ditions は，94℃2 分→(94℃15 秒，60℃30 秒，72℃2 分を 30 セット)→72℃7 分→4℃ とした．

対照として，CMV の滅菌精製水による希釈を Positive Control, PA の滅菌精製水による希釈を PA Negative Control，滅菌精製水のみを Negative Control とした．反応後，電気泳動を行い PCR 産物を確認した．判定は，検出陽性時を＋(CMV ゲノムの破壊なし)，検出陰性時を－(CMV ゲノムの破壊あり)とした．

7. Real-Time PCR 法

Real-Time PCR による CMV-DNA の定量は，定性 PCR で用いた 742 bp サイズでの PCR primer, TaqMan probe(5′-ACCGGTTTGGCAACATTCCATTGTG-3′)および TaqMan Universal PCR Master Mix(Perkin-Elmer Applied Biosystems)を用い，ABI PRISM 7700 Sequence Detector System(Perkin-Elmer)にて行った．cycling conditions は，50℃ 2 分→95℃ 10 分→(95℃ 15 秒，60℃1 分，72℃1 分を 40 セット)→4℃ とした．Positive Control として既知の DNA 量のプラスミドを用いそれとの比較から検体の CMV-DNA 量を算出した．Negative Control として滅菌精製水を用いた．CMV-DNA 量は，copies/mL にて表記した．

結　果

1. CPE の観察による感染価の定量

0.2% PA および 0.3% PA と CMV 懸濁液の等量作用の成績をそれぞれ表 1 および表 2 に示した．CPE における感染価を LD_{50} 値(表中の矢印)にて表記した．0.2% PA の作用では初期感染価に対し，5 分後に $10^{1.58}$ $TCID_{50}$/mL，10 分後に $10^{1.71}$ $TCID_{50}$/mL，30 分後に $10^{2.68}$ $TCID_{50}$/mL の感染価の減少が認められた．また 0.3% PA の作用では初期感染価に対し，5 分後に $10^{1.95}$ $TCID_{50}$/mL，10 分後に $10^{2.71}$ $TCID_{50}$/mL，30 分後に $10^{3.31}$ $TCID_{50}$/mL の感染価の減少が認められた．作用時間の推移により感染価が減少した．

2. 定性 PCR 法による検討

early 遺伝子を含む 742 bp サイズの増幅領域における 0.3% PA と CMV 懸濁液の等量作用の成績を表 3 に示した．同様に 2% GA の成績について表 4 に示した．また，early 遺伝子を含む 2,952 bp サイズの増幅領域における 0.3% PA の成績を表 5 に示した．ウイルス初期濃度時の PCR 産物に対し，742 bp サイズの増幅領域では，PA および GA ともに，10 分後に 25%，30 分後に 50%が消失した．5 分後の短時間作用時では，GA よりも PA のほうが PCR 産物を消失させていた．2,952 bp サイズの増幅領域では，PCR 産物が 5 分後に 75%，10

表 1　0.2%過酢酸と CMV の等量混合での CPE 観察

CMV の希釈	10^{-2} 倍	10^{-3} 倍	10^{-4} 倍	10^{-5} 倍	10^{-6} 倍	LD_{50} titer*1
N.C.*2	0/6	0/6	0/6	0/6	0/6	—
N.C.(0.2% PA*3)	0/6	0/6	0/6	0/6	0/6	—
P.C.*4	12/12	12/12	12/12	12/12	0/12	—
5 分	22/24	18/24 ↓	6/24	2/24	0/24	−3.42
10 分	20/24	17/24 ↓	6/24	0/24	0/24	−3.29
30 分	8/16 ↓	3/16	2/16	0/16	0/16	−2.32

CPE の認められるウェル数/総ウェル数
*1) LD_{50} titer：50% lethal dose (↓で対数表示)
*2) N.C.　：Nagative Control
*3) PA　：Peracetic acid
*4) P.C.　：Positive Control

表 2　0.3%過酢酸と CMV の等量混合での CPE 観察

CMV の希釈	10^{-2} 倍	10^{-3} 倍	10^{-4} 倍	10^{-5} 倍	10^{-6} 倍	LD_{50} titer*1
N.C.*2	0/6	0/6	0/6	0/6	0/6	—
N.C.(0.3% PA*3)	0/6	0/6	0/6	0/6	0/6	—
P.C.*4	12/12	12/12	12/12	12/12	0/12	—
5 分	18/24	15/24 ↓	6/24	0/24	0/24	−3.05
10 分	11/24 ↓	8/24	0/24	0/24	0/24	−2.29
30 分 ↓	1/16	0/16	2/16	0/16	0/16	−1.69

CPE の認められるウェル数/総ウェル数
*1) LD_{50} titer：50% lethal dose (↓で対数表示)
*2) N.C.　：Nagative Control
*3) PA　：Peracetic acid
*4) P.C.　：Positive Control

表3　0.3%過酢酸とCMVの等量混合でのPCR反応結果（分子量742 bp）

10倍希釈系列	10^2倍	10^3倍	10^4倍	10^5倍	10^6倍
N.C.*1	−	−	−	−	−
N.C.(0.3% PA*2)	−	−	−	−	−
P.C.*3	+	+	+	+	−
5分	+	+	+	−	−
10分	+	+	+	−	−
30分	+	+	−	−	−

+：検出陽性　−：検出陰性
*1) N.C.：Nagative Control
*2) PA　：Peracetic acid
*3) P.C.：Positive Control

表4　2%グルタラールとCMVの等量混合でのPCR反応結果（分子量742 bp）

10倍希釈系列	10^2倍	10^3倍	10^4倍	10^5倍	10^6倍
N.C.*1	−	−	−	−	−
N.C.(2% GA*2)	−	−	−	−	−
P.C.*3	+	+	+	+	−
5分	+	+	+	+	−
10分	+	+	+	−	−
30分	+	+	−	−	−

+：検出陽性　−：検出陰性
*1) N.C.：Nagative Control
*2) GA　：Glutaraldehyde
*3) P.C.：Positive Control

表5　0.3%過酢酸とCMVの等量混合でのPCR反応結果（分子量2,952 bp）

10倍希釈系列	10^2倍	10^3倍	10^4倍	10^5倍	10^6倍
N.C.*1	−	−	−	−	−
N.C.(0.3% PA*2)	−	−	−	−	−
P.C.*3	+	+	+	+	−
5分	+	−	−	−	−
10分	−	−	−	−	−
30分	−	−	−	−	−

+：検出陽性　−：検出陰性
*1) N.C.：Nagative Control
*2) PA　：Peracetic acid
*3) P.C.：Positive Control

分後に100%，それぞれ消失した．0.3% PAの作用における増幅領域の大きいサイズでは，PCR産物の破壊や断片化が起きていることが示唆された．

次に，742 bpサイズの増幅領域において，CMV懸濁液に対し0.3% PAを10倍段階増量で作用させたときの成績を表6に示した．同様に2% GAの成績について表7に示した．CMV懸濁液の濃度が薄くなるほど，つまりPA量が増量するほど短時間でPCR産物の消失が顕著に認められた．CMV懸濁液に対し，100倍の0.3% PA量では10分後に，1,000倍の0.3% PA量では5分後にそれぞれ検出陰性であった．これに対しGAは，

表6　0.3%過酢酸とCMV濃度変化時のPCR反応結果（分子量742 bp）

CMV濃度の希釈	10倍	100倍	1,000倍
N.C.*1	−	−	−
N.C.(0.3% PA*2)	−	−	−
P.C.*3	+	+	+
5分	+	+	−
10分	+	−	−
30分	+	−	−

