Available online at www.sciencedirect.com
ScienceDirect
Microbes and Infection 8 (2006) 2872–2879
Microbes and Infection
www.elsevier.com/locate/micinf

Original article

Analysis of HIV-1 sequences before and after co-infecting syphilis

Ichiro Koga [a,*], Takashi Odawara [b], Masakazu Matsuda [c], Wataru Sugiura [c], Mieko Goto [a], Tetsuya Nakamura [b], Aikichi Iwamoto [a,b,d,e]

[a] *Division of Infectious diseases, Advanced Clinical Research Center, The Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, 108-8639 Tokyo, Japan*
[b] *Department of Infectious Diseases and Applied Immunology, Research Hospital, The Institute of Medical Science, The University of Tokyo, Tokyo, Japan*
[c] *AIDS Research Center, National Institute of Infectious Diseases, Tokyo, Japan*
[d] *China-Japan Joint Laboratory of Structural Virology and Immunology, The Institute of Biophysics, The Chinese Academy of Sciences, and The Institute of Medical Science, The University of Tokyo, Tokyo, Japan*
[e] *China-Japan Joint Laboratory of Molecular Immunology and Molecular Microbiology, The Institute of Microbiology, The Chinese Academy of Sciences, and The Institute of Medical Science, The University of Tokyo, Tokyo, Japan*

Received 2 June 2006; accepted 25 September 2006
Available online 23 October 2006

Abstract

Increasing syphilis incidence among men who have sex with men (MSM) has been reported. The index case was a human immunodeficiency virus type 1 (HIV-1)-positive MSM who presented coincidentally with the secondary syphilis and a rebound of plasma viral load after complete suppression of HIV-1 (below 50 copies/ml) for 13 months with potent antiretroviral therapy (PART), suggesting a possibility of HIV-1 superinfection. We analyzed HIV-1 sequences before and after syphilis in four HIV-1-positive patients including the index case to explore drug resistance mutations (DRMs) and a possibility of HIV-1 superinfection. There were patients who obtained DRMs around syphilis infection but no evidence of HIV-1 superinfection was obtained. Our results underline the importance of strict adherence to PART.

Keywords: HIV-1 superinfection; Syphilis; Drug resistance mutations; STI; PART

1. Introduction

Syphilis has been noted as a reemerging infectious disease in large cities in the U.S. and Europe, where the incidence increased steeply among men who have sex with men (MSM) [1,2]. Sexually transmitted infections (STIs) such as syphilis not only imply practice of unsafe sex but actually contribute to the transmission of human immunodeficiency virus type 1 (HIV-1) [3,4]. Over 40% of Japanese HIV-1-positive patients who reported unprotected sex had a history of syphilis proven by *Tremponema pallidum* hemagglutination test (TPHA) [5]. Recently we have been experiencing increasing number of active syphilis cases in our HIV-1 outpatient clinic in Tokyo (Nakamura, H. unpublished).

Infection with drug-resistant HIV-1 is another problem in large cities in the world [6]. Although superinfection may be less frequent than initially concerned [7,8], superinfections with drug-resistant HIV-1 have actually been reported [9].

A patient with acquired immunodeficiency syndrome (AIDS)-lymphoma who had been treated successfully with potent antiretroviral therapy (PART) presented with syphilitic skin rash. Coincidentally his HIV-1 viral load (VL) exploded abruptly to 6.7×10^4/ml and his rebounded plasma HIV-1

Abbreviations for drugs: ddI, didanosine; d4T, stavudine; EFV, efavirenz; LPV, lopinavir; NFV, nelfinavir; RTV, ritonavir; ZDV, zidovudine; 3TC, lamivudine; CHOP, combination therapy with cyclophosphamide, vincristine, doxorubicin and prednisolone.

* Corresponding author. Tel.: +81 3 5449 5338; fax: +81 3 5449 5427.
E-mail address: koga@ims.u-tokyo.ac.jp (I. Koga).

1286-4579/$ - see front matter © 2006 Published by Elsevier Masson SAS.
doi:10.1016/j.micinf.2006.09.003

was found to contain multiple drug-resistant mutations (DRMs). Therefore, a possibility of superinfection with drug resistant HIV-1 was considered in this case. Since it has been reported that syphilis may increase HIV-1 VL [10], it can be a driving force of new mutations for HIV-1. We analyzed HIV-1 sequences before and after syphilis in four HIV-1-positive patients including the index case to explore DRMs and a possibility of HIV-1 superinfection.

2. Patients, materials and methods

2.1. Patient 1

A 34 y/o MSM was referred to our hospital in April 2001. He had been diagnosed as AIDS with extranodular malignant lymphoma as an indicator disease. In parallel with CHOP (cyclophosphamide, vincristine, doxorubicin and prednisolone) therapy to lymphoma, he had been on PART with a combination of zidovudine (ZDV), lamivudine (3TC) and nelfinavir (NFV) since August 2000. As summarized in Fig. 1, his VL had been constantly less than 50 copies/ml for 8 consecutive time points in 13 months including time points 1–1 and 1–2.

He was asymptomatic in July 2002 when periodic blood samples were stored (Time point 1–3). Two months after 1–3 (time point 1–4), he developed skin rush and was examined. Serologic test for syphilis (STS, glass slide test) and TPHA at time point 1–4 was very high and the diagnosis of syphilis was confirmed by a skin biopsy. Retrospective examination using frozen plasma showed that the patient was TPHA-negative at time points 1–1 and 1–2 but turned positive at time point 1–3 indicating syphilis infection before that time point.

2.2. Patient 2

Patient 2 was treated with dual nucleosides (ZDV and 3TC) from 1996 to 1999. After a treatment interruption, he was reinitiated with a combination of stavudine (d4T), didanosine (ddI) and efavirenz (EFV) since July 2001. Although undetectable levels were not attained, his VLs were always under 1000 copies/ml for 20 months including August 2002 (time point 2–1) (Fig. 2). Eight months after time point 2–1 (time point 2–2), he presented with syphilitic skin rash and inguinal lymphadenopathy. At time point 2–2, his VL was unusually higher (4400 copies/ml) than his previous data.

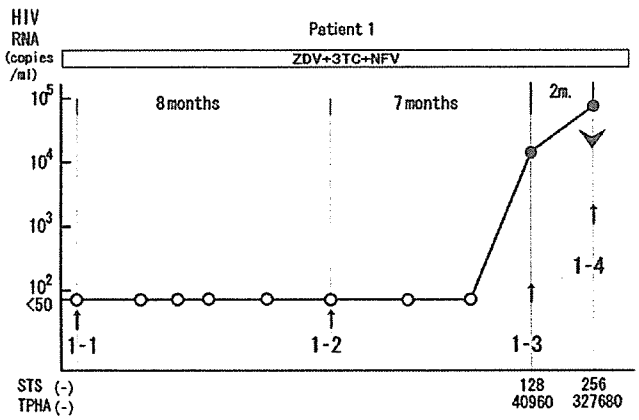

Fig. 1. Clinical course of patient 1. The graph shows VL along the clinical course. Open points indicate undetectable viral load (<50 copies/ml). Names of antiretroviral drugs are shown in the bar on the top. ZDV: zidovudine, 3TC: lamivudine, NFV: nelfinavir. Upward arrows indicate examined time points. A solid downward arrowhead indicates time points when the diagnosis of syphilis was made. STS: Serologic test for syphilis. TPHA: *Treponema pallidum* hemagglutionation test.

2.3. Patient 3

Patient 3 presented with syphilitic rash while he was treated with d4T, 3TC, lopinavir (LPV) and ritonavir (RTV, rtv). His VLs stayed between 100 and 1000 copies/ml three months before (time point 3–1) and at (time point 3–2) syphilis coinfection (Fig. 2).

2.4. Patient 4

Patient 4 was treated with d4T, 3TC and NFV since 1998. His VL became undetectable soon after the initiation of PART and kept undetectable for 41 months until October 2001 (time point 4–1). Although the diagnosis of syphilis was made based on a skin rash between time points 4–2 and 4–3, retrospective TPHA examination tested positive at time point 4–2 (Fig. 2). Except a couple of blips, his VL remained undetectable including time points 4–2, 4–3, and 4–4 after the infection of syphilis.

2.5. Extraction of provirus DNA

Aliquots of blood samples were collected periodically with informed consent. They were centrifuged in Ficoll density gradient and the isolated plasma and peripheral blood mononuclear cells (PBMCs) were preserved at −70 °C. DNA was extracted from frozen PBMC samples with QIAamp DNA mini kit (Qiagen). RNA was extracted from frozen plasma samples with Qiagen Viral RNA mini kit (Qiagen).

2.6. PCR amplification and sequencing

We amplified four regions of HIV-1, i.e., the coding regions of protease (PR), reverse transcriptase (RT), and *env*. For each region, outer polymerase chain reaction (PCR) and inner PCR were performed. Outer PCR, using 0.2–0.3 μg of genomic DNA, was performed in a volume of 50 μl with 1x ExTaq buffer, 0.2 mM of each deoxyribonucleoside mixture, 0.5 μM of each primer, and 1.25 U of Ex-Taq (TaKaRa). Hot start was employed for outer PCR by incubating the reaction mixtures at 94 °C for 1 min, and then the mixtures were subjected to 30 to 40 amplification (94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s) followed by a final incubation at 72 °C for 7 min. Five μl of outer PCR product was used for inner PCR. Inner PCR was performed in a volume of 50 μl under the same conditions as outer PCR. Exceptionally, for not being amplified sufficiently, the third PCR was performed for PR region of 1–2 in patient 1. And for not being amplified in one

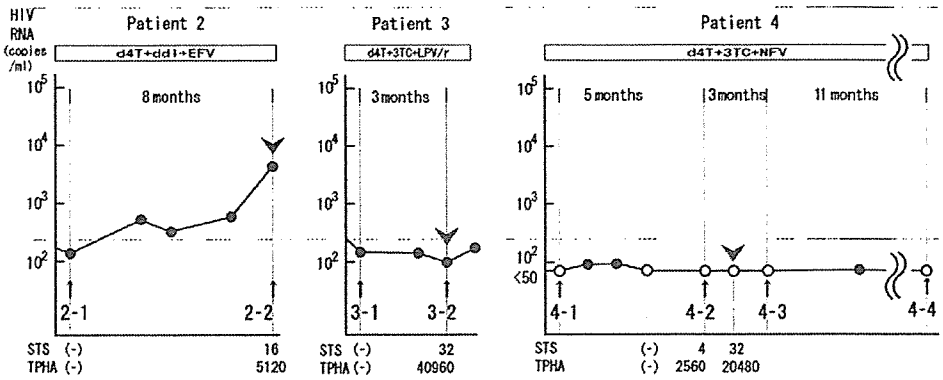

Fig. 2. Clinical course of patient 2, 3 and 4. The graph shows VL along the clinical course. Open points indicate undetectable viral load (<50 copies/ml). Names of antiretroviral drugs are shown in the bar on the top. d4T: stavudine, ddI: didanosine, EFV: efavirenz, 3TC: lamivudine, LPV/r: lopinavir/ritnavir, NFV: nelfinavir. Upward arrows indicate examined time points. A solid downward arrowhead indicates time points when the diagnosis of syphilis was made. STS: Serologic test for syphilis. TPHA: *Treponema pallidum* hemagglutionation test.

fragment, RT region of timepoints 1–2 and 1–4 in patient 1 were separately amplified in two fragments.

The primer sets used are as follows. Outer and inner primer pairs were used for the outer and inner PCR respectively.

2.7. The primer pairs for RT coding region

Outer primer pair: Forward (5′ATGATAGGGGGAAT TGGAGGTTT 3′; SF2 positions 2395–2417) Reverse (5′TACTTCTGTTAGTGCTTTGGTTCC 3′; SF2 positions 3432–3409).

Inner primer pair 1: Forward (5′GACCTACACCTGT CAACATAATTGG 3′; SF2 positions 2492–2516) Reverse (5′TAATCCCTGCATAAATCTGACTTGC 3′; SF2 positions 3379–3355).

Inner primer pair 2: Forward (5′GTACTTTAAATTT CCCCATTAGTCC 3′; SF2 positions 2543–2567) Reverse (5′CAGTCCAGCTGTCTTTTTCTGGC 3′; SF2 positions 3316–3294).

Inner primer pair 3: Forward (5′AACTCAAGACTTCTGG GAAGT 3′; SF2 positions 2805–2825) Reverse (5′TA ATCCCTGCATAAATCTGACTTGC 3′; SF2 positions 3379–3355).

Inner primer pair 4: Forward (5′GACCTACACCTGTCAA CATAATTGG 3′; SF2 positions 2492–2516) Reverse(5′TGG AATATTGCTGGTGATCC 3′; SF2 positions 3038–3019).

(Inner primer pair1 was used for 1–2 and 1–4 of patient 1, all time points of patients 3 and patient 4. Inner primer pairs 2 were used for patient 2. Internal primer pairs 3 and 4 were used for time points 1–2 and 1–4 of patient 1.)

2.8. Primer pairs for Protease coding region

Outer primer pair: Forward (5′AGACAGGYTAATTTTTT AGGGA 3′; SF2 positions 2081–2102) Reverse (5′TATG GATTTTCAGGCCCAATTTTTGA 3′; SF2 positions 2723–2698) (Y: T or C).

Inner primer pair: Forward (5′AGAGCCAACAGCCCC ACCAG 3′; SF2 positions 2155–2174) Reverse (5′ACTTTTG GGCCATCCATTCC 3′; SF2 positions 2618–2599).

3rd PCR primer pair: Forward (5′AGAGAGCTTC AGGTTTGGGG 3′; SF2 positions 2176–2195) Reverse (5′ACTTTTGGGCCATCCATTCC 3′; SF2 positions 2618–2599).

(3rd PCR was employed only for time point 1–2 of patient 1.)

2.9. Primer pairs for env

Outer primer pair: Forward (5′GAAAGAGCAGAAGA CAGTGG 3′; SF2 positions 6211–6230) Reverse (5′GCC CATAGTGCTTCCTGC 3′; SF2 positions 7822–7805).

Inner primer pair 1: Forward (5′GACCATGTACAAATG TCAGC 3′; SF2 positions 6951–6970) Reverse (5′TTCT CCAATTGTCCCTCATATCTCCTCCTCCA 3′; SF2 positions 7667–7636).

Inner primer pair 2: Forward (5′ACATGGAATTAGGCCA 3′; SF2 positions 6985–7000) Reverse (5′ATCTC TTGTTAATAGCAGCC 3′; SF2 positions 7594–7575).

Inner primer pair 3: Forward (5′ACATGGAATTAGGCCA 3′; SF2 positions 6985–7000) Reverse (5′TTCTCCAAT TGTCCCTCATATCTCCTCCTCCA 3′; SF2 positions 7667–7636).

