this integration event, has been the attractive target of anti-HIV drugs. Up to now, a number of inhibitory compounds have been reported, yet none has been successful in clinical treatment usage. In this study we attempted to find new IN inhibitory compounds, and screened a small molecule-compound library.

METHODS: In-house strand-transfer assay was constructed to screen IN inhibitory compounds. In brief, biotinylated 31 bp donor DNA was mixed with recombinant IN, followed by incubation with digoxigenin (DIG), labelled 29 bp target DNA and the test compound. After 1 h incubation at 37°C, integrated product was captured by streptavidin-coated 96 well plate, and quantified by alkaline phosphatase-conjugated anti-DIG antibody and CSPD chemiluminescence detection system. Lineweaver-Burk plot analyses and intercalation assays were performed to clarify the mechanism of inhibitions. To evaluate *in vitro* virus replication suppressions, single replication assays using HeLa/CD4/LTR-EGFP cell line were performed.

RESULTS: We tested 12000 small-molecule compounds and discovered one compound, carbazole derivative, with potent strand-transfer inhibitory activity. To analyse structural determinants of the strand transfer inhibitory activity, we chemically synthesized 15 derivatives with different side chains on the carbazole structural backbone. Among these 15 compounds, eight derivatives have shown potent strand-transfer inhibitions. IC_{50}s of these eight compounds ranged from 0.78 to 5.3 μM. The result of Lineweaver-Burk plot analyses indicated the carbazole derivatives as competitive inhibitor of strand transfer. No intercalation activities were observed. In HeLa/CD4/LTR-EGFP cell culture assay, IC_{50}s of the eight compounds ranged from 0.49 to 1.92 μM. However, these eight derivatives demonstrated cytotoxicity (CC_{50}=1.97 to 5.04 μM) in this HeLa cell culture.

CONCLUSION: We have successfully found novel small-molecule IN inhibitory compounds carbazole derivatives. Though their strong cytotoxicity may limit carbazole derivatives to be used in clinical at this moment, it can be the lead compound for developing novel IN inhibitors. In addition, analysing IN inhibitory mechanisms of carbazole may give more detailed information of HIV-1 IN structure and function.

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Phenotype and function of GM-CSF independent dendritic cells generated by long-term propagation of rat bone marrow cells[☆]

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Abstract

GM-CSF is believed to be an essential factor for growth and differentiation of *myeloid* dendritic cells (DC). Employing a low-density fraction of rat bone marrow cells, we attempted to generate DC with human Flt-3/Flk-2 and IL-6. In this culture system, typical DC gradually appeared without exogenous GM-CSF supplement. Phenotypes and functions of the DC were examined. Evidence provided that the most efficient long-term outgrowth of DC progenitors was obtained by *GM-CSF independent* culture systems with the aid of Flt3/Flk-2 and IL-6, not with c-kit ligand and IL-6. Furthermore, CD103 (OX-62), which is widely used for rat DC separation, was found to be insufficient for enriching DC, due to the down-regulation of the marker. However, the most efficient selection of rat DC was made by CD161a (NKR-P1A), a C-type lectin family. The *GM-CSF independent* DC was functionally active in vitro as well as in vivo assays.
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Keywords: Rat dendritic cell; GM-CSF; IL-4; TNFα; c-kit ligand; Flt3/Flk2 ligand; IL-6; APC; CD161a

1. Introduction

Klinkert and Bowers [1,2] first described a method to generate specialized antigen presenting cells (APC), dendritic cells (DC) [3], and/or veiled-type cells (VC) [4] from low-density fraction of bone marrow cells under a serum free or conditioned medium. DC are known to bear distinguishing morphology and distinct phenotypes from various types of macrophage (Mϕ) populations judged by their phenotypic characteristics [5–7]. Nevertheless, DC are generally difficult to obtain in a substantially large number for many experimental purposes, due to their paucity in the peripheral lymphoid tissues. In this regard, however, Steinman and his colleagues reportedly generated a substantially large number of DC with an in vitro system employing granulocyte–macrophage colony-stimulating factor (GM-CSF). Hence, GM-CSF was believed to support both growth and differentiation from DC-precursor and/or its progeny in mouse BMC and peripheral blood, respectively [8,9]. Additionally, it has also been reported that co-stimulatory factors such as tumor necrosis factor (TNF-α) [10,11] and/or IL-4 were also effective in enhancing human DC induction from BMC culture. Furthermore, it has been shown that pure human DC

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colony was generated from CD34+ cells by additional cytokines such as c-kit ligand with GM-CSF and TNF-α for a long-term BMC culture [12].

DC-like or DC-lineage APC, interstitial DC including skin Langerhans cells, found in many non-lymphoid tissues [13], is believed to play a primary role in initiating immune responses accompanying transplanted tissues and organs. However, inasmuch as the most, if not all, DC or DC-related APC are derived from hematogeneous organs such as BMC, it is important to determine the crucial cytokine that induces cell growth, differentiation and migration of DC from its BMC progeny. In particular, the presence of GM-CSF independent DC subset was demonstrated [14]. Heretofore, subsets of the DC, *myeloid*, *lymphoid*, and/or *plasmacytoid* DC, have been proposed by their phenotypes, differential function to produce specific cytokines, and their growth requirements [14,15]. Thus, it is important to identify a specific factor and a general principle to induce growth and differentiation of DC from their progenitor.

DC play an important role in cellular immunity. However, obtaining a large number of DC for experimental as well as clinical settings requires multi-step separations and time-consuming processes. Thus, it is necessary to establish a simple and efficient culture system to generate a large number of well-characterized DC so that wide audiences in cellular immunology as well as clinical medicine are able to use the DC for their applications.

In this study, we examined recombinant cytokines including GM-CSF and IL-4 to induce the growth and/or differentiation of DC precursors in rat BMC. Furthermore, using Flt-3/Flk-2 and c-kit ligand known as hematopoietic cell growth factors (type III membranous tyrosine kinase), with IL-6, we also attempted to determine a minimal requirement to increase the frequency of DC precursor(s) from hematopoietic stem cells to obtain a practical amount of functionally mature DC from rat BMC culture systems.

Our study demonstrates that unlike mouse systems, GM-CSF per se was not able to support a meaningful growth of rat DC progenitor in BMC, regardless of the cytokine sources, murine, or human. However, we did obtain the outgrowth of the precursors that contained DC progenitors by 1000-fold with combined cytokines, either Flt-3/Flk-2ligand with IL-6 or c-kit ligand (stem cell factor: SCF) with IL-6, the former combination produced far better yields than the latter in terms of outgrowing DC-committed progenitor cells. Thus, a relatively large number of rat DC was obtained from the single step long-term (two or more months) BMC culture without the multi-step and time consuming processes of BMC. Furthermore, our study provided evidence that GM-CSF, TNF-α, and IL-4 promote differentiation of DC and hence down-regulate the growth of DC progenitors.

2. Materials and methods

2.1. Animals

Inbred strain of male and female rats that include LEW (MHC: RT1l), DA (MHC: RT1avl), PVG (MHC: RT1c) and its hybrid (DA × LEW) F1, (LEW × PVG) F1 as well as recombinant strain of PVG.1U (MHC: RT1u) where PVG background and its MHC was derived from WF (MHC: RT1u), were originally obtained from Harlan Olac (Blackthorn, Bicester, England). C57BL/6 (MHC: H-2b) mice were obtained from Tokyo Experimental Animal (Tokyo, Japan). Animals were maintained in our animal facility under the specific pathogen free.

2.2. Reagents

Monoclonal antibodies (mAbs) that included mouse anti-rat FITC-CD103 (MRC-OX62), PE-CD103 (MRC-OX62) used for specific rat DC marker [16] were purchased from Serotec Bioproduct, UK (Dainippon Pharmaceutical, Osaka, Japan). Likewise, mouse anti-rat FITC-CD3, FITC-CD4, PE-CD4, FITC-CD8, PE-CD8, FITC-CD80, FITC-CD86, PE-CD161a (NKR-P1A) were purchased from BD Immunocytometry products (BD Pharmingen International, Fujisawa Pharmaceutical, Osaka, Japan), respectively. Mouse anti-rat hybridoma cloned cell lines that included anti-rat class I (MRC-OX18), class II (MRC-OX6, MRC-OX3), ED-1, ED-2, TcR (R73), CD3, W3/25, MRC-OX35, MRC-OX38 (CD4), MRC-OX8 (CD8), MRC-OX39 (CD25), MRC-OX26 (CD71), MRC-OX7 (CD90), MRC-OX43, HIS24 (CD45R, mouse CD45R/B220 equivalent) were obtained from European Collection of Animal Cell Cultures (Salisbury, United Kingdom). Hybridoma cell line 1A-29 (mouse anti-rat CD54) was donated from Dr. Masayuki Miyasaka (Osaka University, Osaka, Japan), and 3H5 (mouse ant-rat CD80), 24F (mouse ant-rat CD86) were generously provided by Dr. Hideo Yagita (Juntendo University, Tokyo, Japan), respectively.

Cytokines that included recombinant mouse and human GM-CSF, human IL-6 were generously supplied by Kirin Brewer (Maebashi, Gunma, Japan). Likewise, recombinant rat c-kit ligand (SCF) was generously provided by Amgen (Thousand Oaks, CA). Recombinant rat IL-2 and IL-4 were purchased from R&D Systems, USA (Funakoshi, Tokyo, Japan). Recombinant human Flt3/Flk2 ligand, rat GM-CSF were purchased from PeproTech, USA (IBL, Gunma, Japan).