+：検出陽性　−：検出陰性
*1) N.C.：Nagative Control
*2) PA　：Peracetic acid
*3) P.C.：Positive Control

表7　2%グルタラールとCMV濃度変化時のPCR反応結果（分子量742 bp）

CMV濃度の希釈	10倍	100倍	1,000倍
N.C.*1	−	−	−
N.C.(2% GA*2)	−	−	−
P.C.*3	+	+	+
5分	+	+	+
10分	+	+	+
30分	+	+	+

+：検出陽性　−：検出陰性
*1) N.C.：Nagative Control
*2) GA　：Glutaraldehyde
*3) P.C.：Positive Control

CMV濃度や時間経過に依存することなく，30分経過後もほぼ横ばい傾向を示した．

3.　Real-Time PCR法による検討

表6および表7で示したCMV懸濁液に対し0.3% PAを10倍段階増量で作用させたときの成績について，Real-Time PCRにより定量化した．その結果を図1および図2に示した．CMV初期濃度に対し，5分後にはCMV-DNA量を99%以上減少させ，PAの短時間におけるウイルスゲノム破壊作用を顕著に認めた．CMV懸濁液に対し，100倍の0.3% PA量では10分後に，1,000倍の0.3% PA量では5分後に，それぞれCMV-DNA量が検出限界以下を示した．一方，GAではCMV懸濁液に対し，1,000倍の2% GA量において30分経過後も10,500 copies/mLの残存が認められた．GAはCMV濃度や時間経過に依存することなくCMV-DNA量が継続的に検出された．

考　　察

近年，PAはGAの毒性[12,13]や耐性菌[14]の問題を解決する内視鏡や鋼製小物類など医療器具の高度消毒薬として注目されている．

今回我々は巨大なDNAゲノムを有するCMVを供試ウイルスとして選択した．CMVは細胞培養の実験が可

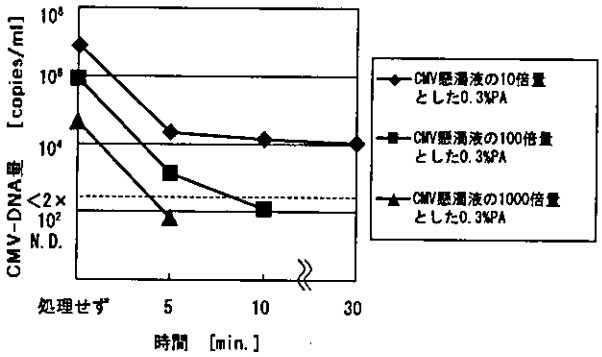


図1　CMV 懸濁液に対し 10 倍段階増量とした 0.3% PA の作用における Real-Time PCR 測定(742 bp サイズの増幅領域)
N.D.　　: Not Detected
処理せず　　: Positive Control
定量測定限界 : CMV-DNA 量が 200 copies/mL 以下とした
0.3% PA の作用では，時間経過に伴い，CMV-DNA 量の顕著な減少を示した

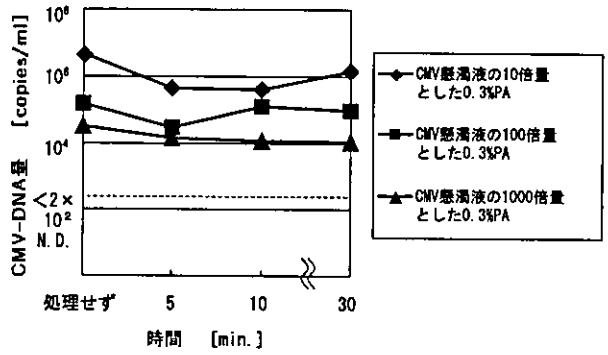


図2　CMV 懸濁液に対し 10 倍段階増量とした 2% GA の作用における Real-Time PCR 測定(742 bp サイズの増幅領域)
N.D.　　: Not Detected
処理せず　　: Positive Control
定量測定限界 : CMV-DNA 量が 200 copies/mL 以下とした
2% GA の作用では，30 分経過後も，CMV-DNA 量がほぼ横ばいを示した

能で，ゲノムの全塩基配列が解読され定量系が確立されていることから利用可能である．本研究では，CMV を用い PA のウイルスゲノムに対する抑制効果を検討するため，PA および CMV 作用液の CPE を指標とした感染価の定量，定性 PCR 法および Real-Time PCR 法による CMV ゲノム破壊作用をそれぞれ検討した．

CPE の観察では，0.3% PA(実用濃度)と CMV 懸濁液(感染価：10^5 $TCID_{50}$/mL)を等量作用させた．その結果，30 分後の感染価が $10^{3.31}$ $TCID_{50}$/mL 減少した．薬剤にはウイルスゲノムに対する抑制効果が現れにくいと考えられた等量混合であるにもかかわらず感染価を有意に減少させた．このことから，PA が CMV の感染増殖能を抑制する働きがあると考えられた．細胞毒性についての検討は，岩沢ら[15]が 0.12% PA における各種細胞への影響を少なからず認められたと報告している．今回の試験では，0.06% PA(PA 原液の 100 倍希釈)における IMR-90 細胞への毒性は確認されなかった．

定性 PCR では，CPE の観察で用いた PA および CMV 作用液から DNA を抽出し特異領域の PCR 産物を検出した．ウイルス初期濃度時の PCR 産物に対し，742 bp サイズの増幅領域では 30 分後に 50%，2,952 bp サイズの増幅領域では 10 分後に 100%，それぞれ消失が認められた．742 bp よりも 2,952 bp といった増幅領域の大きいサイズでは PCR 産物が検出されていないことから，PA が DNA の破壊や断片化に関与していると考えられた．

また，742 bp サイズの増幅領域において，CMV 懸濁液に対し 0.3% PA を 10 倍段階増量として作用させた結果，PA 量を増量するほど短時間で PCR 産物の消失が顕著に認められた．その成績について Real-Time PCR を用いて定量的に検討した結果，5 分後には CMV-DNA 量が 99%以上の減少を認め，PA 量が増えるほど短時間での CMV-DNA 量の減少が顕著であった．すなわち，定性 PCR の成績と Real-Time PCR の成績は一致する結果となった．これに対し 2% GA では，定性 PCR および Real-Time PCR ともにウイルスゲノムの残存が認められた．定量的検討では 30 分後でも CNV-DNA 量がおよそ 10,000 copies/mL 残存していた．これは GA の作用機序である架橋反応による蛋白凝固を起こしたため，ウイルス自体は死滅するもののDNA がほぼ破壊されず残存したと考えられた．GA は CMV のようなヘルペスウイルスに対し短時間での殺ウイルス効果が認められている[8]ことから，必ずしもゲノムの存在がウイルスの生残に結びつくとは考えにくいと推測された．今回の PCR の測定結果と CPE の定量結果において，ウイルスゲノムとウイルス感染価がともに時間経過により減少傾向を示したが，それらが相関するかどうかは解明するまでに至らなかった．

これらの結果から，PA の作用機序がウイルスゲノムの破壊ないしは断片化に関与しているものと推測され，PA が GA よりも短時間で顕著なウイルスゲノム破壊作用を示すことが明らかになった．実際の医療現場では，10 L の 0.3% PA 溶液に 100 mL の CMV 懸濁液(感染価：10^5 $TCID_{50}$/mL 程度)を作用させると，5～10 分でウイルスゲノムまで破壊される計算になる．PA の作用機序は，酢酸(CH_3COOH)，過酸化水素(H_2O_2)および PA(CH_3COOOH)の 3 分子による平衡状態により，酢酸は炭酸塩の除去を，過酸化水素は有機物質の剥離効果を，PA は消毒効果を有するとされている．Tucker[16]らは PA が蛋白質汚れを蓄積させず除去性を高めるとして PA の洗浄効果を報告している．PA のウイルスに対

