are very short in vivo (IL-4 mutant: $t_{1/2}$ = 0·83 h; sIL-4R: $t_{1/2}$ = 4·6 h),[35,36] huge amounts of these molecules are required in plasma to maintain a long period of inhibitory action on allergic inflammation. In fact, administration of these molecules was required many times in high doses from the sensitization to the challenge periods.[35–37] In the present study, we demonstrated a remarkable antagonistic effect of IL-4 mutant DNA applied in a form of vaccination, as a potent new type of immunogene therapy for AD. In previous studies in which gene therapy and DNA vaccines were used in combination with a cytokine gene for tumours or pathogens, effective immune responses to antigen were recognized even in the absence of detectable plasma levels of cytokines. Recently, we also reported that administration of plasmid DNA coding IL-4 cDNA completely inhibited the development of insulitis, which is one of the Th1-type autoimmune diseases, although no IL-4 was detected in plasma.[38] These results suggest that genes applied as a DNA vaccine express and supply products to the host continuously. To occupy the IL-4/IL-13 receptors, a continuous supply of IL-4DM is needed but not bolus application. Therefore, IL-4DM applied as a DNA vaccine might inhibit the allergic inflammation by persistent secretion of mutant IL-4 over a long period in a limited amount.

As we had expected, IL-4DM mitigated phenotypical and histological changes such as severe oedema, inflammatory cell infiltration and epidermal hyperplasia. IL-4DM also significantly decreased the number of dermal mast cells. IL-4 is known to be a potent activator of mast cells. Mast cells, which participate in the inflammatory cascade, serve as an abundant source of Th2 cytokines as well as inflammatory mediators.[39–41] Therefore, inhibition of mast cell activation is another possible mechanism through which IL-4DM ameliorates inflammatory responses in the present model of dermatitis. Eosinophil infiltration into the dermis has been well documented in AD.[42] In this study, an increased number of eosinophils was observed in contact hypersensitivity skin lesions, and was dramatically inhibited by IL-4DM treatment. Inhibition of cellular infiltration in IL-4DM mice may be due to suppression of IL-4-mediated immunological events such as a decreased expression of cellular adhesion molecules on endothelial cells.[43]

Injected IL-4DM and IL-4 DNA are trapped by monocytes/macrophages by phagocytosis. They may migrate to lymph nodes or spleen and show systemic effects. In fact, we could observe a high concentration of IL-4 in cultured splenocytes from IL-4DM DNA injected mice by ELISA. Unfortunately, there is no specific anti-IL-4DM antibody or anti-IL-4DM ELISA. The standard ELISA used in this study could not differentiate the natural mouse IL-4 and mutant IL-4 protein; these findings are consistent with the previous report.[25] The plasma IL-4 levels in the agonistic IL-4DNA-treated mice were consistent with those of the IL-4DM DNA-treated mice. Therefore, we speculate that exogenously applied IL-4DM DNAs were expressed the same as IL-4 DNAs, and showed systemic immunological effects.

IFN-γ production increased systemically and locally in mice treated with IL-4DM DNA. Repeated OX treatments cause expansion both of Th1 and Th2 cells. IL-4DM DNA therapy interfered with the development of the Th2 milieu. Subsequently, IFN-γ production and mRNA expression might become abundant locally and systemically.

Tissue-specific gene transfer could be achieved naturally and effectively through the cell specificity of virus receptors.[44] However, there may be a risk of vector toxicity through viral infection of host cells. Also, the limited size of transgenes is often a serious obstacle. Moreover, immune responses to viral vectors are also induced, and the effects of transgenes are eliminated by immune responses to the vectors. For human applications, the efficacy and safety of any delivery system for gene transfer are always of major concern. Nonviral approaches are advantageous in immunogene therapy. DNA vaccines are capable of inducing potent biological effects in a variety of experimental systems.[45] One of the characteristic features of DNA vaccines is their ability to induce long-lasting immunity. The animals that had been treated with IL-4DM DNA did not develop severe allergic inflammation even before or after antigen sensitization.

In the present study, we showed the beneficial effects of immunogene therapy with IL-4 mutant DNA in an experimental model for AD. An IL-4 mutant DNA vaccination is a potent new tool for the systemic treatment of AD.

Acknowledgments

Grants-in-Aid for Scientific Research and Grants-in-Aid for Core Research Evolutional Science and Technology.

References

1 Paul WE. Interleukin-4: a prototypic immunoregulatory lymphokine. Blood 1991; **77**:1859–70.
2 Mosmann TR, Sad S. The expanding universe of T-cell subsets: Th1, Th2 and more. Immunol Today 1996; **17**:138–46.
3 Romagnani S. The Th1/Th2 paradigm. Immunol Today 1997; **18**:263–6.
4 Zurawski G, de Vries JE. Interleukin 13, an interleukin 4-like cytokine that acts on monocytes and B cells, but not on T cells. Immunol Today 1994; **15**:19–26.
5 Tony HP, Shen BJ, Reusch P et al. Design of human interleukin-4 antagonists inhibiting interleukin-4-dependent and interleukin-13-dependent responses in T-cells and B-cells with high efficiency. Eur J Biochem 1994; **225**:659–65.
6 Smerz-Bertling C, Duschl A. Both interleukin 4 and interleukin 13 induce tyrosine phosphorylation of the 140-kDa subunit of the interleukin 4 receptor. J Biol Chem 1995; **270**:966–70.
7 Kruse N, Tony HP, Sebald W. Conversion of human interleukin-4 into a high affinity antagonist by a single amino acid replacement. EMBO J 1992; **11**:3237–44.
8 Kruse N, Shen BJ, Arnold S et al. Two distinct functional sites of human interleukin 4 are identified by variants impaired in either receptor binding or receptor activation. EMBO J 1993; **12**:5121–9.
9 Zurawski SM, Vega F Jr, Huyghe B et al. Receptors for interleukin-13 and interleukin-4 are complex and share a novel component that functions in signal transduction. EMBO J 1993; **12**:2663–70.

10 Aversa G, Punnonen J, Cocks BG et al. An interleukin 4 (IL-4) mutant protein inhibits both IL-4 or IL-13-induced human immunoglobulin G4 (IgG4) and IgE synthesis and B cell proliferation: support for a common component shared by IL-4 and IL-13 receptors. J Exp Med 1993; **178**:2213–18.
11 Zurawski SM, Chomarat P, Djossou O et al. The primary binding subunit of the human interleukin-4 receptor is also a component of the interleukin-13 receptor. J Biol Chem 1995; **270**:13869–78.
12 Konig B, Fischer A, Konig W. Modulation of cell-bound and soluble CD23, spontaneous and ongoing IgE synthesis of human peripheral blood mononuclear cells by soluble IL-4 receptors and the partial antagonistic IL-4 mutant protein IL-4 (Y124D). Immunology 1995; **85**:604–10.
13 Carballido JM, Schols D, Namikawa R et al. IL-4 induces human B cell maturation and IgE synthesis in SCID-hu mice. Inhibition of ongoing IgE production by in vivo treatment with an IL-4/IL-13 receptor antagonist. J Immunol 1995; **155**:4162–70.
14 Carballido JM, Aversa G, Schols D et al. Inhibition of human IgE synthesis in vitro and in SCID-hu mice by an interleukin-4 receptor antagonist. Int Arch Allergy Immunol 1995; **107**:304–7.
15 Schnyder B, Lugli S, Feng N et al. Interleukin-4 (IL-4) and IL-13 bind to a shared heterodimeric complex on endothelial cells mediating vascular cell adhesion molecule-1 induction in the absence of the common gamma chain. Blood 1996; **87**:4286–95.
16 Sornasse T, Larenas PV, Davis KA et al. Differentiation and stability of T helper 1 and 2 cells derived from naive human neonatal CD4+ T cells, analyzed at the single-cell level. J Exp Med 1996; **184**:473–83.
17 Vannier E, de Waal Malefyt R, Salazar-Montes A et al. Interleukin-13 (IL-13) induces IL-1 receptor antagonist gene expression and protein synthesis in peripheral blood mononuclear cells: inhibition by an IL-4 mutant protein. Blood 1996; **87**:3307–15.
18 Schnarr B, Ezernieks J, Sebald W et al. IL-4 receptor complexes containing or lacking the gamma C chain are inhibited by an overlapping set of antagonistic IL-4 mutant proteins. Int Immunol 1997; **9**:861–8.
19 Grunewald SM, Kunzmann S, Schnarr B et al. A murine interleukin-4 antagonistic mutant protein completely inhibits interleukin-4-induced cell proliferation, differentiation, and signal transduction. J Biol Chem 1997; **272**:1480–3.
20 Andersson A, Grunewald SM, Duschl A et al. Mouse macrophage development in the absence of the common gamma chain: defining receptor complexes responsible for IL-4 and IL-13 signaling. Eur J Immunol 1997; **27**:1762–8.
21 Kitagaki H, Fujisawa S, Watanabe K et al. Immediate-type hypersensitivity response followed by a late reaction is induced by repeated epicutaneous application of contact sensitizing agents in mice. J Invest Dermatol 1995; **105**:749–55.
22 Kitagaki H, Kimishima M, Teraki Y et al. Distinct in vivo and in vitro cytokine profiles of draining lymph node cells in acute and chronic phases of contact hypersensitivity: importance of a type 2 cytokine-rich cutaneous milieu for the development of an early-type response in the chronic phase. J Immunol 1999; **163**:1265–73.
23 Mori H, Yamanaka K, Matsuo K et al. Administration of Ag85B showed therapeutic effects to Th2-type cytokine-mediated acute phase atopic dermatitis by inducing regulatory T cells. Arch Dermatol Res 2009; **301**:151–7.
24 Kopf M, Le Gros G, Bachmann M et al. Disruption of the murine IL-4 gene blocks Th2 cytokine responses. Nature 1993; **362**:245–8.
25 Nishikubo K, Murata Y, Tamaki S et al. A single administration of interleukin-4 antagonistic mutant DNA inhibits allergic airway inflammation in a mouse model of asthma. Gene Ther 2003; **10**:2119–25.
26 Overbergh L, Valckx D, Waer M et al. Quantification of murine cytokine mRNAs using real time quantitative reverse transcriptase PCR. Cytokine 1999; **11**:305–12.
27 Kay AB, Ying S, Varney V et al. Messenger RNA expression of the cytokine gene cluster, interleukin 3 (IL-3), IL-4, IL-5, and granulocyte/macrophage colony-stimulating factor, in allergen-induced late-phase cutaneous reactions in atopic subjects. J Exp Med 1991; **173**:775–8.
28 Herz U, Bunikowski R, Renz H. Role of T cells in atopic dermatitis. New aspects on the dynamics of cytokine production and the contribution of bacterial superantigens. Int Arch Allergy Immunol 1998; **115**:179–90.
29 Akdis CA, Akdis M, Trautmann A et al. Immune regulation in atopic dermatitis. Curr Opin Immunol 2000; **12**:641–6.
30 Ricci M, Matucci A, Rossi O. IL-4 as a key factor influencing the development of allergen-specific Th2-like cells in atopic individuals. J Investig Allergol Clin Immunol 1997; **7**:144–50.
31 Elbe-Burger A, Egyed A, Olt S et al. Overexpression of IL-4 alters the homeostasis in the skin. J Invest Dermatol 2002; **118**:767–78.
32 Zhou CY, Crocker IC, Koenig G et al. Anti-interleukin-4 inhibits immunoglobulin E production in a murine model of atopic asthma. J Asthma 1997; **34**:195–201.
33 Tomaki M, Zhao LL, Lundahl J et al. Eosinophilopoiesis in a murine model of allergic airway eosinophilia: involvement of bone marrow IL-5 and IL-5 receptor alpha. J Immunol 2000; **165**:4040–50.
34 Bost KL, Holton RH, Cain TK et al. In vivo treatment with anti-interleukin-13 antibodies significantly reduces the humoral immune response against an oral immunogen in mice. Immunology 1996; **87**:633–41.
35 Henderson WR Jr, Chi EY, Maliszewski CR. Soluble IL-4 receptor inhibits airway inflammation following allergen challenge in a mouse model of asthma. J Immunol 2000; **164**:1086–95.
36 Tomkinson A, Duez C, Cieslewicz G et al. A murine IL-4 receptor antagonist that inhibits IL-4- and IL-13-induced responses prevents antigen-induced airway eosinophilia and airway hyperresponsiveness. J Immunol 2001; **166**:5792–800.
37 Grunewald SM, Werthmann A, Schnarr B et al. An antagonistic IL-4 mutant prevents type I allergy in the mouse: inhibition of the IL-4/IL-13 receptor system completely abrogates humoral immune response to allergen and development of allergic symptoms in vivo. J Immunol 1998; **160**:4004–9.
38 Hayashi T, Yasutomi Y, Hasegawa K et al. Interleukin-4-expressing plasmid DNA inhibits reovirus type-2-triggered autoimmune insulitis in DBA/1 J suckling mice. Int J Exp Pathol 2003; **84**:101–6.
39 Ruzicka T, Gluck S. Cutaneous histamine levels and histamine releasability from the skin in atopic dermatitis and hyper-IgE-syndrome. Arch Dermatol Res 1983; **275**:41–4.
40 Horsmanheimo L, Harvima IT, Jarvikallio A et al. Mast cells are one major source of interleukin-4 in atopic dermatitis. Br J Dermatol 1994; **131**:348–53.
41 Gibbs BF, Wierecky J, Welker P et al. Human skin mast cells rapidly release preformed and newly generated TNF-alpha and IL-8 following stimulation with anti-IgE and other secretagogues. Exp Dermatol 2001; **10**:312–20.
42 Leung DY. Atopic dermatitis: immunobiology and treatment with immune modulators. Clin Exp Immunol 1997; **107** (Suppl. 1):25–30.
43 Schleimer RP, Sterbinsky SA, Kaiser J et al. IL-4 induces adherence of human eosinophils and basophils but not neutrophils to endothelium. Association with expression of VCAM-1. J Immunol 1992; **148**:1086–92.
44 Mistry AR, Falciola L, Monaco L et al. Recombinant HMG1 protein produced in Pichia pastoris: a nonviral gene delivery agent. BioTechniques 1997; **22**:718–29.
45 Gurunathan S, Klinman DM, Seder RA. DNA vaccines: immunology, application, and optimization. Annu Rev Immunol 2000; **18**:927–74.