2.3. Culture medium

Medium for primary cell preparation was performed by Dulbecco's phosphate-buffered saline (D-PBS) (DAB, OXOID, Basingstoke, Hampshire, UK).

For cell cultures, different medium with or without serum (fetal bovine serum: FBS) were employed.

(1) Serum-free RPMI1640 containing 25 mM Hepes (Whittaker, Walkersville, MD) supplemented with 2 mM L-glutamine and antibiotics was used to determine the effect of single cytokine on the growth of BMC.
(2) For screening purpose to analyze the effects of combined cytokines, complete medium (CM) RPMI1640 supplemented with 10% FBS (Hyclone, Logan, UT), 2-mercaptoethanol (5×10^{-5} M), 2 mM L-glutamine and antibiotics was employed.
(3) For generating a large scale of DC from a long-term culture of rat BMC, GIT (NIHON Pharmaceutical, Tokyo, Japan) supplemented with 2 mM L-glutamine, and antibiotics without 2-mercaptoethanol was employed. This medium was already supplemented with well conditioned-serum components.
(4) For mixed lymphocyte culture (MLC), complete medium (CM) RPMI1640 supplemented with 2.5% LEW rat or 10% FBS (Hyclone, Logan, UT), 2-mercaptoethanol (5×10^{-5} M), 2 mM L-glutamine, and antibiotics was employed.

2.4. Cell preparation

Suspensions of spleen, lymph node, thoracic duct lymphocytes (TDL), and BMC were prepared according to standard procedures [17].

2.5. Preparation of lectin-free conditioned medium

Conditioned medium was prepared according to the method by Kilinkert [2], as described before [5], with several modifications. In brief, spleen cells from LEW rat was stimulated by Concanavalin A (Con A) for 2 h, and were further incubated for 120 h after washing out residual Con A.

2.6. Examination of cytokines

Ten to 10,000 units of recombinant murine (mouse, rat) and human GM-CSF or 15% of the conditioned medium was employed in 1.5 ml RPMI-1640 medium supplemented with 10% FCS, 5×10^{-5} M of 2-mercaptoethanol, 2 mM L-glutamine and antibiotics. The initial cell dose was 7.5×10^6 cells/1.5 ml and cultured in 24-well plates (Nunc, Naperville, IL).

To examine the effect of single or combined cytokines on the growth and differentiation of DC from BMC, RPMI-1640 medium supplemented with 10% FBS, 2-mercaptoethanol (5×10^{-5} M), 2 mM L-glutamine and antibiotic was used.

2.7. Cell surface analysis by flow cytometry

An aliquot of cell suspension was stained with monoclonal antibodies and analyzed by flow cytometry using a FACScan (Becton Dickinson Immunocytometry Systems, San Jose, CA).

2.8. Cell separation by autoMACS systems

According to the manufacturer's procedures (autoMACS systems: Miltenyi Biotec, Bergisch Gladbach, Germany), the positive selection of cells was performed. Briefly, cells were first incubated with FcR blocking antibody for 10 min on ice and then stained with PE-conjugated monoclonal antibody for 30 min on ice. After two times washing by D-PBS, the cells were further incubated with anti-PE magnetic microbeads (10^7 cells/20 μl, Miltenyi Biotec, Bergisch Gladbach, Germany), incubated for 30 min at 4 °C temperature. Cells bound by magnetic-beads were likewise washed carefully and re-suspended in 4 ml buffer solution, and followed by magnetic separation by autoMACS systems.

2.9. Cytospin preparation

To assess the morphological characteristics of proliferating DC, cytospin preparations of 1×10^4 cells were made in a cytocentrifuge (Shandon, Pittsburgh, PA, USA) in 800 rpm for 10 min, stained with May–Gruenwald and Giemsa (Merck Japan, Tokyo, Japan), and examined by light microscopy.

2.10. Skin grafting

The procedure of skin grafting was performed according to standard procedures [18]. In brief, full thickness of donor male trunk skin was removed and the skin muscles were trimmed off by a pair of forceps. Size of the donor skin was 2×2.5 cm; approximately 5 cm^2. Female recipient's skin bed was laterally prepared by trimming off the epidermal layer while keeping the skin muscle by fine optical scissors. Graft skin was sutured, gauzed and bandaged by elastic bandage. Nine days after skin grafting, the bandage was removed and inspected by daily bases for first 30 days and at least twice weekly thereafter.

2.11. Graft versus host (GVH) assay

The popliteal lymph node weight assay [19] was employed for screening purposes and single dose assays were performed. In brief parental donor cell suspensions were prepared at 10×10^6/0.1 ml and injected into each foot pad of 4–6-week-old F1 hybrid. Following the foot pad-injection, draining popliteal lymph node was excised at day 7 and weighed.

2.12. Mixed lymphocyte culture

One source of responder LEW T cells was prepared from LEW rat that had been lethally irradiated (10 Gy) and subjected to the thoracic duct drainage. Normal 1.5×10^9 LEW TDL consisting of 70–80% T cells were collected from three to four LEW donors. After intravenous inoculation of the LEW TDL into the lethally irradiated syngeneic LEW recipient, we collected the first 8–30 h thoracic duct lymph from the lethally irradiated LEW rat. This population is known to contain more than 99% T cells. Tentatively, this T cell population was labeled as LEW_{-LEW}. The other source of responder LEW T cells was likewise labeled LEW_{-DA}. Thus, 1.5×10^9 LEW TDL was injected into the lethally (10 Gy) irradiated DA rat that had been subjected to similar thoracic duct drainage. This LEW_{-DA} population is known to be incapable to cause GvH reactivity to DA in vivo as well as MLC reactivity in vitro, respectively. Employing these responder LEW T cells (LEW_{-LEW} or LEW_{-DA}) syngeneic MLC as well as allogeneic MLC was set up as follows: For syngeneic MLC, 2×10^5/0.1 ml responder LEW T cells were stimulated with a graded dosage of syngeneic LEW DC, from 5×10^4/0.1 to 0/0.1 ml cells. Normally, 0.5 μCi methyl-[^{3}H]thymidine (Amersham International, Amersham, UK) was added to 25 μl of cell suspension at day 4 to day 6 in a 6 h pulse. Likewise, for allogeneic MLC, 2×10^5/0.1 ml responder LEW T cells were stimulated with a graded dosage of allogeneic DA DC.

Standard deviations of [^{3}H]thymidine incorporation were determined from a minimum of four replicated micro-cultures.

2.13. Irradiation

To ensure uniformity of tissue distribution of the radiation dose, rats were rotated in a Perspex box at 15 rpm around a vertical axis in the horizontal beam from two-way X-ray irradiation source (MBR-1520A-TWZ; Hitachi Medico, Tokyo, Japan). The beam was filtered with shaped lead disks to a dose uniformity across the beam of >96%. The dose was delivered at approximately 1 Gy/min.

3. Results

3.1. Effect of GM-CSF on rat bone marrow cell cultures

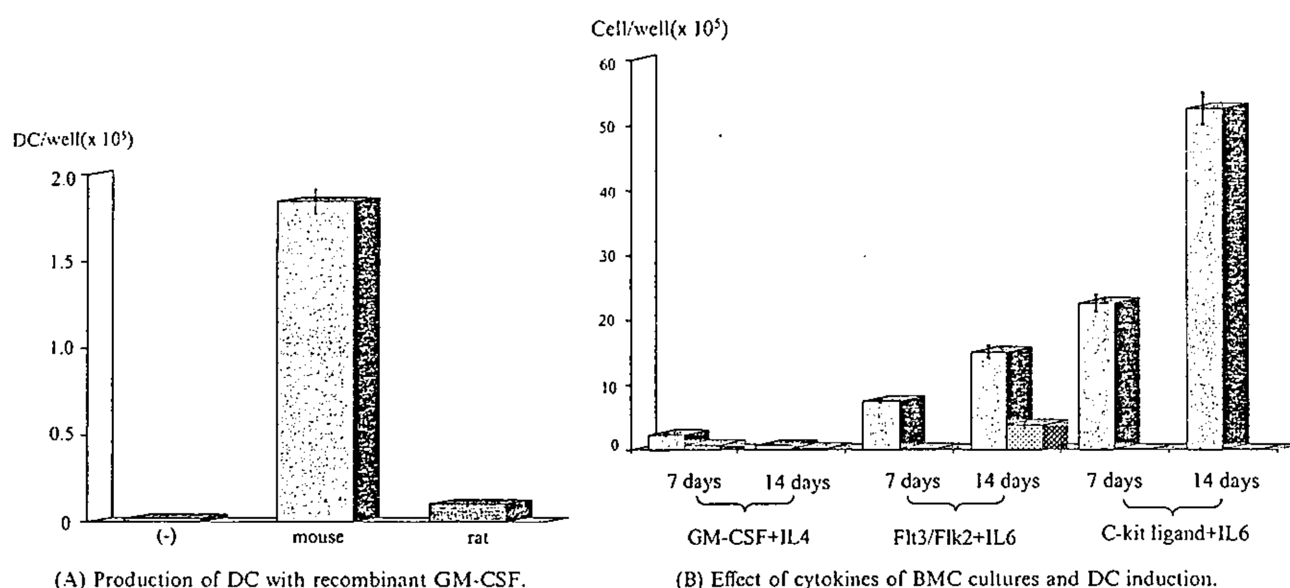
Our attempt to generate rat DC by a serum-free medium supplemented with a conditioned medium [5] based on the early studies [1,2] led to a limited amount of DC recovery, final cell recovery was usually around 0.1% of total BMC input. Hence, attempts were first made to determine whether and to what extent single cytokine GM-CSF is able to increase DC yields in short-term cultures of rat BMC as observed in mouse systems [8]. For this purpose, BMC culture supplemented with single recombinant GM-CSF was compared with those employing serum-free RPMI1640, or those with conditioned medium.