する消毒効果に関する報告はいくつかある[8-10]が，今回のようなウイルスゲノムを指標としてその破壊作用を検討するにはReal-Time PCR法は有用であると考えられた．このことは，定性PCR産物がReal-Time PCRによって定量的に数値化できたことにより，どの程度ウイルスゲノムが破壊されているかを確認する手法として活用できたことを示唆するものである．

以上より，PAはCPEを指標とした感染価の定量からCMVの感染増殖能の抑制を確認することができ，同時にReal-Time PCR法によりゲノム破壊作用を有することが裏付けられた．よって，CMVに対しPAはGAよりも短時間でウイルス抑制効果を示すと考えられた．

本論文の要旨は，2003年2月14～16日に横浜で開催された第18回日本環境感染学会総会において発表し，座長推薦を受けたものである．

謝　辞：稿を終えるにあたり，PCRに関してご協力いただいたApplied Biosystems Japan Ltd.に厚く御礼申し上げます．

文　献

1) 辻　明良，滝沢信寛，谷　昌樹，五島瑳智子：細菌芽胞に対する過酢酸とグルタールアルデヒドの殺菌効果．環境感染 1997; 12: 62.
2) 西　功，浅利誠志，豊川真弘，堀川晶行，西村信哉：臨床使用後の医療器具および臨床分離株に対する過酢酸製剤の殺菌効果．環境感染 2002; 17: 325-8.
3) Lynam PA, Babb JR, Fraise AP: Comparison of the mycobactericidal activity of 2% alkaline glutaraldehyde and 'Nu-Cidex' (0.35% per acetic acid). J Hosp Infect 1995; 30: 237-40.
4) Griffiths PA, Babb JR, Fraise AP. Mycobactericidal activity of selected disinfectants using a quantitative suspension test. J Hosp Infect 1999; 41: 111-21.
5) Sagripanti JL, Bonifacino A: Comparative Sporicidal Effects of Liquid Chemical Agents. Apple Environ Microbial 1996; 62: 545-51.
6) Barbee SL, Weber DJ, Sobsey MD, Rutala WA: Inactivation of Cryptosporidium parvum oocyst infectivity by disinfections and sterilization processes. Gastrointestinal Endscopy 1999; 49: 605-11.
7) Lensing HH, Oei HL: Investigations on the Sporicidal and Fungicidal Activity of Disinfectants. Zbl Bakt Hyg, I Abt Orig 1985; B 181: 487-95.
8) 坂上吉一，勝川千尋，加瀬哲男，久米田裕子，横山　浩他：過酢酸製剤の各種微生物に対する殺菌効果の検討．防菌防黴 1998; 26: 605-10.
9) 土田美由紀，原田容治：強酸性水・ソフト酸化水・過酢酸水の比較．総合消化器ケア 1999; 4: 46-53.
10) Jursch CA, Gerlich WH, Glebe D, Schaefer S, Marie O, Thraenhart O: Molecular approaches to validate disinfectants against human hepatitis B virus. Med Microbiol Immunol 2002; 190: 189-97.
11) 日本組織培養学会編．組織培養の技術．朝倉書店，東京，1982. 117-21.
12) Cowan RE, Manning AP, Ayliffe GAJ, Axon ATR, Causton JS, Cripps NF, *et al.*: Aldehyde disinfectants and health in endoscopy units. Gut 1993; 34: 1641-45.
13) Jachuck SJ, Bound CL, Steel J, Blain PG: Occupational Hazard in Hospital Staff Exposed to 2 per cent Glutaladehyde in an Endoscopy Unit. J Soc Occup Med 1989; 39: 69-71.
14) Manzoor SE, Lambert PA, Griffiths PA, Gill MJ, Fraise AP: Reduced glutaraldehyde susceptibility in *Mycobacterium chelonae* associated with altered cell wall polysaccharides. J Antimicrob Chemother 1999; 43: 759-65.
15) 岩沢篤郎，中村良子：市販酸化剤の抗微生物効果と細胞毒性．環境感染 2000; 15: 183-189.
16) Tucker RC, Lestini BJ, Marchant RE: Surface analysis of clinically used expanded PTFE endoscopic tubing treated by the STERIS PROCESS. *ASAIO J* 1996; 42: 306-13.

〔連絡先：〒113-8655　東京都文京区本郷7-3-1
東京大学医学部附属病院感染制御部　茅野　崇〕

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

Siriphan Saeng-Aroon, MSc, Nuanjun Wichukchinda, MSc,* Lay Myint, MD, PhD,† Panita Pathipvanich, MD,‡ Koya Ariyoshi, MD, PhD,*† Archawin Rojanawiwat, MD,* Masakazu Matsuda,† Pathom Sawanpanyalert, MD, PhD,* Wataru Sugiura, MD, PhD,† and Wattana Auwanit, PhD**

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.

Received December 1, 2003; accepted for publication May 7, 2004.

From *National Institute of Health, Department of Medical Sciences, Ministry of Public Health, Nonthaburi, Thailand; †AIDS Research Center, National Institute of Infectious Diseases, Tokyo, Japan; and ‡Day Care Center, Lampang Hospital, Lampang, Thailand.

Supported by the Department of Medical Sciences, Ministry of Public Health of Thailand, under the national plan for AIDS prevention and control, the Japan International Cooperation Agency (JICA), and the Ministry of Health, Social Welfare and Labour of Japan.

Reprints: Wattana Auwanit, National Institute of Health, Department of Medical Sciences, Ministry of Public Health, Nonthaburi, Thailand 11000 (e-mail: wattana@dmsc.moph.go.th).

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

(*J Acquir Immune Defic Syndr* 2004;36:1051–1056)

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.

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

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	F	K	D	T	K	I	V	V	Q	M	I	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.

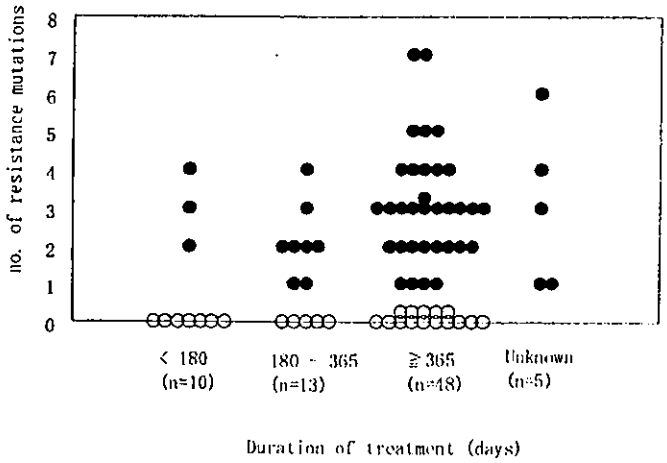


FIGURE 2. Prevalence of AZT, ddI, or ddC resistance mutations in relation to the duration of the dual therapy. Open circles indicate samples without resistance mutations.

Drug Resistance Mutations After Stopping Antiretroviral Drug Therapy

The interval between stopping dual therapy and the time of sampling was also associated with the presence of drug resistance mutations. Twenty-six individuals who had been substantially exposed to dual therapy for >180 days were off therapy at the time of sampling. Seven individuals (27%) were off therapy for >180 days and 19 (73%) were off therapy for <180 days. Drug-resistant viruses were detected in only 1 individual (14%) in the former group but 7 individuals (37%) in the latter group.