Parasitol Res (2009) 105:929–937
DOI 10.1007/s00436-009-1480-3

ORIGINAL PAPER

DNA characterization of simian *Entamoeba histolytica*-like strains to differentiate them from *Entamoeba histolytica*

Jun-ichiro Takano · Hiroshi Tachibana · Miyoko Kato · Toyoko Narita · Tetsuo Yanagi · Yasuhiro Yasutomi · Koji Fujimoto

Received: 27 March 2009 / Accepted: 8 May 2009 / Published online: 27 May 2009

Abstract Two simian *Entamoeba histolytica*-like strains, EHMfas1 and P19-061405, have been suggested to represent a new species based on genetic characterization. Sequence analyses of the hexokinase, glucose phosphate isomerase, and phosphoglucomutase genes supported the previous findings of isoenzyme analyses demonstrating a new zymodeme pattern. Phylogenetic studies of 18S rDNA, 5.8S rDNA, the chaperonin 60 gene, and the pyridine nucleotide transhydrogenase gene showed original clusters of simian *E. histolytica*-like strains below or near *E. histolytica*, respectively. Comparative studies of the chitinase and the serine-rich *E. histolytica* protein genes and locus 1–2 region revealed that most mutated units were shared among the simian *E. histolytica*-like strains. The similarities of each of the repeating units within the simian *E. histolytica*-like strains or *E. histolytica* and the differences of those between the both might be generated by concerted evolution. Our results indicate that EHMfas1 and P19-061405 should be considered to be the same species, despite that they were isolated from different monkey species and different habitats. Simian *E. histolytica*-like amebas may be endemic to macaque monkeys, as a counterpart to *E. histolytica* in humans, and should be differentiated from *E. histolytica* by the revival name *Entamoeba nuttalli*, as proposed for P19-061405.

J.-i. Takano (✉) · M. Kato · T. Narita · K. Fujimoto
The Corporation for Production and Research
of Laboratory Primates,
1-1 Hachimandai,
Tsukuba, Ibaraki 305-0843, Japan
e-mail: takano@primate.or.jp

H. Tachibana
Department of Infectious Diseases,
Tokai University School of Medicine,
143 Shimokasuya,
Isehara, Kanagawa 259-1193, Japan

T. Yanagi
Animal Research Center for Tropical Infections,
Institute of Tropical Medicine, Nagasaki University,
1-12-4 Sakamoto,
Nagasaki, Nagasaki 852-8523, Japan

Y. Yasutomi
Tsukuba Primate Research Center,
National Institute of Biomedical Innovation,
1-1 Hachimandai,
Tsukuba, Ibaraki 305-0843, Japan

Introduction

Entamoeba histolytica causes amoebic colitis and liver abscess, and amoebiasis is one of the most important parasitic diseases in humans. In non-human primates, several cases of *E. histolytica* or *E. histolytica*-like organism infections have been identified by isoenzyme analysis, monoclonal antibody test, or PCR (Tachibana et al. 1990; Verweij et al. 2003; Takano et al. 2005; Tachibana et al. 2007; Suzuki et al. 2007). One of the simian *E. histolytica*-like strains, EHMfas1, has been isolated from a healthy cynomolgus monkey (*Macaca fascicularis*) and identified using species-specific PCR for *E. histolytica* and antigen-capture ELISA (Takano et al. 2005). Another simian *E. histolytica*-like strain, P19-061405, has been isolated from a rhesus monkey (*Macaca mulatta*). Furthermore, the virulence of P19-061405 has also been confirmed by experimental infection of hamsters (Tachibana et al. 2007).

These two simian *E. histolytica*-like strains were suggested to be a new species, because EHMfas1 exhibits differences in the 16S-like small subunit ribosomal RNA

(18S rDNA), chitinase and SREHP genes, and P19-061405 exhibits differences in several DNA sequences, including the 18S rDNA and SREHP genes (Takano et al. 2007; Tachibana et al. 2007). It has been proposed that P19-061405 should be distinguished from *E. histolytica* by revival of the name *Entamoeba nuttalli* (Castellani 1908) that was the first reported *E. histolytica*-like species found in a liver abscess of a monkey (Tachibana et al. 2007). Isoenzyme analyses of these two simian *E. histolytica*-like strains have demonstrated a new and similar zymodeme pattern.

In this study, we compared these two simian *E. histolytica*-like strains based on DNA loci that were used for isoenzyme analysis, phylogenetic investigation, and genotyping to determine the similarity between the simian *E. histolytica*-like strains and the differences between simian *E. histolytica*-like amebas and *E. histolytica*.

Materials and methods

Simian *E. histolytica*-like strains

Trophozoites of the EHMfas1 strain were cultured monoxenically with *Crithidia fasciculata* and axenically in BI-S-33 medium supplemented with 15% adult bovine serum at 37°C (Diamond et al. 1978) and then cloned by limiting dilution, followed by examination using microscopy. Trophozoites of the cloned P19-061405 strain were also axenically cultured in BI-S-33 medium (Tachibana et al. 2007).

Hepatic inoculation of hamsters

Hamsters were inoculated with trophozoites of the EHMfas1 that had been cultured monoxenically with *C. fasciculata* as described by Tachibana et al. (2007).

DNA preparation and sequencing

Total genomic DNA from each cloned trophozoite was extracted using a QIAamp DNA Stool Mini Kit (Qiagen) or a DNeasy tissue kit (Qiagen) according to the manufacturer's instructions.

The genomic DNA from cloned trophozoites was amplified using the primers listed in Table 1. PCR was conducted in a 50 μl reaction mixture containing 2 μl of extracted DNA and 0.1 μg/μl bovine serum albumin using PrimeSTAR Max DNA polymerase (Takara). A total of 35 cycles of PCR were performed, as follows: denaturation at 98°C for 10 s, annealing at 62°C (for pyridine nucleotide transhydrogenase (PNT)) or 56°C (for other genes) for 5 s, and extension at 72°C for 15 s.

Amplified genes for hexokinase (HXK), glucose-6-phosphate isomerase (GPI), and phosphoglucomutase (PGM) from EHMfas1 and chaperonin 60 (Cpn60) were cloned from P19-061405 using a Zero Blunt TOPO PCR cloning kit for sequencing (Invitrogen). Each clone was subjected to sequencing using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) on an ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems), according to the manufacturer's instructions. PCR products for 5.8S rDNA with internal-transcribed spacer (ITS) 1 and ITS 2, Cpn60, and PNT, locus 1–2 from EHMfas1 and PNT, and chitinase and locus 1–2 from P19-061504 were sequenced directly. Sequence data were analyzed using DNASIS Pro ver. 2.08 (Hitachi software). The GenBank accession numbers of the sequences used for comparison with each gene are shown in Figs. 1, 2, 3, and 4.

Phylogenetic analysis

Analysis and multiple alignments of DNA sequences of 18S rDNA, 5.8S rDNA with ITS 1 and ITS 2, Cpn60, and PNT were performed with ClustalX (Thompson et al. 1997), and the phylogenetic trees were constructed using the neighbor-joining method (Saitou and Nei 1987).

Nucleotide sequence accession numbers

The nucleotide sequence data reported here have been submitted to the GenBank/EMBL/DDBJ databases under accession numbers AB454548 to AB454559 and AB480745.

Results

Virulency of EHMfas1 in hamster

EHMfas1 was monoxenically cultured with *C. fasciculata* and inoculated into the livers of hamsters. Liver abscesses were observed in all hamsters at 7 days after inoculation. The presence of trophozoites in the peripheral regions of abscesses in the livers was confirmed in PAS stained tissue slides. No abscesses were observed in the control hamsters, inoculated with *C. fasciculata* alone.

Analysis of zymodeme-related genes

Two HXK genes of EHMfas1 were amplified from the cloned EHMfas1 trophozoites and sequenced after molecular cloning. The calculated molecular masses and isoelectric points (p*I*) were 49.7 kDa and 5.38 in HXK 1 and 49.4 kDa and 4.99 in HXK 2. The deduced amino-acid sequences were compared with those of P19-061405

Table 1 Primers used in this study

Locus	Direction (name of primer)	Sequence (5′-3′)	Reference
HXK	S	ATG CAA GAA ATC ATT GAT CAA TTT	Tachibana et al. 2007
HXK1	AS	TTA GTG TTT ACA TGC AAC AGC A	
HXK2	AS	TTA TTG TTT GCA TGC AAC AGC A	
GPI	S	ATG TTA CCA ACT CTT CCT GAA T	Tachibana et al. 2007
	AS	TTA GTT TTT TCT CAT ATC TTT AAC A	
PGM	S (PGMoutS)	TCG TTG AAC CAG ATC AGT GC	Genome database region on scaffold 00005[a]
	AS (PGMoutAS)	AAG CTT CTC TGG ATG GTG TTG	
ITS1, 5.8S	S (P1)	AGG TGA ACC TGC GGA AGG ATC ATT A	Som et al. 2000
ITS2	AS (P2)	TCA TTC GCC ATT ACT TAA GAA ATC ATT GTT	
Cpn60	S (CpnOf1)	GTT GAA CTT TTC ATA AGG TTG TTT GA	Genome database region on scaffold 00015[a]
	AS (CpnOr1)	CAA AAA TGG GCA GAT GAA CA	
PNT	S (PNT-A)	GTA GGA CTT GCA GCA GTA TT	Bakatselou et al. 2003
	AS (PNT-B)	GGT AAT CTT CCT GCA ACT GG	
Chitinase	S	GGA ACA CCA GGT AAA TGT ATA	Ghosh et al. 2000
	AS	TCT GTA TTG TGC CCA ATT	
Locus 1–2	S (R1)	CTG GTT AGT ATC TTC GCC TGT	Zaki et al. 2002
	AS (R2)	CTT ACA CCC CCA TTA ACA AT	

S sense, *AS* anti-sense

[a] Present study

(GenBank accession numbers: AB282663 for HXK 1 and AB282664 for HXK 2). Glu^{13} and Asn^{32} in HXK 1 of P19-061405 were changed to Gly and Asp, respectively, in HXK 1 of EHMfas1. HXK 2 of EHMfas1was identical to that of P19-061405. The p*I*s for HXK 1 and HXK 2 of EHMfas1 were consistent with those of P19-061405 (Tachibana et al. 2007).

The GPI gene of EHMfas1 was also amplified and sequenced after molecular cloning, and only one GPI gene was obtained from 16 clones. The calculated molecular mass and p*I* for GPI of EHMfas1, 61.4 kDa and 6.60, were also consistent with those of P19-061405. In the deduced amino-acid sequence, Val^{232} in GPI 1 of P19-061405 (GenBank accession number: AB282665) was changed to Ile, and Val^{330} in the GPI 2 of P19-061405 (GenBank accession number: AB282666) was changed to Ala in EHMfas1.

One PGM gene from EHMfas1 was detected in clones from EHMfas1. The calculated molecular mass and p*I* for PGM were 60.8 kDa and 5.99, respectively. In the deduced amino-acid sequence of PGM from EHMfas1, there were four and 13 differences compared with *E. histolytica* (GenBank accession number: Y14444) and *Entamoeba dispar* (GenBank accession number: Y14445), respectively. Although the isoenzyme pattern of PGM in EHMfas1 was identical to *E. dispar*, the amino-acid sequences of PGM in EHMfas1 were more similar to *E. histolytica* than *E. dispar*.