2.10. Primer pairs for gag

Outer primer pair: Forward (5′CCAAATGAGAGAACC AAGG 3′; SF2 positions 1474–1492) Reverse (5′TCTTA CTTTGATAAAACCTCC 3′; SF2 positions 2430–2410).

inner primer pair 1: Forward (5′CCACCTATCCCAGT AGGAG 3′; SF2 positions 1556–1574) Reverse (5′GGTGG GGCTGTTGGCTC 3′; SF2 positions 2172–2156).

inner primer pair 2: Forward (5′CCACCTATCCCAGTA GGAG 3′; SF2 positions 1556–1574) Reverse (5′TTC CCTAAAAAATTAGCCTG 3′; SF2 positions 2103–2084).

PCR fragments were sequenced directly or sub-cloned into pGEM-T Easy vector (Promega) and sequenced bi-directionally. All the nucleotide sequences were determined by using Big dye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems) and an ABI-377 sequencer (Applied Biosystems).

The reaction solution consisted of 2 μl of Ready Reaction Premix, 1 μl of BigDye Sequencing Buffer, 0.5 μM of a primer, 2 to 4 μl of template DNA, filled with distilled water to 10 μl of final volume.

2.11. Phylogenetic analysis and evolutionary distance calculation

Alignments of the sequences and the phylogenetic reconstructions using the neighbor joining method with a maximum likelihood distance matrix were performed by CLUSTAL W program [11]. The final graphical output was created with the program TREEVIEW version 1.6.6 [12]. Consensus sequences for the analysis were extracted from Updated Proposal of Reference Sequences of HIV-1 Genetic Subtypes [13]. We also added some sequences determined as subtype B from previous study in our laboratory [14]. We used Stanford drug resostamce database (http://hivdb.stanford.edu/) for the definition of resistant mutations.

3. Results

3.1. Resistant mutations in patient 1

The initial genotypic assay of the plasma HIV-RNA was performed at time point 1–4, since a rebound of VL at the previous visit (time point 1–3) was recognized. The genotype revealed five nucleoside analogue reverse transcriptase inhibitor (NRTI)-associated mutations and four protease inhibitor (PI)-associated mutations.

Cloning and sequence analysis of proviral DNA at the baseline (time point 1–1) revealed a mixture of wild type and DRMs (Fig. 3). In RT region, 5 out of 16 clones (31%) at amino acid position 41 and all 16 clones at amino acid position 210 contained single point mutation, which confer DRMs known as M41L (31%) and L210W (100%), respectively. There were two nucleotide mutations at amino acid position 215 in all 16 clones, which would convert the amino acid at this position from the wild type Threonine (T) into Asparatic acid (D). This change (T215D) is known as ZDV sensitive but suggestive of back-mutation from T215Y [15,16]. In PR region, polymorphic change at amino acid position 63 (L63P) was detected [17]. At time point 1–2, when VL was still undetectable, populations of M41L elevated to 100% (20/20 clones). Third PCR was necessary to amplify the PR region at time point 1–2, presumably because the amount of proviral DNA in PBMC declined after one year of PART. However, a single point mutation which confers DRM at amino acid position 46 in PR region (M46I) were detected in 100% (13/13) of clones sequenced.

Proviral DNA contained nine DRMs at time point 1–3 when abrupt elevation of VL was first detected. Therefore, five DRMs (D67N, M184V, T215Y in RT and D30N, N88D in PR) had accumulated between time points 1–2 and 1–3. The great majority of proviral DNA sequences at time point 1–4 contained DRMs in accordance with the direct sequence data of plasma viruses described above. However, we could detect wild type sequences by cloning and sequencing at amino acid positions 67 (D), 184 (M) in RT and at amino acid positions 46 (M), 88 (N) in PR. Of note was the reemergence of T215D in 4 clones while ZDV-containing treatment was continued.

3.2. Analysis of env sequences in patient 1

To examine a possibility of superinfection by drug-resistant HIV-1 strain in patient 1, we compared nucleotide sequences of V3 region (105 bases) in *env* gene at four time points. 20 clones from time point 1–1, 15 clones from time point 1–2 and 17 clones from time point 1–3, and the direct sequence data from time point 1–4 were used. V3 sequences were most diverse at time point 1–1, in which we detected 8 different sequences among 20 clones. We detected 5 different sequences at time point 1–2. However, a clonal sequence (17/17) which was closely related to some sequences at time point 1–1 dominated at time point 1–3 (17/17) (Fig. 4).

3.3. Drug resistant mutations and env sequences in patient 2

Although PART with a combination of d4T/ddI/EFV was not completely suppressive in patient 2, his VL was always below before time-point 2–2. His VL rose to 4400 copies/ml at time point 2–2 coincidentally with syphilitic rash. We compared the nucleotide sequences of the proviral DNA looking for DRMs (Fig. 5). One non-nucleoside analogue reverse transcriptase inhibitor (NNRTI)-related mutation at amino acid 179 (V179D), was already observed at time point 2–1 when he was seronegative for syphilis. Although we could not detect any new DRMs at 2–2 by direct sequencing of the PCR product, cloning and sequencing revealed minor clones with DRMs at amino acid positions 106 (V106M) and 184 (M184V). It should be noted that wild type clones at amino acid 179 (V179) showed up at time point 2–2. Interestingly, V179D and M184V were not observed in the same clone.

Env V3 region sequences at time points 2–1 and 2–2 clustered together within a large cluster of many reference sequences of subtype B viruses (Fig. 4). There was no evidence of superinfection.

3.4. Drug resistant mutations and env sequences before and after syphilis co-infection in patient 3 and 4

VLs in patients 3 and 4 were well suppressed before and after syphilis co-infection (Fig. 2). We did not observe any drug-resistant mutations in RT at indicated time points (data not shown). Although we did not observe DRMs in PR in patient 4, amino acid substitution at position 36 (M36I) was observed in patient 3 both before and after syphilis co-infection (data not shown). V3 region sequences in patient 3 clustered together with reference sequences of AE recombinant viruses suggesting that the virus in this patient is AE recombinant in origin (3–1 and 3–2 in Fig. 4). Therefore, it is inferred that M36I in patient 3 was not a DRM but a polymorphism of

A. Nucleotide sequence of proviral DNA

		RT coding region							PR coding region				
Amino acid number		41	67		184	210	215		30	46	63	88	
Wt nucleotide		ATG	GAC		ATG	TTG	ACC		GAT	ATG	CTC	AAT	
Time point													
1-1	Nucleotide	...	...	11/16*	...	.G.	GA.	11/16*	...	...	.C.	...	16/16
	Nucleotide	C..	...	5/16*									
1-2	Nucleotide	C..	...	20/20	...	.G.	GA.	19/20	...	..A	.C.	...	13/13
	Nucleotide				.C.	.G.	GA.	1/20					
1-3	Nucleotide	C..	A..	20/20*	G..	.G.	TA.	20/20*	A..	..A	.C.	G..	20/20
1-4	Nucleotide	C..	A..	19/20	G..	.G.	TA.	15/20	A..	..A	.C.	G..	12/20
	Nucleotide	C..	...	1/20	...	.G.	GA.	4/20	A..	..A	.C.	...	2/20
	Nucleotide				...	.G.	TA.	1/20	...	..A	.C.	G..	1/20
	Nucleotide								A..	...	.C.	...	1/20
	Nucleotide								...	...	.C.	G..	1/20
	Nucleotide								...	...	.C.	...	3/20

B. Amino acid sequence of proviral DNA

		RT coding region							PR coding region				
Amino acid number		41	67		184	210	215		30	46	63	88	
Wt amino acid		M	D		M	L	T		D	M	L	N	
Time point													
1-1	Amino acid	-	-	11/16*	-	W	D	11/16*	-	-	P	-	16/16
	Amino acid	L	-	5/16*									
1-2	Amino acid	L	-	20/20	-	W	D	19/20	-	I	P	-	13/13
	Amino acid				T	W	D	1/20					
1-3	Amino acid	L	N	20/20*	V	W	Y	20/20*	N	I	P	D	20/20
1-4	Amino acid	L	N	19/20	V	W	Y	15/20	N	I	P	D	12/20
	Amino acid	L	-	1/20	-	W	D	4/20	N	I	P	-	2/20
	Amino acid				-	W	Y	1/20	-	I	P	D	1/20
	Amino acid								N	-	P	-	1/20
	Amino acid								-	-	P	D	1/20
	Amino acid								-	-	P	-	3/20

Fig. 3. Drug resistant mutations in patient 1. Wild type residues are shown on the top. Residues identical to the wild type are shown by dots (nucleotides) or dashes (amino acids). Squared capital letters indicate amino acid mutations related to the drug resistance. Fraction numbers are the number of subclones with the amino acid change indicated per total number analyzed subclones. Examined time points are shown on the left. RT at 1–2 and 1–4 were separately analyzed.

HIV-1 [18,19]. Since V3 sequences of patient 4 clustered together with reference sequences of subtype B viruses, it was likely that patient 4 was infected by a subtype B virus. From the serologic tests, it was likely that patient 4 had syphilis co-infection before time point 4–2 (Fig. 2). It is intriguing that a transient but substantial shift of V3 sequences was observed at time point 4–2 (Figs. 4 and 6). Since all V3 clones of patient 4 clustered compactly by themselves, however, it is unlikely that viruses at time point 4–2 were originated from superinfection.

4. Discussion

The coincidence of active syphilis and a break through of drug-resistant HIV-1 in a patient suggested us a possibility of superinfection at first. Therefore, we studied this case longitudinally using frozen samples. He was referred to our hospital after the initiation of PART. At time point 1–1, he was on his initial PART with ZDV, 3TC and NFV and VL was undetectable. However, the sequences of proviruses at time point 1–1 showed that a couple of thymidine analog-related mutations(TAMs) such as L210W and M41L had already been present in RT region. T215D was also present. T215D, referred to as a fossil mutation, is drug-sensitive but suggestive of a reversion from a drug resistant mutation T215Y [15,16]. L210W and M41L are TAMs that usually occur in the virus harboring T215Y [20]. Co-presence of L210W and M41L together with a fossil mutation T215D suggests that patient 1 had originally been infected by a ZDV-resistant virus, although we cannot rule out the possibility that these TAMs accumulated gradually while the patient had undetectable VL [21]. Proviruses at time point 1–2 contained increasing number of drug-resistant mutations while the patient's VL was still undetectable; all the 20 clones were now M41L in RT region, and M46I in PR region was also detected. The concomitant presence of M46I and L63P tends to lead the acquisition of D30N and N88D whose combination reduces susceptibility to NFV some 50 fold [22]. Actually both D30N and N88D were proven in patient 1 at time point 1–3 when the patient developed full-blown resistance. Analysis of serial V3 region sequences excluded the possibility of superinfection.

At time point 1–4 when VL went up higher, reversions towards the wild type residues were detected by cloning and sequencing. Reversions were observed in different classes of anti-retroviral drugs (ARVs) (thymidine and cytidine analogue-related mutations and protease inhibitor-related

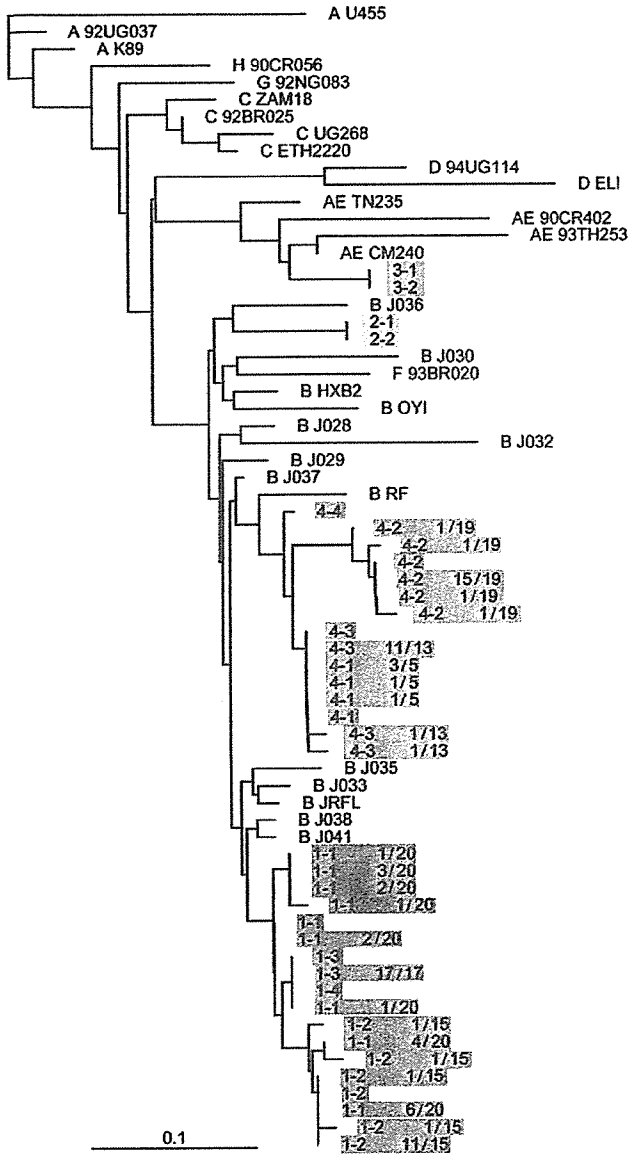

Fig. 4. Phylogenetic tree of V3 sequences in four patients. The phylogenetic tree was drawn according to the nucleotide sequences of *env* V3 regions (105 base). Time points with direct sequence indicate those derived from the direct sequencing. Time points with fraction numbers indicate the sequences from subclones per total numbers analyzed. The first or the first two capital letters of each consensus sequence indicates its subtype (e.g. B: subtype B, AE: AE recombinant).

mutations), which suggest general decrease of selective pressure by ARVs. Although he declared strict adherence to the treatment throughout the course, intended or unintended drug holidays were likely to be the cause of full-blown drug resistance mutations at time point 1–3 and subsequent appearance of revertants at time point 1–4. The importance of adherence to PART should not be underestimated.

The revertants at time point 1–4 could have occurred by simple back-mutation. However, it is noteworthy that the reversions were seen at only those mutations which occurred after time point 1–2 and not at mutations already present at time point 1–1. There could be a possibility that clones harboring wild type residues arose from latently infected reservoir cells. Although both simple back-mutation and reemergence of latent viruses could occur under the decreased selective pressure of ARVs, it was recently reported that back-mutation usually did not take place for more than a year among drug-resistant mutations transmitted at primary HIV-1 infection [23,24].