Evaluation of M41L and K70R MS-PCR in Detecting AZT-Resistant Strains

We then compared direct sequence methods with M41L and K70R MS-PCR in 96 antiretroviral drug–experienced individuals for whom both sequence and MS-PCR results were available (Table 2). Overall concordant rate for codon 41 was 96.9% (93/96) where M41I was regarded as a mutant type and concordant rate for codon 70 was 92.7% (89/96). Discordant results were seen mainly in the samples that were determined as mutant type by the MS-PCR and as wild type by the sequencing method.

To study the sensitivity of M41L and K70R MS-PCR as a screening strategy in detecting AZT-resistant strains, we defined the AZT-resistant strains as viruses with at least one AZT resistance mutation, which was detected by the sequencing method. Out of the 96 plasma samples that were tested by both the sequencing and the MS-PCR methods, 52 samples had no AZT resistance mutation and 44 samples had at least one AZT resistance mutation and were regarded as containing AZT-resistant viruses. Of the 44 samples with AZT-resistant viruses, the M41L and K70R MS-PCR detected either M41L or K70R mutation in 38 samples, resulting in the sensitivity of the M41L and K70R MS-PCR in detecting the AZT-resistant viruses at 86.4%. The number of AZT resistance mutations related to the detection rate of AZT resistance mutations by the MS-PCR (Table 3). When the viruses had multiple mutations, the sensitivity of the M41L and K70R MS-PCR was considerably higher. Of 39 samples containing HIV-1 with more than one AZT resistance mutation, 37 samples (94.5%) were diagnosed as having resistant viruses by the M41L and K70R MS-PCR.

TABLE 2. Comparison Between MS-PCR and Sequencing Results

Codon 41 Mutations			
	Sequencing Results		
	M	L	I
MS-PCR results			
Wild	84	1*	0
Mutant	2	7	2

*Sequence result of this patient showed a mixed type of M and L; it turned out to be mutant type when MS-PCR experiment was repeated.

Codon 70 Mutations		
	Sequencing Results	
	K	R
MS-PCR results		
Wild	65	1
Mutant	6	24

Screening AZT-Resistant Viruses Among Antiretroviral Drug–Naive Individuals in Northern Thailand

We applied the M41L and K70R MS-PCR to the screening of 292 antiretroviral drug–naive HIV-1–infected individu-

TABLE 3. The Sensitivity of M41L and K70R MS-PCR in Detecting AZT Resistance Mutations

AZT Resistance Mutations, n	Total	M41L and K70R MS-PCR Results		
		Wild	Mutant	Sensitivity
0	52	52	0	—
1	5	4	1	20%
2	14	1	13	92.9%
3	11	1	10	90.9%
≥4	14	0	14	100%

als attending the Lampang Hospital for the existence of AZT drug-resistant viruses. There were 271 individuals (92.8%) who were known to be infected with HIV-1 via the heterosexual route. We found 2 patients (0.7%) who carried mutant viruses: one had M41L and the other had K70R mutation. Later it was noted that these 2 patients, as well as their spouses, had never received any antiretroviral drugs but both had participated in clinical trials of herbal medicine in the past.

DISCUSSION

Our observation showed that AZT, ddI, or ddC resistance mutations were found in >50% of individuals who had received dual therapy. The prevalence of drug-resistant viruses was higher among individuals who had received the drugs for a longer period, as previously reported.[18,19] We attribute the high prevalence of resistant viruses to the fact that the dual therapy was suboptimal. Clinicians working in government hospitals, however, did not have other options because the more efficient antiretroviral therapy such as triple or quadruple therapy was not affordable for most patients when this study was conducted.[7] Recently, access to multiple antiretroviral drugs has been dramatically improved, because the Government Pharmaceutical Organization (GPO) started the production of generic antiretroviral drugs known as "GPOvir," which is a combined tablet of stavudine, lamivudine, and nevirapine. We nevertheless anticipate that individuals who had already had viruses resistant to NRTI dual therapy may not gain as much benefit from the generic medicine as antiretroviral drug–naive individuals do.

The most common mutations observed in this study were D67N, K70R, and T215Y/F, and we found few mutations at codons 65, 74, 108, 151, and 184. Such patterns of NRTI resistance mutations are similar to the patterns in CRF01_AE infection as well as in subtype B infections that have been reported in our previous report.[4] M184V mutation was often found in our previous study but not in the current study. We think that this difference reflects on the rare use of 3TC in Thailand when this study was conducted. Our current study, though a cross-sectional observation, showed several associations among resistance mutations such as D67N and M41L or K70R, T215Y/F and M41L in Thai strains as known in subtype B infection.[20,21]

We found a high concordance rate of MS-PCR with the sequencing method in detecting M41L and K70R point mutations. The finding is compatible with previous papers.[16,17] Discordant results between the MS-PCR and sequencing method were seen in some samples, most of which showed mutant type by the MS-PCR but wild type by the sequencing method. We think that such discordances are due to the greater sensitivity of MS-PCR for detecting a minor virus population than the sequencing method. However, a high sensitivity and specificity of detecting 2 particular point mutations do not specifically justify the application of M41L and K70R MS-PCR for the screening of AZT-resistant viruses in the field. D67N and T215Y/F mutations are very common but it is technically difficult to establish MS-PCR specific for these mutations due to a higher degree of polymorphism around the mutation sites. Our data showed that these mutations were frequently accompanied by M41L and/or K70R as previously reported in subtype B.[22] Furthermore, we evaluated how efficiently the M41L and K70R MS-PCR could detect AZT-resistant viruses that were detected by the sequencing. The overall sensitivity was reasonably high particularly among the viruses with multiple drug resistance mutations.

This is the first report that addressed the transmission of drug-resistant HIV-1 using a large number of samples in Thailand. We found that the prevalence of HIV-1 strains with either M41L or K70R mutation was as low as 0.7% among our drug-naive population. Considering that the overall sensitivity of the MS-PCR for detecting HIV-1 with any AZT resistance mutation was 86.4%, the prevalence of AZT-resistant HIV-1 was estimated to be 0.8%, which is still very low. There is still the concern that the low prevalence of resistant virus could be a consequence of the fact that the resistance to AZT in the drug-naive population was often associated with mutations at codon 60 or 215. To exclude this possibility, we further tested 60 samples, which were randomly selected from the drug-naive samples and confirmed that none had drug resistance mutations at these sites. The majority (127/292) of drug-naive individuals (43.5%) were initially diagnosed as HIV infected in 1997 or before, when the PMTCT program started in the region, and many were likely to have been infected several years prior to their first diagnosis of HIV infection. Thus, our result may not show an effect, which could have been triggered by the PMTCT program. A report from the United Kingdom suggests that transmission of drug-resistant HIV-1 is increasing.[23] We believe that our report is important in providing the baseline information on AZT-resistant HIV-1.

There has not been a consensus on the strategy of monitoring the transmission of drug-resistant HIV-1 in developing countries. Detecting individuals with primary viremia is ideal but not practical. In our study, we surveyed a drug-naive population for the presence of drug-resistant viruses. One concern with this approach is that drug-resistant viruses, which are generally less fit, might have been overwhelmed by the wild-type viruses in the absence of antiretroviral drug pressure because drug-resistant viruses among drug-treated individuals disappear following the interruption of antiretroviral therapy.[24] However, a recently published paper showed 2 cases of transmission of drug-resistant HIV-1 in which the resistant genotypes remained as a dominant population for a prolonged period in the absence of antiretroviral therapy.[25] Another way to monitor the spread of antiretroviral drug–resistant viruses is to screen infected individuals shortly after they receive antiretroviral therapy, which selects a minor population of insidious

resistant viruses, before de novo resistance mutations occur. Further studies are needed.