Analyses and phylogenic studies of ribosomal RNA genes and mitosome genes

Comparison of the 18S rDNA sequences reported for EHMfas1 (Takano et al. 2007) and P19-061405 (Tachibana et al. 2007) showed a difference of 0.1% (two of 1,945). A reconstructed phylogenetic tree showed the original cluster of simian *E. histolytica*-like strains beside the *E. histolytica* branch (Fig. 1a). The 5.8S rDNA, with ITS 1 and 2 regions of EHMfas1, was sequenced directly. Comparison of this region of EHMfas1 and P19-061405 (Tachibana et al. 2007) showed no differences throughout the length. The constructed phylogenetic tree of this region also showed that the simian isolates were located beside the *E. histolytica* branch (Fig. 1b). The Cpn60 and PNT genes of EHMfas1 and P19-061405 were directly sequenced. Although DNAs were extracted from cloned trophozoites, obvious mixed sequences were confirmed by direct sequencing of Cpn60 gene from P19-061405. The Cpn60 gene from P19-061405 was cloned, and we obtained two Cpn60 gene alleles from six clones. In the nucleotide sequence of Cpn60 from EHMfas1, five and four differences were present compared with P19-061405 alleles 1 and 2, respectively. In the deduced amino-acid sequence, all Cpn60 from EHMfas1 and P19-061405 were consistent with *E. histolytica*. The phylogenetic relationship of the Cpn60 genes among *Entamoeba* species was also reconstructed (Fig. 1c). In

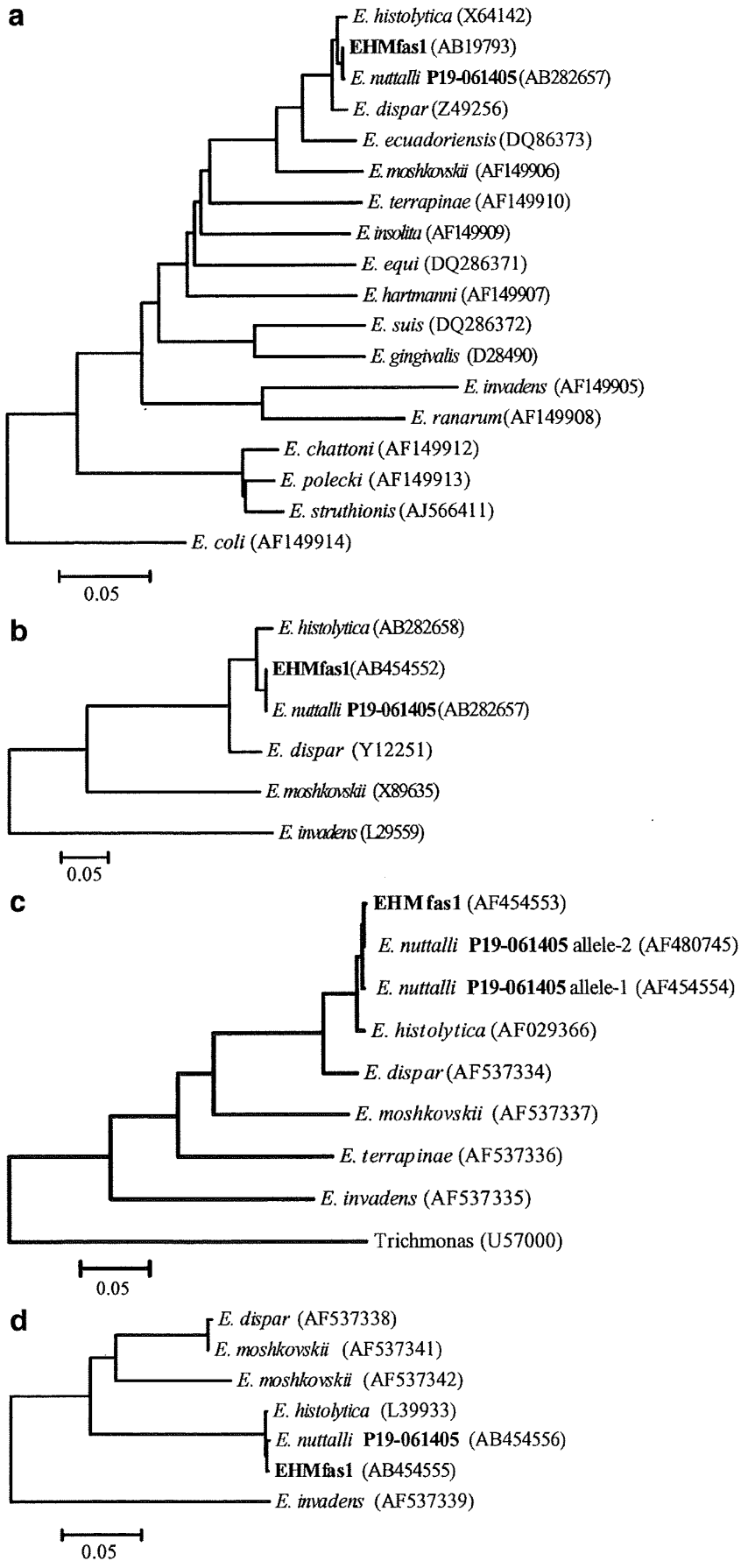

Fig. 1 Phylogenetic relationships among simian *E. histolytica*-like strains and other *Entamoeba* species. 18S rDNA sequences (**a**), 5.8S rDNA with ITS1 and ITS2 sequences (**b**), the Cpn60 gene sequences (**c**), and the PNT gene sequences (**d**). Branch lengths are proportional to estimated number of substitutions per site, which represent the evolutional distance

the reconstructed phylogenetic tree of the Cpn60 genes, the cluster of simian *E. histolytica*-like strains was located under the *E. histolytica* branch. PNT gene sequences were also compared between EHMfas1 and P19-061405, and a difference of 0.23% (one of 432) was evident. *E. histolytica*, EHMfas1 and P19-061405, was categorized in the same cluster (Fig. 1d).

Comparison of polymorphic loci: the chitinase gene, SREHP gene, and locus 1–2

The chitinase gene of P19-061405 was directly sequenced and compared with the reported sequences of EHMfas1, KU3 (genotype A) and KU15 (genotype D) of *E. histolytica* (Fig. 2; de la Vega et al. 1997; Ghosh et al. 2000; Haghighi et al. 2002, 2003). Each unit of the nucleotide and deduced amino-acid sequences was tentatively given a number, as in a previous study (Takano et al. 2007). All known EHMfas1-specific units, CN3, CN5, CN7, and CN3′C2, were also observed in P19-061405; however, the combination pattern of the repeating unit was different. Although P19-061405 was not classified into any known genotype, based on the nucleotide sequence, it was classified into genotype D based on the deduced amino-acid sequence in the polymorphic region.

Many SREHP genes of *E. histolytica* have been sequenced for genotyping (Li et al. 1992; Clark and Diamond 1993; Kohler and Tannich 1993; Stanley et al. 1990; Ayeh-Kumi et al. 2001; Haghighi et al. 2002, 2003). The reported SREHP gene sequences of EHMfas1, P19-061405 and *E. histolytica* KU27 (genotype I), were compared (Fig. 3). Each unit of the nucleotide and deduced amino-acid sequences was tentatively given a number, as in a previous study (Takano et al. 2007). All known EHMfas1-specific units, SN2, SN5, SN9, SN17, SN20, and SN3′C2, were also observed in P19-061405, but the combination pattern was different. The SN16 and DEE insertion, i.e., the second EHMfas1-specific insertion, were not observed in P19-061405. The nucleotide and deduced amino-acid sequences of P19-061405 were not classified into any of the known genotypes.

Locus 1–2 (also known as a non-coding short tandem repeat, D-A; Ali et al. 2005) of EHMfas1 and P19-061405 was directly sequenced and compared with the reported genotypes of *E. histolytica* (Zaki and Clark 2001; Haghighi et al. 2002, 2003). A number was tentatively assigned to each unit of the nucleotide sequences (Fig. 4). The nucleotide sequences of locus 1–2 were constructed from combinations of the 5′-conserved region, an 8 bp-repeating polymorphic region (8L1, 8L2, 8L3, and 8L4), intra-conserved region 1 (CL1, CL2, CL3, CL4, CL5,

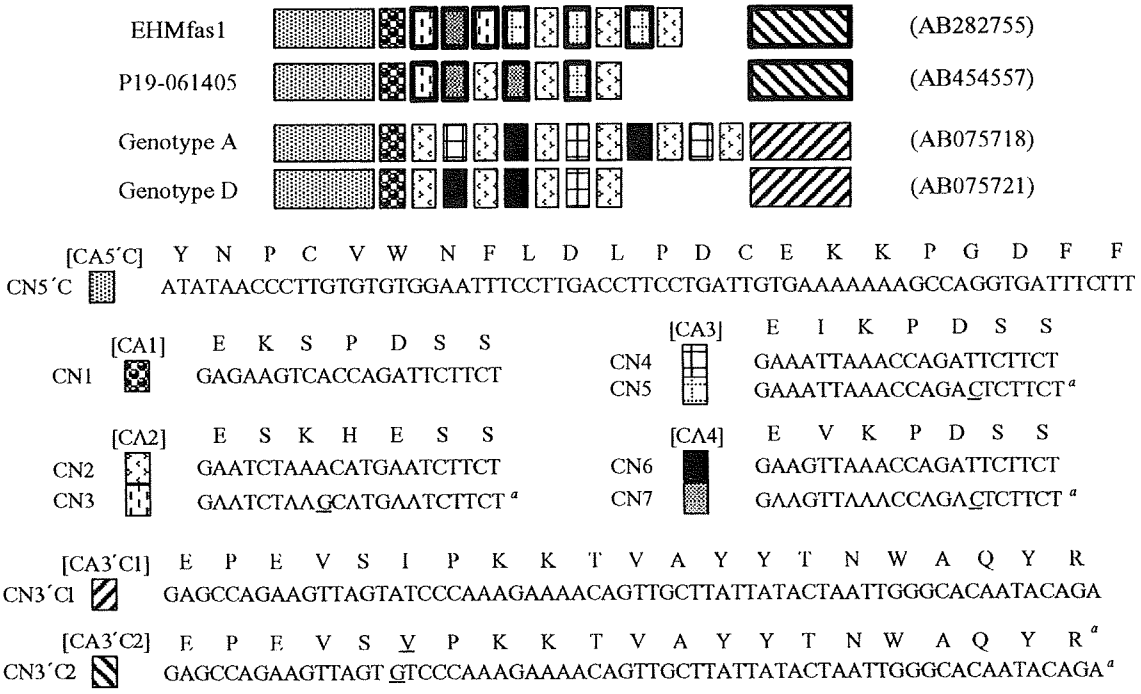

Fig. 2 Schematic representation of polymorphism in the repeat-containing region of the chitinase gene among simian *E. histolytica*-like strains and genotype A and D of *E. histolytica*. Nucleotide sequences pattern was shown. Each of nucleotide and deduced amino-acid sequences of unit was tentatively given a number. Nucleotide and deduced amino-acid sequences of these units are also shown. *Enclosed units with bold line* were simian *E. histolytica*-like strain-specific units. Simian *E. histolytica*-like strain-specific mutations in nucleotide and deduced amino-acid sequences are *underlined*. *a* Simian *E. histolytica*-like strain-specific unit sequences

