Houghton M, Weiner A, Han J, Kuo G, Choo QL. 1991. Molecular biology of the hepatitis C viruses: Implications for diagnosis, development and control of viral disease. Hepatology 14:381–388.

Katayama Y, Barzaga NG, Alipio A, Soetjipto XX, Doi H, Ishido S, Hotta H. 1996. Genotype analysis of hepatitis C virus among blood donors and inmates in Metro Manila, The Philippines. Microbiol Immunol 40:525–529.

Lauer GM, Walker BD. 2001. Hepatitis C virus infection. N Engl J Med 345:41–52.

Leano PS, Kageyama S, Espantaleon A, Maniar J, Iwasaki M, Saple D, Yoshihara N, Kurimura T, Agdamag DM. 2003. Introduction of human immunodeficiency virus type 2 infection in the Philippines. J Clin Microbiol 41:516–518.

Martell M, Esteban JI, Quer J, Genesca J, Weiner A, Esteban R, Guardia J, Gomez J. 1992. Hepatitis C virus (HCV) circulates as a population of different but closely related genomes: Quasispecies nature of HCV genome distribution. J Virol 66:3225–3229.

Okamoto H, Okada S, Sugiyama Y, Kurai K, Iizuka H, Machida A, Miyakawa Y, Mayumi M. 1991. Nucleotide sequence of the genomic RNA of hepatitis C virus isolated from a human carrier: Comparison with Reported isolates for conserved and divergent regions. J Gen Virol 72:2697–2704.

Paladin FJ, Monzon OT, Tsuchie H, Aplasca MR, Learn GH Jr., Kurimura T. 1998. Genetic subtypes of HIV-1 in the Philippines. AIDS 12:291–300.

Perriere G, Gouy M. 1996. WWW-query: An on-line retrieval system for biological sequence banks. Biochimie 78:364–369.

Royce RA, Sena A, Cates W Jr., Cohen MS. 1997. Sexual transmission of HIV. N Engl J Med 336:1072–1078.

Ruxrungtham K, Brown T, Phanuphak P. 2004. HIV/AIDS in Asia. Lancet 364:69–82.

Santiago ML, Santiago EG, Hafalla JC, Manalo MA, Orantia L, Cajimat MN, Martin C, Cuaresma C, Dominguez CE, Borromeo ME, De Groot AS, Flanigan TP, Carpenter CC, Mayer KH, Ramirez BL. 1998. Molecular epidemiology of HIV-1 infection in the Philippines, 1985 to 1997: Transmission of subtypes B and E and potential emergence of subtypes C and F. J Acquir Immune Defic Syndr Hum Retrovirol 18:260–269.

Soriano V, Sulkowski M, Bergin C, Hatzakis A, Cacoub P, Katlama C, Cargnel A, Mauss S, Dieterich D, Moreno S, Ferrari C, Poynard T, Rockstroh J. 2002. Care of patients with chronic hepatitis C and HIV co-infection: Recommendations from the HIV-HCV International Panel. AIDS 16:813–828.

Thompson JD, Higgins DG, Gibson TJ. 1994. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680.

Van Ameijden EJ, Van den Hoek JA, Mientjes GH, Coutinho RA. 1993. A longitudinal study on the incidence and transmission patterns of HIV, HBV and HCV infection among drug users in Amsterdam. Eur J Epidemiol 9:255–262.

van Asten L, Verhaest I, Lamzira S, Hernandez-Aguado I, Zangerle R, Boufassa F, Rezza G, Broers B, Robertson JR, Brettle RP, McMenamin J, Prins M, Cochrane A, Simmonds P, Coutinho RA, Bruisten S. 2004. Spread of hepatitis C virus among European injection drug users infected with HIV: A phylogenetic analysis. J Infect Dis 189:292–302. Epub 2004 Jan 2008.

van den Hoek JA, van Haastrecht HJ, Goudsmit J, de Wolf F, Coutinho RA. 1990. Prevalence, incidence, and risk factors of hepatitis C virus infection among drug users in Amsterdam. J Infect Dis 162:823–826.

White PA, Zhai X, Carter I, Zhao Y, Rawlinson WD. 2000. Simplified hepatitis C virus genotyping by heteroduplex mobility analysis. J Clin Microbiol 38:477–482.

Zuckerman AJ, Zuckerman JN. 1995. Prospects for hepatitis C vaccine. J Hepatol 22:97–100.