Patient 2 also obtained drug-resistant mutations around syphilis infection. One NNRTI- related mutation V179D was already present in 100% of the populations before syphilis infection, but another NNRTI-related mutation V106M emerged in 15% of the populations after syphilis infection. Similarly to time point 1–4 in patient 1, reversion of V179D to wild type residue was seen in about half of the populations after syphilis, suggesting a decreased selective pressure of ARVs in this case also.

Of interest was the emergence of M184V mutation, which is known to cause high level resistance to 3TC [25]. This patient was not taking 3TC during the examined period, although he had previously been treated with dual nucleoside therapy with ZDV and 3TC. Among the ARVs he took during the period, ddI may be a responsible drug for M184V mutation, although M184V's selective advantage over ddI is much lower

A. Nucleotide sequence of proviral DNA

Amino acid position	106	179	184	
Wt nucleotide	GTA	GTT	ATG	
Time point				
2-1				
Nucleotide	...	.A.	...	20/20
2-2				
Nucleotide	...	.A.	...	6/20
Nucleotide	A.G	.A.	...	2/20
Nucleotide	A.G	...	G..	1/20
Nucleotide	...	...	G..	4/20
Nucleotide	...	...	...	7/20

B. Amino acid sequence of proviral DNA

Amino acid position	106	179	184	
Wt amino acid	V	V	M	
Time point				
2-1				
Amino acid	-	[D]	-	20/20
2-2				
Amino acid	-	[D]	-	6/20
Amino acid	[M]	[D]	-	2/20
Amino acid	[M]	-	[V]	1/20
Amino acid	-	-	[V]	4/20
Amino acid	-	-	-	7/20

Fig. 5. Drug resistant mutations in patient 2. Wild type residues are shown on the top. Residues identical to the wild type are shown by dots (nucleotides) or dashes (amino acids). Squared capital letters indicate amino acid mutations related to the drug resistance. Fraction numbers are the number of subclones with the amino acid change indicated per total number analyzed subclones. Examined time points are shown on the left.

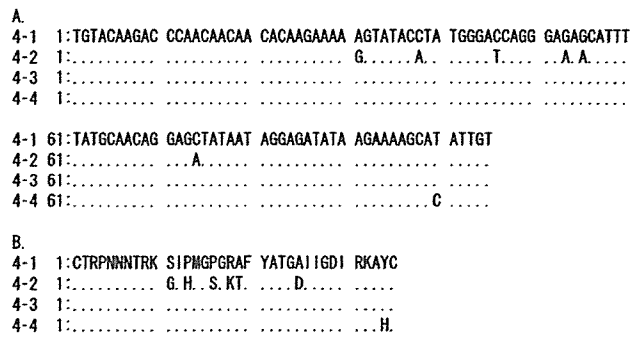

Fig. 6. Nucleotide (a); and amino acid (b) change in *env* V3 region in patient 4.

than over 3TC (1.4 fold versus more than 200 fold) [26]. Furthermore none of the clones containing M184V had V179D which was observed in all the clones at time point 2–1. Thus, it is likely that the mutants harboring M184V were derived from latently infected reservoir cells which had been established while the patient had taken 3TC without NNRTI. Syphilis infection may have predisposed the reactivation of these proviruses [10,27].

In both patients 1 and 2, decreased selective pressure appeared to be the principal driving factor for the emergence of drug-resistant mutants and the concomitant appearance of revertants and/or latently infected viruses. Our results may suggest that deteriorated adherence to ARV and committing unprotected sexual intercourse may be linked at least in some patients. Clinicians taking care of patients on ARV should pay special attention on ARV adherence when their patients get new STI.

It was recently reported that syphilis infection increased HIV-1 VL [10]. This increase was reported to be more prominent for patients without PART than patients under PART. Among the four cases under PART that we examined here, VL increased more than one log after syphilis co-infection in two patients. Both of them had already some DRMs before syphilis co-infection, and accumulated further DRMs after syphilis co-infection. The other two patients without any DRMs did not show significant changes in VL. It is possible that syphilis co-infection had some effect on the increase of VL for the former two cases that had some DRMs at baseline. However, as discussed above, a decrease of selective pressure was more probable cause of VL increase in these patients.

In patients 3 and 4, we could not detect any DRMs before and after syphilis co-infection, but longitudinal analysis of provirus sequences in patient 4 revealed a transient dynamic shift of env V3 region just after syphilis co-infection (time point 4–2). This transient shift of V3 sequences might be also attributable to the reactivation of latent proviruses in reservoir cells. However, the sequence shift was only seen in *env* V3 region, and no remarkable nucleotide changes were observed in *gag* (data not shown) or *pol* sequences. The reason for the dynamic change only in V3 region waits for further explanations.

In conclusion, we examined longitudinally the changes of provirus sequences of four patients under PART before and after syphilis co-infection. Two patients who had already some DRMs at baseline revealed VL increase and further accumulation of mutations around syphilis co-infection, whereas the other two that were devoid of any DRMs did not show VL increase or mutation accumulation. Reactivation of latent proviruses was suspected in some cases, but no evidence of superinfection was observed in any patient. HIV-1 superinfection appears to be less common than presumed before [7,8]. However, the prevalence of syphilis co-infection among HIV-1-infected patients indicates a spread of unprotected sexual behavior which may lead to HIV-1 superinfection as well as syphilis and other STIs. The importance of practicing safer sex should be underscored. Since it is most likely that the accumulation of DRMs in our cases was due to loosened adherence to ARVs, the importance of strict adherence should be emphasized.

References

[1] CDC, Trends in primary and secondary syphilis and HIV infections in men who have sex with men—San Francisco and Los Angeles, California, 1998–2002, MMWR Morb. Mortal Wkly Rep. 53 (2004) 575–578.
[2] M. Hourihan, H. Wheeler, R. Houghton, B.T. Goh, Lessons from the syphilis outbreak in homosexual men in east London. Sex Transm. Infect. 80 (2004) 509–511.
[3] M.S. Cohen, Sexually transmitted diseases enhance HIV transmission: no longer a hypothesis, Lancet 351 (Suppl. 3) (1998) 5–7.
[4] T.K. Collis, C.L. Celum, The clinical manifestations and treatment of sexually transmitted diseases in human immunodeficiency virus-positive men, Clin. Infect. Dis. 32 (2001) 611–612.
[5] T. Fujii, H. Taguchi, H. Katano, S. Mori, T. Nakamura, N. Nojiri, K. Nakajima, K. Tadokoro, T. Juji, A. Iwamoto, Seroprevalence of human herpesvirus 8 in human immunodeficiency virus 1-positive and human immunodeficiency virus 1-negative populations in Japan, J. Med. Virol. 57 (1999) 159–162.
[6] S.J. Little, S. Holte, J.P. Routy, E.S. Daar, M. Markowitz, A.C. Collier, R.A. Koup, J.W. Mellors, E. Connick, B. Conway, M. Kilby, L. Wang, J.M. Whitcomb, N.S. Hellmann, D.D. Richman, Antiretroviral-drug resistance among patients recently infected with HIV, N. Engl. J. Med. 347 (2002) 385–394.
[7] M.J. Gonzales, E. Delwart, S.Y. Rhee, R. Tsui, A.R. Zolopa, J. Taylor, R.W. Shafer, Lack of detectable human immunodeficiency virus type 1 superinfection during 1072 person-years of observation, J. Infect. Dis. 188 (2003) 397–405.
[8] R. Tsui, B.L. Herring, J.D. Barbour, R.M. Grant, P. Bacchetti, A. Kral, B.R. Edlin, E.L. Delwart, Human immunodeficiency virus type 1 superinfection was not detected following 215 years of injection drug user exposure, J. Virol. 78 (2004) 94–103.
[9] D.M. Smith, J.K. Wong, G.K. Hightower, C.C. Ignacio, K.K. Koelsch, E.S. Daar, D.D. Richman, S.J. Little, Incidence of HIV superinfection following primary infection, JAMA 292 (2004) 1177–1178.
[10] K. Buchacz, P. Patel, M. Taylor, P.R. Kerndt, R.H. Byers, S.D. Holmberg, J.D. Klausner, Syphilis increases HIV viral load and decreases CD4 cell counts in HIV-infected patients with new syphilis infections, Aids 18 (2004) 2075–2079.
[11] J.D. Thompson, D.G. Higgins, T.J. Gibson, CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice, Nucleic Acids Res. 22 (1994) 4673–4680.
[12] R.D. Page, TreeView: an application to display phylogenetic trees on personal computers, Comput. Appl. Biosci. 12 (1996) 357–358.
[13] T. Leitner, Updated proposal of reference sequences of HIV-1 genetic subtypes.www.hiv.lanl.gov/content/hiv-db/COMPENDIUM/1997/partIII/Leitner.pdf (1997).

[14] T. Furutsuki, N. Hosoya, A. Kawana-Tachikawa, M. Tomizawa, T. Odawara, M. Goto, Y. Kitamura, T. Nakamura, A.D. Kelleher, D.A. Cooper, A. Iwamoto, Frequent transmission of cytotoxic-T-lymphocyte escape mutants of human immunodeficiency virus type 1 in the highly HLA-A24-positive Japanese population, J. Virol. 78 (2004) 8437–8445.

[15] J.G. Garcia-Lerma, S. Nidtha, K. Blumoff, H. Weinstock, W. Heneine, Increased ability for selection of zidovudine resistance in a distinct class of wild-type HIV-1 from drug-naive persons, Proc. Natl. Acad. Sci. U.S.A. 98 (2001) 13907–13912.

[16] D.R. Kuritzkes, A fossil record of zidovudine resistance in transmitted isolates of HIV-1, Proc. Natl. Acad. Sci. U.S.A. 98 (2001) 13485–13487.

[17] M.J. Kozal, N. Shah, N. Shen, R. Yang, R. Fucini, T.C. Merigan, D.D. Richman, D. Morris, E. Hubbell, M. Chee, T.R. Gingeras, Extensive polymorphisms observed in HIV-1 clade B protease gene using high-density oligonucleotide arrays, Nat. Med. 2 (1996) 753–759.

[18] J.C. Clemente, R. Hemrajani, L.E. Blum, M.M. Goodenow, B.M. Dunn, Secondary mutations M36I and A71V in the human immunodeficiency virus type 1 protease can provide an advantage for the emergence of the primary mutation D30N, Biochemistry 42 (2003) 15029–15035.

[19] L. Vergne, M. Peeters, E. Mpoudi-Ngole, A. Bourgeois, F. Liegeois, C. Toure-Kane, S. Mboup, C. Mulanga-Kabeya, E. Saman, J. Jourdan, J. Reynes, E. Delaporte, Genetic diversity of protease and reverse transcriptase sequences in non-subtype-B human immunodeficiency virus type 1 strains: evidence of many minor drug resistance mutations in treatment-naive patients, J. Clin. Microbiol. 38 (2000) 3919–3925.

[20] N. Yahi, C. Tamalet, C. Tourres, N. Tivoli, J. Fantini, Mutation L210W of HIV-1 reverse transcriptase in patients receiving combination therapy. Incidence, association with other mutations, and effects on the structure of mutated reverse transcriptase, J. Biomed. Sci. 7 (2000) 507–513.

[21] A. de Ronde, M. van Dooren, L. van Der Hoek, D. Bouwhuis, E. de Rooij, B. van Gemen, R. de Boer, J. Goudsmit, Establishment of new transmissible and drug-sensitive human immunodeficiency virus type 1 wild types due to transmission of nucleoside analogue-resistant virus, J. Virol. 75 (2001) 595–602.

[22] A.K. Patick, M. Duran, Y. Cao, D. Shugarts, M.R. Keller, E. Mazabel, M. Knowles, S. Chapman, D.R. Kuritzkes, M. Markowitz, Genotypic and phenotypic characterization of human immunodeficiency virus type 1 variants isolated from patients treated with the protease inhibitor nelfinavir, Antimicrob. Agents Chemother. 42 (1998) 2637–2644.

[23] R.T. Gandhi, A. Wurcel, E.S. Rosenberg, M.N. Johnston, N. Hellmann, M. Bates, M.S. Hirsch, B.D. Walker, Progressive reversion of human immunodeficiency virus type 1 resistance mutations in vivo after transmission of a multiply drug-resistant virus, Clin. Infect. Dis. 37 (2003) 1693–1698.

[24] D. Pao, U. Andrady, J. Clarke, G. Dean, S. Drake, M. Fisher, T. Green, S. Kumar, M. Murphy, A. Tang, S. Taylor, D. White, G. Underhill, D. Pillay, P. Cane, Long-term persistence of primary genotypic resistance after HIV-1 seroconversion, J. Acquir. Immune Defic. Syndr. 37 (2004) 1570–1573.

[25] Y. Quan, Z. Gu, X. Li, Z. Li, C.D. Morrow, M.A. Wainberg, Endogenous reverse transcription assays reveal high-level resistance to the triphosphate of (−)2′-dideoxy-3′-thiacytidine by mutated M184V human immunodeficiency virus type 1, J. Virol. 70 (1996) 5642–5645.

[26] L. Ross, N. Parkin, C. Chappey, R. Fisher, M.S. Clair, M. Bates, M. Tisdale, E.R. Lanier, Phenotypic impact of HIV reverse transcriptase M184I/V mutations in combination with single thymidine analog mutations on nucleoside reverse transcriptase inhibitor resistance, Aids 18 (2004) 1691–1696.

[27] J. Podwinska, M. Lusiak, R. Zaba, J. Bowszyc, The pattern and level of cytokines secreted by Th1 and Th2 lymphocytes of syphilitic patients correlate to the progression of the disease, FEMS Immunol. Med. Microbiol. 28 (2000) 1–14.