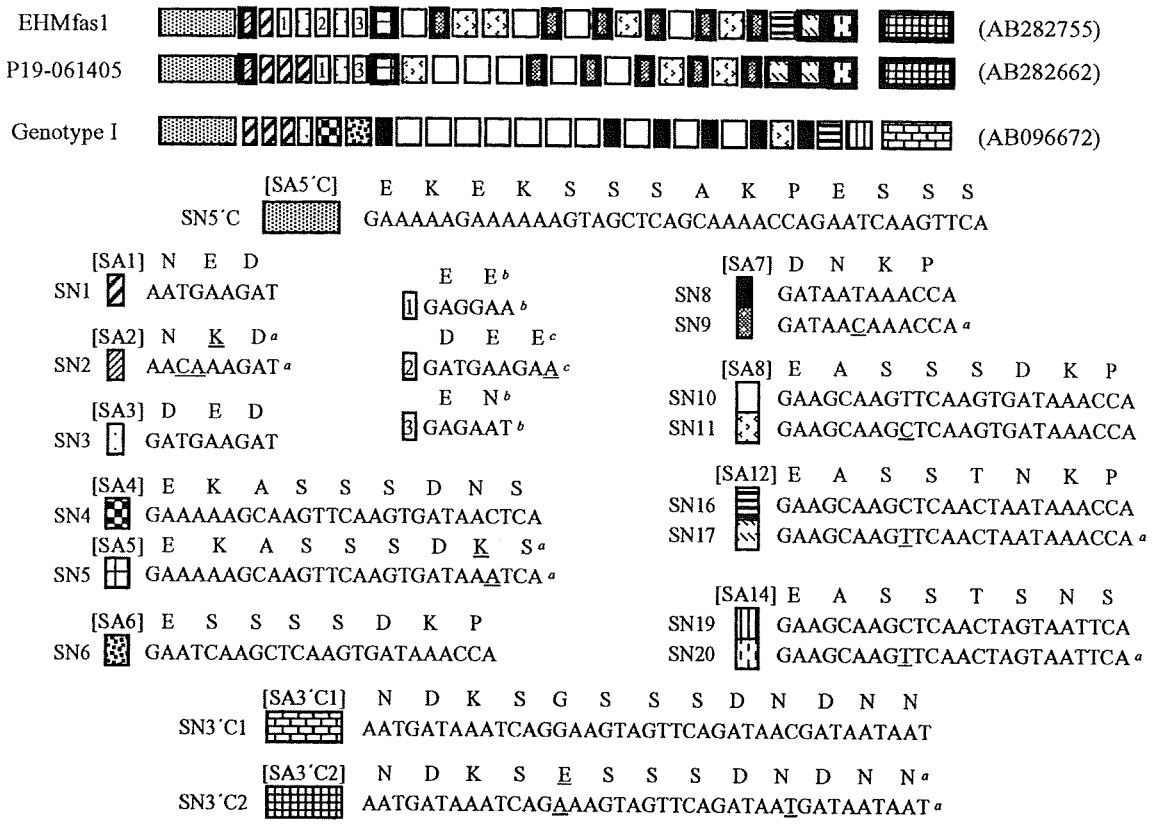

Fig. 3 Schematic representation of polymorphism in the repeat-containing region of the SREHP gene among simian *E. histolytica*-like strains and genotype I of *E. histolytica*. Nucleotide sequences pattern was shown. Each nucleotide and deduced amino-acid sequence of unit was tentatively given a number. Nucleotide and deduced amino-acid sequences of these units are also shown. ***Enclosed units with bold line*** were simian *E. histolytica*-like strain-specific units. Simian *E. histolytica*-like strain-specific mutations in nucleotide and deduced amino-acid sequences are *underlined*. *a* Simian *E. histolytica*-like strain-specific unit sequences. *b* Simian *E. histolytica*-like strain-specific block insertions. *c* EHMfas1-specific block insertion

CL6, and CL7), a 9-bp-repeating polymorphic region (9L1, 9L2, 9L3, 9L4, 9L5, and 9L6), intra-conserved region 2 (CL6, CL9, CL10, and CL11), a 12-bp-repeating polymorphic region (12L1, 12L2, 12L3, and 12L4), and the 3′-conserved region. The 8L1, 8L3, CL1, CL5, CL6, 9L1, 9L2, 9L4, and 12L3 units were common to simian *E. histolytica*-like strains and *E. histolytica*. On the other hand, 8L4, CL2, CL4, 9L3, 9L5, CL11, 12L2, and 12L4, were simian *E. histolytica*-like strain-specific mutated units. These mutated units corresponded to the 8L3, CL1, CL3, 9L2, 9L4, CL10, 12L1, and 12L3 units, with a single-nucleotide substitution in each unit, respectively. The CL8 was also a simian *E. histolytica*-like strain-specific unit that corresponded to CL7 with a four-nucleotide deletion (CCCT). Most units observed in *E. histolytica* were also observed in simian *E. histolytica*-like strains; however, all 12L1 were changed to 12L2 in the simian *E. histolytica*-like strains, and the CL9 was missing in the simian *E. histolytica*-like strains. Furthermore, 8L2 was observed only in EHMfas1 and corresponded to 8L1 with a double-nucleotide substitution.

Discussion

In this study, genetic similarities between EHMfas1 and P19-061405 and differences between simian *E. histolytica*-like strains and *E. histolytica* were revealed. EHMfas1 was also a virulent strain as P19-061405. The similar zymodeme patterns of EHMfas1 and P19-061405 have not been classified into any known patterns (Takano et al. 2007; Tachibana et al. 2007). Although some deduced amino acids were changed, the genetic similarities of HXK, GPI, and PGM supported the results of the isoenzyme analyses. HXK, GPI, and PGM of the simian *E. histolytica*-like strains were obviously more similar to *E. histolytica* than *E. dispar*. It is considered that EHMfas1 and P19-061405 are the same species, despite the fact that these strains were isolated from different host monkey species and a different habitat. Thus, simian *E. histolytica*-like amebas likely naturally infect macaques as a counterpart to *E. histolytica* in human.

Phylogenetic studies also indicated that EHMfas1 and P19-061405 are the same species and are closely related to *E. histolytica*. Nucleotide sequences of the 18S rDNA

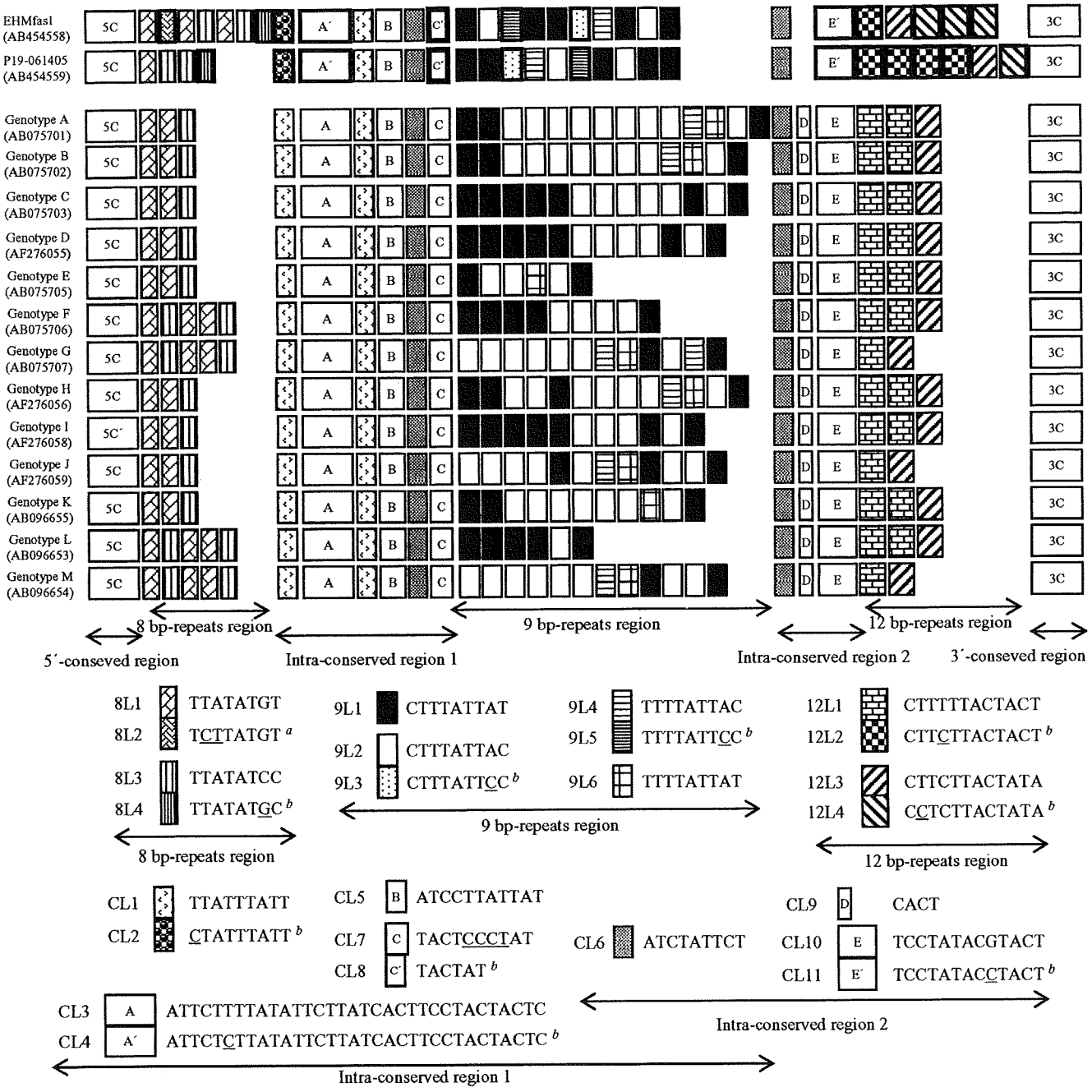

Fig. 4 Schematic representation of polymorphism in the repeat-containing region of the locus 1–2 region among simian *E. histolytica*-like strains and available *E. histolytica*. Nucleotide sequences pattern was shown. Each nucleotide sequence of unit was tentatively given a number. Nucleotide sequences of these units are also shown. *Enclosed units with bold line* were EHMfas1-specific or simian *E. histolytica*-like strain-specific units. EHMfas1-specific or simian *E. histolytica*-like strain-specific mutations in nucleotide sequences are *underlined*. Simian *E. histolytica*-like strain-specific deletion from human isolates is underlined at comparative sequence. *a* EHMfas1-specific unit sequence. *b* Simian *E. histolytica*-like strain-specific unit sequences

and 5.8S rDNA with ITS 1 and ITS 2 and Cpn60 and PNT genes of EHMfas1 and P19-061405 have been used for previous phylogenetic studies of the genus *Entamoeba* (Clark and Diamond 1997; Silberman et al. 1999; Som et al. 2000; Bakatselou et al. 2003; Clark et al. 2006). All phylogenetic studies indicated that EHMfas1 and P19-061405 generated an original cluster beside or under *E. histolytica*, including the PNT gene, which is known to contribute to an anomalous-shaped phylogenetic tree (Bakatselou et al. 2003).

Comparisons of polymorphic loci used for genotyping revealed notable differences from *E. histolytica* and

similarities between simian *E. histolytica*-like strains in sequence variation of the constructing units (Figs. 2, 3, and 4). The chitinase genes, the SREHP genes, and the locus 1–2 sequences of *E. histolytica* have been categorized into seven, 37, and 13 genotypes, respectively, from 79 strains, based on the combination pattern of constructing units in the polymorphic regions, respectively (Haghighi et al. 2002, 2003). The most kinds of mutation units were observed in locus 1–2, although the most kinds of genotype have been reported in the SREHP gene. In all polymorphic loci, most mutated units that were observed in EHMfas1 and P19-061405 were shared as simian *E. histolytica*-like strain-specific units, including deletions, among EHMfas1 and P19-061405. These mutated units had single-nucleotide substitutions relative to the corresponding units in *E. histolytica*. On the other hand, unshared mutated units were also observed. In the SREHP gene, SN16 was not observed in P19-061405, and the second insertion unit (GATGAA-GAA; DEE) was only observed in EHMfas1. In locus 1–2, 8L2 was specific for EHMfas1. The EHMfas1-specific insertion unit was likely derived from the non-repeating SN3 (GATGAAGAT; DED), with a single-nucleotide substitution. These slight disparities may reflect the differences between the host monkey species or the habitats of EHMfas1 and P19-061405; however, not enough strains have been studied to date. Analyses of these polymorphic loci also indicated that simian *E. histolytica*-like strains can also be classified by genotyping with original mutated units as *E. histolytica*.

It is interesting that *E. dispar* exhibit the same repetitive units as *E. histolytica* in the chitinase (CN2, CN4, and CN6) and the SREHP (SN8 and SN16) genes (Ghosh et al. 2000). Although *E. dispar* exhibits *E. dispar*-specific units, most of those mutated units were not repetitive. On the other hand, EHMfas1 and P19-061405 exhibited many repetitive mutated units, CN3, CN5, CN7, SN9, and SN17. EHMfas1 and P19-061405 exhibited not only mutated CN3 and SN17 but also the corresponding CN2 and SN16. However, all CN4, CN6, and SN8 were changed to CN5, CN7, and SN9. These findings indicate that *E. histolytica* is more closely related to *E. dispar* than the simian *E. histolytica*-like strains, in contrast to the results of other sequence analyses. It is considered that these contradictory findings are probably caused by specific effects, other than general evolution, because SREHP, in particular, is known to be a trophozoite surface antigen (Stanley et al. 1995). In other words, these contradictory findings may indicate differences in the manner of immune escape between different host species. On the other hand, locus 1–2 is a non-coding region and has the most kinds of mutation units. These polymorphic loci must be exposed to other evolutionary effects. This specific contradictory finding is likely a consequence of "concerted evolution" (Dover 1982, 1993; Dover and Tautz 1986; Wilkinson and Chapman 1991; Jinks-Robertson and Petes 1993; Liao 1999).