Journal of General Virology (2005), 86, 2047–2056 DOI 10.1099/vir.0.80922-0

A new subtype (subgenotype) Ac (A3) of hepatitis B virus and recombination between genotypes A and E in Cameroon

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Blood samples ($n=544$) from two different populations (Pygmies and Bantus) in Cameroon, West Africa, were analysed. Serological tests indicated that the anti-hepatitis C virus (HCV) prevalence in Bantus (20·3 %) was higher than that in Pygmies (2·3 %, $P<0·0001$), whereas the distribution of hepatitis B virus (HBV) serological markers was equally high in both populations: in total, 9·4, 17·3 and 86·8 % for HBsAg, anti-HBs and anti-HBc, respectively. HBV genotype A (HBV/A) and HBV/E were predominant (43·5 % each) in both populations, and HBV/D was found in a minority (13 %). The preS/S region was sequenced in nine cases (five HBV/A and four HBV/E) and the complete genome in six cases (four HBV/A and two HBV/E). Subsequent phylogenetic analysis revealed that the HBV/A strains were distinct from the subtypes (subgenotypes) described previously, Ae (A2) and Aa (A1), and in the preS/S region they clustered with previously reported sequences from Cameroon. Based on the nucleotide difference from Aa (A1) and Ae (A2), more than 4 % in the complete genome, the Cameroonian strains were suggested to represent a new subtype (subgenotype), designated HBV/Ac (A3). A high (3·9 %) nucleotide divergence in HBV/Ac (A3) strains suggested that the subtype (subgenotype) has a long natural history in the population of Cameroon. One of the HBV/Ac (A3) strains was found to be a recombinant with an HBV/E-specific sequence in the polymerase reverse transcriptase domain. Further cohort studies will be required to assess detailed epidemiological, virological and clinical characteristics of HBV/Ac (A3), as well as its recombinant form.

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INTRODUCTION

According to the World Health Organization, hepatitis B virus (HBV) infection is one of the major global public health problems. Of the two billion people who have been infected with HBV worldwide, more than 350 million are at risk of developing cirrhosis and hepatocellular carcinoma due to chronic infection (Kane, 1995).

Based on a genomic sequence divergence in the entire genome exceeding 8 %, HBV strains have been classified into seven genotypes, denoted A (HBV/A) to G (HBV/G) (Norder *et al.*, 1994; Okamoto *et al.*, 1988; Stuyver *et al.*, 2000). A possible eighth genotype has been proposed with the tentative designation 'H' (Arauz-Ruiz *et al.*, 2002), which is, however, closely related to genotype F phylogenetically, with a complete genome difference of around 8 % (Kato *et al.*, 2005).

Research on HBV genotypes during the last decade has

The GenBank/EMBL/DDBJ accession numbers for the nucleotide sequences determined in this study are AB194947–AB194955.

demonstrated significant associations between the HBV genotypes and the severity of liver disease, clinical outcomes and the response to antiviral therapies (Kramvis & Kew, 2005). Moreover, it was also demonstrated that the clinical and virological characteristics may also differ among patients infected with the same genotype (Miyakawa & Mizokami, 2003). The existence of different subtypes (subgenotypes) within same genotype helps to explain this for HBV/B, where one of the subtypes (subgenotypes) (widespread in Asia; Ba) possesses a recombination with genotype HBV/C, while another (indigenous to Japan; Bj) does not (Sugauchi *et al.*, 2003). Similarly, two subtypes (subgenotypes) have been reported for HBV/A: one of them, Aa (A'/A1) prevails in sub-Saharan Africa and South Asia, while the other, Ae (A2), is widely distributed in Europe and the USA (Bowyer *et al.*, 1997; Kramvis *et al.*, 2002; Sugauchi *et al.*, 2004). The subtypes (subgenotypes) of HBV/A show no evidence of distinguishing recombination; nevertheless, they are associated with differences in replicative activity, and in the mechanisms of HBeAg seroconversion as a result of specific nucleotide substitutions in the core promoter and precore regions (Kimbi *et al.*, 2004; Sugauchi *et al.*, 2004; Tanaka *et al.*, 2004).

The characterization of isolates from indigenous populations, especially in Africa where HBV is hyperendemic, may assist in revealing the origin of HBV and clarify the many questions about its evolutionary history (Kramvis *et al.*, 2005). The genetic diversity and distribution of HBV genotypes in Central West Africa, particularly in Cameroon, are poorly documented. No data were available for the HBV strains from Pygmies in this region. The objectives of the present study were to assess the prevalence of HBV and hepatitis C virus (HCV) markers among Bantus and Pygmies, to compare the distribution of HBV genotypes and to analyse the genomic characteristics of the HBV/A strain in Cameroon. Six full genome sequences, including four representing a new subtype (subgenotype) of HBV/A and two HBV/E strains from the Cameroonian Pygmies, were analysed.

METHODS

Blood serum samples. Blood serum samples were collected in 1994 from 544 voluntary donors, including representatives of two relatively isolated populations (Bantu and Pygmies) in Cameroon, Central West Africa. The Pygmies enrolled were from two forest encampments in the East province, and the Bantu were enrolled from five provinces across the country (Central, South, North, West and East). None of the donors had clinical symptoms of liver disease. Written informed consent was obtained from all subjects enrolled. After isolation of the serum fraction from whole blood, the samples were stored at −40 °C until use. The number of subjects studied in each group, their ages and sexes are summarized in Table 1.

Serological assays for hepatitis virus markers and HBV genotyping. HCV (anti-HCV) and HBV serological markers (HBsAg, HBeAg, anti-HBs and anti-HBc) were examined using a chemiluminescent immunoassay (Ortho Clinical Diagnostics).