Un-manipulated whole BMC at 7.5×10^6/1.5 ml were cultured for one week. On day 7, free-floating cells were harvested, and counted under phase-contrast microscopy. In this particular experimental setting, unlike mouse systems, even species-matched GM-CSF was unable to generate a significant increase of rat DC recovery, as shown in Fig. 1A. Although we examined a wide range of GM-CSF concentrations from 1 to 1000 ng/ml, the results were not significantly altered, and final DC recovery in mouse systems was found to be, at most, 1.8×10^5 DC/7.5×10^6 BMC/well (final DC recovery was approximately 2.3 ± 0.4% of the initial BMC input compared with that of 0.2 ± 0.03% in rat DC recovery). It should be noted that rat GM-CSF per se has nearly equal activity for granulocyte–macrophage (GM) colony formation under an agar culture system for both mouse and rat BMC, however, the mouse GM-colony was significantly larger than those of rats (data not shown), and most of the BMC appeared to proliferate and adhere to the culture dishes. Despite the growth-promoting activity, final DC recovery by GM-CSF was not as high as was expected.

3.2. Effect of GM-CSF and IL-4 on rat bone marrow cell cultures and comparison of other putative cytokines for DC induction

Based on the studies by others, we examined three sets of culture systems in an effort to seek better culture systems to obtain a large number of DC from rat BMC progenitors. Thus, one culture system adopted an FBS-supplemented RPMI1640 medium containing two cytokine combinations, mouse GM-CSF and rat IL-4 [20]. The other two sets of culture systems were based on the reports that employed type III membranous tyrosine kinase, as a growth factor for hematopoietic stem cell, c-kit ligand or Flt3 ligand. It has been shown that the former type III membranous tyrosine kinase c-kit ligand generated pure human DC colony from CD34+ cells of BMC with GM-CSF and TNFα [12], and the latter Flt3 ligand IL-6 combinations have been shown as growth factors for primitive multipotential hematopoietic progenitor cells [21].

The single step of low-density separation method (*d*; 1.077) was chosen. Inasmuch as our preliminary experiments repeatedly demonstrated that a single step of two-layers separation which employed two layers of Ficoll-based separation medium, i.e., the light density (*d*; 1.077) medium over the high density (*d*; 1.094) medium and each layer was cultured separately, resulted in a



(A) Production of DC with recombinant GM-CSF.

(B) Effect of cytokines of BMC cultures and DC induction.

Fig. 1. (A) About 7.5×10^6/1.5 ml rat bone marrow cells (BMC) and mouse BMC was cultured for one week with recombinant rat GM-CSF and mouse GM-CSF under various concentrations of GM-CSF under serum free medium, respectively. At day 7, survived, free-floating cells were harvested and the number of typical veiled shaped DC was counted by phase-contrast microscopy. Each column was expressed by an average of quadruplicated culture wells. Figure showed representative results of BMC culture employing GM-CSF at the dosage of 20 ng/ml. Left column was from serum free RPMI1640 medium supplemented lectin-free condition medium described in Section 2. Central column expressed final DC recovery from mouse BMC culture with mouse GM-CSF. Likewise, right column expressed the number of DC from rat BMC culture supplemented with rat GM-CSF. (B) 7.5×10^6/1.5 ml low-density (*d*; 1.077) rat bone marrow cells were cultured for two weeks with three different conditions under 10% FBS supplemented-RPMI 1640 medium. Namely (1) rat GM-CSF (20 ng/ml) and rat IL-4 (10 ng/ml) (2) human Flt3/Flk2 ligand (100 ng/ml) and human IL-6 (10 ng/ml) (3) rat c-kit ligand (100 ng/ml) and human IL-6 (10 ng/ml). At day 7 and day 14, survived, free-floating cells were harvested and the number of typical veiled shaped DC was counted by phase-contrast microscopy, respectively. Total cells (open column) and content of DC (shaded column) were compared with each experiment. Each column was expressed by average of quadruplicated culture well.

far higher yield of DC recovery in the low-density fraction of BMC (data not shown).

Thus, the low-density (*d*; 1.077) rat bone marrow cells were cultured for two weeks with three different conditions under 10% FBS supplemented RPMI 1640 medium. Namely,

(1) rat GM-CSF and rat IL-4,
(2) human Flt3/Flk2 ligand and human IL-6,
(3) rat c-kit ligand and human IL-6.

At day 7 and day 14, survived, free-floating cells were harvested and the number of typical veiled shaped DC was counted by phase-contrast microscopy, respectively. Total cells and content of DC were compared with each experiment.

As shown in Fig. 1B, the Flt3/Flk2 ligand with IL-6-supplemented culture medium was found to produce the best recovery of veiled-type DC at 14 days, compared with other culture conditions. During the course of BMC culture, the DC recovered by BMC culturing was often dependent upon FBS regardless of the cytokine concentration. Indeed, DC induction was never succeeded by a single cytokine such as Flt3/Flk2 without FBS supplementation (data not shown). To standardize and simplify the culture systems and to avoid the lot difference of FBS, we employed the well-defined culture medium, GIT supplemented with freshly prepared L-glutamine and single antibiotics for further study. Under the conditions, generation of DC from rat BMC appeared to be stable. Thus, we performed phenotypic and functional analyses with GIT-based culture systems instead of the RPMI1640 culture medium.

3.3. Histological examination and FACS analysis of DC

Fig. 2A (fresh BMC) depicts representative flow cytometric analysis of low-density BMC, and Fig. 2B (at eight weeks) depicts that of DC induced by GIT based medium supplemented with human Flt3 ligand and IL-6. When DC were generated by Flt3/Flk2 ligand and IL-6 for eight-week's culturing, the most prominent picture was evident in anti-CD161a (NKR-P1A), a C type lectin family [22], which has been described as a marker for rat NK cells [23]. Additionally, anti-CD70 (thy1.1) with two phases of dull and bright population, CD80 and CD86 of B7 family, and CD54 (ICAM-1) of co-accessory molecules with a single peak, were found to be consistently positive. By contrast, CD11b/c (MRC-OX42) expressed on most of the typical Mϕ, and CD45R/B220 (HIS24) which is considered to be a counter part of mouse B220, a marker for *plasmacytoid* DC, was down-regulated and stained as