Concerted evolution is a universal biological phenomenon for repetition of DNA elements and has been observed in many repetitive DNA sequences and multi-gene families. Concerted evolution is thought to result from various mechanisms of DNA turnover, including unequal crossing-over, DNA amplification, gene conversion, and replication slippage. In *Entamoeba* species, concerted evolution has been discussed as it relates to the 5.8S rRNA gene and rRNA-linked *E. histolytica* short tandem repeats (Som et al. 2000; Tawari et al. 2008). It is suggested that diversification of the chitinase gene, the SREHP gene, and locus 1–2 can be caused by replication slippage (Ghosh et al. 2000; Bhattacharya et al. 2005). The repetitive units developing by concerted evolution are homogeneous within species, but differ somewhat between species. The similarities of each kind of unit in the chitinase and SREHP genes, locus 1-2 within simian *E. histolytica*-like strains or *E. histolytica*, and the differences of those between the both are accountable by concerted evolution. Concerted evolution of these polymorphic loci indicates that the repetitive simian *E. histolytica*-like strain-specific units had been amplified and conserved according to speciation as opposed to general DNA evolution, whereas some of these units had been eliminated or were not apparent in *E. histolytica* and *E. dispar*.

Tachibana et al. (2007) proposed that P19-061405 should be distinguished from *E. histolytica* by revival of the named *E. nuttalli* (Castellani 1908). Because EHMfas1 exhibited slight differences but had the same genetic characteristics as P19-061405, EHMfas1 also should be identified as *E. nuttalli*. This study indicates that *E. nuttalli* is endemic to monkey but we cannot exclude the possibility of zoonotic infection from monkey to human. We believe that further comparative study of *E. nuttalli* will contribute to more insights into the phylogeny and pathogenicity of *Entamoeba* species because *E. nuttalli* is very closely related to *E. histolytica* and *E. nuttalli* is the only pathogenic species other than *E. histolytica* among the *Entamoeba* species.

Acknowledgment This work was supported by a Grant-in-Aid for Scientific Research from the Japanese Society for the Promotion of Science.

References

Ali IKM, Zaki M, Clark CG (2005) Use of PCR amplification of tRNA gene-linked short tandem repeats for genotyping *Entamoeba histolytica*. J Clin Microbiol 43:5842–5847

Ayeh-Kumi PF, Ali IM, Lockhart LA, Gilchrist CA, Petri WA Jr, Haque R (2001) *Entamoeba histolytica*: genetic diversity of

clinical isolates from Bangladesh as demonstrated by polymorphisms in the serine-rich gene. Exp Parasitol 99:80–88

Bakatselou C, Beste D, Kadri AO, Somanath S, Clarc CG (2003) Analysis of genes of mitochondrial origin in the genus *Entamoeba*. J Eukaryot Microbiol 50:210–214

Bhattacharya D, Haque R, Singh U (2005) Coding and noncoding genomic regions of *Entamoeba histolytica* have significantly different rates of sequence polymorphisms: implications for epidemiological studies. J Clin Microbiol 43:4815–4819

Castellani A (1908) Note on a liver abscess of amoebic origin in a monkey. Parasitology 1:101–102

Clark CG, Diamond LS (1993) *Entamoeba histolytica*: a method for isolate identification. Exp Parasitol 77:450–455

Clark CG, Diamond LS (1997) Inraspecific variation and phylogenetic relationships in the genus *Entamoeba* as revealed by riboprinting. J Euk Microbiol 44:142–154

Clark CG, Kaffashian F, Tawari B, Windsor JJ, Twigg-Flesner A, Davies-Morel MC, Blessmann J, Ebert F, Peschel B, Le Van A, Jackson CJ, Macfarlane L, Tannich E (2006) New insights into the phylogeny of *Entamoeba* species provided by analysis of four new small-subunit rRNA genes. Int J Syst Evol Microbiol 56:2235–2239

Diamond LS, Harlow DR, Cunnick CC (1978) A new medium for the axenic cultivation of *Entamoeba histolytica* and other *Entamoeba*. Trans R Soc Trop Med Hyg 72:431–432

de la Vega H, Specht CA, Semio CE, Robbins PW, Eichinger D, Caplivski D, Ghosh S, Samuelson J (1997) Cloning and expression of chitinases of *Entamoebae*. Mol Biochem Parasitol 85:139–147

Dover G (1982) Molecular drive: a cohesive mode of species evolution. Nature 299:111–117

Dover GA (1993) Evolution of genetic redundancy for advanced players. Curr Opin Genet Dev 3:902–910

Dover GA, Tautz D (1986) Conservation and divergence in multigene families: alternatives to selection and drift. Philos Trans R Soc Lond B Biol Sci 312:275–289

Ghosh S, Frisardi M, Ramirez-Avila DS, Sturm-Ramirez K, Newton Sanchez OA, Sntos-Preciado JI, Ganguly C, Lohia A, Reed S, Samuelson J (2000) Molecular epidemiology of *Entamoeba* spp.: evidence of a bottleneck (Demographic Sweep) and transcontinental spread of diploid parasites. J Clin Microbiol 38:3815–3821

Haghighi A, Kobayashi S, Takeuchi T, Masuda G, Nozaki T (2002) Remarkable genetic polymorphism among *Entamoeba histolytica* isolates from a limited geographic area. J Clin Microbiol 40:4081–4090

Haghighi A, Kobayashi S, Takeuchi T, Thammapalerd N, Nozaki T (2003) Geographic diversity among genotypes of *Entamoeba histolytica* field isolates. J Clin Microbiol 41:3748–3756

Jinks-Robertson S, Petes TD (1993) Experimental determination of rates of concerted evolution. Methods Enzymol 224:631–646

Kohler S, Tannich E (1993) A family of transcripts (K2) of *Entamoeba histolytica* contains polymorphic repetitive regions with highly conserved elements. Mol Biochem Parasitol 59:49–58

Li E, Kunz-Jenkins C, Stanley SL Jr (1992) Isolation and characterization of genomic clones encoding a serine-rich *Entamoeba histolytica* protein. Mol Biochem Parasitol 50:355–358

Liao D (1999) Concerted evolution: molecular mechanism and biological implications. Am J Hum Genet 64:24–30

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425

Silberman JD, Clark CG, Diamond LS, Sogin ML (1999) Phylogeny of the genera *Entamoeba* and *Endolimax* as deduced from small-subunit ribosomal RNA sequence. Mol Biol Evol 16:1740–1751

Som I, Azam A, Bhattacharya A, Bhattacharya S (2000) Inter- and intra-strain variation in the 5.8S ribosomal RNA and internal transcribed spacer sequences of *Entamoeba histolytica* and comparison with *Entamoeba dispar*, *Entamoeba moshkovskii* and *Entamoeba invadens*. Int J Parasitol 30:723–728

Stanley SL Jr, Becker A, Kunz-Jenkins C, Foster L, Li E (1990) Cloning and expression of a membrane antigen of *Entamoeba histolytica* possessing multiple tandem repeats. Proc Natl Acad Sci U S A 87:4976–4980

Stanley SL Jr, Tian K, Koester JP, Li E (1995) The serine-rich *Entamoeba histolytica* protein is a phosphorylated membrane protein containing O-linked terminal N-acetylglucosamine residues. J Biol Chem 270:4121–4126

Suzuki J, Kobayashi S, Murata R, Yanagawa Y, Takeuchi T (2007) Profiles of a pathogenic *Entamoeba histolytica*-like variant with variations in the nucleotide sequence of the small subunit ribosomal RNA isolated from a primate (De Brazza's guenon). J Zoo Wildl Med 38:471–474

Tachibana H, Kobayashi S, Kato Y, Nagakura K, Kaneda Y, Takeuchi T (1990) Identification of a pathogenic isolate-specific 30, 000-Mr antigen of Entamoeba histolytica by using a monoclonal antibody. Infect Immun 58:955–960

Tachibana H, Yanagi T, Pandey K, Cheng XJ, Kobayashi S, Sherchand JB, Kanbara H (2007) An *Entamoeba* sp. strain isolated from rhesus monkey is virulent but genetically different from *Entamoeba histolytica*. Mol Biochem Parasitol 153:107–114

Takano J, Narita T, Tachibana H, Shimizu T, Komatsubara H, Terao K, Fujimoto K (2005) *Entamoeba histolytica* and *Entamoeba dispar* infections in cynomolgus monkeys imported into Japan for research. Parasitol Res 97:255–257

Takano J, Narita T, Tachibana H, Terao K, Fujimoto K (2007) Comparison of *Entamoeba histolytica* DNA isolated from a cynomolgus monkey with human isolates. Parasitol Res 101:539–546

Tawari B, Ali IK, Scott C, Quail MA, Berriman M, Hall N, Clark CG (2008) Patterns of evolution in the unique tRNA gene arrays of the genus *Entamoeba*. Mol Biol Evol 25:187–198

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Research 25:4876–4882

Verweij JJ, Vermeer J, Brienen EAT, Blotkamp C, Laeijendecker D, Lieshout LV, Polderman AM (2003) *Entamoeba histolytica* infections in captive primates. Parasitol Res 90:100–103

Wilkinson GS, Chapman AM (1991) Length and sequence variation in evening bat D-loop mtDNA. Genetics 128:607–617

Zaki M, Clark CG (2001) Isolation and characterization of polymorphic DNA from *Entamoeba histolytica*. J Clin Microbiol 39:897–905

Zaki M, Meelu P, Sun W, Clark CG (2002) Simultaneous differentiation and typing of *Entamoeba histolytica* and *Entamoeba dispar*. J Clin Microbiol 40:1271–1276

Int J Hematol (2010) 91:140–145
DOI 10.1007/s12185-009-0467-2

CASE REPORT

Successful unrelated bone marrow transplantation for a human immunodeficiency virus type-1-seropositive acute myelogenous leukemia patient following HAART

Yoko Oka · Haruko Tashiro · Mitsuho Mizutani-Noguchi · Ichiro Koga · Toshihiko Sugao · Ryosuke Shirasaki · Toshiyuki Miura · Nobu Akiyama · Kazuo Kawasugi · Shin Fujimori · Naoki Shirafuji

Received: 23 May 2009 / Revised: 10 November 2009 / Accepted: 2 December 2009 / Published online: 25 December 2009

Abstract The availability of highly active anti-retroviral therapy (HAART) has greatly improved the outcome of human immunodeficiency virus type-1 (HIV-1) infection and disease. We report here on a case of an HIV-1-seropositive patient with acute myelogenous leukemia who underwent a successful allogeneic unrelated bone marrow transplantation following HAART. A 40-year-old Japanese HIV-seropositive man underwent allogeneic unrelated bone marrow transplantation using a myeloablative pre-transplant-conditioning regimen. Neutrophil engraftment occurred on day +18, and donor chimerism was achieved on day +27. During pre- and post- transplantation, the HAART was not interrupted. Over 1 year after transplantation, the patient is alive and in continuous complete remission with undetectable levels of HIV-1 RNA. HAART can lead to a successful hematopoietic stem cell transplantation without severe opportunistic infections.

Keywords HIV-1 · AML · HAART · Unrelated bone marrow transplantation

Y. Oka · I. Koga · S. Fujimori
Department of Internal Medicine, Teikyo University School of Medicine, 2-11-1 Kaga, Itabashi-ku, Tokyo 173-8606, Japan

Y. Oka · H. Tashiro · M. Mizutani-Noguchi · T. Sugao · R. Shirasaki · N. Akiyama · K. Kawasugi · N. Shirafuji (✉)
Department of Hematology/Oncology, Teikyo University School of Medicine, 2-11-1 Kaga, Itabashi-ku, Tokyo 173-8606, Japan
e-mail: ramuji@med.teikyo-u.ac.jp

T. Miura
Department of Infectious Disease Control, International Research Center for Infectious Disease, The Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8606, Japan

1 Introduction

Highly active anti-retroviral therapy (HAART) has greatly improved the outcome of human immunodeficiency virus type-1 (HIV-1) infection and disease [1]. Before the introduction of HAART, a high rate of unsuccessful autologous and allogeneic hematopoietic stem cell transplantation (HSCT) for HIV-1-seropositive patients was reported [2–5]. Thus, HIV-1-seropositive patients had been generally excluded from HSCT, even if they had hematological malignancies that would have been treated with HSCT in HIV-1-negative patients. However, successful autologous HSCT for hematological malignancies following HAART with a reduction in HIV-1 load and control of the CD4+ cell count has been reported, especially for HIV-1-associated lymphoma [6, 7]. On the other hand, allogeneic HSCT is an established therapy for some kinds of hematological disorders with curative potential; however, only a few cases of allogeneic HSCT for HIV-1-seropositive patients have been described since HAART became available [8–12].

HIV-1-seropositive patients have an increased risk of developing various kinds of malignancies, including non-Hodgkin's lymphoma and Kaposi's sarcoma [13, 14]. There are a few reports on the relationship between acute myeloid leukemia (AML) and HIV-1 infection. Sutton et al. [15] reported that the estimated risk of AML among HIV-1-infected adults was twice as that in the general population. We report here on an AML patient infected with HIV-1, who underwent unrelated bone marrow transplantation and is alive and well over one year after the transplant. To our knowledge, this is the first report on unrelated bone marrow transplantation for an AML patient with HIV-1.