HBsAg-positive samples were subjected to HBV genotyping using an enzyme-linked immunoassay (EIA) with monoclonal antibodies to type-specific epitopes of the preS2 region (Usuda *et al.*, 1999), using commercial kits (HBV Genotype EIA; Institute of Immunology Co.).

Table 1. The distribution of HBV and HCV serological markers and HBV genotypes among two populations in Cameroon

Population	Bantu (n=370)	Pygmies (n=174)	P
Male/Female*	177/188	87/82	NS†
Age (years); mean ± SD	34·2 ± 14·5	29·9 ± 9·2	<0·05
Anti-HCV	75 (20·3 %)	4 (2·3 %)	<0·0001
HBsAg	33 (8·9 %)	13 (7·5 %)	NS
HBeAg	5 (15·2 %)	0	NS
Anti-HBs	77 (20·8 %)	17 (9·8 %)	0·001
Anti-HBc	322 (87·0 %)	150 (86·2 %)	NS
HBV/A	15 (45·5 %)	5 (38·5 %)	NS
HBV/E	13 (39·4 %)	7 (53·8 %)	NS
HBV/D	5 (15·1 %)	1 (7·7 %)	NS

*Gender and age data were not available for some of the specimens.
†NS, Not significant.

Amplification, quantification of HBV DNA and nucleotide sequencing. DNA was extracted from 27 serum samples, in which HBV/A and HBV/E had been identified by genotyping EIA: 20 (15 from Bantu and five from Pygmies) and seven (only from Pygmies), respectively. Total DNA was extracted from 100 µl serum using a QIAamp DNA mini kit (Qiagen) and suspended in 100 µl storage buffer (supplied by the kit manufacturer). A real-time PCR assay, allowing detection of up to 100 viral DNA copies ml^{-1} (Abe *et al.*, 1999), with slight modifications (Tanaka *et al.*, 2004), was used for HBV DNA screening.

Two overlapping HBV DNA fragments covering the entire genome sequence were amplified using specific primers and PCR conditions that have been described previously (Sugauchi *et al.*, 2001). Amplified HBV DNA fragments were sequenced directly using a Prism Big Dye v3.0 kit (Applied Biosystems) on an ABI 3100 DNA automated sequencer (Applied Biosystems). All sequences were analysed in both the forward and reverse directions. Complete and partial HBV genomes were assembled using GENETYX v11.0 (Software Development). The nucleotide sequence data reported in this paper appear in the GenBank/EMBL/DDBJ nucleotide sequence databases with the accession numbers AB194947–AB194955.

Sequence analysis. Sequences were aligned using the CLUSTAL W software program (Thompson *et al.*, 1997). Phylogenetic trees were constructed using neighbour-joining (NJ) analysis incorporating the six-parameter distance correction method (Gojobori *et al.*, 1982) with bootstrap test confirmation performed on 1000 resamplings using the Online Hepatitis virus database (http://s2as02.genes.nig.ac.jp/). Preliminary trees were constructed for Cameroonian HBV strains obtained in this study and corresponding data of 632 HBV genome sequences available from the GenBank/DDBJ databases (the trees are available from the authors). The final trees presented herein were constructed for Cameroonian strains together with the selected GenBank/DDBJ references including the HBV/A strains of various geographical origins, and representatives of other known human HBV genotypes.

Nucleotide divergence over complete genomes was calculated using the CLUSTAL method implemented in the MEGALIGN software (Clewley & Arnold, 1997).

Detection of recombination. All Cameroonian strains' complete genome sequences were examined for the presence of recombination with other HBV genotypes, as described previously (Robertson *et al.*, 1995). Bootscan analysis implemented in the SimPlot software program (Lole *et al.*, 1999) was performed for each of the strains.

Statistical analysis. All statistical values were calculated using the Mann–Whitney U test, Fisher's exact test and the χ^2 test with Yate's correction, implemented in the STATA v8.0 software program (Stata). Differences were considered significant for P values less than 0·05.

RESULTS

Hepatitis virus serological markers and HBV genotypes in Cameroon

Table 1 summarizes results of the serological screening and HBV genotyping. The overall anti-HCV seroprevalence was very high (14·5 %), and was significantly higher in Bantus (20·3 %) than in Pygmies (2·3 %, $P<0·0001$), demonstrating that transmission networks of the infection are relatively isolated between two populations (blood transfusion and medical procedures probably contributed to transmission in the Bantus but not in the Pygmies; Kowo *et al.*, 1995). Nevertheless, HBsAg prevalence was equally high in both