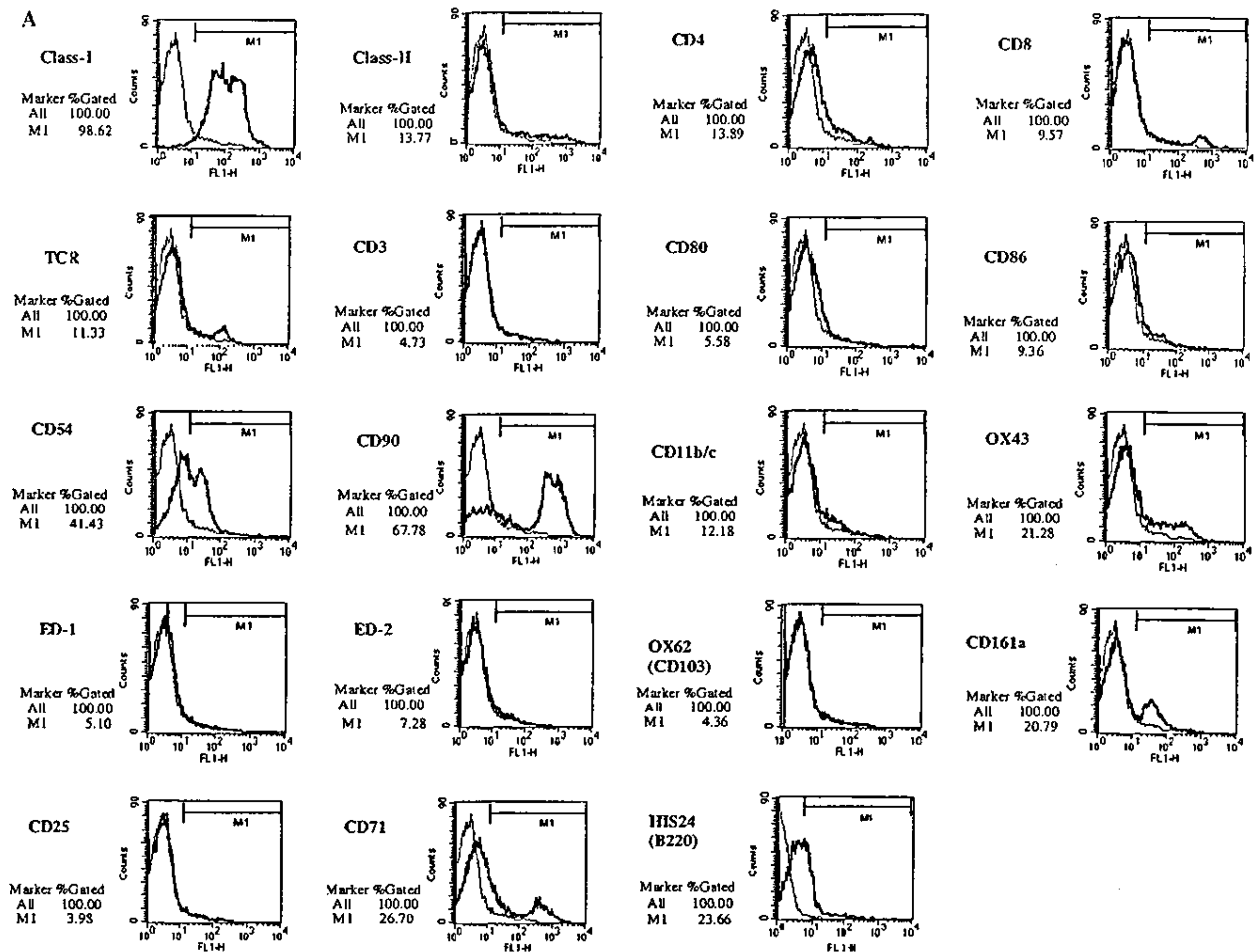


Fig. 2. (A) Cell surface phenotypes of LEW fresh bone marrow cells (BMC) analyzed by various mAbs which are related with antigen presenting cells (APC). These include mAbs specific for class I (MRC-OX18), class II (MRC-OX6) and co-accessory molecules for B7.1 (CD80), B7.2 (CD86) and for functional cell surface molecules of Mϕ for complement $CD11_{b/c}$ and CD161a (NKR-P1A) of the C-type lectin family expressed on NK cells. (B) Cell surface phenotypes of outgrowing DC derived from LEW bone marrow cell cultures for eight weeks supplemented with human Flt3/Flk2 and human IL-6, were analyzed by various mAbs with a unique and restricted region where DC accumulated. Antibodies used are mainly concerning with antigen presenting cells (APC). These include mAbs specific for class I (MRC-OX18), class II (MRC-OX6) and co-accessory molecules for ICAM-1 (CD54), B7.1 (CD80), B7.2 (CD86) and for functional cell surface molecules of Mϕ for complement $CD11_{b/c}$ and one of the C-type lectin family expressed on NK cells, CD161a (NKRP1A). Rat DC specific MRC-OX62 (CD103), rat Mϕ specific CD4, $CD11_{b/c}$, ED1, ED2, OX43 were all negative or extremely dull. Consistent finding was most of DC under the culture conditions expressed CD161a (NKR-P1A) at a relatively high level. (C) Representative flowcytometric analysis of cell surface markers of outgrowing DC derived from LEW BMC; BMC cultures supplemented with human Flt3/Flk2 and human IL-6 at two weeks and three weeks, were positively selected by AutoMACS cell separation systems with PE-CD161a (NKR-P1A) and anti-PE micromagnetic beads described in Section 2. Cells were analyzed by various FITC-mAbs. Antibodies used are mainly concerning with antigen presenting cells (APC). These include mAbs specific for class II (MRC-OX6) and co-accessory molecules for B7.2 (CD86) and for functional cell surface molecules of $CD11_{b/c}$ (MRC-OX42) and rat DC specific MRC-OX62 (CD103), CD4 specific for a subset of rat Mϕ as well as CD8. Consistent finding was that most of CD103+ (MRC-OX62) DC, if not all, was down-regulated or lost the marker during the extended culture and hence became dull or null (see B).

nearly null cells. As for the CD4 as well as CD8 that has been used to define *lymphoid* DC in splenic and thymic *lymphoid* DC in mice, likewise, not expressed. Furthermore, unlike co-accessory molecules that appeared to be up-regulated during the culture, CD103 (OX-62) which has been widely used for one of the rat DC specific marker was found to be down-regulated during the culture (Fig. 2C, at two weeks vs. at three weeks, and B).

Based on these profiles, positive selection of fully mature DC by CD54, CD71, CD80, CD86, and/or CD161a was considered for final purification of DC. Nevertheless, inasmuch as co-accessory molecules such as CD54, CD80 and CD86 play important roles in T cell activation, use of these markers for DC separation may not be suitable for the subsequent examination of purified DC as APC in in vitro as well as in vivo studies. Therefore, a population of $CD161a^{+}$ DC was enriched in high purity (>98%) by automatic cell separation system as described in Section 2. Their functional aspect as antigen-presenting cells (APC) were analyzed in the

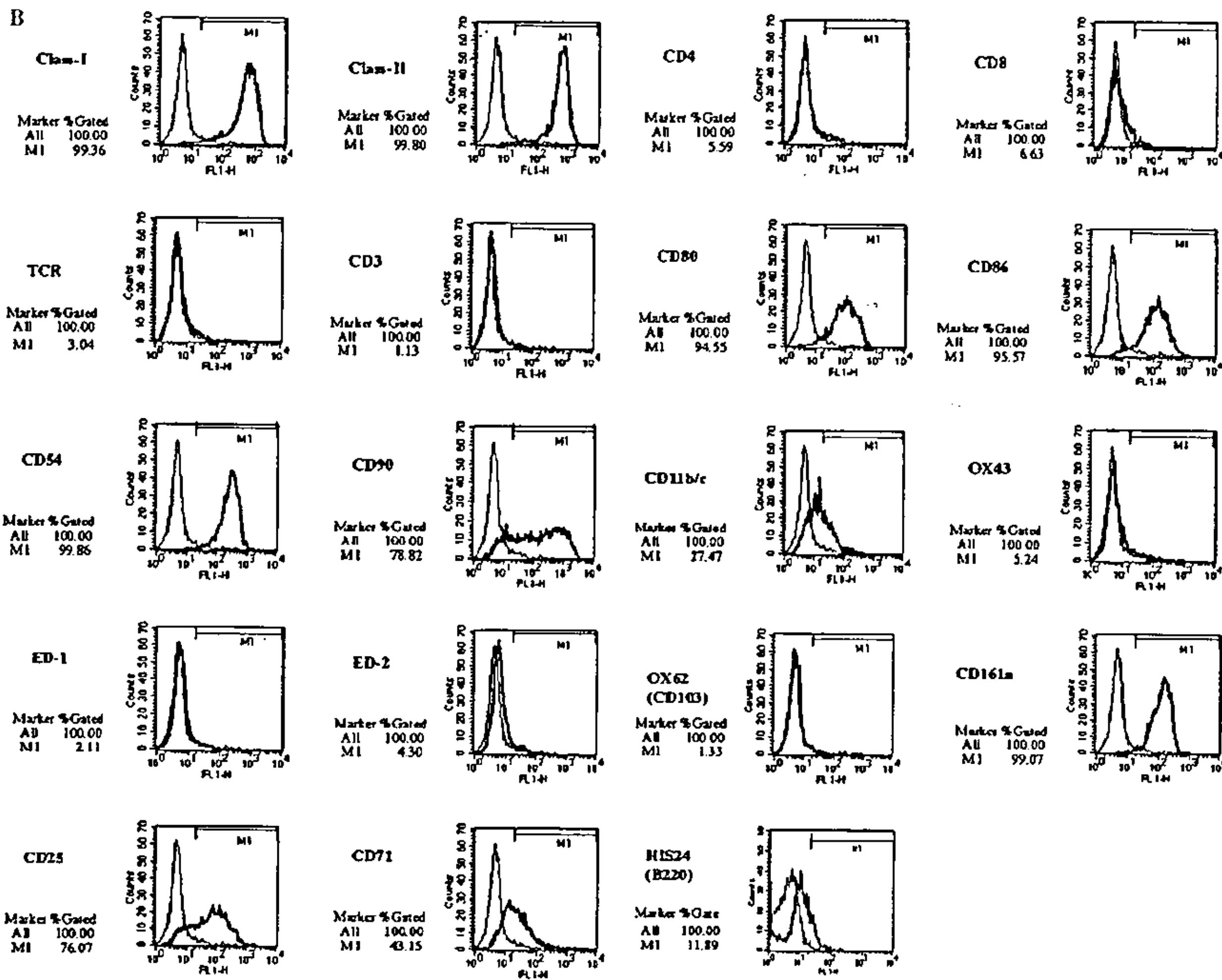


Fig. 2. (*continued*)

following experiments on in vitro as well as in vivo systems.

Fig. 3 illustrates typical figures of DC colonies under phase-contrast microscopic observation (A). Following autoMACS cell separation of CD161a$^+$ DC, described in Section 2, its cytospin preparation (B), phase contrast outlook of cell suspension (C), and May-Gruenwald and Giemsa staining (D) were shown.

3.4. Functional analysis of CD161+ DC in vitro

The most typical characteristic of DC as a specialized APC in vitro is that mature DC has profound capability to activate autologous or syngeneic T cells, in particular the CD4+ T cell subset, generating the so-called autologous or syngeneic MLC reaction. In allogeneic MLC it has become evident that both CD4+ and CD8+ T cells equally contribute to the responses, whereas in syngeneic MLC the contribution of CD8+ T cells is minimal or none. This is because fully matured DC expresses an enormous amount of class II on the cell surface, compared with any other known cells. In contrast to class II expression, the level of class I expression on fully matured DC appears not to be as high as class II, and skin Langerhans cells express nearly null class I in vivo [24]. Thus, in syngeneic MLC, CD4+ T cell–DC not CD8+ T cell–DC interaction [25] is unique and results in non-specific polyclonal activation of the CD4+ T subset in vitro. To examine the APC-activity of CD161a-selected DC in vitro, in particular for syngeneic MLC, we prepared two types of in vivo purified T cells from TDL. As described in Section 2, one source of TDL was prepared from LEW rat that had been lethally irradiated and subjected to the thoracic duct drainage, and further inoculated with normal LEW TDL. This population is known to contain more than 99% T cells [26]. Tentatively, this T cell population was labeled as LEW$_{\text{-LEW}}$. The other source of TDL was likewise labeled LEW$_{\text{-DA}}$. The latter LEW$_{\text{-DA}}$ T cell preparation is important to evaluate whether and to what extent the LEW$_{\text{-DA}}$ T cells respond to nominal antigens such as heterologous serum such as FBS in the presence of allogeneic APC. Due to the MHC restriction, LEW$_{\text{-DA}}$ T should not respond to any peptides in allogeneic MHC such as RT1avl of DA. Thus, LEW