CLINICAL AND VACCINE IMMUNOLOGY, Feb. 2007, p. 168–172 Vol. 14, No. 2
1556-6811/07/$08.00+0 doi:10.1128/CVI.00224-06

Detecting Immunoglobulin M Antibodies against Microsporidian *Encephalitozoon cuniculi* Polar Tubes in Sera from Healthy and Human Immunodeficiency Virus-Infected Persons in Japan▽

Mako Omura,[1] Koji Furuya,[1]* Shinichi Kudo,[2] Wataru Sugiura,[3] and Hiroshi Azuma[4]

Department of Parasitology, National Institute of Infectious Diseases, Tokyo, Japan[1]; Hokkaido Institute of Public Health, Sapporo, Japan[2]; AIDS Research Center, National Institute of Infectious Diseases, Tokyo, Japan[3]; and Hokkaido Red Cross Blood Center, Sapporo, Japan[4]

Received 18 June 2006/Returned for modification 1 September 2006/Accepted 6 November 2006

Encephalitozoon cuniculi*, a spore-forming obligate intracellular parasitic pathogen belonging to the phylum Microsporidia, has a unique and highly specialized organelle called the polar tube. Using an enzyme immunostaining assay in which germinated *E. cuniculi* spores were coated onto plastic surfaces, we tested healthy and human immunodeficiency virus (HIV)-infected individuals in Japan for anti-polar tube antibodies of each immunoglobulin (Ig) class. Anti-polar tube IgG was detected in just 4 of 380 healthy individuals; no anti-polar tube IgA was detected in any individuals; however, unexpectedly, anti-polar tube IgM antibodies were detected in 138 individuals (36%). When the healthy individuals were grouped by age, the highest rate of positivity to anti-polar tube IgM antibodies was seen in individuals aged 20 years old or younger. Fifty-nine percent (24/41) of the individuals aged 20 years or younger were anti-polar tube IgM antibody positive. This rate tended to decrease among individuals in older age groups. However, no anti-polar tube IgM antibodies were detected in 21 HIV-infected persons who were younger than 30 years of age and who had CD4 cell levels below 250/μl. These seroepidemiological results clearly indicate that circulating anti-polar tube IgM antibodies that are capable of strongly reacting with filaments extruded from geminated spores exist and suggest that such antibodies may play a part in protective immunity.

Encephalitozoon cuniculi is a microsporidian parasitic pathogen listed in a 1996 WHO report as an emerging infectious agent (17). The pathogen is also considered a zoonotic parasite (4). Various animals can be naturally infected by *E. cuniculi*, and its geographical distribution is worldwide (3). In Japan, *E. cuniculi* infection in rabbits and in squirrel monkeys in zoos is of current concern (1, 5). The rate of infection is considered to increase each year, and the infection has now spread throughout Japan. However, to the best of our knowledge, the only case of human microsporidiosis reported in Japan was in a 9-year-old boy in 1958 (10). Although immunological conditions of the Japanese case were not recorded, almost all other patients infected with this pathogen in other nations have been immunocompromised groups of human immunodeficiency virus (HIV)-infected patients (16). A few cases have also been found among renal transplant recipients (6, 11). *E. cuniculi* can thus be regarded as an opportunistic pathogen (2). Cases of HIV-associated infections with *E. cuniculi* are increasingly reported, although they remain less common than those due to *Encephalitozoon bieneusi* and *Encephalitozoon intestinalis* (2). Many reports on the seroprevalence of human *E. cuniculi* infection have been published (3, 8). However, the reported rates of microsporidial seropositivity vary greatly, depending on the serological technique used, probably due to the use of antigens unsuitable for measurement of specific antibodies and the use of secondary antibodies without differential specificities.

Recently, specific immunoglobulin G (IgG) antibodies against the polar tube (PT) of *E. cuniculi* were demonstrated in a healthy laboratory worker accidentally infected with *E. cuniculi* (14). The PT is a typical microsporidian spore structure with an extrusion that is essential for invasion of a host cell, as sporoplasm flows through the discharged PT and into the host cell (13).

We have recently developed an enzyme immunostaining assay (EIA) for measuring anti-*E. cuniculi* PT antibodies using 96-well microplates coated with germinated spores. This method allows us to screen human sera for anti-PT antibodies on a large scale for seroepidemiological analysis. This study reports on the screening of sera from 380 healthy persons and 78 HIV-infected persons seroepidemiologically analyzed by this particular EIA, which is capable of measuring anti-PT antibodies of each Ig class, that is, IgM, IgG, and IgA.

MATERIALS AND METHODS

Serum samples. For this study we used serum samples from 380 healthy people living in Hokkaido Prefecture, Japan; serum samples from 180 residents who underwent a serological test for parasitosis in 2000 but who showed negative results; and serum samples from 200 blood donors collected in 2005.

Serum samples from 78 HIV-infected persons, collected in 1999 from the Kanto region of Japan, were also provided for this study. These included sera from 51 persons with CD4 cell levels below 250/μl and sera from 27 persons with CD4 cell levels between 251 and 900/μl. The 51 persons in the former group were of various ages, while the 27 persons in the latter group were younger than 30 years of age. Tests for HIV infection and determination of CD4 lymphocyte counts were performed by standard laboratory protocols.

, *E. cuniculi* spores. For this study we used the *E. cuniculi* HF strain, isolated from a rabbit with encephalitozoonosis. Strain HF was then cultivated in RK-13

* Corresponding author. Mailing address: Department of Parasitology, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan. Phone: 81(3)5285-1111. Fax: 81(3)5285-1173. E-mail: kfuruya@nih.go.jp.

▽ Published ahead of print on 15 November 2006.

TABLE 1. Results of serological detection of anti-*E. cuniculi* PT antibodies in healthy residents, blood donors, and HIV-infected persons

Subject group and antibody	No. (%[a]) of individuals with antibody titer of:							
	<1/50	1/50	1/100	1/200	1/400	1/800	1/1,600	Total
Healthy residents								
IgM	114 (63.3)	26 (14.4)	16 (8.9)	16 (8.9)	5 (2.8)	3 (1.7)	0	180
IgG	177	3	0	0	0	0	0	180
IgA	180	0	0	0	0	0	0	180
Blood donors								
IgM	128 (64)	29 (14.5)	27 (13.5)	11 (5.5)	2 (1.0)	2 (1.0)	1 (0.5)	200
IgG	199	0	0	0	0	0	1	200
IgA	200	0	0	0	0	0	0	200
HIV-infected persons with CD4 cell counts below 250/μl								
IgM	47	2	1	1	0	0	0	51
IgG	49	0	1	1	0	0	0	51
IgA	51	0	0	0	0	0	0	51

[a] Rates of positivity for anti-PT IgM antibodies.

cells (ATCC CCL-37) (5). Culture supernatants of HF-infected RK-13 cells were collected, centrifuged, and used for serological tests.

Strain HF was genetically analyzed beforehand by PCR, followed by direct DNA sequencing (1). The internal transcribed spacer gene sequence revealed that strain HF was classified into genotype I, since it contained three GTTT repeats. Sequence analysis of the spore wall protein I gene revealed that the strain belonged to genotype Ia because of the amplification of a 399-bp PCR product.

Microplate enzyme immunostaining assay. Sediments containing germinated spores, nongerminated spores, and heavily infected cells detached from cell sheets, were suspended in Gibco minimal essential medium including Earle's salts and glutamine (Invitrogen Corporation, Grand Island, NY) and supplemented with 1,000 U/ml penicillin G, 1,000 μg/ml streptomycin, and 10% fetal bovine serum; this medium was also used for cultures of RK-13 and BS-C-1 (ATCC CCL-26) cells, as described below. Approximately 4×10^6 free spores (containing detached cells) were inoculated into each well of a 96-well flat bottom microplate (high-binding polystyrene; Corning Incorporated, NY) and cultured for 3 days at 35°C in an incubator with 5% CO_2. Subsequently, the wells were washed once with phosphate-buffered saline (PBS; pH 7.2) and fixed with 10% formalin in PBS for 1 h at room temperature (RT). The wells were then washed twice with PBS, treated with PBS containing 1% Tween 20 for 1 h at RT, and washed twice with PBS. The wells were further treated with blocking buffer (SuperBlock; Pierce, Rockford, IL) for 1 h at RT and were finally washed twice with PBS. The plates with wells coated with more than 100 germinated spores per well were then studied.

Twofold dilutions of each serum sample were made by using PBS containing 0.05% Tween 20 (PBS-T), starting from a 1:50 dilution; 100 μl of each of the dilutions was added to each coated well. The wells were incubated for 90 min at RT and then washed five times with 200 μl PBS-T. Subsequently, the wells were incubated with 100 μl of the secondary antibody or protein A/G, incubated for 60 min at RT, and washed five times with PBS-T. A 1:3,000 dilution of protein A/G labeled with peroxidase (PO) (Prozyme Inc., San Leandro, CA), a 1:5,000 dilution of anti-human IgM (μ-chain specific) labeled with PO (QED Bioscience Inc., San Diego, CA), and a 1:3,000 dilution of anti-human IgA (Fc specific) labeled with PO (Nordic Immunology, The Netherlands) were used to capture IgG, IgM, and IgA antibodies, respectively. Finally, the signals of PO bound to human Ig antibodies were visualized by using the substrate aminoethyl carbazole (Zymed Laboratories Inc., San Francisco, CA), according to the manufacturer's instructions. After the wells were washed with pure water, the results were observed with a light microscope. In this assay, only judgments on the serological reactions to filaments extruded from germinated spores (i.e., PTs) were made, while the reactions to the spore walls and the host cells were recorded as reference data.

The concentrations of each secondary antibody, noted above, were determined beforehand by using a dot immunoassay and the corresponding purified Ig. Sera from rabbits with encephalitozoonosis were used as positive controls (5). PO-labeled protein A/G was used for detection of rabbit IgG antibodies as the secondary antibody.

BS-C-1 cells were infected with *E. intestinalis* (ATCC 5057) and *E. hellem* (ATCC 50451) spores. The resultant germinated spores were examined by the procedures mentioned above.

Ethical considerations. The protocol for this study was approved by the Committee for Research on Human Subjects of the National Institute of Infectious Diseases, Tokyo, Japan. Written informed consent was obtained from the HIV-positive subjects. The use of healthy residents' sera and the use of blood donors' sera were approved by the Institutional Review Board of the Hokkaido Institute of Public Health and the Institutional Review Board of the Hokkaido Red Cross Blood Center, respectively. All serum samples included in this study were processed to protect personal information. For all serum samples, the only specific clinical information available was the sex, age, and health condition.

Statistical analysis. The sera of the healthy residents and blood donors were each divided into six groups according to age. The relationship between anti-PT IgM prevalence in each age group and the year of blood collection (2000 for healthy residents and 2005 for blood donors) was analyzed by the Mantel-Haenszel method. The relationship between increasing age and decreasing rate of positivity for anti-PT IgM antibodies in healthy subjects (healthy residents plus blood donors) was analyzed by the Cochran-Armitage test. The statistical significance of the presence of anti-PT IgM antibodies by gender was determined by the chi-square test. P values of <0.01 were considered statistically significant. Excel Statistics 2006 software for Windows (release 6.7.1; Social Survey Research Information Co. Ltd., Tokyo, Japan) was used for statistical analysis.

RESULTS

Table 1 summarizes the results of the EIA for the detection of anti-*E. cuniculi* PT antibodies in samples from healthy residents, blood donors, and HIV-infected persons. Anti-PT IgG antibodies were detected in only 3 of 180 serum samples from healthy individuals; the titers were 1:50, which was significantly lower than those for the controls (naturally infected rabbit sera), which showed titers of 1:6,400 to 1:102,400. When the 200 donor serum samples were examined by EIA, anti-*E. cuniculi* PT IgG antibodies were detected in only 1 serum sample; the titer was 1:1,600, but the positive signals were very weak even at the lower dilutions.

No anti-PT IgA antibody was detected in any of the 380 serum samples with titers below 1:50. Furthermore, 2 of the 51 HIV-infected persons with CD4 cell levels below 250/μl had anti-PT IgG antibodies, and anti-PT IgA antibodies were not detected in any of the 51 HIV-infected persons.

On the other hand, when the same serum samples described above were examined for the presence or absence of anti-PT IgM antibodies, the results were quite different from those for

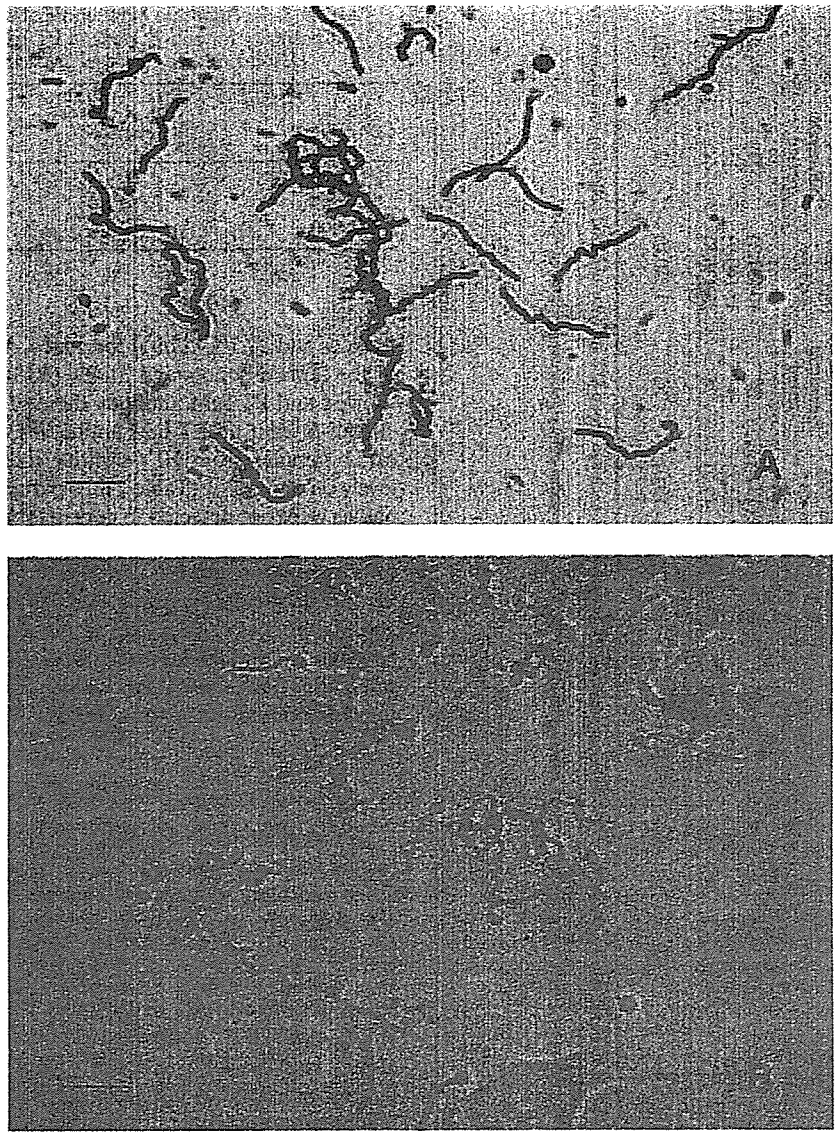

FIG. 1. Immunostaining of filaments (PTs) extruded from *E. cuniculi*-geminated spores with human IgM antibodies or rabbit IgG antibodies. (A) Positive results obtained from the serum sample from donor 197 diluted 1:200. Note the strongly positive signals on the filaments extruded from the germinated spores but the unstained spore walls. (B) Positive results obtained from a serum sample from a symptomatic rabbit with natural *E cuniculi* infection diluted 1:400. Note the positive signals on the spore wall and the filament. Bars, 10 μm.

anti-PT IgG and IgA antibodies. Anti-PT IgM antibodies were detected in 66 (36.7%) of the 180 serum samples from healthy persons, which showed titers of 1:50 to 1:800; and in 72 (36%) of the 200 serum samples from the blood donors, which showed titers of 1:50 to 1:1,600. The reactivities of the IgM antibodies with filaments were considered to be typical of IgM, because of the low titers (below 1:1,600), but the IgM antibodies had stronger reactivities than the rabbit anti-*E. cuniculi* PT IgG antibodies (Fig. 1A and B). The four serum samples from healthy persons and donors with anti-PT IgG activities also had anti-PT IgM activities.