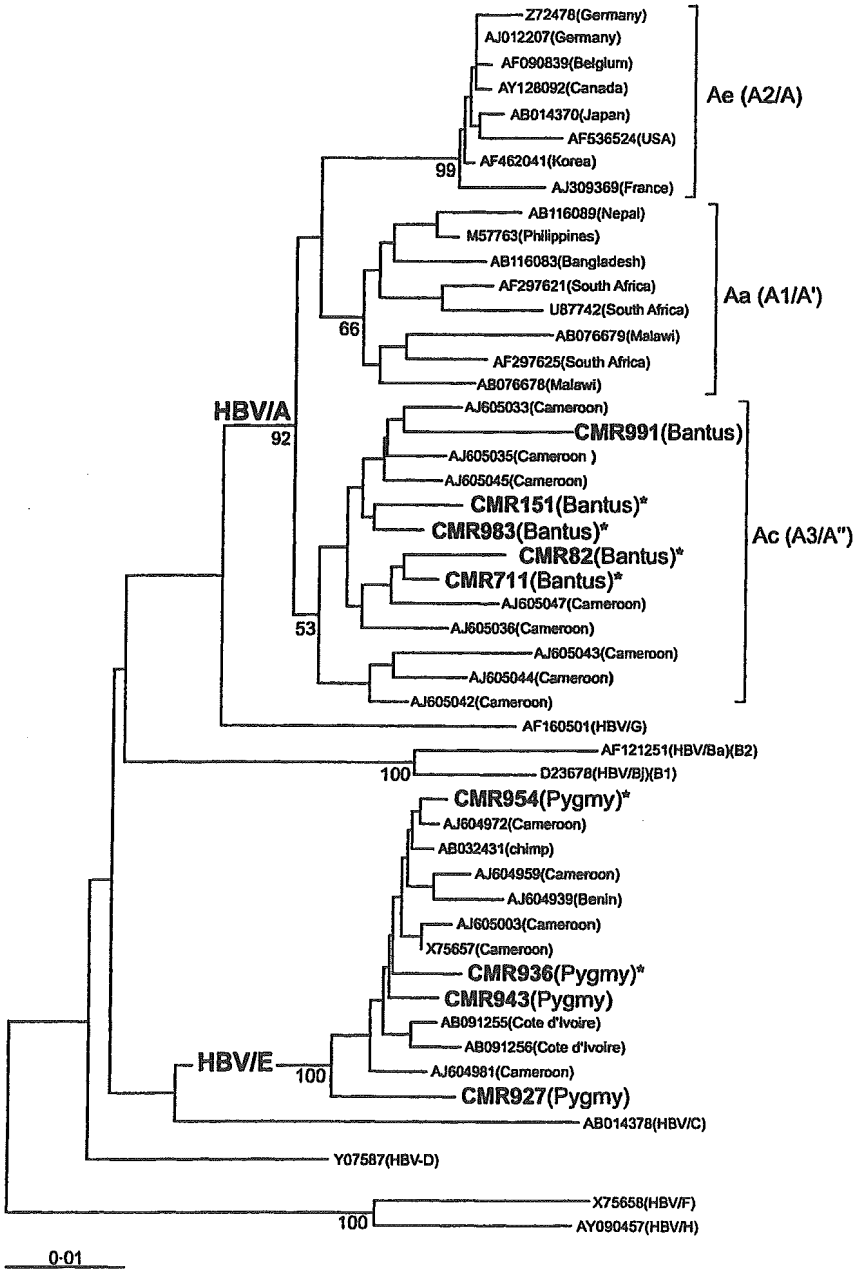


Fig. 1. A phylogenetic NJ tree constructed using the HBV preS2/S nucleotide sequences. Nine strains from Cameroon isolated in this study are indicated in bold. Reference sequences were retrieved from GenBank/EMBL/DDBJ with their accession numbers and origin (in parentheses) indicated. Bootstrap values are indicated in the tree roots. Asterisk (*) marked strains, four HBV/A and two HBV/E, from Cameroon were used for further analyses based on the complete genome sequences.

populations (8·9–7·5 %). HBeAg examined among HBsAg-positive carriers was determined in 10·9 % of cases, all of which were in the Bantus infected with HBV/E (mean age 21·2 years, range 1–30 years). The mean age of HBeAg-negative carriers in the Bantus was 31·6 years, range 17–90 years. All HBsAg-positive carriers among the Pygmies were negative for HBeAg (mean age 29·5 years, range 27–38 years). Thus, the mean age of the HBeAg-negative group was relatively young for HBsAg carriers in both populations in Cameroon, suggesting early HBeAg seroconversion. Anti-HBc seroprevalence was very high in both populations (mean 86·7 %), with no significant difference (86·2 vs 87 %), concordant with a previous report (Ndumbe *et al.*, 1993) and indicating a high incidence of HBV infection in both populations, probably attributable to effective horizontal transmission at a young age, as reported previously in African countries (Kramvis *et al.*, 2005). There was no significant difference in the distribution of the examined viral markers among the Bantu population in different provinces, or among the Pygmies population in the different encampments. A total of 46 serum samples found to be positive for HBsAg were subjected to HBV genotyping using the EIA method. Genotypes A and E identified in 43·5 % of cases were equally predominant in both of the populations, and genotype D was found in a minority (13 %) of cases. No significant difference in distribution of the genotypes was found within the same population in different provinces or between the two populations.