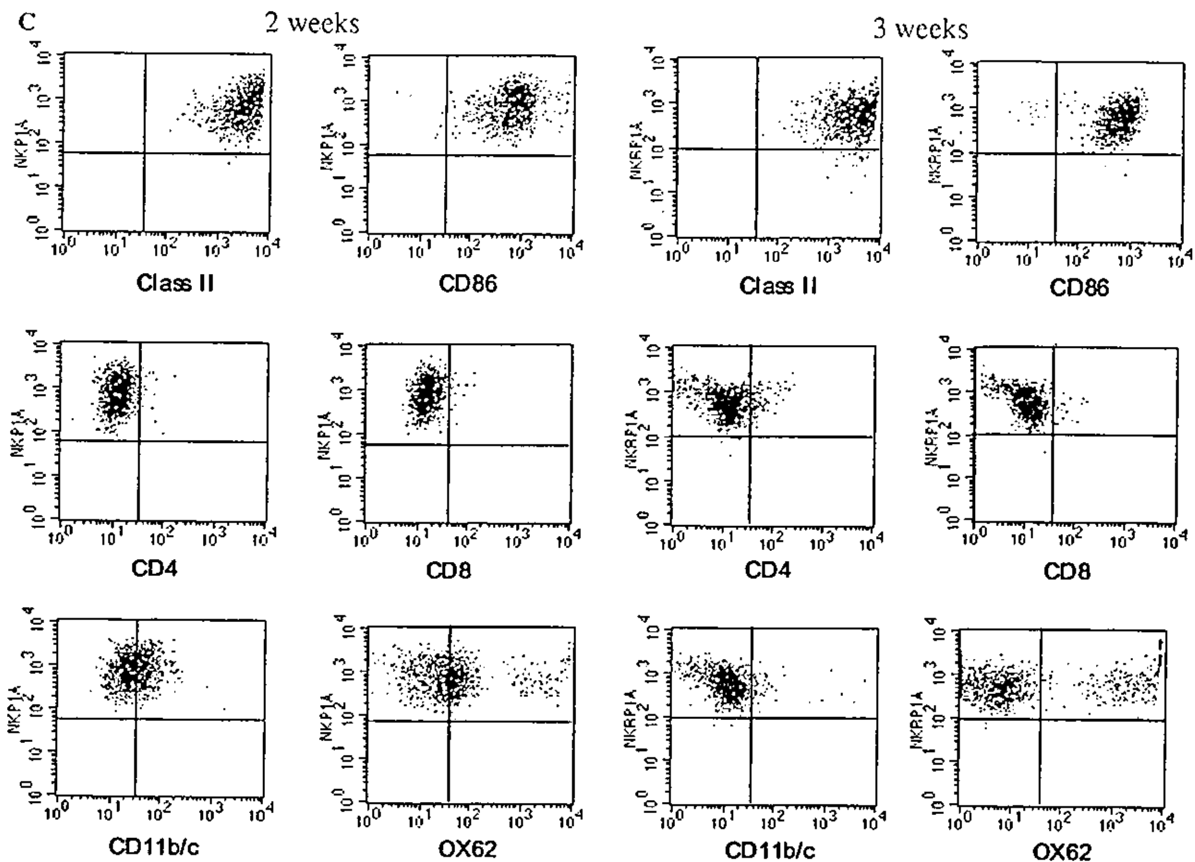


Fig. 2. (*continued*)

TDL was injected into the lethally irradiated DA rat that had been subjected to similar thoracic duct drainage.

Classical local GvH assay demonstrated that LEW$_{\text{-LEW}}$ T cells were active to both allogeneic DA (RT1avl) in (DA × LEW) F1 and PVG (RT1c) in (LEW × PVG) F1, whereas LEW$_{\text{-DA}}$ T cells were functionally inactive with respect to allogeneic MHC of DA, however, the same cells retained their reactivity to third-party allogeneic MHC of RT1c in (LEW × PVG) F1 (Table 1).

In Fig. 4A (2.5% rat serum) and Fig. 4B (10% FBS) demonstrate that in vivo purified LEW$_{\text{-LEW}}$ T cells responded in syngeneic MLC [27] were observed regardless of serum sources (syngeneic or xenogeneic). Likewise both in vivo purified LEW$_{\text{-LEW}}$ T cells and LEW$_{\text{-DA}}$ T cells were equally active in syngeneic MLC (Fig. 4C). Unexpectedly, for allogeneic MLC, CD161a$^+$ DC from DA was found to be able to activate LEW$_{\text{-DA}}$ T cells significantly in the absence of heterologous serum such as FBS, although it was far less strong and dose-dependent manner than that of syngeneic CD161a$^+$ LEW DC (Fig. 4D).

3.5. *Functional analysis of CD161a$^+$ DC in vivo*

Lastly, we examined the in vivo function of CD161a$^+$ DC as a specialized APC. Employing weak histocompatibility antigen difference, rat H-Y antigen, we immunized female rats with isogeneic male DC and subsequently challenged them by male isografts and compared with female rats immunized with fresh BMC. Based on our previous studies and for screening purposes, a single dose of assays was performed. As shown in Table 2, regardless of the cell administration route, as few as 1×10^5 CD161a$^+$ male LEW DC were found to sensitize female rats with male-specific antigen H-Y, hence, female LEW rats rejected male isografts. However, intravenous administration of CD161a$^+$ DC failed to have this effect in DA rats under the immunization protocols. It should be noted that regardless of the route (subcutaneous, intra-peritoneal or intravenous) or sources of cells (fresh BMC or DC described as above), PVG.1U (RT1u), which is known genetically to be non-responder, failed to sensitize with H-Y antigen.

4. Discussion

Several findings emerge from our study.

First, unlike mouse systems [8], rat DC progenitor in BMC did not fully respond to GM-CSF in terms of the growth of DC progenitor. Murine GM-CSF, both mouse and rat origin, do have a similar capacity to form granulocyte and Mϕ colonies in agar medium cultures

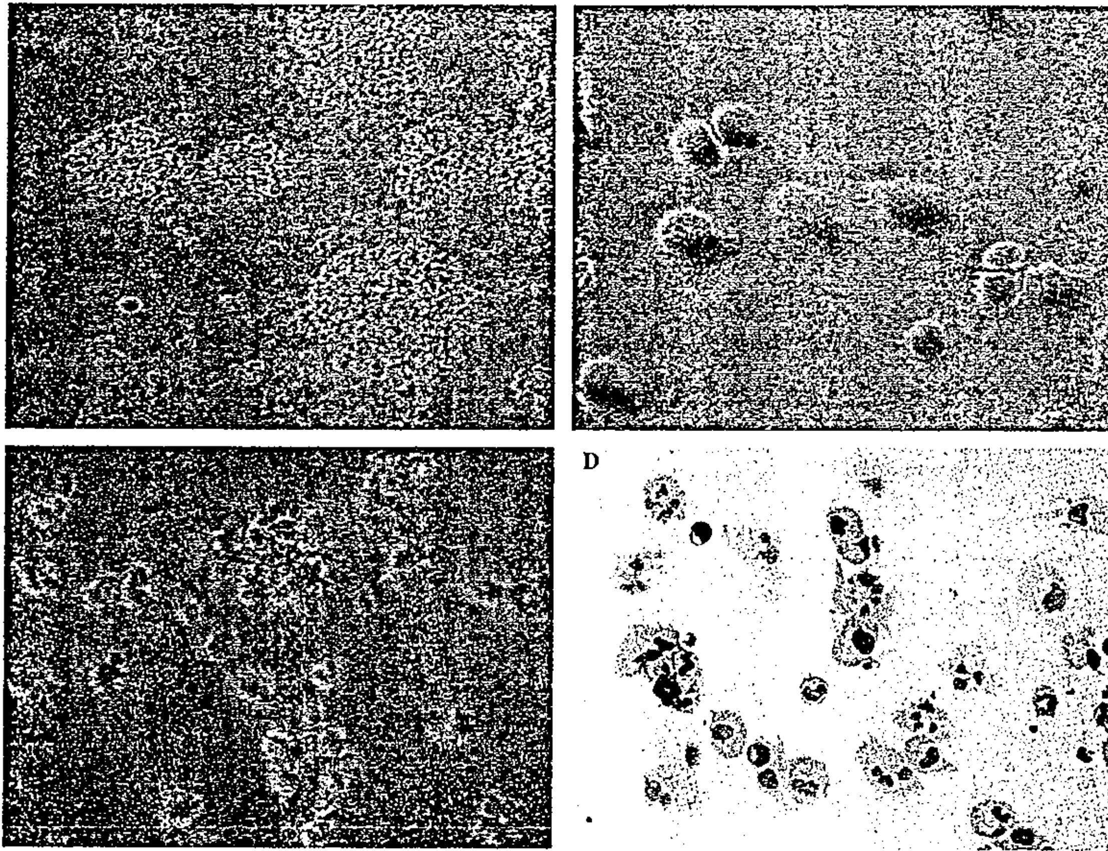


Fig. 3. Typical DC culture generated by Flt3/Flk2 ligand and IL-6 for eight weeks culturing was shown. When DC were generated spontaneously by Flt3/Flk2 ligand and IL-6, the most prominent picture was seen under the microscope. Large DC cell cluster or colony-like cell cluster appeared (A). With anti-CD161a (NKR-P1A), one of the C type lectin family, BMC-derived DC was positively separated by autoMACS systems as described in Section 2, and cytospin preparation was made following separation. Its fresh cytospin preparation with phase contrast image revealed the interference of phase contrast by magnetic bead attached on cell surface of DC. Hence its appearance looked as much smooth and as glossy (B). Phase contrast outlook of cell suspension for a short-term culture after the separation (C), and its staining by May–Grünwald and Giemsa was shown (D).