By using almost the same procedures used for the EIA used for detection of anti-*E. cuniculi* PT IgM antibodies, sera with titers of more than 1:200 were examined for cross-reactions with PTs of *E. hellem* and *E. intestinalis*; however, no antibody activity against the filaments was detected (data not shown).

A decreasing trend in positivity rates for anti-PT IgM antibodies was observed when subjects (healthy residents plus blood donors) were grouped according to age. As seen from Table 2, the rate of positivity for anti-PT IgM antibodies was the highest among people aged 19 years or younger: 59% of healthy subjects. The seropositivity rates clearly tended to decrease among the older subjects ($P < 0.01$). The rates of positivity for anti-PT IgM antibodies in each age group were irrelevant to the year that the serum samples were collected, i.e., in 2000 (healthy residents) and 2005 (blood donors). The rate of positivity for anti-PT IgM antibodies among females was a little higher than that among males: 43.8% (39/89) for

TABLE 2. Age distribution of cases with anti-*E. cuniculi* PT IgM antibodies of healthy and HIV-infected persons

Subject group	Value for age group											
	19 yr or younger		20–29 yr		30–39 yr		40–49 yr		50–59 yr		60 yr or older	
	No. of individuals tested	No. (%) positive	No. of individuals tested	No. (%) positive	No. of individuals tested	No. (%) positive	No. of individuals tested	No. (%) positive	No. of individuals tested	No. (%) positive	No. of individuals tested	No. (%) positive
Healthy residents[a]	31	18 (58.1)	29	13 (44.8)	26	12 (46.2)	31	4 (12.9)	32	10 (31.3)	31	9 (29.0)
Blood donors[a]	10	6 (60.0)	46	19 (41.3)	54	19 (35.2)	33	7 (21.2)	44	18 (40.9)	13	3 (23.1)
Healthy residents and blood donors[b]	41	24 (58.5)	75	32 (42.7)	80	31 (38.8)	64	11 (17.2)	76	28 (36.8)	44	12 (27.3)
HIV-infected persons with CD4 cell counts below 250/μl	4	0 (0)	17	0 (0)	16	2 (12.5)	10	1 (10.0)	2	1 (50.0)	2	0 (0)

[a] Serum samples were collected in 2000 from healthy residents and in 2005 from blood donors. The relationship between anti-PT IgM prevalence in each age group and the year of blood collection was analyzed by the Mantel-Haenszel method, which did not give statistically significantly different values.

[b] An association between increasing age and a decreasing rate of positivity for anti-PT IgM was analyzed by the Cochran-Armitage test, which gave statistically significant *P* values of <0.01.

TABLE 3. Relationship between number of CD4 cells and rate of positivity for anti-*E. cuniculi* PT IgM antibodies among HIV-infected persons younger than 30 years of age

No. of CD4 cells/μl	No. of persons examined	No. (%) of persons with anti-*E. cuniculi* PT IgM antibodies
<250	21	0 (0)
251–399	12	3 (25)
400–900	15	9 (60)

females and 29.7% (27/91) for males. A total of 41.1% (39/95) of the female donors and 31.4% (33/105) of the male donors showed anti-PT IgM antibody titers of 1:50 or more.

Anti-*E. cuniculi* PT IgM antibodies were detected in only 4 of 51 samples from HIV-positive individuals with CD4 cell levels below 250/μl (Table 1). In particular, anti-PT IgM antibodies were not detected in persons younger than age 30 years and with <250 CD4 cells/μl (Table 2). Interestingly, a high rate of positivity for anti-PT IgM antibodies was observed among the 27 HIV-positive persons with CD4 cell counts between 251 and 900/μl; i.e., 25% (3/12) of people with CD4 cell counts between 251 and 399/μl and in 60% (9/15) of people with CD4 cell counts between 400 and 900/μl (Table 3); furthermore, all these individuals were younger than 30 years of age.

DISCUSSION

Our present results indicate that anti-*E. cuniculi* PT antibodies could be detected in 36% of the people, healthy residents and blood donors, who should be considered immunocompetent. In respect to antibodies against *Encephalitozoon* PT among immunocompetent persons, it has been reported that anti-*E. intestinalis* PT was demonstrated in 8% of Dutch blood donors and 5% of pregnant French women (15). Our anti-*E. cuniculi* PT antibodies were detected by EIA, while the anti-*E. intestinalis* PT antibodies were detected by an indirect fluorescent-antibody test (15). The sensitivities of enzyme immunoassays are generally believed to be fairly higher than those of immunofluorescence assays.

No cross-reactive relationship between human anti-*E. cuniculi* PT and human anti-*E. intestinalis* PT has been proved. We clearly showed that our human sera containing anti-PT IgM antibodies did not cross-react with filaments extruded from germinated spores of *E. intestinalis* and *E. hellem*. Our finding is in agreement with previous ones that human sera containing anti-*E. intestinalis* PT antibody activity did not immunostain the filaments extruded from *E. cuniculi* (13).

Anti-*E. cuniculi* PT antibodies, unlike the immunoglobulin class of anti-*E. intestinalis* PT antibodies, which was shown to be IgG (13, 15), were not of the IgG class but were of the IgM class. Anti-*E. cuniculi* PT IgG antibodies were detected in the sera of just four persons (one had a titer of 1:1,600, and the others showed low titers of 1:50) (Table 1). Their reactivities probably resulted from anti-*E. cuniculi* PT IgM antibodies. In our experiments, we used protein A/G instead of anti-human IgG to capture IgG antibodies. It is known that protein A strongly binds to IgG molecules but also weakly binds to some IgM molecules (9). In fact, the four serum samples all exhib-

ited elevated titers of anti-PT IgM antibodies, but their ability to stain extruded filaments was not so strong.

There was no significant difference in the rate of positivity for anti-PT IgM antibodies by gender, although the rate of positivity for anti-PT IgM antibodies was slightly higher among females than among males. However, some relationship between the prevalence of anti-PT IgM antibodies and age would be expected. The rate of positivity for anti-PT IgM antibodies was significantly higher among people <20 years of age than among people in older groups (Table 2). However, this activity for anti-PT IgM antibodies was not found among the 21 HIV-positive persons younger than 30 years of age (Table 2), all of whom had CD4 lymphocyte levels below 250/μl, indicating that they were severely immunocompromised. However, when CD4 counts were between 400 and 900 cells/μl, anti-PT IgM antibodies were detectable in 60% (9/15) of HIV-positive persons younger than 30 years of age (Table 3). Thus, we were surprised to find that increasing age and decreasing numbers of CD4 lymphocytes, factors that can induce immunosuppression, influence the production of anti-PT IgM antibodies.

Anti-PT IgG antibodies were not detectable in most of our subjects, as noted above. The only human case demonstrating elevated anti-PT IgG antibodies involved an accidental *E. cuniculi* infection (14). In respect to the specific immune responses to the *E. cuniculi* infection, anti-spore wall IgG was observed to precede anti-PT IgG (14). Most of our subjects were negative for anti-spore wall IgM and IgG (data not shown). Additionally, the rate of positivity for anti-PT IgM antibodies for the serum samples collected from blood donors in 2005 was almost the same as that for the serum samples collected from healthy residents in 2000 (Table 1). These findings suggest that most of our anti-PT IgM antibodies do not belong to the class of early IgM antibodies found after infection by *E. cuniculi*. Although anti-PT IgM reacted only with the *E. cuniculi* PTs of the *Encephalitozoon* sp. tested, as described above, further research concerning the specificities and immunoreactivities of human anti-*E. cuniculi* PT IgM antibodies needs to be undertaken.

E. cuniculi infection in immunocompromised patients results in disseminated disease that is clinically manifested in symptoms such as keratoconjunctivitis, hepatitis, and peritonitis (16). However, no symptomatic cases of infection with *E. cuniculi* among immunocompetent persons have been described (16), apart from the accidentally infected French individual, who had severe keratoconjunctivitis (14). A Japanese child with encephalitozoonosis due to *E. cuniculi* infection in 1958 is considered the only case due to natural infection, but unfortunately, the patient's immune status was not recorded (10). A few cases of *E. cuniculi* infection have also been reported in transplant patients (6, 11). Thus, apart from some extremely rare situations, it is most unlikely that *E. cuniculi* causes microsporidiosis in immunocompetent persons (12, 16). Considering that almost all human encephalitozoonosis cases occurred in immunocompromised patients infected with HIV (16), one can speculate that protective immunity plays a very important role against *E. cuniculi* infection. In experimental models, the protective immune response against *E. cuniculi* is noted to be mediated by cytotoxic $CD8^+$ T cells (7). Also, the in vitro infectivity of microsporidia has been observed to be reduced by treatment with monoclonal and polyclonal antibodies to the polar tube protein (7), suggesting that anti-PT antibody may constitute a first line of defense against infection by *E. cuniculi*. Our study clearly indicates that there are circulating IgM antibodies that are capable of strongly reacting with the filaments that extrude from germinated *E. cuniculi* spores. We believe that this is the first study to provide seroepidemiological data on human anti-PT IgM antibodies. Further studies focused on human anti-*E. cuniculi* PT IgM antibodies need to be performed from the parallel perspectives of protective immunity and preventive medicine.

ACKNOWLEDGMENTS

We appreciate the assistance with serological testing and cell culture given by Tokiko Asakura.

This work was supported by grant-in-aid no. 16390177 from the Ministry of Education, Science, Sports and Culture of Japan.

REFERENCES

1. **Asakura, T., S. Nakamura, M. Ohta, Y. Une, and K. Furuya.** 2006. Genetically unique microsporidian *Encephalitozoon cuniculi* strain type III isolated from squirrel monkeys. Parasitol. Int. **55**:159–162.
2. **Bryan, R. T.** 1995. Microsporidiosis as an AIDS-related opportunistic infection. Clin. Infect. Dis. **21**(Suppl. 1):S62–S65.
3. **Canning, E. U., and W. S. Hollister.** 1987. Microsporidia of mammals—widespread pathogens or opportunistic curiosities? Parasitol. Today **3**:267–273.
4. **Deplazes, P., A. Mathis, R. Baumgartner, L. Tanner, and R. Weber.** 1996. Immunologic and molecular characteristics of *Encephalitozoon*-like microsporidia isolated from humans and rabbits indicate that *Encephalitozoon cuniculi* is a zoonotic parasite. Clin. Infect. Dis. **22**:557–559.
5. **Furuya, K., D. Fukui, M. Yamaguchi, Y. Nakaoka, G. Bando, and M. Kosuge.** 2001. Isolation of *Encephalitozoon cuniculi* using primary tissue culture techniques from a rabbit in a colony showing encephalitozoonosis. J. Vet. Med. Sci. **63**:203–206.
6. **Gamboa-Dominguez, A., J. De Anda, J. Donis, F. Ruiz-Maza, G. S. Visvesvara, and H. Diliz.** 2003. Disseminated *Encephalitozoon cuniculi* infection in a Mexican kidney transplant recipient. Transplantation **75**:1898–1900.
7. **Khan, I. A., M. Moretto, and L. M. Weiss.** 2001. Immune response to *Encephalitozoon cuniculi* infection. Microbes Infect. **3**:401–405.
8. **Kucerova-Pospisilova, Z., and O. Ditrich.** 1998. The serological surveillance of several groups of patients using antigens of *Encephalitozoon hellem* and *E. cuniculi* antibodies to microsporidia in patients. Folia Parasitol. (Praha) **45**:108–112.
9. **Langone, J. J.** 1982. Protein A of *Staphylococcus aureus* and related immunoglobulin receptors produced by streptococci and pneumonococci. Adv. Immunol. **32**:157–252.
10. **Matsubayashi, H., T. Koike, I. Mikata, H. Takei, and S. Hagiwara.** 1959. A case of *Encephalitozoon*-like body infection in man. Arch. Pathol. **67**:181–187.
11. **Mohindra, A. R., M. W. Lee, G. Visvesvara, H. Moura, R. Parasuraman, G. J. Leitch, L. Xiao, J. Yee, and R. del Busto.** 2002. Disseminated microsporidiosis in a renal transplant recipient. Transplant. Infect. Dis. **4**:102–107.
12. **Orenstein, J. M., H. P. Gaetz, A. T. Yachnis, S. S. Frankel, R. B. Mertens, and E. S. Didier.** 1997. Disseminated microsporidiosis in AIDS: are any organs spared? AIDS **11**:385–386.
13. **Peek, R., F. Delbac, D. Speijer, V. Polonais, S. Greve, E. Wentink-Bonnema, J. Ringrose, and T. van Gool.** 2005. Carbohydrate moieties of microsporidian polar tube proteins are targeted by immunoglobulin G in immunocompetent individuals. Infect. Immun. **73**:7906–7913.
14. **van Gool, T., C. Biderre, F. Delbac, E. Wentink-Bonnema, R. Peek, and C. P. Vivares.** 2004. Serodiagnostic studies in an immunocompetent individual infected with *Encephalitozoon cuniculi*. J. Infect. Dis. **189**:2243–2249.
15. **van Gool, T., J. C. M. Vetter, B. Weinmayr, A. Van Dam, F. Derouin, and J. Dankert.** 1997. High seroprevalence of *Encephalitozoon* species in immunocompetent subjects. J. Infect. Dis. **175**:1020–1024.
16. **Weber, R., D. A. Schwartz, and P. Deplazes.** 1999. Laboratory diagnosis of microsporidiosis, p. 315–362. *In* M. Wittner and L. M. Weiss (ed.), The microsporidia and microsporidiosis. ASM Press, Washington, DC.
17. **World Health Organization.** 1996. Fighting disease fostering development, p. 112. *In* The world health report. World Health Organization, Geneva, Switzerland.