This study demonstrates that it is feasible to apply MS-PCR techniques for screening a large number of field samples for the presence of AZT-resistant viruses in Thailand. Taking into account the enormous benefits of MS-PCR such as much lower cost, ease of use, no requirement of automated sequencers, and higher sensitivity of detecting a minor virus population, we think that the M41L and K70R MS-PCR is a useful technique for the screening of AZT-resistant HIV-1 in epidemiologic surveys in developing countries. Recently, GPOvir has become widely available in Thailand. As the patterns of drug-resistant mutations against 3TC and nevirapine are relatively simple, we propose that MS-PCR technique should be considered for monitoring viruses resistant to this combination of antiretroviral drugs.

ACKNOWLEDGMENTS

We thank Ms. Suthira Kasemusuk, Ms. Sriprai Seneewong-na-ayoottaya, Ms. Anong-nard Ariyakrua, Ms. Nutira Boonna, and Mr. Praphan Wrongramnong for their field works; Ms. Kanha Lor-yont, Mr. Wicharn Khaewkarnka, Mr. Sithichai Umnajsirisuk, Mr. Somchai Ni-yom-thai, and Ms. Jirawan Srisawat for their laboratory assistance. Finally, we would like to thank the HIV-infected individuals who participated in this study.

REFERENCES

1. Hirsch MS, Brun-Vezinet F, Clotet B, et al. Antiretroviral drug resistance testing in adults infected with human immunodeficiency virus type 1: 2003 recommendations of an international AIDS society-USA panel. *Clin Infect Dis*. 2003;37:113–128.
2. Robertson DL, Anderson JP, Bradac JA, et al. HIV-1 nomenclature proposal, 1999. Available at: http://hiv-web.lanl.gov/conent/hiv-db/REVIEWS/review.html. Accessed on October 31, 2000.
3. Grossman Z, Vardinon N, Chemtob D, et al. Genotypic variation of HIV-1 reverse transcriptase and protease: comparative analysis of clade C and clade B. *AIDS*. 2001;15:1453–1460.
4. Ariyoshi K, Matsuda M, Miura H, et al. Patterns of point mutations associated with anti-retroviral drug resistance in CRF01_AE (subtype E) infection differ from subtype B infection. *J Acquir Immune Defic Syndr*. 2003;33:336–342.
5. Brenner B, Turner D, Oliveira M, et al. A V106M mutation in HIV-1 clade C viruses exposed to efavirenz confers cross-resistance to non-nucleoside reverse transcriptase inhibitors. *AIDS*. 2003;17:F1–F5.
6. The Thai Working Group on HIV/AIDS Projection. Projections for HIV/AIDS in Thailand: 2000–2020. 2001. Karnsana Printing Press, Bangkok.
7. Pathipvanich P, Ariyosh K, Rojanawiwat A, et al. Survival benefit from non-highly active antiretroviral therapy in a resource-constrained setting. *J Acquir Immune Defic Syndr*. 2003;32:157–160.
8. Kanshana S, Simond RJ. National program for preventing mother-child HIV transmission in Thailand: successful implementation and lessons learned. *AIDS*. 2002;16:953–959.
9. Hecht FM, Grant RM, Petropoulos CJ, et al. Sexual transmission of an HIV-1 variant resistant to multiple reverse-transcriptase and protease inhibitors. *N Engl J Med*. 1998;339:307–311.
10. Yerly S, Kaiser L, Race E, et al. Transmission of antiretroviral-drug-resistant HIV-1 variants. *Lancet*. 1999;354:729–733.
11. Salomon H, Wainberg MA, Brenner B, et al. Prevalence of HIV-1 resistant to antiretroviral drugs in 81 individuals newly infected by sexual contact or injecting drug use. Investigators of the Quebec Primary Infection Study. *AIDS*. 2000;14:F17–F23.
12. Frenkel LM, Wagner LE 2nd, Demeter LM, et al. Effects of zidovudine use during pregnancy on resistance and vertical transmission of human immunodeficiency virus type 1. *Clin Infect Dis*. 1995;20:1321–1326.
13. Richman DD, Guatelli JC, Grimes J, et al. Detection of mutations associated with zidovudine resistance in human immunodeficiency virus by use of the polymerase chain reaction. *J Infect Dis*. 1991;164:1075–1081.
14. Eastman PS, Urdea M, Besemer D, et al. Comparison of selective polymerase chain reaction primers and differential probe hybridization of polymerase chain reaction products for determination of relative amounts of codon 215 mutant and wild-type HIV-1 populations. *J Acquir Immune Defic Syndr*. 1995;9:264–273.
15. Rust S, Funke H, Assmann G. Mutagenically separated PCR (MS-PCR): a high specific one step procedure for easy mutation detection. *Nucleic Acids Res*. 1993;21:3623–3629.
16. Frater AJ, Chaput CC, Beddows S, et al. Simple detection of point mutations associated with HIV-1 drug resistance. *J Virol Methods*. 2001;93:145–156.
17. Myint L, Ariyoshi K, Yan H, et al. Mutagenically separated PCR assay for rapid detection of M41L and K70R zidovudine resistance mutations in CRF01_AE (subtype E) human immunodeficiency virus type 1. *Antimicrob Agents Chemother*. 2002;46:3861–3868.
18. Birch C, Middleton T, Hales G, et al. Limited evolution of HIV antiretroviral drug resistance-associated mutations during the performance of drug resistance testing. *J Acquir Immune Defic Syndr*. 2003;32:57–61.
19. Decamps D, Flandre P, Joly V, et al. Effect of zidovudine resistance mutations on virologic response to treatment with zidovudine or stavudine, each in combination with lamivudine and indinavir. *J Acquir Immune Defic Syndr*. 2002;31:464–471.
20. Larder BA, Kemp SD. Multiple mutations in HIV-1 reverse transcriptase confer high-level resistance to zidovudine (AZT). *Science*. 1989;246:1155–1158.
21. Kellam P, Boucher CA, Larder BA. Fifth mutation in human immunodeficiency virus type 1 reverse transcriptase contributes to the development of high-level resistance to zidovudine. *Proc Natl Acad Sci U S A*. 1992;89:1934–1938.
22. Kellam P, Boucher CAB, Tijanagel JMGH, et al. Zidovudine treatment results in the selection of human immunodeficiency virus type 1 variants whose genotypes confer increasing levels of drug resistance. *J Gen Virol*. 1994;75:341–351.
23. UK Collaborative Group on Monitoring the Transmission of HIV Drug Resistance. Analysis of prevalence of HIV-1 drug resistance in primary infections in the United Kingdom. *BMJ*. 2001;322:1087–1088.
24. Hance AJ, Lemiale V, Izopet J, et al. Changes in human immunodeficiency virus type 1 populations after treatment interruption in patients failing antiretroviral therapy. *J Virol*. 2001;75:6410–6417.
25. Chan K, Galli R, Montaner J, et al. Prolonged retention of drug resistance mutations and rapid disease progression in the absence of therapy after primary HIV infection. *AIDS*. 2003;17:1256–1258.

Available online at www.sciencedirect.com
SCIENCE DIRECT®
Cellular Immunology 229 (2004) 117–129
Cellular Immunology
www.elsevier.com/locate/ycimm

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

Hua Yan[a,b,c], Tohko Miyagi[a], Eigo Satoh[a,d], Wataru Sugiura[b], Naoki Yamamoto[b,c], Hiromitsu Kimura[a,*]

[a] *Department of Research Surgery, National Center for Child Health and Development, Tokyo, Japan*
[b] *Aids Research Center, National Institute of Infectious Disease, Tokyo, Japan*
[c] *Department of Molecular Virology, Graduate School, Tokyo Medical and Dental University, Japan*
[d] *Department of Surgery, Graduate School, Tokyo Medical and Dental University, Japan*

Received 3 February 2004; accepted 24 July 2004

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.