2 Case report

In December 2006, a 39-year-old Japanese man was diagnosed with AML (FAB classification: M4). The white blood cell count of the patient was 131,000/μl. Bone marrow examination revealed that myelomonoblasts were expanded to 94%, which expressed CD13, CD33, CD34, and HLA-DR. Chromosomal analysis showed a normal karyotype. Screening for HIV-1 antibody by enzyme-linked immunosorbent assay (ELISA) was not performed at diagnosis. The patient underwent induction and consolidation chemotherapy in accordance with the JALSG AML 97 protocol [16] and achieved complete remission (CR). Because FLT3 internal tandem duplication of the bone marrow sample at the onset was positive, and it had continued to be detected, we planned an allogeneic bone marrow transplantation (BMT) during the first CR [17]. The patient had no HLA-identical sibling donor, so was registered for the Japan marrow donor program (JMDP) and underwent a screening test for HSCT. In October 2007, we found out that the patient was seropositive for HIV-1 by ELISA using his blood after obtaining informed consent. He described that he was a gay. We confirmed the presence of HIV-1 antibody by Western blot analysis, which detected bands against HIV-1 gp160, gp110/120, p68, p55, p52, gp 41, p34, and p24/25. At that time, the patient's HIV-1 load and CD4+ cell count were 2.9×10^4 copies/ml and 374/μl, respectively. The patient did not have any opportunistic infections. We started HAART consisting of emtricitabine (FTC) 200 mg, tenofovir (TDF) 300 mg, and efavirenz (EFV) 600 mg, which was determined with antiviral effects. The HIV-1 RNA load rapidly decreased to 61 copies/ml by December 2007. Retrospective analysis of the bone marrow sample showed that the patient had been infected with HIV-1 since he was diagnosed with AML (Fig. 1) [18, 19]; however, during standard chemotherapies before transplantation no severe infections were complicated.

In January 2008, an allogeneic BMT was performed during the first CR, using an unrelated HLA-matched male donor (Fig. 2). The number of transplanted nucleated cells was 3.1×10^8/kg. The patient was conditioned with a myeloablative regimen consisting of 12 Gy total body irradiation, 30 mg/kg intravenous etoposide, and 120 mg/kg intravenous cyclophosphamide. As prophylaxis for acute graft-versus-host disease (GVHD), administration of 3 mg/kg of cyclosporine A (CSP) was started on day −1, and short-term methotrexate was given on days +1, +3, +6, and +11. On HAART it was concerned that the patient could not take medication due to the pretransplant regimen-related toxicity. EFV shows a slow turnover (half time over 50 h); thus, in such a case only EFV was remaining in blood, which might lead to an induction of drug-refractory

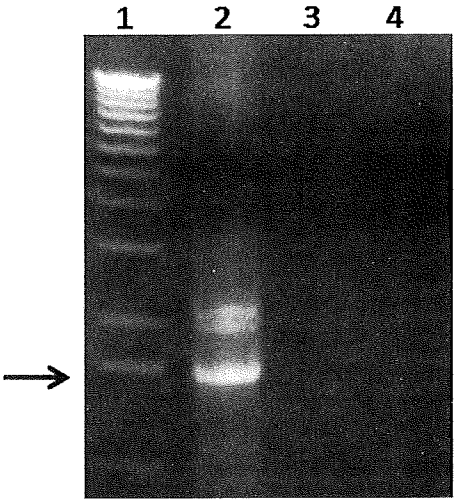

Fig. 1 Genomic PCR analysis. Total genomic DNA was isolated using Qiagen DNA blood mini kit (Qiagen Inc., Valencia, CA), and was subjected to PCR amplification. HIV-1 proviral gene sequence was amplified using nested PCR to generate *gag* products [18, 19]. Hot start was employed for the first and second PCR by incubating the reaction mixtures at 95°C for 2 min. Then, 35 cycles of amplification (94°C for 30 s, 60°C for 30 s, 72°C for 30 s) followed by a final incubation at 72°C for 2 min was applied for the first PCR. The second PCR involved the same conditions except for the amplification temperature (62°C). The primer pairs were: outer forward primer (5′-AAATCTCTA GCAGTGGCGCCCGAACAG-3′; positions 623–649 on HXB2); and reverse (5′-TAACCCTGCGGGATGTGGTATTCC-3′; positions 2849–2826); and inner forward primer (5′-GCGGCG ACTGGTGAGTA CGCC-3′, positions 734–754 on HXB2); and reverse (5′-TCCTTTAG TTGCCCCCCTATC-3′, positions 2314–2294 on HXB2). *Lane 1* MW marker, *Lane 2* bone marrow samples taken at diagnosis of the patient, *Lane 3* bone marrow samples at first CR and soon after the start of HAART (Dec. 2007), and *Lane 4* negative control. PCR products were analyzed by 2.5% TBE gel electrophoresis

HIV-1. We exchanged EFV for lopinavir (LPV) and ritonavir (RPV) referred to a half time.

Polymyxin-B sulfate was administered orally as prophylaxis for bacterial infection when the pretransplant conditioning started. 200 mg Fluconazole was given as prophylaxis against fungal infection. Acyclovir was used against herpes simplex virus infection until day +35. For the prophylaxis of *Pneumocystis Jiroveci*, trimethoprim-sulfamethoxazole was used. Cytomegalovirus (CMV) prophylaxis was started with 5000 mg of globulin every week from day −1. Ganciclovir as prophylaxis was not administered. Surveillance for CMV was performed by taking blood samples every week. The patient was received 5 μg/kg of granulocyte colony-stimulating factor (G-CSF) by continuous drip injection from day +1.

Febrile neutropenia developed requiring antibiotic therapy on day +6. Neutrophil, reticulocyte, and platelet engraftment was achieved on days +18, +18, and +22, respectively. Donor chimerism was achieved on day +27 in

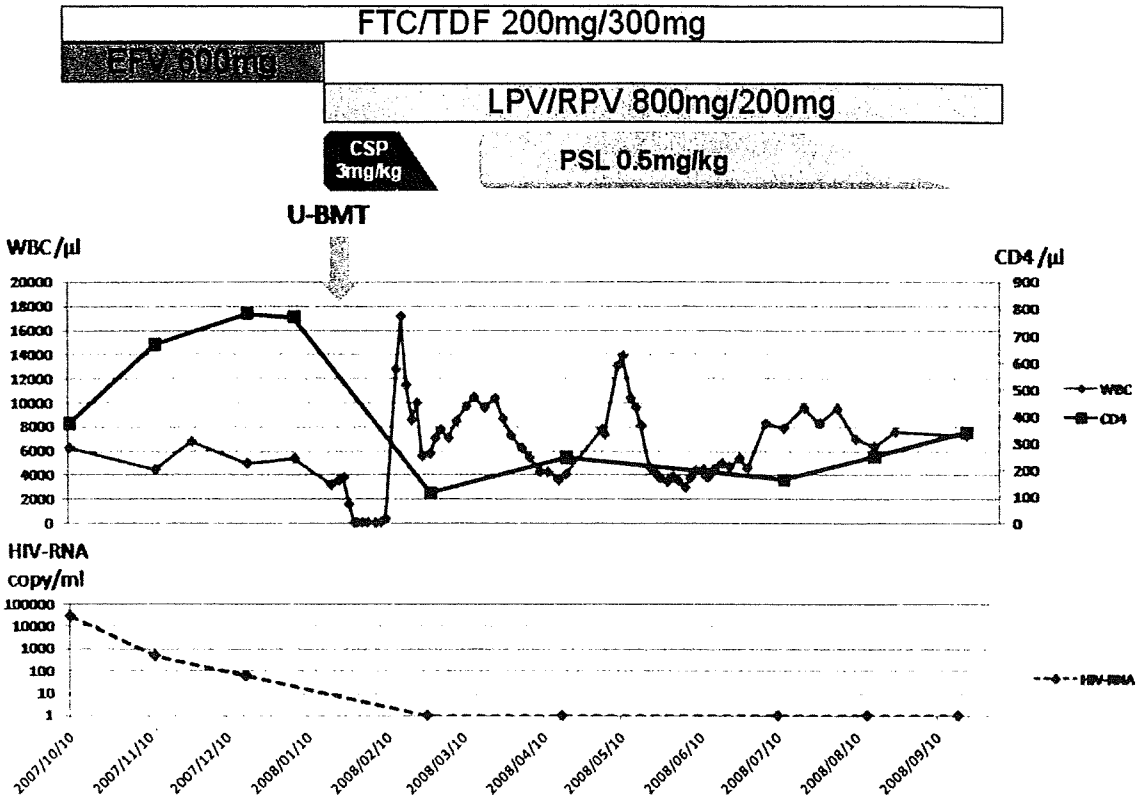

Fig. 2 Counts of HIV-1 viral load, CD4+ T-cells, and total white blood cells observed in the patient pre- and post-transplantation. *FTC* emtricitabine, *TDF* tenofovir, *EFV* efavirenz, *LPV* lopinavir, *RPV* ritonavir, *CSP* cyclosporine A, *PSL* predonisolone, *U-BMT* unrelated bone marrow transplantation

whole blood. Mild impairment of renal function developed (serum creatinine (Cr) level of 0.73 mg/dl on day 10, and 0.93 mg/dl on day 18). Owing to the rapid elevation of the serum level of CSP (971 ng/ml on day 10), which was probably due to interaction between CSP, LPV, RPV, and TDF, the administered dose of CSP was reduced. However, the patient's renal function worsened (Cr 1.14 mg/dl on day 20), and administration of CSP was stopped on day +21. Serum Cr level was 1.66 mg/dl on day 31, and the level was decreased thereafter. On day +53, CMV viremia developed without any evidence of CMV disease. We started intravenous administration of 10 mg/kg of ganciclovir, which promptly resolved the CMV viremia. On day +37, the patient developed acute skin GVHD (stage 2) and high fever without evidence of infection, and 0.5 mg/kg of predonisolone (PSL) was administered. After PSL treatment, the patient gradually recovered and was discharged on day +84.

Despite severe nausea, the patient could take all anti-HIV-1 drugs orally during pre- and post-transplantation. HIV-1 has not been detectable from day +31 until day +650. Although CD4+ cell count decreased to 114/μl on day +31, CD4+ cell count remained above 200/μl from day +202. Immunoglobulin level was sustained at the lower level (600–700 mg/dl after 6 months to 21 months after the transplantation). After 6 months from the transplantation, chronic extensive GVHD was observed, and PSL (1 mg/kg) with CSP was continued to be administered; however, no severe opportunistic infections were observed. At 21 months post-transplantation, the patient is alive and in continuous CR with undetectable levels of plasma HIV-1 RNA.

3 Discussion

In the HAART era, high-dose chemotherapy and autologous HSCT for HIV-1-seropositive patients can be successful [5–7]. Krishnan et al. [20] reviewed autologous transplantation for HIV-1-positive patients with lymphoma, and concluded that high-dose therapy with autologous stem cell rescue is feasible in HIV-1-infected patients with lymphoma following HAART. During chemotherapy, in combination with HAART, prompt reduction of HIV-1 load was reported [21, 22]. In our case, when HAART was introduced, prompt disappearance of HIV-1 was observed at PCR level (lane 3 of Fig. 1).

There are a few reports about allogeneic HSCT for HIV-1 carrier patients [8–12], and a brief summary is shown in Table 1. Recently, the Center for International Blood and Marrow Transplant Research (CIBMTR) reported allogeneic hematopoietic cell transplantation in human immunodeficiency virus-positive patients with hematological disorders [23]. Some problems are unresolved in allogeneic HSCT for HIV-1-seropositive patients treated with HAART, such as engraftment, GVHD prophylaxis and drug interaction, and opportunistic infection, in which the most important point is to check HIV-1 when a new patient suffering from a hematological malignancy is administered. In our case we fortunately found out his HIV-1 infection. CIBMTR reports that 31% of the HIV-1-infected patients with hematological malignancy who underwent HSCT were diagnosed before or at the onset of hematological malignancy. After this case we check HIV-1 antibody in all the new patients with hematological malignancies.

Among HIV-1-negative allogeneic BMT patients at our institute, the median time of myeloid, erythroid, and platelet engraftment is day +17.5, +21, and +23, respectively (unpublished). In this HIV-1-seropositive case, myeloid, erythroid, and platelet engraftments were similar to those of HIV-1-negative allogeneic BMT recipients. Tomonari et al. described a successful second unrelated cord blood transplantation for an HIV-1-seropositive patient using HAART, although the first cord blood transplantation showed prolonged bone marrow suppression and probable engraftment failure [10], in which HAART might have influenced the engraftment. Other reports describe similar engraftment periods for HIV-1-positive and negative patients who received allogeneic HSCT [8–12, 23, 24].

Previously, before the HAART era, some reports describing life-threatening opportunistic infections that often occur upon HSCT were published [2, 3]. Nowadays, HAART can lead to successful HSCT without severe opportunistic infections, control of the CD4+ cell count, and a decreased HIV-1 load. In our patient, cytomegalovirus reactivation was observed, which was promptly resolved by the administration of ganciclovir without CMV disease; other opportunistic infections did not occur. Sutton et al. [15] reported that a CD4+ cell count above 200/μl at the onset of AML was predictive of longer survival. Also, there are reported that before HAART era CD4 recovered very slowly after chemotherapy, and autologous and allogeneic transplantation [2–5, 23]. In HSCT, it seems likely that CD4+ cell count is as an important predictor of survival.