Table 1
Local GvH assay by recirculating T cells

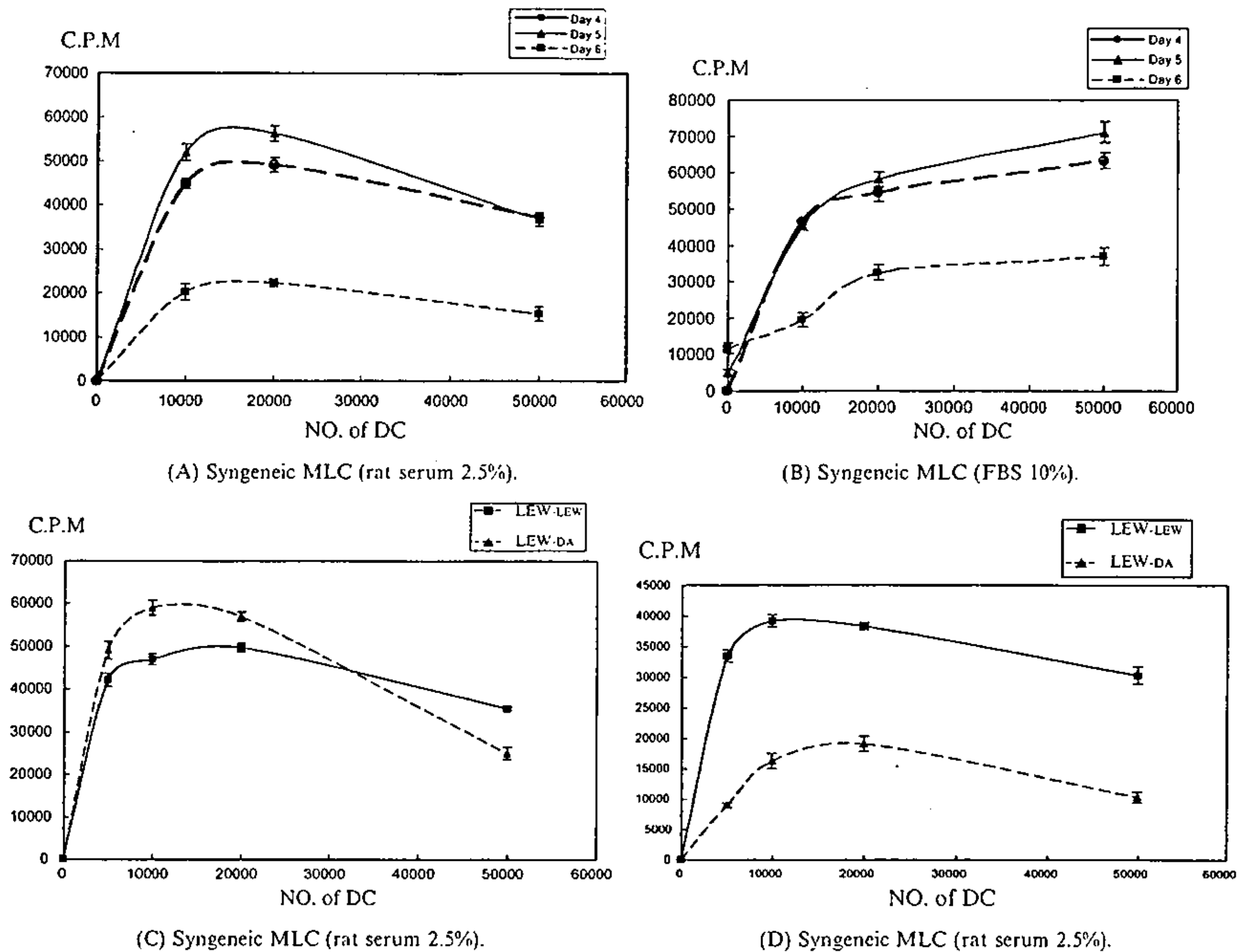
Source of cell population	F1 hybrid	LN weight ± SE (mg)[a]	
		Injected side	Control
LEW_{-LEW} TDL[b]	DA × LEW	128 ± 21	5.1 ± 0.4
	LEW × PVG	99 ± 8	4.7 ± 0.2
LEW_{-DA} TDL	DA × LEW	7.0 ± 0.8	4.2 ± 0.1
	LEW × PVG	92 ± 6	4.8 ± 0.3

[a] Each estimates is the mean of four assay animals with 10×10^6.
[b] 10^7/0.1 ml of re-circulating T cells (>99% purity) were injected into foot pads of F1 recipients at day 7, popliteal lymph nodes were removed and weighed.

containing mouse and rat GM-CSF (data not shown). However, culturing rat BMC with a single supplement of GM-CSF failed to significantly increase DC yield. It appears that GM-CSF per se facilitates the differentiation of DC progeny rather than the growth of DC progenitors.

Second, combined cytokines of rat c-kit ligand and human IL-6 are able to support undefined BMC precursors by five hundred folds to one thousand folds within one month by in vitro culture [12], however, they fail to increase DC progeny of rat BMC. Indeed, the early expanded smooth-surfaced, round cells rapidly changed into large, irregular and spindled cells following a GM-CSF-containing medium, however, these cells were unable to differentiate into typical veiled-typed DC. Heretofore most undefined adherent cells were not fully characterized, how GM-CSF, TNF-α, and IL-4 influence the induction of DC is not known.

Third, combined Flt3/Flk2 ligand and IL-6 [21] appeared to support the growth of a low-density fraction BMC for at least the first four week's culture. It maintained the proliferation of round smooth-surfaced cells along with other adherent cells. Regarding the undefined adherent irregularly shaped cells, combined Flt3/Flk2 ligand and IL-6 culture systems resulted in far fewer adherent cells compared with those of GM-CSF supplemented cultures. At any time points of cultures, switching into the cocktails of cytokines such as IL-4 or TNF-α (data not shown), down-regulated the proliferation activity of smooth round-shaped cells. Thus,



(A) Syngeneic MLC (rat serum 2.5%).

(B) Syngeneic MLC (FBS 10%).

(C) Syngeneic MLC (rat serum 2.5%).

(D) Syngeneic MLC (rat serum 2.5%).

Fig. 4. (A) Highly purified LEW T cells (LEW_{-LEW}) (>99%) were prepared by collecting thoracic duct lymph filtration in vivo through lethally irradiated LEW rat that had been under the thoracic duct drainage and injected with 1.5×10^9 normal LEW TDL as described in Section 2. About 2×10^5 LEW_{-LEW} T cells were cultured with various numbers of $CD161a^+$ LEW DC generated with Flt3/Flk2 ligand and IL-6 from long-term bone marrow cell (BMC) cultures. During the syngeneic MLC cultures at day 4, day 5, and day 6, 0.5 μCi methyl-[^{3}H]thymidine of 25 μl was added to cell suspension in a 6 h pulse. In this culture 2.5% syngeneic LEW rat serum was employed. Optimal cell ratio and maximal activation occurred around 20T cell/1 DC and at day 4 to day 5, respectively. (B) Highly purified LEW T cells (LEW_{-LEW}) (>99%) were prepared by collecting thoracic duct lymph filtration in vivo through lethally irradiated LEW rat that had been under the thoracic duct drainage (LEW_{-LEW}) and injected with 1.5×10^9 normal LEW TDL as described in Section 2. About 2×10^5 LEW_{-LEW} T cells were cultured with various number of $CD161a^+$ LEW DC generated with Flt3/Flk2 ligand and IL-6 from long-term bone marrow cell cultures. During the syngeneic MLC cultures at day 4, 5, and 6, 0.5 μCi methyl-[^{3}H]thymidine of 25 μl was added to cell suspension in a 6 h pulse. In this culture 10% FBS was employed. In the presence of heterologous serum such as FBS, optimal cell ratio was not determined. However maximal activation likewise occurred at day 4 to 5. (C) In order to determine the relative difference between purified LEW T cells filtered through syngeneic LEW or allogeneic DA rat with syngeneic MLC reaction, reactivity was estimated at single day 4 that appears to be maximal [^{3}H]thymidine incorporation. Thus 2×10^5 LEW_{-LEW} and LEW_{-DA} T cells were likewise cultured with various numbers of $CD161a^+$ LEW DC generated with Flt3/Flk2 ligand and IL-6 from long-term bone marrow cell cultures. (D) In order to determine the functional difference between purified LEW T cells filtered through syngeneic LEW or allogeneic DA rat with allogeneic MLC reaction, reactivity was estimated at single day 5 that appears to be maximal [^{3}H]thymidine incorporation. Thus 2×10^5 LEW_{-LEW} and LEW_{-DA} T cells were likewise cultured with various number of $CD161a^+$ DA DC generated with Flt3/Flk2 ligand and IL-6 from long-term bone marrow cell cultures. Although it was weaker than LEW_{-LEW}, LEW_{-DA} T cells were significantly activated with a wide range of $CD161a^+$ allogeneic DA DC. This was sharp contrast to in vivo reactivity of LEW_{-DA} to DA alloantigen.

typical veiled-shaped, free-floating VC or DC appeared in substantially large numbers along with large irregularly shaped adherent cells that outnumbered the DC colony. A population of DC-committed progeny of BMC appeared to account for a small percentage of cells compared with those of Mϕ-committed progeny. It should be noted that additional cytokines such as GM-CSF, IL-4 and/or TNF-α rendered proliferating free round cells into irregularly shaped adherent cells. Differentiation of DC-committed progeny into fully mature DC, however, was found not to be totally dependent on these cytokines. Rather these cytokine combinations appear to facilitate the DC progeny to differentiate further into fully mature DC.