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

☆ This study was supported by a Research Grant from the Ministry of Health and Welfare of Japan, a Grant-in-Aid from the Ministry of Education, Science and Culture of Japan, and the Human Science Foundation of Japan.
* Corresponding author. Fax: +81 3 3414 8121.
E-mail address: chimera@nch.go.jp (H. Kimura).

0008-8749/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.cellimm.2004.07.006

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 IA-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

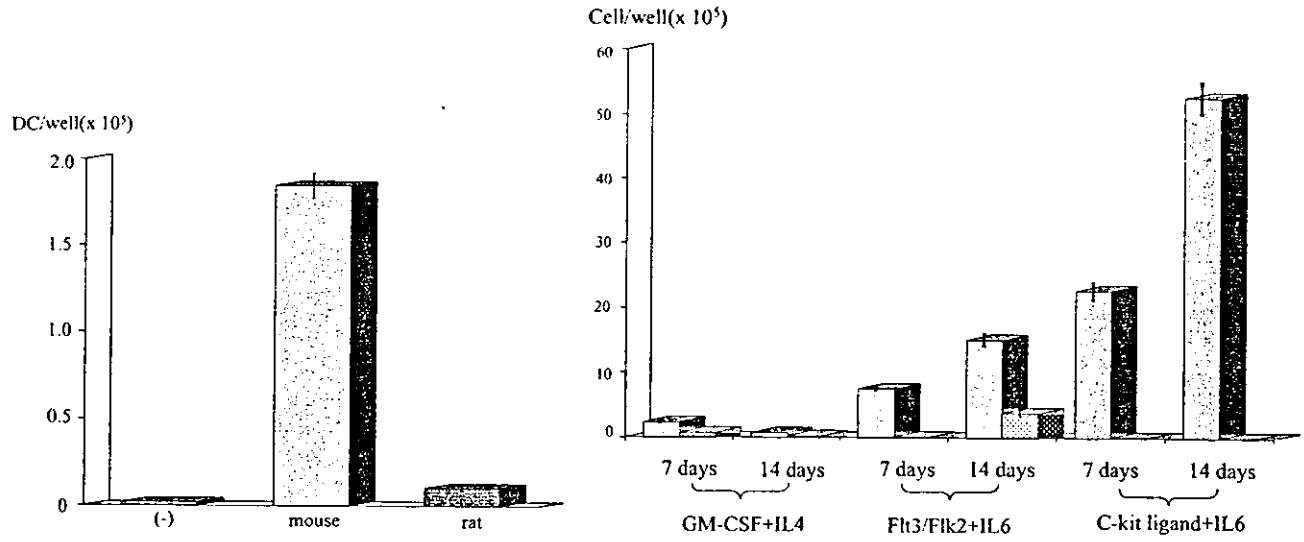
Our attempt to generate rat DC by a serum-free medium supplemented with a conditioned medium [5] based on the early studies [1,2] led to a limited amount of DC recovery, final cell recovery was usually around 0.1% of total BMC input. Hence, attempts were first made to determine whether and to what extent 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

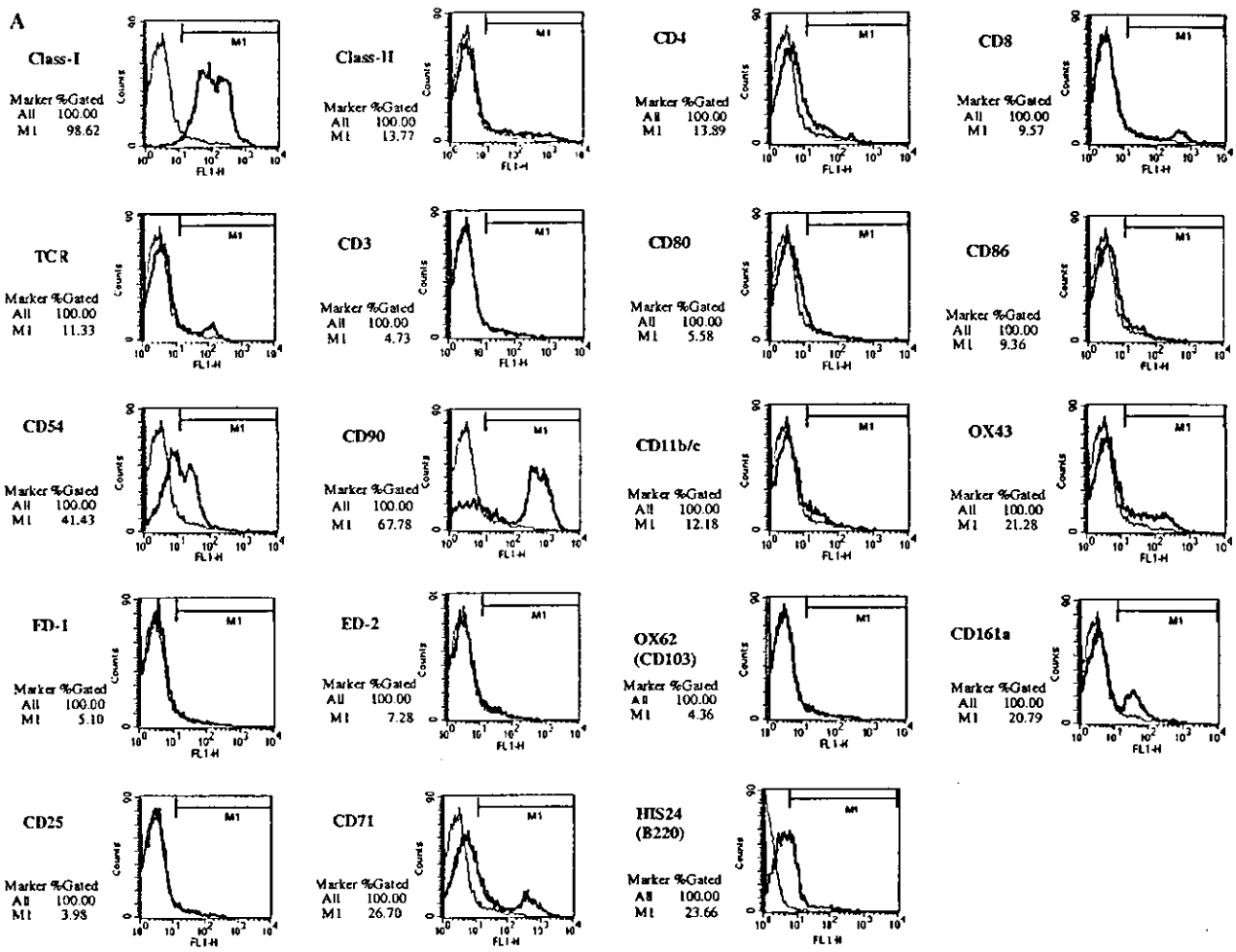


Fig. 2. (A) Cell surface phenotypes of LEW fresh bone marrow cells (BMC) analyzed by various mAbs which are related with antigen presenting cells (APC). These include mAbs specific for class I (MRC-OX18), class II (MRC-OX6) and co-accessory molecules for B7.1 (CD80), B7.2 (CD86) and for functional cell 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