JOURNAL OF CLINICAL MICROBIOLOGY, Feb. 2007, p. 477–487 Vol. 45, No. 2
0095-1137/07/$08.00+0 doi:10.1128/JCM.01708-06

Use of New T-Cell-Based Cell Lines Expressing Two Luciferase Reporters for Accurately Evaluating Susceptibility to Anti-Human Immunodeficiency Virus Type 1 Drugs▽

Tomoko Chiba-Mizutani,[1,2] Hideka Miura,[1] Masakazu Matsuda,[1] Zene Matsuda,[1] Yoshiyuki Yokomaku,[1] Kosuke Miyauchi,[1] Masako Nishizawa,[1] Naoki Yamamoto,[1,2] and Wataru Sugiura[1]*

AIDS Research Center, National Institute of Infectious Diseases, Tokyo, Japan,[1] and Department of Molecular Virology and Microbiology, Tokyo Medical and Dental University, Tokyo, Japan[2]

Received 17 August 2006/Returned for modification 25 October 2006/Accepted 11 December 2006

Two new T-cell-based reporter cell lines were established to measure human immunodeficiency virus type 1 (HIV-1) infectivity. One cell line naturally expresses CD4 and CXCR4, making it susceptible to X4-tropic viruses, and the other cell line, in which a CCR5 expression vector was introduced, is susceptible to both X4- and R5-tropic viruses. Reporter cells were constructed by transfecting the human T-cell line HPB-Ma, which demonstrates high susceptibility to HIV-1, with genomes expressing two different luciferase reporters, HIV-1 long terminal repeat-driven firefly luciferase and cytomegalovirus promoter-driven renilla luciferase. Upon HIV infection, the cells expressed firefly luciferase at levels that were highly correlated (r^2 = 0.91 to 0.98) with the production of the capsid antigen p24. The cells also constitutively expressed renilla luciferase, which was used to monitor cell numbers and viability. The reliability of the cell lines for two in vitro applications, drug resistance phenotyping and drug screening, was confirmed. As HIV-1 efficiently replicated in these cells, they could be used for multiple-round replication assays as an alternative method to a single-cycle replication protocol. Coefficients of variation for drug susceptibility evaluated with the cell lines ranged from 17 to 41%. The new cell lines were beneficial for evaluating antiretroviral drug resistance. Firefly luciferase gave a wider dynamic range for evaluating virus infectivity, and the introduction of renilla luciferase improved assay reproducibility. The cell lines were also beneficial for screening new antiretroviral agents, as false inhibition caused by the cytotoxicity of test compounds was easily detected by monitoring renilla luciferase activity.

Drug resistance assays have been accepted as standard clinical tests to guide the antiretroviral therapy of human immunodeficiency virus (HIV)-infected patients who have developed resistance to drug treatment or drug-naïve patients infected with drug-resistant virus. These tests have been shown to improve treatment outcomes by selecting the most effective drugs and by minimizing the risk of treatment failure (2, 5–7, 9, 34). Drug resistance has been determined by two approaches. One is drug resistance genotyping, in which drug resistance is evaluated by sequencing the viral genes targeted by the drug, such as the HIV-1 protease and reverse transcriptase (RT) genes. The level of drug resistance is estimated by using observed mutation patterns and interpretation algorithms (23). Several protocols have been used for drug resistance genotyping, including in-house sequencing (10, 13, 38). Although these protocols differ in some aspects, e.g., the design of primers, the length of analyses, and amplification procedures, all are based on the same technical approach, modified Sanger sequencing.

The other approach to drug resistance assays is phenotyping. In this method, the levels of drug resistance of patient-derived viral isolates are evaluated by using in vitro bioassays (17, 26). Two advantages of the phenotyping assay are its ability to directly evaluate the drug susceptibilities of patient-derived viruses and the ease of interpreting its results compared to those from genotyping. This assay is especially useful in cases with a high degree of exposure to antiretroviral drugs, therefore involving many mutations. In these cases, the evaluation of resistance levels by genotyping alone may be difficult (35). In addition, the resistance levels determined by phenotyping provide important information for updating interpretation algorithms used in genotyping.

Although peripheral blood mononuclear cells (PBMC) are the natural target of HIV type 1 (HIV-1) and hence are the best candidates for host cells in phenotyping assays, reporter cell systems are more commonly used in drug susceptibility assays (1, 12, 15, 31). Reporter systems are preferred because their susceptibility to HIV-1 is stable and their output is both rapidly measured and highly reproducible compared to that of PBMC assays. Several kinds of reporter cells have been used with different reporter proteins, such as MAGI cells with β-galactosidase (21), GHOST cells with enhanced green fluorescent protein (36), MOCHA cells with secreted alkaline phosphatase (24), and CEM.NKR-CCR5-Luc cells with luciferase (31). Although these systems use different cell lines, their basic strategies for evaluating HIV infectivity are similar (21, 36). The cell lines carry a reporter protein gene regulated by the HIV-1 long terminal repeat (LTR) promoter, inducing them to

* Corresponding author. Mailing address: AIDS Research Center, National Institute of Infectious Diseases, 4-7-1 Gakuenn, Musashimurayama, Tokyo 2080011, Japan. Phone: 81-42-561-0771. Fax: 81-42-561-7746. E-mail: wsugiura@nih.go.jp.

▽ Published ahead of print on 20 December 2006.

produce the reporter protein when they are infected with HIV-1. Which reporter system is used depends on the properties of the original cell line and the installed reporter protein.

Reporter systems using MAGI and GHOST cells have the advantages of high sensitivity and rapidity in determining infectivity. However, MAGI and GHOST cells have been established from HeLa cells (21) and human osteosarcoma cells (36), respectively, which are not naturally susceptible to HIV-1. Therefore, these cells cannot propagate viruses efficiently. On the other hand, MOCHA and CEM.NKR-CCR5-Luc cell lines were established from T-cell lines and secreted alkaline phosphatase and luciferase, respectively, were installed as reporters. These reporter systems allow for the evaluation of HIV-1 infectivity by using enzymatic reactions and demonstrate greater reproducibility with wider dynamic ranges of reporter proteins. However, for these cells to produce sufficient reporter protein for accurate determinations, they must be cultured for 5 to 7 days, longer than MAGI and GHOST cells. Longer culture periods allow reporter cells to divide, which may affect the accuracy of the quantification.

Given the advantages and limitations of previously constructed reporter cell lines, we designed and tested two new reporter cell lines with dual chemokine receptors for use in drug resistance phenotypic assays and other HIV infectivity assays. The cell lines we designed have unique characteristics in that they originate from the human T-cell line HPB-Ma (16, 29, 40) and were engineered to express the CCR5 receptor and two different marker proteins, firefly luciferase (FL) and renilla luciferase (RL). FL, which is under HIV-1 LTR promoter regulation, is produced upon HIV-1 infection. Therefore, firefly luciferase activity can be used as a marker for virus infectivity. RL, which is under cytomegalovirus (CMV) promoter control, is constitutively expressed in the cells. Therefore, renilla luciferase activity can be used as a marker for cell number and viability.

MATERIALS AND METHODS

Construction of luciferase and CCR5 expression vectors. Two different luciferase expression vectors were constructed. The first luciferase construct comprised HIV-1 Tat-regulated FL and the red fluorescent protein (DsRed) construct 53LTRN-lucneo^r#1. The HIV-1 Tat-responsive reporter construct 53LTRN-lucneo^r#1 was constructed based on the expression vector pGEM-7Zf(+) (Promega, Madison, WI). Initially, a parent vector was constructed, 53LTRNCNS, which has a rabbit β-globin unit under the control of the HIV-1 LTR derived from strain HXB2. In this construct, a gene of interest can be cloned within the second exon of the β-globin gene and the polyadenylation signal is provided by the β-globin unit. A neomycin expression module was prepared by PCR and cloned upstream of the HIV-1 LTR region to generate 53LTRCNSneo. The reporter gene employed here was a fusion between an FL gene and a DsRed gene. The FL gene allows HIV-1 replication to be quantitatively evaluated by using luciferase activity when the LTR is activated by HIV-derived Tat, and the DsRed gene allows transfected and HIV-infected cells to be identified by red fluorescence. The FL portion was derived from pGLuc5 (Promega), and the DsRed portion was derived from pDsRed1N-1 (Clontech). Both genes were prepared by PCR, fused, and cloned into the β-globin unit by using NcoI and NotI restriction sites.

The second luciferase construct, pRenillaPac, was constructed using the plasmid pPUR (Clontech). The PCR-amplified RL gene, derived from phRL-CMV (Promega), was spliced into the upstream region of the *pac* gene. This hybrid gene manifests both RL activity and resistance to puromycin. Expression of the fusion gene was constitutive under the control of a CMV promoter.

A CCR5 expression vector, pCCR5/CEP4, was constructed based on the pCEP4 expression vector (Invitrogen), which possesses the Epstein-Barr nuclear antigen 1 episomal-expression gene. The CCR5 gene was inserted into the vector by using NotI and SnaB I restriction sites on the vector. Expression of the CCR5 gene was constitutive under the control of a CMV promoter.

Selection of host cell line and establishment of new reporter cell lines. To design new reporter cell lines for quantifying HIV-1 replication, we selected the murine leukemia virus-transformed human T-cell line HPB-Ma, established by Y. K. Shimizu and H. Yoshikura (16, 29, 40), because of its high susceptibility to HIV-1 and its stable expression of CD4 and CXCR4. HPB-Ma cells were maintained at 37°C in 5% CO_2 in complete RPMI 1640 medium (Sigma, Tokyo, Japan) supplemented with 10% fetal calf serum (HyClone, Logan, UT) and 1% penicillin-streptomycin (Invitrogen, Tokyo, Japan). Cells were transfected by electroporation with the two luciferase expression vectors, 53LTRN-lucneo^r#1 and pRenillaPac. Plasmid DNA (10 μg) was mixed with HPB-Ma cells (5×10^6 cells in 500 μl phosphate-buffered saline), and the mixture was incubated for 5 min at 4°C and electropulsed with a Gene Pulser II apparatus (Bio-Rad, Hercules, CA) at 250 V and 950 μF. After electroporation, the cells were resuspended in complete medium and incubated at 37°C in 5% CO_2. Subsequently, cells with incorporated plasmids were selected with 0.1 μg/ml puromycin (BD Biosciences, San Jose, CA) and 250 μg/ml Geneticin (Invitrogen), maintained in complete medium for several weeks, and enriched with cell populations expressing high levels of CD4 and CXCR4 by fluorescence-activated cell sorting with a FACSVantage system (BD Biosciences). Finally, clones were generated by limiting dilution and selected if they showed high sensitivity to HIV-1 and low spontaneous expression of FL and DsRed.

Since the parent HPB-Ma cell line expresses only the CXCR4 receptor, we extended the spectrum of the reporter cell lines to include R5-tropic viral isolates by transfecting cells by electroporation with a CCR5 expression plasmid. Clones were selected by incubating for several weeks with 0.1 μg/ml puromycin, 250 μg/ml Geneticin, and 150 μg/ml hygromycin B. Selected cells were recloned, and the expression of cell surface markers was confirmed by using FACSCaliber (Becton Dickinson, San Jose, CA). CD4, CXCR4, and CCR5 receptors were stained with SK-3-Cy5.5, 12G5-phycoerythrin, and 2D7-fluorescein isothiocyanate monoclonal antibodies, respectively (all from BD Biosciences, San Jose, CA).

Evaluation of introduced reporter gene functions. To confirm the ability of FL activity to reliably measure virus titer and production, established cell lines were plated into 96-well plates at 10^5 cells per well and inoculated with 50 to 400 50% tissue culture infective doses ($TCID_{50}$) of HXB2 or JRCSF. After 7 days of culture with the test viruses, cells were harvested and lysed in 75 μl of luciferase assay reagent. FL activity was quantified using a Dual-Glo luciferase reporter assay system (Promega, Madison, WI) and an LMax microplate luminometer (Molecular Devices, Sunnyvale, CA). Virus production was also quantified by using the p24 antigen enzyme-linked immunosorbent assay RETROtek kit (ZeproMetrix Co., Buffalo, NY) and compared with FL activity.

The validity of using RL activity to monitor MaRBLE cell numbers was evaluated by measuring RL activity in various numbers of cells and determining the correlation between RL activity and cell numbers. The correlation between RL activity and cell viability was also confirmed in cell killing assays with two anticancer drugs, hygromycin B (Invitrogen, Tokyo, Japan) and blasticidin S (Funakoshi, Tokyo, Japan). Target cells were plated into 96-well plates at 10^5 cells per well, and hygromycin B (15.6 to 500 μg/ml) and blasticidin S (1.25 to 20 μg/ml) were added. After 7 days of culture, cells were harvested and RL activity was measured by using the Dual-Glo luciferase reporter assay system (Promega) and the percentage of cell killing was determined by trypan blue staining.

Preparation of recombinant and patient-derived viruses. Recombinant viruses with point mutations were constructed as described elsewhere (25). In brief, drug resistance mutations were introduced into the RT and protease genes of the HXB2 clone by site-directed mutagenesis (28). MT-2 cells (5×10^6 human T-lymphoblastoid cells) were then transfected by electroporation with the recombinant virus plasmids, and the cells were maintained in 10 ml of complete medium for 7 to 14 days. Half the culture supernatant was harvested and replaced with fresh medium every other day. Viral replication was monitored by measuring RT activity in the supernatant, and the sample with the highest RT activity was used in subsequent studies.

Eight clinical samples were selected randomly from patient blood specimens sent for routine HIV-1 drug resistance testing to the AIDS Research Center, National Institute of Infectious Diseases, Tokyo, Japan. Patient viruses were isolated by a standard coculture method described elsewhere (18). In brief, 2×10^7 patient PBMC were mixed with the same number of phytohemagglutinin-stimulated normal human PBMC and the mixture was cultured for 2 weeks. Half the culture supernatant was collected and replaced with the same amount of fresh culture medium every other day. Viral replication was monitored by mea-

suring RT activity in the supernatant, and the sample with peak RT activity was selected and used for infection experiments afterward. RT assays were performed as previously described (37). Viral RNAs in collected supernatants were sequenced, and drug resistance mutation patterns were determined.

For the reconstructed virus, viral RNA was extracted from 200 μl of patient plasma by using a High Pure viral RNA kit (Roche, Basel, Switzerland) according to the manufacturer's instructions. Subsequently, a 1.8-kb *gag-pol* fragment, encoding the region from $p2^{gag}$ to whole protease, and the 5′ half of the RT gene fragment were amplified and inserted into the HXB2 backbone. MT-2 cells (5×10^6) were then transfected by electroporation with the plasmid, and the cells were maintained in 10 ml of complete medium for 7 to 14 days. Half the culture supernatant was harvested and replaced with fresh medium every other day. Viral replication was monitored by measuring RT activity in the supernatant, and the sample with the highest RT activity was selected for use in subsequent studies. Viral RNAs in collected supernatants were sequenced, and the drug resistance mutation patterns were confirmed. For both the patient-derived and reconstructed viruses, HIV infectivity ($TCID_{50}$) in the target cell lines was assayed by the Reed-Muench method (27).