The optimum conditions for HAART before and during transplantation have not been established. Referred to the previous treatment-guidelines, no indication of HAART was determined to our case if he was not suffering from AML, because CD4+ cell count was above 350/μl; however, for the treatment of AML we used chemotherapy,

Table 1 Reported allogeneic HSCT cases for HIV-1-seropositive patients following HAART

	Diagnosis	Disease status	HAART	Transplant graft	Pretransplant conditioning	Engraftment	Outcome
Kang et al. [8]	t-AML	CR	I, S, L	Sibling PB	CY/Flu		Alive in CR: 2 years
Kang et al. [8]	HD	Primary refractory	Nf, ABC, L	Sibling PB	CY/Flu		Death: day +180 due to relapse
Sora et al. [9]	AML	CR	S, L, I→L, AZT, R	Sibling PB	BU/CY	Day +9	Alive in CR: 39 months
Tomonari et al. [10]	Ph ALL	CR	S, L, E	Unrelated CB	TBI/CY	Engraftment failure	To second CBT
Tomonari et al. [10]		Engraftment failure	(–)	Unrelated CB	Flu	Day +27	Alive in CR: 15 months
Wolf et al. [11]	AA		ABC, L, SQV, R → paused	Unrelated PB	CY/Flu	Day +18	Alive: 8 months
Woolfrey et al. [12]	AML	N/A	E, ABC, T	Unrelated PB	TBI/Flu		Death: day +301 due to GVHD/BO
Woolfrey et al. [12]	AML	N/A	E, ABC, L	Sibling PB	TBI/Flu		Alive: day +180
Our case	AML	CR	FTC/TDF, LPV/R	Unrelated BM	TBI/ETP/CY	Day +18	Alive in CR: 12 months

I inducates indinavir, *S* stavudine, *L* lamivudine, *Nf* nelfinavir, *ABC* abacavir, *AZT* zidovudine, *R* ritonavir, *E* efavirenz, *SQV* saquinavir, *T* tenofovir, *ALL* acute lymphocytic leukemia, *Ph* Philadelphia, *AA* aplastic anemia, *AML* acute myelogenous leukemia, *t-AML* treatment-related AML, *HD* Hodgkin's lymphoma, *CR* complete remission, *N/A* not described, *CB* umbilical cord blood, *PB* peripheral blood stem cell, *TBI* total body irradiation, *CY* cyclophosphamide, *Flu* fludarabine, *ETP* etoposide, *BU* busulfan, *GVHD* graft-versus-host disease, *BO* bronchiolitis obliterance

which induced CD4+ cells to decrease below 50/μl. To prevent opportunistic infections we started HAART when his HIV-1 infection was documented.

On GVHD prophylaxis, the interaction between HAART and CSP or tacrolimus makes control of the serum concentration of CSP or tacrolimus difficult. Owing to the interruption of HAART due to regimen-related toxicity, it should be adjusted cautiously by monitoring the concentration of CSP or tacrolimus; however, in the present case, we could not prevent renal dysfunction in the patient which was possibly induced by the administration of TDF and CSP. The patient's acute GVHD was controlled by 0.5 mg/kg PSL without CSP.

During transplantation, our patient was able to undergo HAART fortunately; however, it is reported that most HIV-1-positive patients who undergo allogeneic transplantation discontinue HAART due to nausea and vomiting. Some cases in which HAART was interrupted during transplantation resulted in an increase in HIV-1 load after transplantation and the development of acute HIV infection [8, 11]. These observations reveal the importance of continuous HAART following transplantation.

Allogeneic unrelated BMT may be feasible for HIV-1-seropositive patients with hematological malignancies, even in the absence of related donors. Recently Hütter et al. [25] reported that after allogeneic stem cell transplantation, HIV-1 was cleared in the long term. In their study, a polymorphism in the chemokine receptor 5 gene between the recipient and the donor plays an important role in the clearance of HIV-1. Thus, post-engrafted anti-HIV-1 effect may be possible on allogeneic transplantation for HIV-1-positive hematological malignancy. Further investigation on the selection of antiretroviral drugs and their interaction with other drugs is required, which will lead to achieving more feasible transplantation.

References

1. Palella FJ Jr, Delaney KM, Moorman AC, et al. Declining morbidity and mortality among patients with advanced human immunodeficiency virus infection. HIV Outpatient Study Investigators. N Engl J Med. 1998;338:853–60.
2. Angelucci E, Lucarelli G, Baronciani D, et al. Bone marrow transplantation in an HIV positive thalassemic child following therapy with azidothymidine. Haematologica. 1990;75:285–7.
3. Giri N, Vowels MR, Ziegler JB. Failure of allogeneic bone marrow transplantation to benefit HIV infection. J Paediatr Child Health. 1992;28:331–3.
4. Schneider E, Lambermont M, Voorenv J-P V, et al. Autologous stem cell infusion for acute myeloblastic leukemia in an HIV-1 carrier. Bone Marrow Transplant. 1997;20:611–2.
5. Gabarre J, Leblond V, Sutton L, et al. Autologous bone marrow transplantation in relapsed HIV-1 related non-Hodgkin's lymphoma. Bone Marrow Transplant. 1996;18:1195–7.
6. Re A, Cattaneo C, Michieli M, et al. High-dose therapy and autologous peripheral-blood stem-cell transplantation as salvage treatment for HIV-associated lymphoma in patients receiving highly active antiretroviral therapy. J Clin Oncol. 2003;21:4423–7.
7. Gabriel I, Apperley J, Bower M, Chaidos A, Gazzard B, Giles C, et al. A long-term durable remission with high-dose therapy autologous stem cell transplant for stage IVB HIV-associated Hodgkins disease. AIDS. 2008;22:539–44.
8. Kang EM, Witte Md, Malech H, Morgan RA, Phang S, Carter C, et al. Nonmyeloablative conditioning followed by transplantation of genetically modified HLA-matched peripheral blood progenitor cells for hematologic malignancies in patients with acquired immunodeficiency syndrome. Blood. 2002;99:698–701.
9. Sora F, Antinori A, Picirillo N, De Luca A, Chiusolo P, Cingolani A, et al. Highly active antiretoroviral therapy and allogeneic CD34+ peripheral blood progenitor cells transplantation in an HIV/HCV coinfected patient with acute myeloid leukemia. Exp Hematol. 2002;30:279–84.
10. Tomonari A, Takahashi S, Shimohakamada Y, Ooi J, Takasugi K, Ohno N, et al. Unrelated cord blood transplantation for a human immunodeficiency virus-1-seropositive patient with acute lymphoblastic leukemia. Bone Marrow Transplant. 2005;36:261–2.
11. Wolf T, Rickerts V, Staszewski S, Kriener S, Wassmann B, Bug G, et al. First case of successful allogeneic stem cell transplantation in an HIV-patient who acquired sever aplastic anemia. Haematologica. 2007;92:e56–8.
12. Woolfrey AE, Malhotra U, Harrington RD, McNevin J, Manley TJ, Riddell SR, et al. Generation of HIV-1-specific CD8+ cell responses following allogeneic hematopoietic cell transplantation. Blood. 2008;112:3484–7.
13. Monfardini S, Vaccher E, Pizzocaro G, et al. Unusual malignant tumors with HIV infection. AIDS. 1989;3:449–52.
14. Biggar RJ, Curtis RE, Cote TR, Rabkin CS, Melbye M. Risk of other cancers following Kaposi's sarcoma: relation to acquired immunodeficiency syndrome. Am J Epidemiol. 1994;139:362–8.
15. Sutton L, Guenel P, Tanguy M-L, Rio B, Dhedin N, Casassus P, et al. Acute myeloid leukaemia in human immunodeficiency virus-infected adults: epidemiology, treatment feasibility and outcome. Br J Hematol. 2001;112:900–8.
16. Miyawaki S, Sakamaki H, Ohtake S, et al. A randomized, postremission comparison of four courses of standard-dose consolidation therapy without maintenance therapy versus three courses of standard-dose consolidation with maintenance therapy in adults with acute myeloid leukemia: the Japan Adult Leukemia Study Group AML 97 Study. Cancer. 2005;104:2726–34.
17. Thiede C, Steudel C, Mohr B, Schaich M, Schakel U, Wermke MW, et al. Analysis of FLT3-activating mutations in 979 patients with acute myelogenous leukemia: association with FAB subtype and identification of subgroup with poor prognosis. Blood. 2002;99:4326–35.
18. Koga I, Odawara T, Matsuda M, Sugiura W, Goto M, Nakamura T, et al. Analysis of HIV-1 sequences before and after co-infecting syphilis. Microbes Infect. 2006;8:2872–9.
19. Miura T, Brockman MA, Brumme CJ, Brumme ZL, Carlson JM, Pereyra F, et al. Genetic characterization of human immunodeficiency virus type 1 in elite controllers: lack of gross genetic defects or common amino acid changes. J Virol. 2008;82:8422–30.
20. Krishnan A, Zaia J, Forman SJ. Should HIV-positive patients with lymphoma be offered stem cell transplants? Bone Marrow Transplant. 2003;32:741–8.
21. Antinori A, Cingolani A, Alba L, Ammassari A, Serraino D, Ciancio BC, et al. Better response to chemotherapy and prolonged survival in AIDS-related lymphomas responding to highly active antiretroviral therapy. AIDS. 2001;15:1483–91.

22. Ratner L, Lee J, Tang S, Redden D, Hamzeh F, Herndier B, et al. Chemotherapy for human immunodeficiency virus-associated non-Hodgkin's lymphoma in combination with highly active antiretroviral therapy. J Clin Oncol. 2001;19:2171–8.
23. Gupta V, Tomblyon M, Pedersen TL, Atkins HL, Battiwalla M, Gress RE, et al. Allogeneic hematopoietic cell transplantation in human immunodeficiency virus-positive patients with hematologic disorders: a report from the center for international blood and marrow transplant research. Biol Blood Marrow Transpl. 2009;15:864–71.
24. Schlegel P, Beaty P, Halvorsen P, McCune J. Successful allogeneic bone marrow transplant in an HIV-1-positive man with chronic myelogeneous leukemia. J AIDS. 2000;24:289–90.
25. Hütter G, Nowak D, Mossner M, Ganepola S, Müβig A, Allers K, et al. Long-term control of HIV by CCR5 delta32/delta32 stem cell transplantation. N Engl J Med. 2009;360:692–8.

MAJOR ARTICLE

Persistent Low-Level Viremia in HIV-1 Elite Controllers and Relationship to Immunologic Parameters

Florencia Pereyra,[1,2] Sarah Palmer,[3,7] Toshiyuki Miura,[1,6] Brian L. Block,[1] Ann Wiegand,[3] Alissa C. Rothchild,[1] Brett Baker,[1] Rachel Rosenberg,[1] Emily Cutrell,[1] Michael S. Seaman,[4] John M. Coffin,[5] and Bruce D. Walker[1,6]

[1]The Ragon Institute of Massachusetts General Hospital, Massachusetts Institute of Technology, and Harvard, [2]Brigham and Women's Hospital, Division of Infectious Diseases, [4]Division of Viral Pathogenesis, Beth Israel Deaconess Medical Center, and [5]Department of Molecular Biology and Microbiology, Tufts University, Boston Massachusetts; [3]HIV Drug Resistance Program, National Cancer Institute, National Institutes of Health, Frederick, and [6]Howard Hughes Medical Institute, Chevy Chase, Maryland; and [7]Virology Department, Swedish Institute for Infectious Disease Control and Karolinska Institute, Solna, Sweden

Background. Human immunodeficiency virus type 1 (HIV-1) elite controllers are able to control virus replication to levels below the limits of detection by commercial assays, but the actual level of viremia in these individuals is not well defined. Here, we quantify plasma HIV-1 RNA in elite controllers and correlate this with specific immunologic parameters.

Methods. Plasma HIV-1 RNA levels were quantified in 90 elite controllers with use of a real time reverse-transcriptase polymerase chain reaction assay with a sensitivity of 0.2 copies/mL. HIV-1–specific immune responses and longitudinal $CD4^+$ T cell counts were examined.

Results. The median plasma HIV-1 RNA level was 2 copies/mL (interquartile range, 0.2–14 copies/mL). A longitudinal analysis of 31 elite controllers demonstrated 2–5–fold fluctuations in viremia in the majority of individuals; 6 had persistent levels below 1 copy/mL. Viremia correlated directly with HIV-1–specific neutralizing antibodies and Western blot reactivity but not with $CD8^+$ T cell responses. Absolute $CD4^+$ T cell decrease was more common among individuals with detectable viremia ($P = .04$).