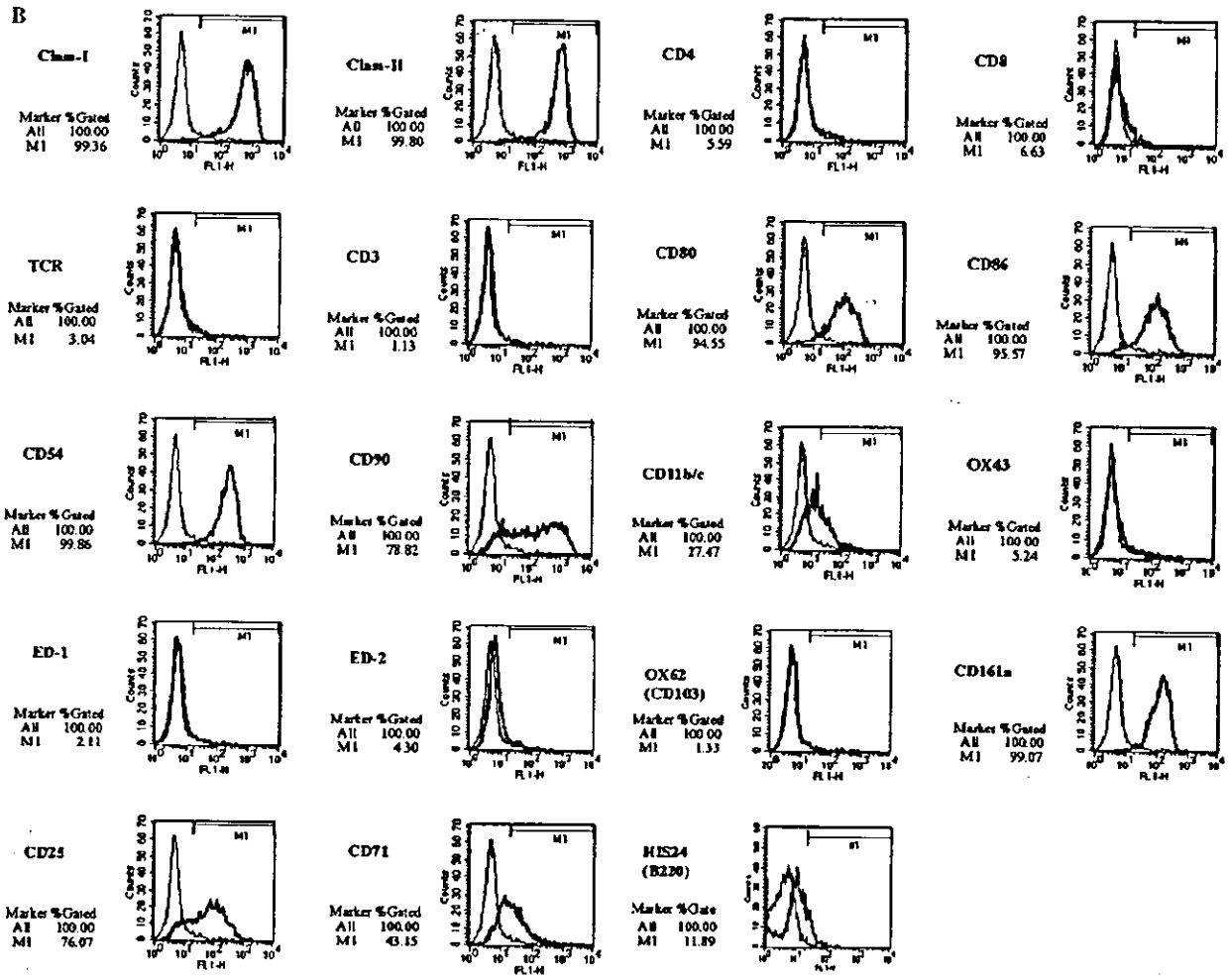


Fig. 2. (*continued*)

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_{-LEW}. The other source of TDL was likewise labeled LEW_{-DA}. The latter LEW_{-DA} T cell preparation is important to evaluate whether and to what extent the LEW_{-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_{-DA} T should not respond to any peptides in allogeneic MHC such as RT1avl of DA. Thus, LEW

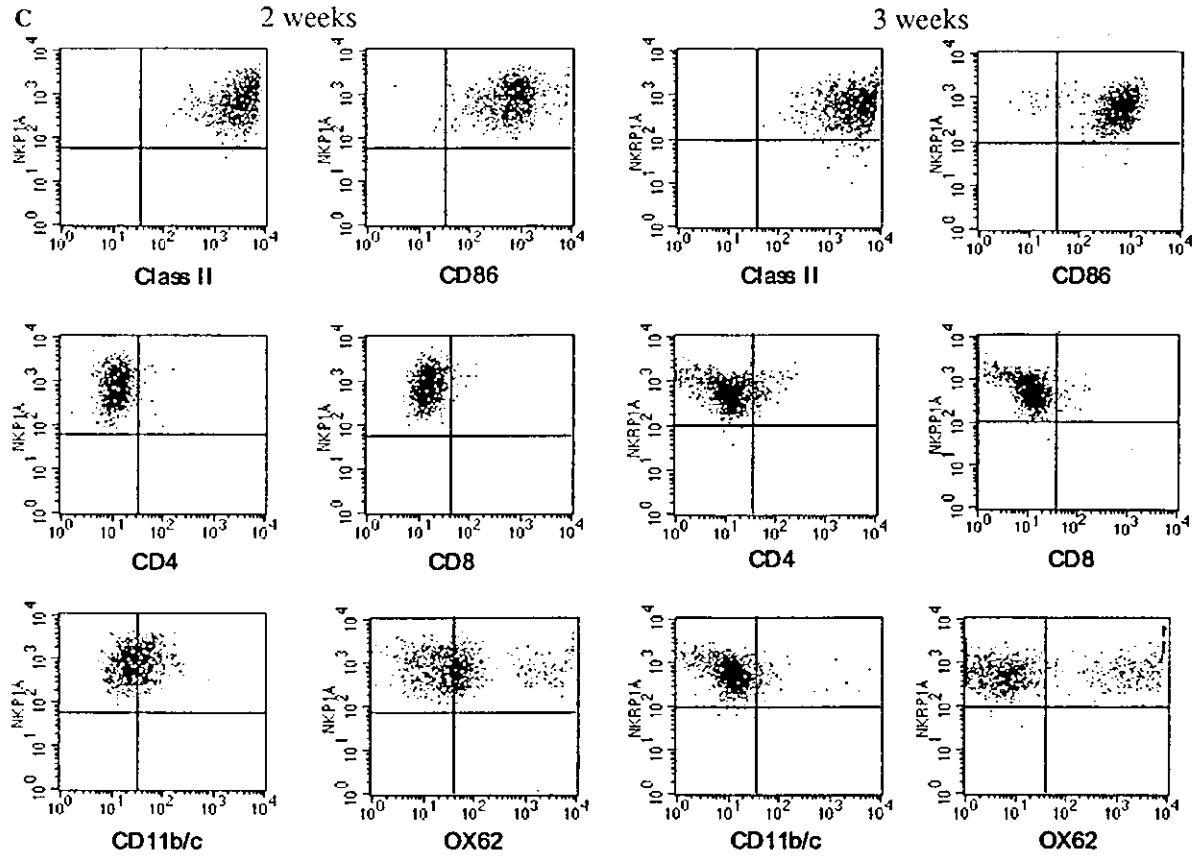


Fig. 2. (*continued*)

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_{-LEW} T cells were active to both allogeneic DA (RT1avl) in (DA × LEW) F1 and PVG (RT1c) in (LEW × PVG) F1, whereas LEW_{-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_{-LEW} T cells responded in syngeneic MLC [27] were observed regardless of serum sources (syngeneic or xenogeneic). Likewise both in vivo purified LEW_{-LEW} T cells and LEW_{-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_{-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