Regarding the phenotype of DC specific markers, no single-cell surface marker has been able to define DC

Table 2
Comparison of the effect of fresh BMC and DC prepared from male donor on sensitization of female recipients

Experimental animals	Source of cell used for sensitization	Source of cells	Graft survivals (days)[a]	Median of survival times (MST)
LEW ♂ → ♀ LEW	IV[b]	Fresh BMC	12, 12, 13, 15, 16, 17	15
	SC[b]		14, 14, 15, 17, 78	14
	IV	DC[c]	21, 27, 42, 58, > 100	42
	SC		10, 11, 12, 18, 23, 57	15
DA ♂ → ♀ DA	IV	Fresh BMC	68, 9× > 100	100
	SC		19, 21, 23, 28, 2× > 100	25.5
	IV	DC	23, 17× > 100	100
	SC		17, 18, 24, 33, 48, 3× > 100	40.5
PVG.RT1U ♂ → ♀ PVG.RT1U		Fresh BMC	ND[d]	
	IV	DC	4× > 100	100
	SC		8× > 100	100

[a] Following the immunization of female rats with male cells, one month later the recipient rats received male skin isografts as described in Section 2. The fate of the graft was followed.
[b] Fresh male BMC without culture were prepared as described in Section 2. Two doses of 20×10^6/0.1 ml of cells were injected either in the foot pads (subcutaneous, SC) or through the tail vien (IV).
[c] Male DC from the cultured BMC as described in Section 2 were prepared. Two doses of 0.1×10^6/0.1 ml of cells were injected either in the foot pads (SC) or through the tail vien (IV).
[d] ND: not determined.

specifically. Although in mouse DC, CD11c is widely used to define a subset of DC, equivalent rat CD11b/c (OX42) was expressed at an extremely low level or null on rat DC described here. Furthermore, classification of mouse *plasmacytoid* DC generated by Flt3 ligand is characterized by CD11c expression with B220 (CD45R), and proposed by others to distinguish the *plasmacytoid* or *lymphoid* DC from myeloid DC. The latter differentially expressed CD11b but not B220 [28]. With this respect, the rat DC generated in this study appears to belong to neither typical plasmacytoid nor lymphoid DC subsets.

As for the DC specific marker, CD103 (MRC-OX62) has been characterized by Brenan and Puklavec [16] and has been considered to be a specific marker for a subset of DC. Two subsets of rat DC have been reported; (i) $CD103^-$ phenotype consisting of DC in the epidermal Langerhans cells [16], and (ii) $CD103^+$ phenotype consisting of DC-like veiled cells in the thoracic duct lymph (TDL) [16,29,30]. Additionally, $CD161a^+$ (also defined as NKR-P1A [23,31]) was found to be positive for a subset of rat DC. It is tempting to define rat DC by two markers, CD103 and CD161a. By this criteria, DC driven by Flt3/Flk2 ligand and/or IL-6 appear to be defined $CD161a^+$ $CD103^-$. In this study, we confirmed and extended the study by Brissette-Storkus et al. [32]. These investigators also described a short-term rat BMC culture driven by *Flt3/Flk2 ligand* in FBS based culture medium resulting in $CD161a^+$ $CD103^-$ phenotype DC, although the expression level of CD161a appears to be far below that of our study. Nevertheless, the level of CD161a as well as class II expression on rat DC cultured in the present study gradually increased during a long-term culture. It appears that the level of CD161a is intimately related to the maturation processes of DC progeny. Whether CD161a expression of DC during the maturation processes is a general feature of the DC subset or only a unique characteristic of rat DC remains to be determined.

The view of that GM-CSF plays a primary role in growth and differentiation of DC precursor cells, was first suggested by Steinman and his colleagues in mouse systems [8,9,33]. However, based on our study, it is tempting to postulate that GM-CSF per se has a limited capacity to increase DC progenitors of BMC but instead acts as a differentiation factor in general. It should be noted that both the GM-CSF deficient mouse and the GM-CSF receptor deficient mouse contained a substantial number of DC, although the level of the cell number was significantly lower than both the normal mouse and the GM-CSF transgenic mouse [34]. In this regard, our preliminary and on-going studies (manuscript in preparation) demonstrate that our culture systems are likewise applied to generate *myeloid DC* from BMC, not only from the GM-CSF deficient mouse and GM-CSF receptor deficient mouse [35], but also in the human system. Thus, our culture systems demonstrate that Flt3/Flk2 ligand combined with IL-6 is able to replace the effect of GM-CSF on the outgrowth of DC precursors from the BMC culture. These results strongly suggest a common pathway, which is *GM-CSF independent myeloid DC* development [15,36,37]. Different subsets of DC-precursors might require different cytokines, depending on the cell-differentiation stages. Nevertheless, at least in the rat system, the absolute requirement of exogenous GM-CSF for BMC-derived *myeloid DC induction* appears to be less likely.

As for the function of *GM-CSF independent myeloid DC*, the APC function of CD161a$^+$ DC generated by *GM-CSF independent* rat BMC culture driven by Flt3/Flk2 ligand and IL-6 was verified by in vitro syngeneic MLC as well as in vivo sensitization of minor histocompatibility antigen H-Y, which is considered to be a prototype of tumor immunology. Thus, the simple culture methods described here would facilitate the acquisition of a large number of highly uniform subsets of DC for cellular immunology research including transplantation biology as well as tumor immunology.

Heretofore, valuable information and knowledge have been accumulated on the prerequisite cytokines for DC development and classification of DC subsets. However, it remains to be determined whether and to what extent each cytokine, or cytokines in concert, play a crucial role in growth and/or differentiation of particular DC subsets from hematopoietic stem cells, and whether to what extent so-called DC specific markers are stably expressed during the their life span. Our simplified culture method described here may be applied to further investigation of the functional and developmental aspects of DC subset.

In summary, we have demonstrated that GM-CSF per se is not able to support a significant growth of DC progenitors in rat BMC, regardless of the sources (mouse, rat, human). Combined with Flt3/Flk2 ligand and IL-6, not with c-kit ligand and IL-6, undefined DC progenitors emerge, and these cytokines appear to be able to increase DC progeny from rat BMC. Thus, these expanded DC precursors are able to differentiate into fully mature DC in both phenotype and function as specialized APC. Functionally full-mature DC express at a high level of CD161a and hence the mature DC were highly purified by this single cell surface marker. With additional cytokines such as murine GM-CSF, TNF-α and IL-4, it appears that heterogeneity of phenotypic and functional DC [38–40] is generated. These additional cytokines in concert, not a single cytokine, might enable generating phenotypic and functional diversity of DC in vivo.

Acknowledgments

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Study of Antiretroviral Drug–Resistant HIV-1 Genotypes in Northern Thailand: Role of Mutagenically Separated Polymerase Chain Reaction as a Tool for Monitoring Zidovudine-Resistant HIV-1 in Resource-Limited Settings

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Summary: As the number of HIV-1–infected individuals receiving antiretroviral drugs has been rapidly increasing in developing countries, there is an urgent need for drug resistance genotype information of non-B subtype HIV-1 and for the establishment of a practical system of monitoring drug-resistant viruses. This study first sequenced the reverse transcriptase region of HIV-1 in 112 infected individuals who had been treated with zidovudine (AZT)/didanosine or AZT/zalcitabine as dual therapy at a government hospital in northern Thailand and then compared the above sequence method with mutagenically separated polymerase chain reaction (MS-PCR) for detecting M41L and K70R mutations. Concordant rates of detecting M41L and K70R mutations by the 2 methods were 96.9% (93/96) and 92.7% (89/96), respectively. The M41L and K70R MS-PCR could detect 86.4% of AZT-resistant strains with any resistance mutation, which was determined by the sequencing method. Then 292 drug-naive individuals were screened for the presence of drug-resistant HIV-1 by the MS-PCR assay and it was found that 2 individuals (0.7%) carried viruses with either the M41L or K70R mutation. It is feasible to test a large number of samples with MS-PCR, which is sensitive, cheap, and easy to perform and does not require sophisticated equipment. The M41L and K70R MS-PCR is potentially a useful tool to monitor the spread of AZT-resistant HIV-1 in resource-limited countries.

Key Words: HIV, CRF01_AE, Thailand, antiretroviral drug, drug resistance

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HIV-1 has tremendous ability to mutate swiftly and to develop resistance to almost all clinically used antiretroviral drugs. Reduced sensitivity to nucleoside reverse transcriptase inhibitors (NRTIs), non-NRTI (NNRTIs), and protease inhibitors has been studied extensively and is linked to specific point mutations in either the reverse transcriptase or the protease gene.[1] Most current knowledge for interpreting these genotypic changes has been derived from studies on HIV-1 subtype B viruses. Worldwide, however, the majority of HIV-1–infected people live in developing countries and most of them are infected with non-B subtypes. Non-B subtypes differ from subtype B in *pol* gene by 10–15%.[2] We and other groups have published data showing some discrete differences in the patterns of drug resistance mutations between subtypes.[3–5] With a growing demand for access to antiretroviral therapy in resource-limited countries, the resistance patterns of non-B subtype viruses to antiretroviral drugs are becoming an important issue.