Conclusions. Low-level viremia is present in the majority of elite controllers and is associated with higher HIV-1–specific antibody responses. Absolute $CD4^+$ T cell loss is more common among viremic individuals, suggesting that even very low-level viremia has negative consequences over time.

In light of the recent failure of a prophylactic human immunodeficiency virus type 1 (HIV-1) vaccine trial [1], the rationale for current T cell–based HIV-1 vaccine approaches has been questioned. Understanding the relationships between low-level viremia, adaptive immune responses, and disease progression is of importance, particularly in situations of natural HIV-1 control that are relevant to vaccine approaches based on protection from disease progression. Such situations of control of HIV-1 are seen in elite controllers, individuals who spontaneously control virus replication without antiretroviral drugs, to levels below the limits of detection by commercial assays [2]. Although some recent studies have indicated low-level viremia in these persons, the relationship to immunologic parameters, including $CD4^+$ T cell decrease remains to be determined [3–5].

We previously reported that low-level viremia can be detected by ultrasensitive quantitative polymerase chain reaction (PCR) in elite controllers and is associated with the expression of immunoregulatory receptors that affect $CD4^+$ T cell function [6]. In that study, the relationship between low-level viremia and other immunologic parameters was not examined. Low-level viremia and its relationship to HIV-1 antibody lev-

Received 29 January 2009; accepted 21 April 2009; electronically published 4 August 2009.

Potential conflicts of interest: none reported

Presented in part: 15th Conference on Retroviruses and Opportunistic Infections, Boston, Massachusetts, February 2008 (abstract 349).

Financial support: National Institutes of Health (AI28568 and AI30914 to B.D.W.), the Bill and Melinda Gates Foundation, the International AIDS Vaccine Initiative, and the Mark and Lisa Schwartz Foundation.

Reprints or correspondence: Florencia Pereyra, MD, Ragon Institute, Massachusetts General Hospital, 149 13th St, Charlestown, MA 02129 (fpereyra@partners.org).

The Journal of Infectious Diseases 2009;200:984–90

0022-1899/2009/20006-0019$15.00

DOI: 10.1086/605446

els have recently been described in elite controllers, but semiquantitative methods were used to measure both HIV-1 RNA and HIV-1 antibody levels, so the number of copies per mL of plasma was not defined [4]. Furthermore, the relationship between residual low-level viremia and Western blot reactivity, HIV-1–specific neutralizing antibodies, and $CD8^+$ T cell responses remains unknown.

Here, we used an assay described elsewhere [7] for the detection of HIV-1 RNA with single-copy sensitivity to quantitatively characterize the level of viral RNA in plasma of elite controllers. We examined the relationship between virus load, absolute $CD4^+$ T cell counts, and HIV-1–specific immune responses. We find that the majority of elite controllers maintain detectable levels of plasma viremia, with a median virus load of 2 copies/mL. Moreover, we show that this extremely low level of viremia correlates positively with HIV-1–specific neutralizing antibodies and full Western blot reactivity. We also show that $CD4^+$ T cell decrease over time is more common among individuals with low-level viremia.

METHODS

Study subjects. HIV-1 elite controllers were randomly selected from a local cohort [8]. These subjects maintained plasma HIV-1 RNA levels below the limit of detection by commercial assays (<50 copies, by ultrasensitive PCR) without antiretroviral therapy. Definition of elite control was as described elsewhere [8]. The Massachusetts General Hospital Institutional Review Board approved all studies, and written informed consent was obtained from all participants prior to enrollment. Plasma and peripheral blood mononuclear cells (PBMC) were obtained as described elsewhere [8].

HIV-1 RNA determination. A single-copy assay for HIV-1 RNA detection was performed as described elsewhere [7], starting with 9 mL of plasma, which afforded a lower limit of detection of 0.2 RNA copies/mL. PCR amplification and sequencing of the HIV-1 *gag* region was performed for each subject from either plasma or PBMC samples, as described elsewhere [9], to rule out inefficient amplification by single-copy assay attributable to possible polymorphisms in the probe and/or primer sequences. Further details of optimum amplification conditions and performance characteristics, as well as quality control procedures to prevent artifactual amplification, are described elsewhere [7].

Assessment of HIV-1–specific $CD8^+$ T cell responses. In 53 individuals with available fresh PBMCs, interferon (IFN)-γ enzyme-linked immunospot (ELISPOT) assays were performed as described elsewhere [10, 11]; the assays included 410 peptides 16–19 amino acids in length, overlapping by 10 amino acids, spanning the entire HIV-1 proteome. The same time point was used for ELISPOT and HIV-1 RNA detection. Positive responses were confirmed in a second assay after cell incubation for 24 h. Responses were considered to be positive if experimental wells had at least 3 times the mean number of spot forming cells (SFCs) in the 6 negative control wells, there were at least 5 spots present in the experimental well, and the number of SFCs per 1×10^6 PBMCs was >55. Responses to peptides were previously shown to be largely mediated by $CD8^+$ T cells [11, 12].

Assessment of HIV-1–specific antibody responses. Western blot was performed according to the manufacturer's instructions (GS HIV 1 Western Blot; Bio-Rad) for qualitative detection of HIV-1 antibodies to specific viral proteins (gp160, gp120, p65, p55, p51, gp41, p40, p31, p24, and p18). Neutralizing antibody responses against HIV-1 Env-pseudoviruses were measured as described elsewhere [4, 10]. Briefly, 3-fold serial dilutions of plasma samples were performed in a 96-well flat bottom plate, 200 median tissue culture infective doses (TCID50) of the virus was added to each well, and the plates were incubated for 1 h at 37°C. TZM.bl cells were then added. Following a 48-h incubation at 37°C, 100 μL of Bright-Glo luciferase reagent (Promega) was added. The cells were allowed to lyse for 2 min, then the lysate was transferred to a 96-well black solid plate, and luminescence was measured using a Victor 3 luminometer (Perkin Elmer). The 80% inhibitory dose (ID80) titer was calculated as the serum dilution that caused an 80% reduction in relative luminescence units, compared with the virus control wells, after subtraction of cell control relative luminescence units.

Statistical analysis. Statistical analyses and graphical presentations were performed using GraphPad Prism, version 5.0 (GraphPad Software). A nonparametric Spearman test was used to calculate correlations. Significance when comparing 2 groups was determined with a 2-tailed nonparametric Mann Whitney *U* test. Changes in absolute $CD4^+$ T cell count over time were calculated using a linear regression model to calculate the value of the slope.

RESULTS

Low-level plasma viremia is detectable in the majority of HIV-1 elite controllers. HIV-1 RNA levels were measured at a single time point in 90 HIV-1 elite controllers. Amplification of *gag* sequences was successfully obtained in 62 individuals. Polymorphisms in the primer and/or probe sequences that could affect amplification were present in 7 (11%) of 62 persons, which is consistent with our experience with this assay in large datasets [13]. All individuals with confirmed primer and/or probe mismatches were excluded, leaving 83 individuals in the analysis. The level of plasma viremia varied over a wide distribution (figure 1*A*), with a median of 2.3 copies/mL (interquartile range, 0.2-14 copies/mL). In the majority of individuals ($n = 79$), the average HIV-1 RNA levels measured by single-copy assay were consistent with results of previous ul-

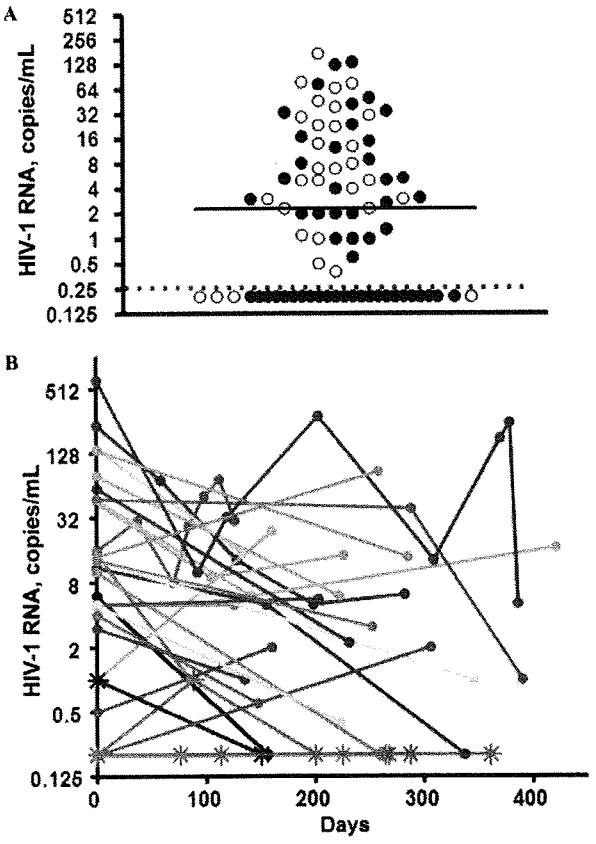

Figure 1. Plasma human immunodeficiency virus type 1 (HIV-1) RNA load distribution among 83 elite controllers *(A)*. The cut off level of 0.2 copies/mL is represented by the dotted red line. The median values are indicated with the black horizontal bar. The arithmetic mean HIV-1 RNA virus load for each subject is shown. Open circles represent patients with longitudinal HIV-1 RNA values; filled circles represent subjects with single time point virus load determinations. *B*, Longitudinal HIV-1 RNA virus load in 31 elite controllers. Each line represents 1 study subject. Subjects with all HIV-1 RNA measurements <1 copy/mL are shown with asterisks ($n = 6$).

trasensitive commercial assays, whereas 4 individuals had apparent "blips" >50 RNA copies/mL at the time the plasma was obtained for the single-copy assay. Thirty (36%) of 83 individuals had HIV-1 RNA levels of ≤1 copies/mL; in 25 (30%) individuals, virus could not be detected with a single measurement (<0.2 copies/mL).

We next used the single-copy assay to measure longitudinal plasma samples from 31 of these individuals, including 11 in whom the initial viral load measurement result was <0.2 copies/mL. Follow-up ranged from 85 to 420 days (mean duration, 232 days) and included 2–8 measurements (mean, 3 measurements) per subject (figure 1*B*). The majority of individuals showed fluctuations in plasma HIV-1 RNA levels, 5 had transient viral "blips" >50 copies/mL, as described elsewhere in elite controllers [8]. Of 11 persons with RNA levels below the limit of detection by the single-copy assay who were studied longitudinally, 6 had levels <1 copy/mL at all time points measured. For 4 of these, the values were all <0.2 copies/mL, whereas 2 subjects had arithmetic mean RNA values of 0.4 and 0.6 copies/mL, respectively. Of the 4 with undetectable RNA levels in this assay (<0.2 copies/mL), we were able to sequence virus from 2 and confirm that there were no primer mismatches. These data show that viremia can be detected in the majority of elite controllers, but there are rare individuals in whom the level of plasma virus is persistently below the ability to measure, even by an assay that is 250-fold more sensitive than current commercial assays.

The breadth and potency of HIV-1 antibody responses inversely correlates with plasma viremia. We previously demonstrated that elite controllers have very low levels of heterologous neutralizing antibodies, compared with individuals with higher virus loads, suggesting that HIV-1 replication drives the production of heterologous neutralizing antibodies [14]. To determine the effect of extremely low-level viremia on HIV-1–specific antibody responses, Western blot was performed in all individuals, and detection of antibodies to gp120, gp160, gp41, p18, p24, p31, p40, p51, p55, and/or p65 was recorded. All elite controllers had detectable responses to multiple proteins (range, 2 to 10 proteins), and the majority of individuals (77%) had antibodies against all proteins tested. The most common antibodies detected were gp120 and gp160, which were detected in all individuals, followed by p24, p40, and gp41, which were present in 98%, 96%, and 93% of individuals, respectively. The least commonly detected antibodies were p18, p31, and p65, which were found in 80%, 84%, and 86% of individuals, respectively. One individual had only 2 antibodies detected (gp120 and gp160) in 2 tests performed over a 76-day period, at a time when the individual's level of plasma viremia was <0.2 copies/mL. Analysis of all individuals together indicated that the number of bands detected correlated directly with plasma HIV-1 RNA levels and that full Western blot reactivity was present in all persons with plasma viral loads of >13 copies/mL ($r = 0.38$; $P \leq .01$) (figure 2*A*).

Marked heterogeneity in neutralizing antibody responses among elite controllers has also been described elsewhere, with some individuals having broad responses and others having minimal or no neutralizing antibodies [8]. To determine whether the level of plasma HIV-1 RNA was associated with differences in neutralizing antibodies, we tested plasma samples from all subjects against a standard reference panel of 12 primary clade B HIV-1 viruses [15]. Despite high-level inhibition of the neutralization-sensitive laboratory strain SF162 (data not shown), elite controllers' plasma exhibited limited cross-neutralization against nearly all of the primary reference viruses. ID80 titers against a MuLV-negative control pseudovirus were below the cut off of <20 in all individuals. The average ID80