In order to study the molecular genetic characteristics of the prevalent HBV genotypes in Cameroon, 20 HBV/A and seven HBV/E samples, for which sufficient volume was available, were subjected to further investigation. Of the

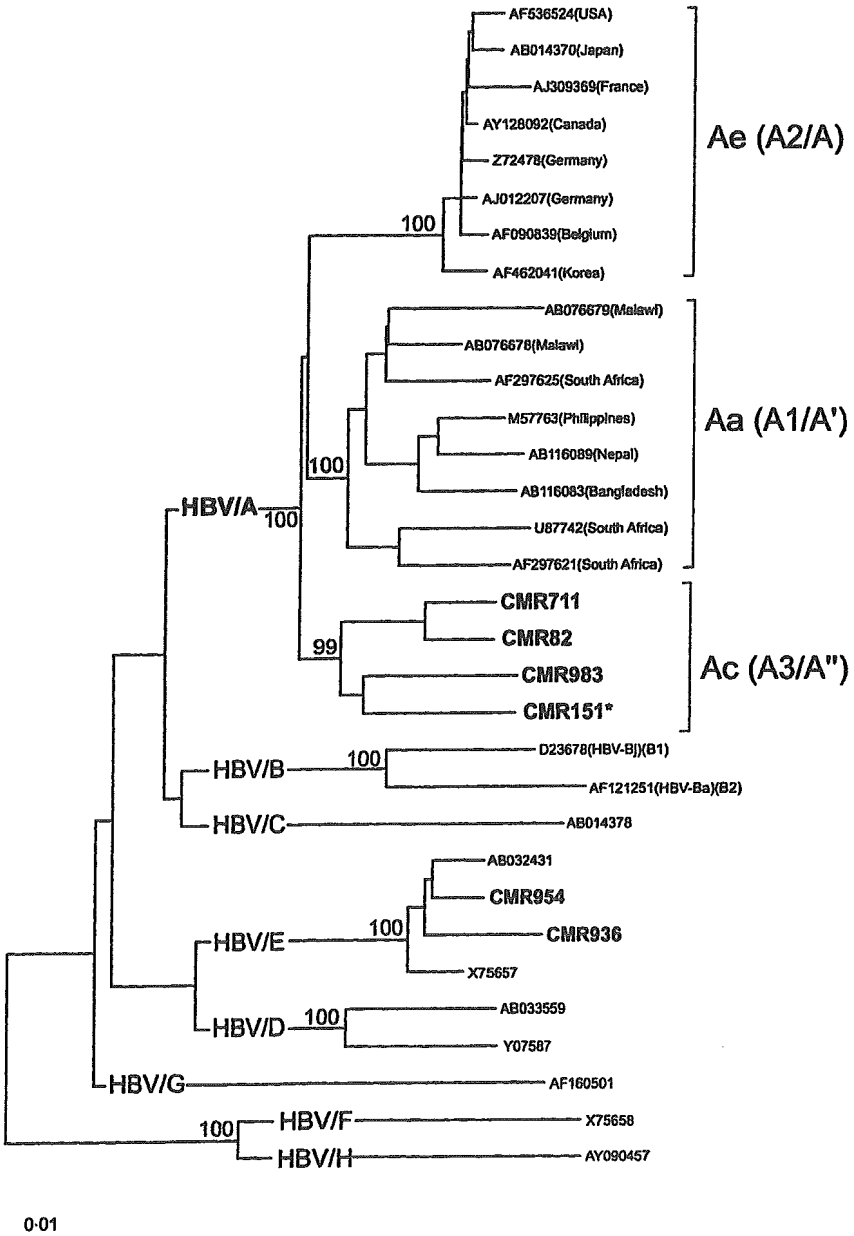
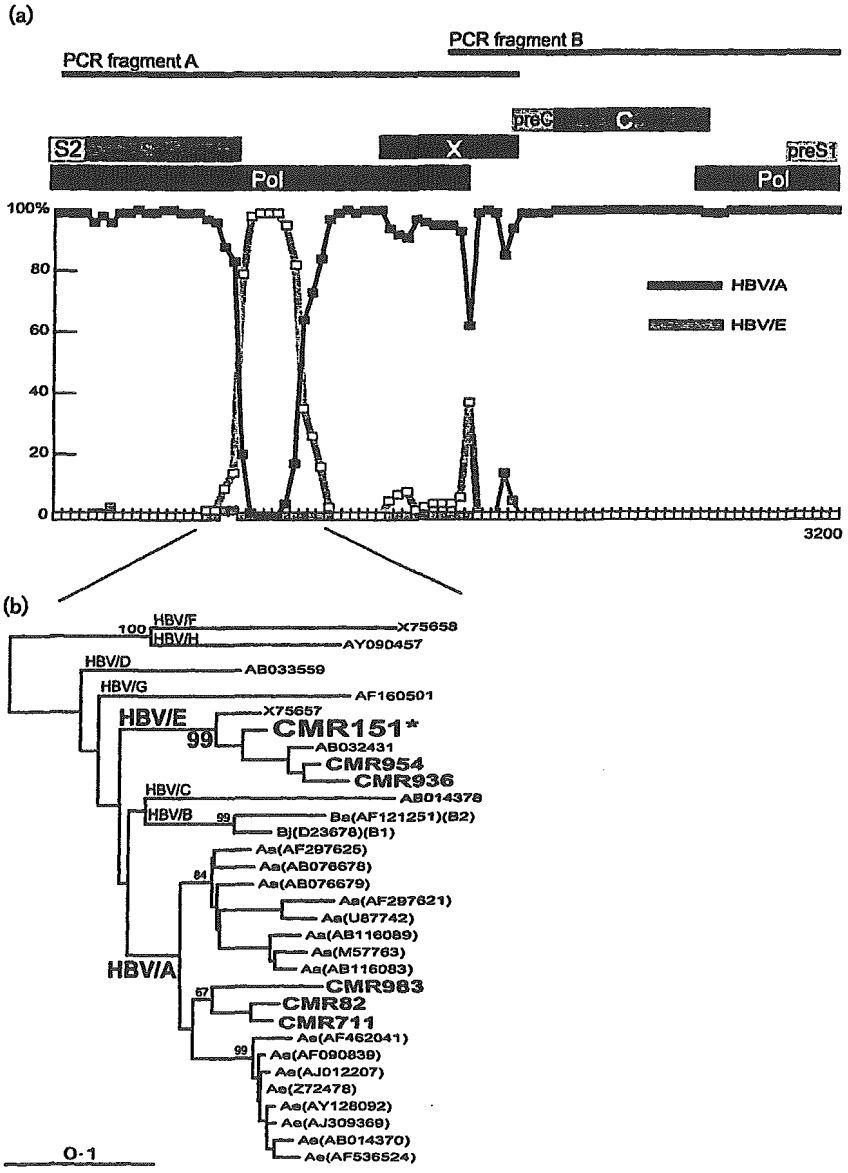