Drug resistance genotyping. HIV-1 RNA was extracted from 200 μl of patient plasma using a High Pure viral RNA kit according to the manufacturer's instructions. For amplification of the 500-bp protease gene fragment, DRPRO5 (5′-AGA-CAG-GYT-AAT-TTT-TTA-GGG-A) and DRPRO2L (5′-TAT-GGA-TTT-TCA-GGC-CCA-ATT-TTT-GA) were used for reverse transcription and the first PCR and DRPRO1M (5′-AGA-GCC-AAC-AGC-CCC-ACC-AG) and DRPRO6 (5′-ACT-TTT-GGG-CCA-TCC-ATT-CC) were used for the second PCR. For amplification of the 800-bp RT gene fragment, DRRT1L (5′-ATG-ATA-GGG-GGA-ATT-GGA-GGT-TT) and DRRT4L (5′-TAC-TTC-TGT-TAG-TGC-TTT-GGT-TCC) were used for reverse transcription and the first PCR and DRRT7L (5′-GAC-CTA-CAC-CTG-TCA-ACA-TAA-TTG-G) and DRRT6L (5′-TAA-TCC-CTG-CAT-AAA-TCT-GAC-TTG-C) were used for the second PCR. The amplicons were purified by using a MultiScreen PCR filter plate (Millipore), and sequence reactions were performed by using the BigDye Terminator v3.1 cycle sequencing kit, followed by electrophoresis using an ABI-3730 auto sequencer (Applied Biosystems, Foster City, CA).

HIV-1 replication kinetics analyses and drug susceptibility assays. To analyze the replication kinetics of clinically derived HIV-1 isolates, target cells were plated into 96-well plates at 10^5 cells per well and infected with 100 $TCID_{50}$ of test viruses per well. At days 3, 5, and 7, the culture supernatant of each well was collected and RT activity was measured as previously described (37).

To evaluate anti-HIV-1 drug susceptibility, 10^7 cells were infected with 10,000 $TCID_{50}$ of wild-type control or test viruses in 50-ml tubes and incubated for 2 h at 37°C. Infected cells were resuspended in culture medium and plated into 96-well plates at 10^5 cells per well. At 2 and 48 h after infection, serial RT inhibitor dilutions and serial protease inhibitor (PI) dilutions were added, respectively. Each drug was prepared in a fivefold serial dilution and tested over different dose ranges, as follows. Didanosine, abacavir, and nevirapine were tested at concentrations from 25.0×10^1 μm to 3.2×10^{-4} μM. Lamivudine and stavudine were tested at concentrations from 5.0×10^1 μm to 6.4×10^{-5} μM. Zidovudine, zalcitabine, and the five PIs (saquinavir, indinavir, nelfinavir, lopinavir, and amprenavir) were tested at concentrations from 1.0×10^1 μm to 12.8×10^{-6} μM. Efavirenz was tested at concentrations from 0.2×10^1 μm to 25.6×10^{-7} μM. All samples were tested in triplicate. The following manufacturers kindly supplied anti-HIV drugs: GlaxoSmithKline, Middlesex, United Kingdom (zidovudine, lamivudine, and abacavir); Bristol-Myers Squibb, New York, NY (didanosine, stavudine, and efavirenz); Roche, Basel, Switzerland (zalcitabine and saquinavir); Boehringer Ingelheim, Ingelheim, Germany (nevirapine); Merck Research Laboratories, Rahway, NJ (indinavir); Japan Tobacco, Tokyo, Japan (nelfinavir); Vertex Pharmaceuticals, Cambridge, MA (amprenavir); and Abbott Laboratories, Abbott Park, IL (lopinavir).

After 7 days of culture with test drugs and test viruses, cells were harvested and lysed in 75 μl of luciferase assay reagent. Firefly and RL activities were sequentially quantified using a dual-luciferase reporter assay system (Promega) and an LMax microplate luminometer (Molecular Devices). Data were displayed by plotting the percentage of luciferase activity versus the $\log_{10}$ drug concentration. The concentration at which 50% of viral replication was inhibited (IC_{50}) was determined by plotting curves defined by the four-parametric sigmoidal equation $f(x) = A + ([B - A])/(1 + [C/x]^D)$ using XLfit4 software (CTC Laboratory Systems Corporation, Tokyo, Japan). To determine susceptibility or resistance, results for test viruses were compared to those for wild-type HIV-1 and evaluated by Student's *t* test.

RESULTS

Establishment of new T-cell-based cell lines with two luciferase reporter proteins. Two luciferase expression vectors were successfully constructed and used for transfection of the HPB-Ma cell line. These vectors were 53LTRN-lucneo^r#1, with FL under HIV-1 LTR regulation, and pRenillaPac, with RL under CMV promoter regulation. HPB-Ma cells with these vectors were subjected to several rounds of selection for cells resistant to Geneticin and puromycin and were enriched by flow cytometry with populations expressing high levels of CD4 and CXCR4 to establish the new cell line HPB-Ma/LTR-FL/CMV-RL (X4-MaRBLE).

Since the parent HPB-Ma cell line expresses only CXCR4, the spectrum of the X4-MaRBLE cell line was extended to include R5-tropic viruses by transfection with a CCR5 expression plasmid, thus establishing the R5-MaRBLE cell line. Expression levels of CD4 were comparable among the parent HPB-Ma, X4-MaRBLE, and R5-MaRBLE cell lines (Fig. 1a to c), whereas the proportion of CXCR4-positive cell populations and CXCR4 expression levels were slightly higher in X4- and R5-MaRBLE cells than in the parent HPB-Ma cell line (Fig. 1d to f). This difference is due to the cell sorter's selecting for populations expressing high levels of CXCR4. As for CCR5 expression, HPB-Ma and X4-MaRBLE cells did not significantly express the receptor (Fig. 1g and h). On the other hand, more than 76% of the R5-MaRBLE cell population expressed CCR5 (Fig. 1i).

To confirm the susceptibility of cell lines to X4- and R5-tropic viruses, each cell line was inoculated with HXB2 (X4-tropic) and JRCSF (R5-tropic) viruses. X4-MaRBLE cells inoculated with HXB2 expressed FL activity in a dose-dependent manner but did not show any FL activity after inoculation with JRCSF (Fig. 2a). On the other hand, R5-MaRBLE cells were susceptible to both HXB2 and JRCSF, which induced FL activity in a dose-dependent manner (Fig. 2b). To validate the use of FL activity to evaluate viral production, FL levels were compared to amounts of the viral capsid antigen, p24, in both X4- and R5-MaRBLE cell lines, and the correlation between FL levels and the amounts of p24 was determined. As shown in Fig. 2c, FL activity in cell lysates and the amount of p24 antigen in the culture supernatant were positively and linearly correlated in X4-MaRBLE cells infected with HXB2 ($r^2 = 0.98$), in R5-MaRBLE cells infected with HXB2 ($r^2 = 0.91$), and in R5-MaRBLE cells infected with JRCSF ($r^2 = 0.94$). These results verify that FL activity expressed by both X4- and R5-MaRBLE cell lines accurately represents the levels of viral replication and production. These good correlations also indicate a small likelihood of interference between the two LTRs in infected cells, the one driving luciferase and the other contained in the infecting virus.

The second type of luciferase, RL, was inserted into MaRBLE cells to monitor and evaluate their number and viability. As shown in Fig. 3a, RL activity demonstrated a positive, linear correlation with cell number ($r^2 = 0.99$). Thus, RL activity can be used to assess cell number in culture. Another useful parameter evaluated by RL activity was the cytotoxicity of test compounds added to cultures. To confirm the relationship between RL activity and cell viability, cell killing assays were performed with two anticancer drugs, hygromycin

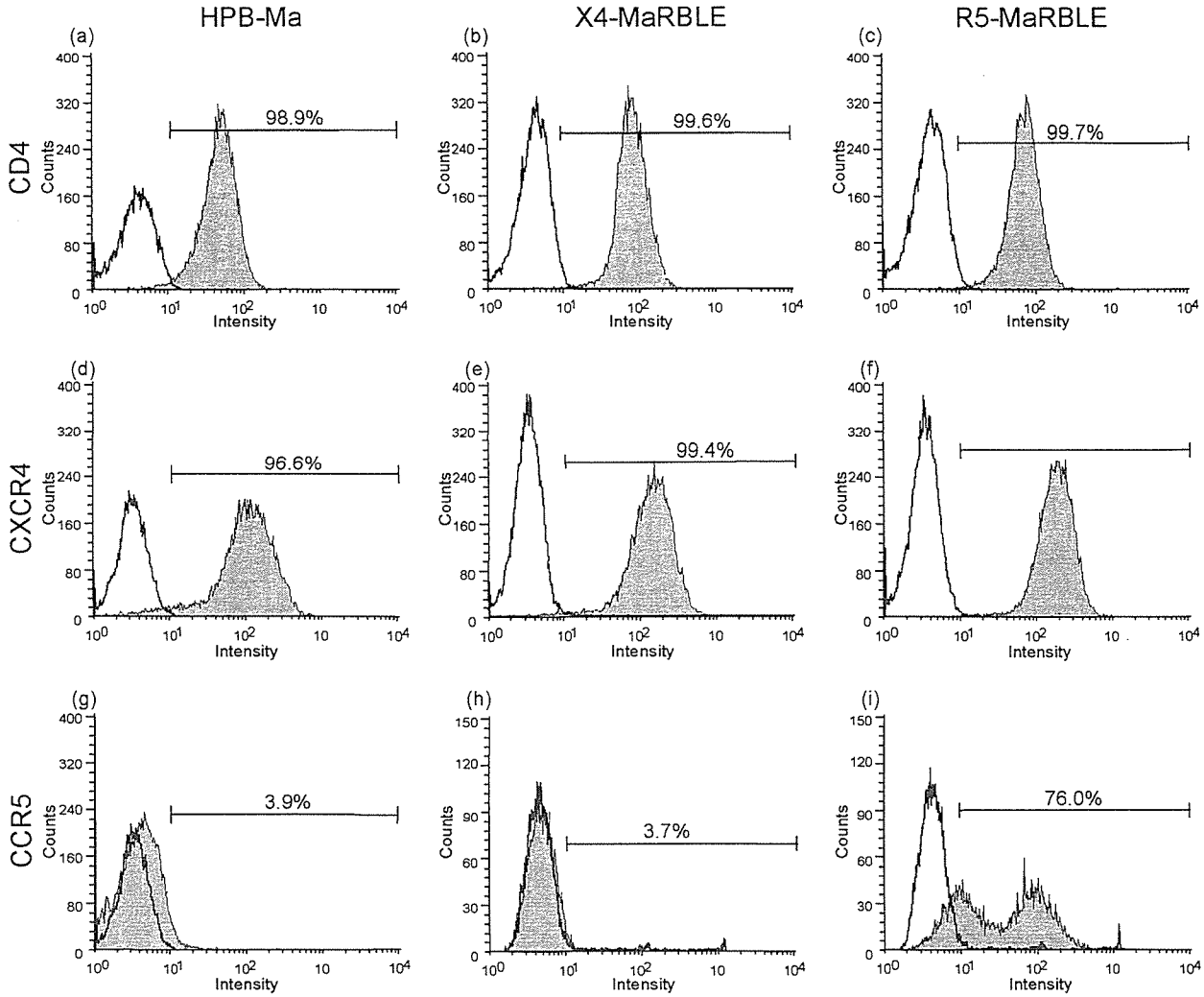

FIG. 1. Levels of expression of CD4, CXCR4, and CCR5 in parent HPB-Ma cells and X4-MaRBLE and R5-MaRBLE cells. Parent HPB-Ma (a, d, g), X4-MaRBLE (b, e, h), and R5-MaRBLE (c, f, i) cells were stained with monoclonal antibodies to CD4 (a, b, c), CXCR4 (d, e, f), and CCR5 (g, h, i). To calculate the percentage of each population positive for the expression of cytokine receptors (bars), 2,000 to 5,000 cells were analyzed by fluorescence-activated cell sorting. To calculate the percentage of each population positive for expression of CD4 and cytokine receptors (bar), 2,000 to 5,000 cells were analyzed by FACSCalibur and compared with fluorescence-negative control cells. Histograms with gray shading indicate cell populations stained with each monoclonal antibody; histograms without shading indicate negative control populations.

B (Fig. 3b) and blasticidin S (Fig. 3c). As the percentages of cells killed by both test chemicals increased, RL activity declined (Fig. 3b and c). The concentrations of hygromycin B and blasticidin S needed to kill 50% of the cells were 100 μg/ml and 2 μg/ml, respectively, in agreement with data from previous reports (3, 32). Thus, RL activity can be used to measure cytotoxicity.

In addition, the replication of two patient-derived viral isolates, 8 and 9, in R5-MaRBLE cells was compared to that in PBMC. As shown in Fig. 4, the two clinical isolates efficiently replicated in R5-MaRBLE cells. Isolate 8 replicated more efficiently in R5-MaRBLE cells than in PBMC, as indicated by the 10-fold-higher RT activity at day 7 in R5-MaRBLE cells (Fig. 4a). Isolate 9 had comparable day 7 RT activities in R5-MaRBLE cells and PBMC (Fig. 4b). These data clearly show that R5-MaRBLE cells can efficiently propagate clinical isolates.

Evaluation of HIV-1 drug susceptibility using X4- and R5-MaRBLE cells is highly reproducible. Having confirmed the quantitative reliability of FL expressed by HIV-infected X4- and R5-MaRBLE cell lines, we next used the cell lines to evaluate HIV-1 susceptibility to antiretroviral drugs. First, we evaluated the precision of phenotyping using the X4- and R5-MaRBLE cell lines. By using the wild-type HXB2 strain as a target virus for both X4- and R5-MaRBLE cells and JRCSF as a target virus for R5-MaRBLE cells, the IC_{50}s of four representative drugs from three classes of antiretroviral agents (zidovudine, lamivudine, efavirenz, and lopinavir) were determined by measuring FL activity. As shown in Fig. 5, well-characterized dose-response curves were obtained for the four drugs. The mean IC_{50}s, standard deviations (SD), and coefficients of variation (CV) for each cell line are summarized in Table 1. The CV ranged from 17 to 41%, demonstrating high reproducibility for drug susceptibility assays using both HIV-

FIG. 2. FL reporter activity in MaRBLE cells accurately represents viral production\chatn. (a) X4-MaRBLE cells are susceptible to HXB2 (X4-tropic) but not JRCSF (R5-tropic) viruses. FL activity was confirmed as a reliable measure of X4-tropic HIV-1 in X4-MaRBLE cells by inoculating the cells with various amounts of HXB2 or JRCSF and reading FL activity 7 days later. Solid and dashed lines indicate HXB2 and JRCSF, respectively. (b) R5-MaRBLE cells are susceptible to both HXB2 (X4-tropic) and JRCSF (R5-tropic) viruses. FL activity was confirmed as a reliable measure of X4- and R5-tropic HIV-1 in R5-MaRBLE cells by inoculating the cells with various amounts of HXB2 or JRCSF and reading FL activity 7 days later. Solid and dashed lines indicate HXB2 and JRCSF, respectively. (c) FL activity and the amount of capsid antigen p24 are correlated in HIV-infected MaRBLE cells. The reliability of using FL activity instead of the amount of p24 to quantify HIV-1 production was evaluated by measuring intracellular FL activity and the amount of p24 antigen in the supernatant from the same culture. Solid, dashed, and dotted lines indicate HXB2-infected X4-MaRBLE cells, HXB2-infected R5-MaRBLE cells, and JRCSF-infected R5-MaRBLE cells, respectively. Percentages of FL activity and of p24 production were calculated from the following formula: percentage = (observed value with the drug − background value)/(observed value without the drug − background value) × 100. RU, relative units.