In Thailand, with a population of approximately 62 million, it was estimated that 695,000 people were living with HIV-1 infection. Of these, the majority are infected with CRF01_AE (previously known as subtype E), and 55,000 people had AIDS in 2000.[6] In the past, only a small minority of HIV-1–infected patients could afford antiretroviral drugs due to the high monthly price; thus most were either not treated or were treated with suboptimal antiretroviral regimens, mostly dual therapy.[7] When patients are treated suboptimally, HIV-1 acquires resistance to drugs more quickly.

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Furthermore, the prevention of mother-to-child HIV-1 transmission (PMTCT) program, which uses a short-course zidovudine (AZT) regimen, was expanded first in northern Thailand in 1997 and later throughout the country.[8] This expanded program could also trigger the emergence of AZT drug resistance. Studies of HIV-1–infected individuals with primary HIV infection have shown that drug-resistant HIV strains can be transmitted from one adult to another and occasionally transmitted vertically from mother to child.[9–12] To control the spread of drug-resistant HIV-1, a monitoring system of antiretroviral drug-resistant HIV-1 in an epidemiologic scale is urgently needed.

The *pol* gene has been commonly sequenced for testing drug resistance in many HIV laboratories of developed countries. However, access to the sequencing test in developing countries is limited due to the relatively high costs of reagents and unavailability of expensive equipment such as an automated sequencer. Polymerase chain reaction (PCR)-based assays are an alternative method of detecting point mutations, having the advantage of increased sensitivity and low cost. Allele-specific primer extension assays have been applied to detect drug-resistant HIV-1; however, they have not been adequately specific for widespread application.[13,14] Conversely, mutagenically separated PCR (MS-PCR) is a PCR-based point mutation assay that overcomes this specificity limitation and has been successfully applied to detect drug-resistant HIV-1 of non-B subtypes.[15–17] Previous papers evaluated the performance of MS-PCR in developed countries, but it has not yet been used in resource-limited settings.

We conducted this study with the following objectives: to evaluate the performance of MS-PCR specific for M41L and K70R mutations in detecting AZT-resistant HIV-1 strains in Thailand; to apply the MS-PCR to the screening of AZT-resistant HIV-1 among drug-naive HIV-1–infected Thais; and to investigate the patterns and prevalence of drug-resistant genotypes among HIV-1–infected Thai individuals who had been treated with suboptimal antiretroviral regimens by sequencing the *pol* gene.

PATIENTS AND METHODS

Study Population

We used samples obtained from HIV-1–infected individuals who attended the Day Care Center clinic at the Lampang Hospital from July 6, 2000, to July 15, 2001 and gave a written informed consent. The Lampang HIV study was approved by the Thai government ethics committee. The Lampang Hospital is a government referral hospital with approximately 800 beds, situated in the center of Lampang province, which is 100 km south of Chiang Mai in northern Thailand. Plasma samples were collected from these individuals and stored at −80°C until their use. Viral load measurement was conducted using a commercial kit (Amplicor HIV-1 Monitor Test, version 1.5; Roche Diagnostics, Branchburg, NJ). CD4+ cell count was measured by flow cytometory (FACScan; Becton-Dickinson, Franklin Lakes, NJ).

Direct Sequencing

After viral load measurement, the residual RNA was used for drug resistance genotyping by sequencing *pol* gene as previously described.[17] Briefly, an 888-basepair (bp) reverse transcriptase fragment (base number of nucleotide: 2485-3372) was amplified by PCR after a reverse transcription (RT) reaction from the RNA by an RNA-PCR kit (AMV One Step RNA PCR Kit; Takara, Osaka, Japan). Primary PCR products were further amplified with a high-fidelity DNA polymerase (KOD DNA polymerase; Toyobo, Osaka, Japan). Sequencing was performed using an autosequencer ABI-3100 (Applied Biosystems, Foster City, CA) with dye terminators (BigDye Terminator Cycle Sequencing Ready Reaction Kit; Applied Biosystems). The sequence results were assembled and aligned on the reference HIV sequence of HIV-1 HXB2 (GenBank accession number M38432) by ABI Prism SeqScape Software (Applied Biosystems). They were submitted to Stanford HIV RT and Protease Sequence Database (http://hivdb.stanford.edu/) for drug resistance genotyping.

M41L and K70R MS-PCR

On measuring the viral load, we used the residual RNA for the MS-PCR experiments. The method of MS-PCR for detecting M41L and K70R AZT resistance mutations in CRF01_AE has been described in a previously published paper.[17] Briefly, the first-round RT-PCR was conducted to amplify a 370-bp RT region, which spans codon 41 and 70. The second- and third-round PCR were conducted with 1 common forward primer and 2 reverse primers, which are allele specific. The size of the wild-type specific primer was designed about 20 nucleotide bases longer than the mutant type-specific primer so that the wild-type PCR product could be easily differentiated from the mutant-type PCR product by electrophoresis with a 3% agarose gel. We used clinical samples, of which drug-resistant genotypes were confirmed by the sequencing method used for positive controls, and included them for every experiment. When an MS-PCR result showed double bands, of which one was faint, we retested the sample.

Statistical Methods

We compared proportions by the χ^2 test. Means of continuous variables were compared by a nonparametric test, the Kruskal-Wallis one-way analysis of variance. The data management and statistical analysis were conducted using Epi Info version 6.04.

RESULTS

A total of 489 HIV-1–infected individuals attended the clinic during the observation period. History of antiretroviral

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drug therapy was available from 487 infected individuals, in whom 336 were drug naive and 151 were drug experienced; 22 individuals were exposed to a single NRTI, 114 to two NRTIs, 14 to three or more antiretroviral drugs including protease inhibitors, and one did not have regimen information.

Patterns and Prevalence of Drug Resistance Mutations Among Suboptimally Treated Individuals

Out of the 114 individuals who had been exposed to 2 NRTIs, we first analyzed 112 individuals who had received AZT/didanosine (ddI) or AZT/zalcitabine (ddC) dual therapy for the sequencing of RT region; the other 2 individuals had received either d4T/ddC or AZT/lamivudine (3TC). A total of 108 individuals (96.4%) were infected via the heterosexual route. The median (range) duration of dual therapy was 621 (28–1600) days; 76 individuals were on therapy and 36 patients were off therapy at the time of sampling; 28 individuals were exposed to AZT/ddI, 76 to AZT/ddC, and 8 to both AZT/ddI and AZT/ddC. Sequence data were obtained from 99 individuals, of whom 57 (57.6%) had mutations that are known to be associated with AZT, ddI, or ddC resistance. We could not obtain sequence data from 13 individuals, mainly because of their low viral load; 10 had a viral load under the detectable level (<400 copies/mL). Assuming that the individuals without sequence data did not have any drug-resistant virus, the prevalence of drug-resistant viruses detected by the sequencing method was 57/112 (50.9%) among those who had received dual therapy. If confined to the 76 individuals on therapy, 48 (63.2%) had drug-resistant viruses. Figure 1 summarizes amino acid variations at the sites, which are known to be associated with AZT, ddI, or ddC resistance mutations in subtype B infection. The most common drug resistance mutations were D67N, followed by K70R and T215Y/F. Other mutations were also commonly seen at codon 41, 210, and 219. No mutation of Q151M was found. We also analyzed associations among these specific mutations. The presence of D67N was strongly associated with K70R and less significantly with M41L; 31 (86.1%) out of 36 individuals with D67N mutation had either a K70R or M41L mutation (Table 1). The presence of T215Y/F was strongly associated with M41L but not with K70R; 22 (71%) of 31 individuals with T215Y/F mutation had K70R or M41L mutation (Table 1).

TABLE 1. Associations of D67N and T215Y/F With M41L and K70R Mutations

	Codon 67 Mutation					
	M41L*		K70R†		M41L or K70R*	
	Wild	Mutant	Wild	Mutant	Wild	Mutant
Codon 67						
D	57	6	55	8	49	14
N	26	10	12	24	5	31

*$P < 0.0001$, †$P = 0.017$.

	Codon 215 Mutation					
	M41L*		K70R		M41L or K70R†	
	Wild	Mutant	Wild	Mutant	Wild	Mutant
Codon 215						
T	66	2	46	22	45	23
Y/F	17	14	21	10	9	22

*$P < 0.0001$, †$P = 0.0005$.

Drug Resistance Mutations in Relation to the Duration of Antiretroviral Therapy

The prevalence of drug resistance mutations correlated with the duration of dual therapy among 76 individuals on therapy. Among those with the duration of therapy for <180 days, 180–365 days, and >365 days, drug-resistant viruses were found to be predominant in 3 (30%), 8 (62%), and 33 (69%) individuals, respectively, with the median numbers of drug resistance mutations of 0, 2, and 2, respectively (Fig. 2). The number of drug resistance mutations was significantly associated with the median level of viral load: the median (interquartile range, IQR) viral load of individuals with no mutation, with 1–4 mutations, and with ≧5 mutations was 7412 (<400–62,432); 37,871 (7866–105,105); and 156,989 (32,682–184,767) copies/mL, respectively ($P = 0.018$ by Kruskal Wallis one-way analysis of variance).

Codon	41	44	65	67	69	70	74	108	118	151	184	210	215	219
Consensus B	M	E	K	D	T	K	L	V	V	Q	M	L	T	K
N=99	L(12) V(1) I(2)	D(3)	V(1)	N(33) E(2) G(2)	D(1) N(2) S(1) S_SG(1)	R(29)	V(1)	-	I(6)	-	-	W(13) F(2) M(1)	Y/ F(28) I(10) S(2) N(1)	Q/ E(20) N(1)

FIGURE 1. Patterns of AZT, ddI, or ddC resistance mutations. This figure shows amino acid variation at known AZT, ddI, or ddC resistance mutation sites. The frequency of each substitution is shown in parentheses. Reported drug resistance–associated mutations are shown in bold.

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