Fig. 2. A phylogenetic NJ tree constructed using the complete HBV genome. Six strains from Cameroon isolated in this study are indicated in bold. Accession numbers are given for reference sequences retrieved from GenBank/EMBL/DDBJ. The origins of the previously published HBV/A strains are indicated in parentheses. Bootstrap values are indicated in the tree roots. The strain from Cameroon with the recombination between HBV/A and HBV/E is marked with an asterisk (*).

samples, only 1/27 was HBeAg-positive (HBV/E by EIA), which was obtained from a 1-year-old child, and the rest (26/27) of the HBsAg-positive carriers had undergone HBeAg seroconversion.

HBV DNA quantification, sequencing, phylogenetic relation and genetic diversity of HBV/A subtypes (subgenotypes)

HBV DNA was detected in only 10/27 serum samples: 5/15 Bantus and 5/12 Pygmies. The highest HBV DNA level ($3{\cdot}4 \times 10^{10}$ copies ml^{-1}) was detected in the sample obtained from a 1-year-old child. The rest of the nine positive samples were obtained from (mean) 26-year-old carriers (range 21–30 years), with HBV DNA levels ranging from $1{\cdot}1 \times 10^{3}$ to $7{\cdot}8 \times 10^{5}$ copies ml^{-1}. HBV DNA-negative carriers were (mean) 30·4 years old, range 17–50 years, showing a general tendency of HBV DNA level to decline with age (not statistically significant, probably due to small numbers). HBV large S coding region sequences were successfully amplified from 9/10 samples. The sequences were subjected to a similarity search throughout GenBank/DDBJ using the BLAST search engine, and the most similar strains were used for phylogenetic analysis together with the reference sequences of all known human HBV genotypes. The phylogenetic relationship of the ~800 nt (positions 31–835) preS2/S sequences of the HBV strains is represented in Fig. 1. Within the HBV/A phylogenetic cluster, the HBV/Aa (A1) and HBV/Ae (A2) strains separated out into two clusters and the five Cameroonian strains sequenced in this study together with other Cameroonian strains retrieved from GenBank/DDBJ clustered separately. The Cameroonian strains retrieved from GenBank/DDBJ were previously designated A″ cluster according to partial (Large S) genome sequence (Mulders *et al.*, 2004). The Cameroonian and HBV/Aa (A1) subclusters, however, did not have significant bootstrap indexes.