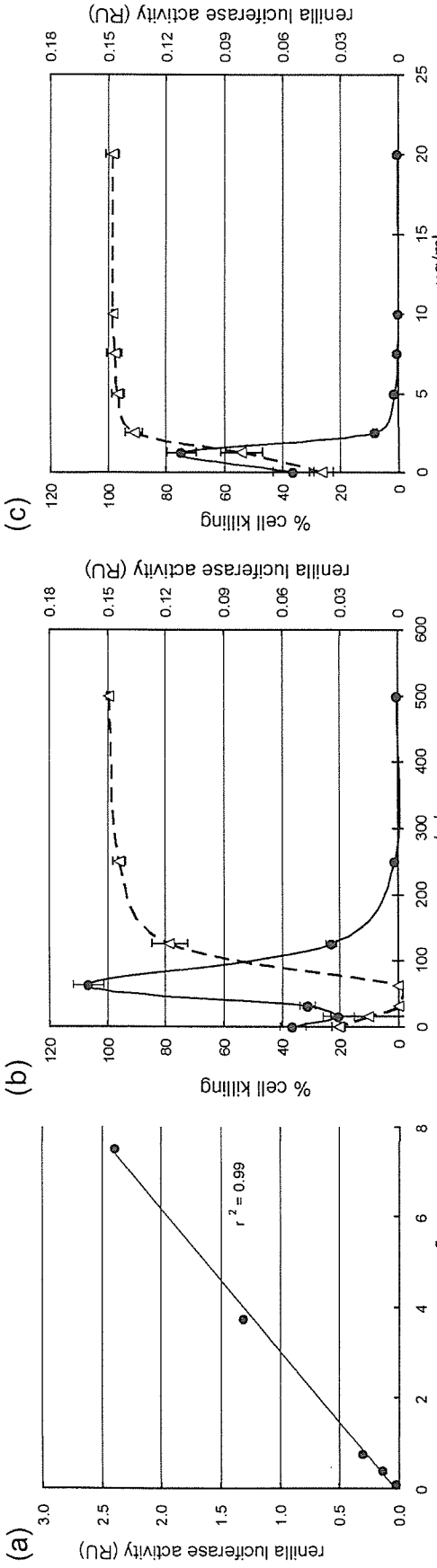

FIG. 3. Constitutively expressed RL in MaRBLE cells provides a reliable measure of cell number and viability. (a) RL activity accurately indicates MaRBLE cell numbers. The validity of using RL activity to monitor MaRBLE cell numbers was evaluated by measuring RL activity in various numbers of cells and plotting the corresponding values. RL activity and cell number were positively and linearly correlated ($r^2 = 0.99$). (b and c) RL activity reliably indicates hygromycin B and blasticidin S cytotoxicity in MaRBLE cells. The reliability of RL activity as a marker of cytotoxicity was evaluated for hygromycin B (b) and blasticidin S HCl (c). Cells were cultured for 1 week with serial dilutions of each drug and lysed, and their RL activities were determined. In graphs in both panels b and c, solid lines represent the RL activities of cell lysates and dashed lines indicate percentages of dead cells as determined by trypan blue staining. RU, relative units.

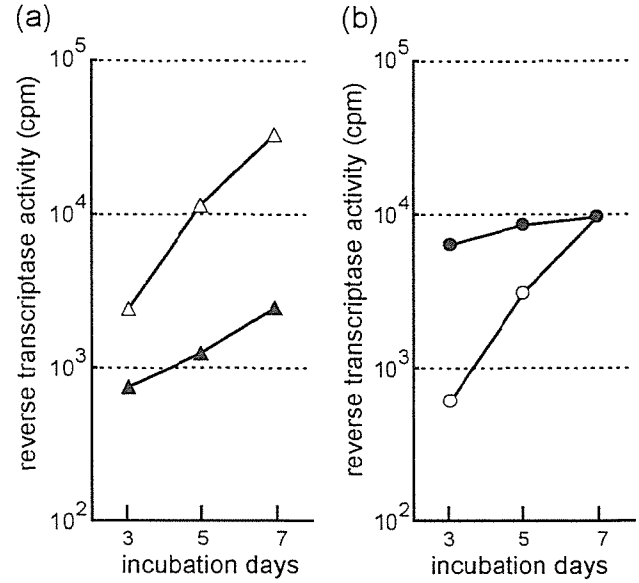

FIG. 4. Clinically derived isolates replicate in R5-MaRBLE cells as efficiently as in PBMC. The replication kinetics of two clinical isolates, 8 and 9, were compared after inoculation into both R5-MaRBLE cells and human PBMC. (a) Replication kinetics of isolate 8. Open and closed triangles indicate kinetics in R5-MaRBLE cells and PBMC, respectively. (b) Replication kinetics of isolate 9. Open and closed circles indicate kinetics in R5-MaRBLE cells and PBMC, respectively. cpm, counts per minute.

1-inoculated X4- and R5-MaRBLE cells. Interestingly, while the efavirenz and lopinavir susceptibilities of wild-type HXB2 were identical in evaluations with both X4- and R5-MaRBLE cells, the IC_{50}s for HXB2 and JRCSF were significantly different ($P < 0.001$) in the R5-MaRBLE cell line. Thus, in our assay, JRCSF appeared to be more susceptible than the HXB2 HIV-1 strain to efavirenz and slightly less resistant to lopinavir.

Drug susceptibility of drug-resistant HIV-1 can be evaluated using X4- and R5-MaRBLE cell lines. Given the accuracy and reproducibility of assays using MaRBLE cells to determine the drug susceptibilities of wild-type HXB2 and JRCSF, we then evaluated the reliability of using the cell lines for drug resistance phenotyping. Recombinant viruses with representative drug resistance mutations were constructed, and the drug resistance levels of the viruses were determined using X4-MaRBLE cells. The drug resistance levels associated with five patterns of nucleoside RT inhibitor (NRTI) resistance mutations are summarized in Table 2. Of the five mutant viral clones tested, four showed significant resistance to zidovudine, with resistance levels in the following order from lowest to highest: M41L/M184V/T215Y ≤ D67N/K70R < M41L/T215Y < M41L/D67N/K70R/T215Y. Thus, zidovudine resistance increased with the accumulation of thymidine analogue mutations (TAMs), and the M184V mutation caused reversion to the zidovudine resistance phenotype in the M41L/T215Y mutant, with a change in the susceptibility level of 12.5- to 3.5-fold relative to that of the wild-type virus, similar to results in previous reports (11, 19). Two clones with the M184V mutation demonstrated over 500-fold (>533.7- and >1,339.3-fold)-greater resistance to lamivudine but no significant resistance to didanosine and zalcitabine, although M184V has been re-

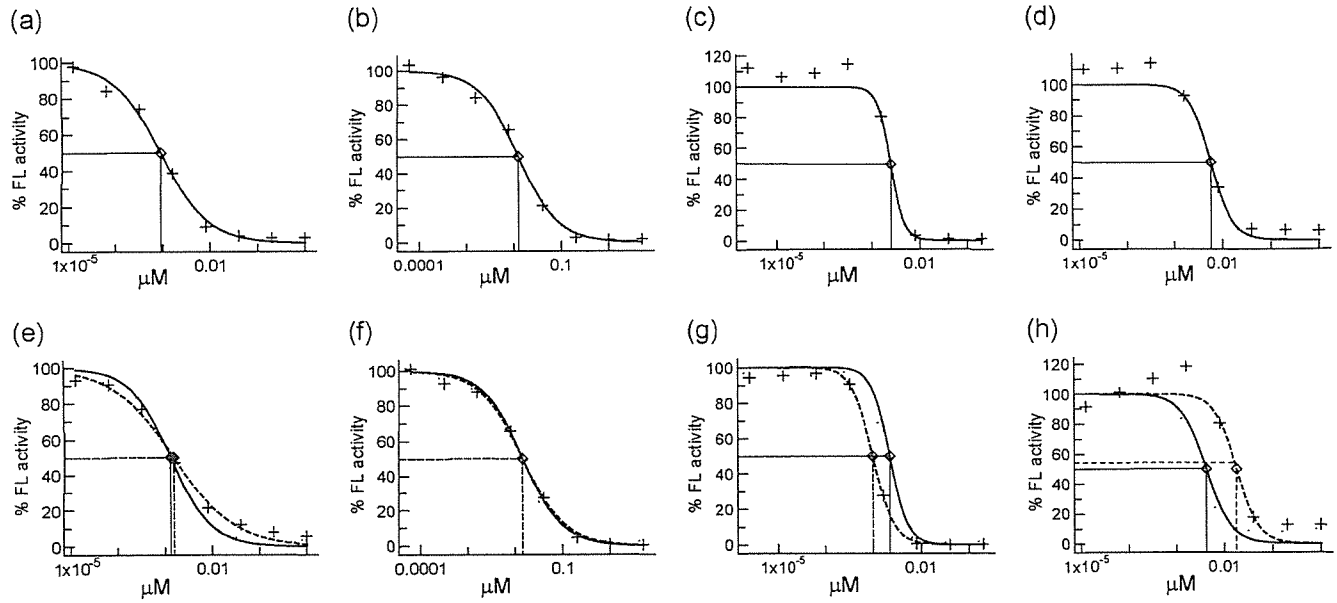

FIG. 5. Results of assays for HIV-1 drug susceptibility with MaRBLE reporter cell lines are highly reproducible. Dose-response curves for four representative agents against wild-type HXB2 and JRCSF are shown. Solid and dotted lines indicate HXB2 and JRCSF, respectively. (a to d) Results of assays for susceptibility to zidovudine, lamivudine, efavirenz, and lopinavir, respectively, using X4-MaRBLE cells. (e to h) Results of assays for susceptibility to zidovudine, lamivudine, efavirenz, and lopinavir, respectively, using R5-MaRBLE cells. The percentage of inhibition was calculated as follows: percentage = (observed FL activity with the drug − background FL activity)/(FL activity without the drug − background FL activity) × 100.

ported to confer a three- to fivefold increase in the level of resistance (14). Our data confirm the recent revalidation of using didanosine for cases involving the M184V mutation (39). Low-level but significant stavudine resistance in M41L/T215Y (change, 2.2-fold), M41L/M184V/T215Y (change, 2.9-fold), and M41L/D67N/K70R/T215Y (change, 3.8-fold) was found, consistent with data from previous reports (22, 33). All five clones demonstrated significant resistance to abacavir. The highest resistance was observed in the M41L/M184V/T215Y mutant, consistent with findings in previous reports that TAMs with M184V reduce susceptibility to abacavir 10-fold (1).

The drug resistance levels associated with the two most common nonnucleoside RT inhibitor (NNRTI) resistance mutations (K103N and Y181C) are summarized in Table 3. The K103N mutant virus demonstrated reduced susceptibility to both nevirapine (change, 78.6-fold) and efavirenz (change, 54.7-fold), whereas the Y181C virus was resistant only to nevirapine (change, 47.5-fold) but remained susceptible to efavirenz (change, 1.5-fold).

TABLE 1. Susceptibility of wild-type HXB2 and JRCSF to representative antiretrovirals as determined using X4- and R5-MaRBLE cells

Cell line	Agent	Mean IC_{50} (nM) ± SD (CV [%]) for[a]: HXB2 (n = 18)	HXB2 (n = 21)	JRCSF (n = 24)
X4-MaRBLE	Zidovudine	0.9 ± 0.4 (41)		
	Lamivudine	12.3 ± 3.9 (32)		
	Efavirenz	2.7 ± 1.0 (37)		
	Lopinavir	6.0 ± 1.0 (17)		
R5-MaRBLE	Zidovudine		1.3 ± 0.4 (31)	1.5 ± 0.6 (40)
	Lamivudine		13.6 ± 4.8 (35)	13.4 ± 3.6 (27)
	Efavirenz		2.1 ± 0.5[b] (24)	1.0 ± 0.3[b] (30)
	Lopinavir		4.2 ± 1.4[b] (33)	17.6 ± 6.9[b] (39)

[a] n, number of isolates of the indicated virus strain.
[b] IC_{50}s of efavirenz and lopinavir were significantly different for HXB2 and JRCSF in R5-MaRBLE cells ($P < 0.001$).

The drug resistance levels of three PI-resistant mutant clones (M46I, V82T, and L90M) are summarized in Table 4. Clones with M46I and L90M mutations did not demonstrate significant resistance to any PI tested, except for nelfinavir, to which the L90M clone demonstrated low-level (change, 3.3-fold) resistance. The clone with the V82T mutation demonstrated low-level resistance to indinavir (change, 3.8-fold), nelfinavir (change, 5.4-fold), amprenavir (change, 2.9-fold), and lopinavir (change, 5.0-fold), consistent with results in previous reports (8). Thus, the drug susceptibilities of viruses with resistance mutations evaluated with the MaRBLE cell lines matched those from previous reports of drug resistance, indicating the reliability of using the new cell lines to evaluate drug resistance.

To assess the reliability of using R5-MaRBLE cells to evaluate the levels of drug resistance of viral isolates from patients for whom treatment failed, seven cases were selected and viruses were isolated by coculture with normal human PBMC. Among the isolates from these seven cases, isolate 7 did not yield measurable virus by coculture. Therefore, a protease-RT gene fragment was amplified by RT-PCR and inserted into the HXB2 backbone. As shown in Table 5, isolate 1 had three minor mutations in the protease region and the virus was susceptible to all four inhibitors tested. The increase in resistance, calculated by comparison to the drug resistance of JRCSF, was <1.0-fold for zidovudine, lamivudine, and lopina-