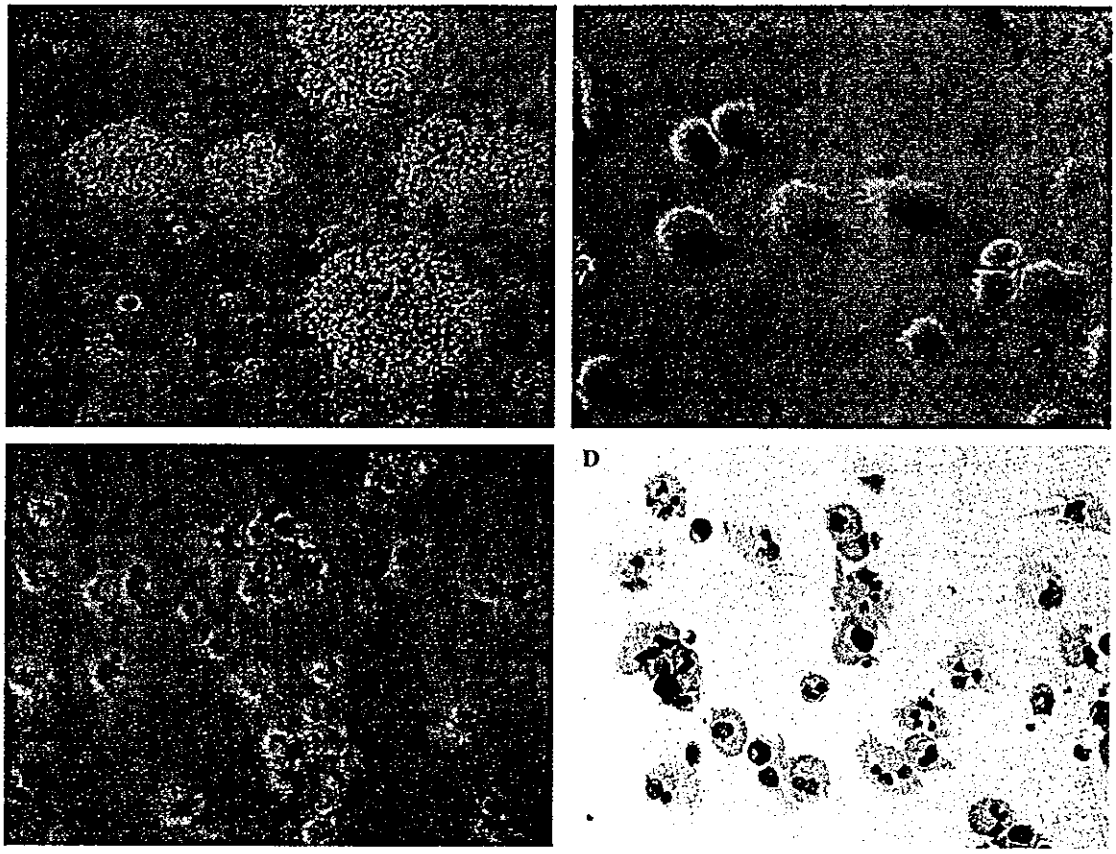


Fig. 3. Typical DC culture generated by Flt3/Flk2 ligand and IL-6 for eight weeks culturing was shown. When DC were generated spontaneously by Flt3/Flk2 ligand and IL-6, the most prominent picture was seen under the microscope. Large DC cell cluster or colony-like cell cluster appeared (A). With anti-CD161a (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,

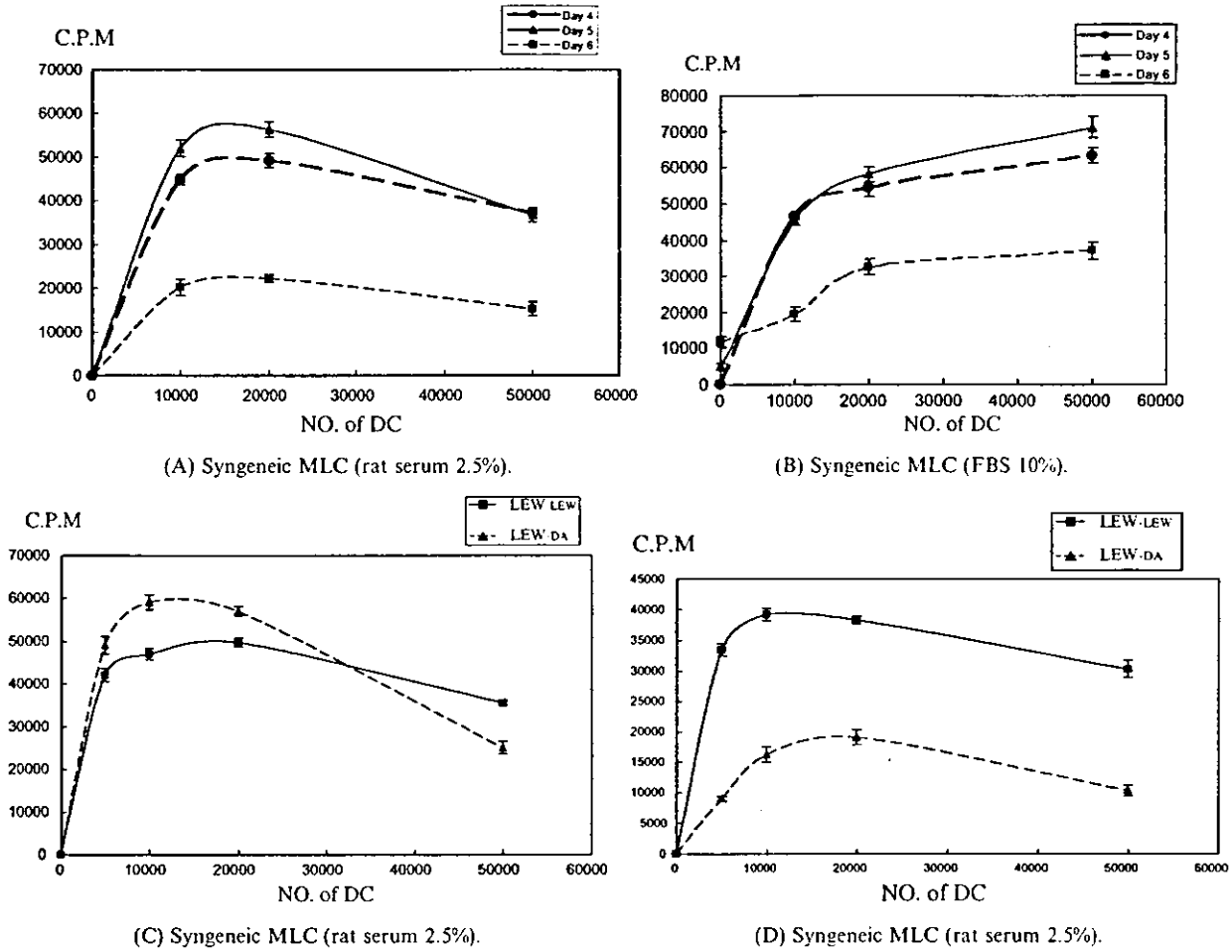


Fig. 4. (A) Highly purified LEW T cells (LEW_{-LEW}) (>99%) were prepared by collecting thoracic duct lymph filtration in vivo through lethally irradiated LEW rat that had been under the thoracic duct drainage and injected with 1.5×10^9 normal LEW TDL as described in Section 2. About 2×10^5 LEW_{-LEW} 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