The complete genome of six strains (four HBV/A and two HBV/E) were sequenced successfully (marked by asterisks in Fig. 1). The lengths of the complete genomes corresponding to HBV/A and HBV/E were 3221 and 3212 nt, respectively. The phylogenetic analysis of the complete genome sequences (Fig. 2) revealed three distinct bootstrap test supported groups within the HBV/A cluster: HBV/Aa (A1), HBV/Ae (A2), and the third group formed by samples from Cameroon. We denoted the third phylogenetic group as 'HBV/Ac', where 'c' stands for Cameroon and Central Africa. The distinctive grouping of HBV/Ac (A3) strains was also confirmed when preS1/S2, preC/C, and complete Pol genes were analysed phylogenetically. S and X genes were phylogenetically related between the HBV/Aa (A1) and HBV/Ac (A3) groups. Estimated inter-group percentage nucleotide divergence over complete genome sequences consisted of [mean ± SD (range)]: 4·9 ± 0·4 (4·2–6·1), Aa (A1) versus Ae (A2); 5·1 ± 0·5 (4·0–6·0), Aa (A1) versus Ac (A3); and 5·2 ± 0·3 (4·7–5·8), Ae (A2) versus Ac (A3). On the other hand, intra-group percentage nucleotide divergence was similar for HBV/Aa (A1) and HBV/Ac (A3) [mean ± SD (range)]: 3·6 ± 0·8 (4·0–4·6) and 3·9 ± 1·1 (1·8–4·8), respectively, and lowest for Ae (A2): 0·9 ± 0·3 (0·4–1·6) (P<0·0001).

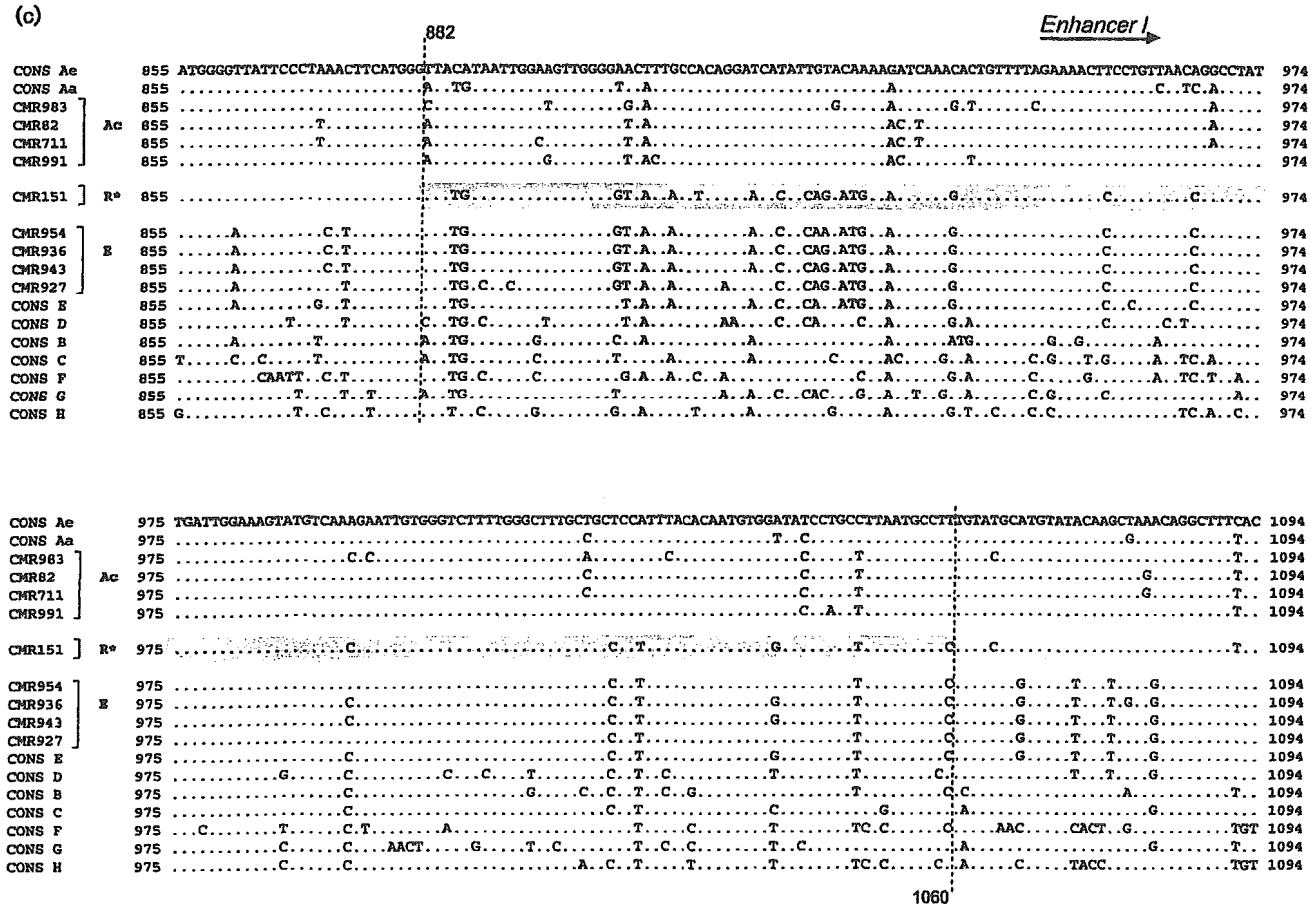


Fig. 3. (a) SimPlot analysis demonstrating the recombination in the non-overlapping part of the polymerase coding region of the CMR151 strain. The strain was subjected to bootscan analysis over the entire genome using the SimPlot program (Lole *et al.*, 1999) with a window size of 300 bp and a step size of 30 bp, under the F84 (ML) model, with bootstrap resampling performed 1000 times. Initially, consensus sequences of each human HBV genotype were used as references; after manual confirmation of the sequence alignment, the final plot was constructed using the consensus of the HBV/A, HBV/E and HBV/D genotypes only. The sequences were obtained from two overlapping PCR fragments, indicated by two lines ('A' and 'B'). HBV genome coding regions are indicated by standard abbreviations (S2, S, Pol, X, preC, C and preS1). (b) The phylogenetic NJ tree constructed using the recombinant segment. Six strains from Cameroon in this study are indicated in bold. Accession numbers are given for reference sequences retrieved from GenBank/EMBL/DDBJ. (c) Alignment of all human HBV genotype genome nucleotide sequences in the region corresponding to the recombination in the Cameroonian strain CMR151 (shaded in grey). Nucleotide positions correspond to the HBV genome reference sequence, GenBank accession no. NC_003977. Dashed lines at 882 and 1060 represent the breakpoints.

Recombination

Evidence of recombination between HBV/A and HBV/E was observed in one of the Cameroonian strains (CMR151, marked by an asterisk in Fig. 2). The result of the bootscan analysis for the complete genome sequence of the strain is presented in Fig. 3(a). The phylogenetic tree constructed using the corresponding sequence segment confirmed the grouping of the CMR151 strain together with the HBV/E strains, with strong bootstrap support (Fig. 3b). The recombinant segment corresponded to a part of the non-overlapping HBV DNA polymerase in the reverse transcriptase (RT) domain and a part of the enhancer I–X promoter. (Fig. 3c). The breakpoints at nucleotide positions 882 and 1060 were estimated by mapping the informative sites and using χ^2 confirmation (Robertson *et al.*, 1995).

Enhancer/promoter elements and amino acid characteristics of the HBV/Ac (A3) strains

A comparison of the nucleotide substitutions within the *cis*-acting elements among the four HBV/Ac (A3) strains and the consensus sequences of the HBV/Aa (A1) and HBV/Ae (A2) subtypes (subgenotypes) as well as the other HBV genotypes (including HBV/Ba, Bj, B2, B1) are summarized in Table 2. Nine specific nucleotide substitutions were found in HBV/Ac (A3) strains: $G^{1173}A$ (enhancer I–X promoter), $C^{1473}G$, $G^{1512}A$ and $C^{1703}T$ (enhancer II–core promoter), $A^{2742}G$ (S1–promoter), $C^{3021}T$, $C^{3042}T$,

Table 2. Subtype (subgenotype) specific sites (bold) within enhancers and promoter regions of HBV/Aa (A1), HBV/Ac (A3) and HBV/Ae (A2)

Nucleotide positions correspond to the HBV genome reference sequence, GenBank accession no. NC_003977. Consensus sequences were composed according to 60% or higher incidence at the corresponding nucleotide position.

Region	Position (nt)	HBV/Aa (A1)	HBV/Ac (A3)				HBV/Ae (A2)
			CMR711	CMR82	CMR983	CMR151	
Enhancer I–X promoter (950–1350)	963	C	T	T	T	T	T
	1041	T	A	A	A	G	A
	1173	G	–	A	A	A	–
	1320	A	A	A	A	A	G
	1350	T	–	–	–	–	C
Enhancer II–core promoter (1400–1850)	1404	T	–	–	–	–	C
	1464	G	T	T	T	T	T
	1473	C	G	G	–	T	–
	1484	A	–	–	–	–	C
	1511	G	–	–	–	–	A
	1512	T	A	A	–	A	G
	1703	C	T	T	T	A	–
	1727	A	–	–	–	–	G
	1740	T	–	–	–	–	C
	1809–1812	TCAT	-TC-	--C-	----	G--C	G--C
Encapsidation signal (1846–1908)	1888	A	G	G	G	G	G
S1–Promoter (2716–2806)	2720	A	T	T	T	T	G
	2742	A	G	G	G	G	–
	2744	C	A	A	–	–	–
	2777	G	C	T	T	T	T
S2–Promoter (2999–3219)	3013–3014	CA	--	--	--	--	GC
	3021	C	T	T	T	T	–
	3042	C	T	T	T	–	–
	3052	T	–	–	–	–	C
	3057/60	T/C	C/T	C/T	C/T	C/T	C/T
	3069	A	–	–	–	–	C
	3072–3073	TG	A-	C-	--	--	-A
	3076	T	C	C	C	C	–
	3111	T	T	T	T	T	C
	3118	C	–	–	–	–	T
	3121	G	–	–	–	–	A
	3124	G	–	–	–	–	A

$T^{3076}C$ and $C^{3111}T$ (S2–promoter). Interestingly, three of four strains had substitutions in the Kozak sequence (1809–1812) (Ahn *et al.*, 2003; Tanaka *et al.*, 2004), and two had a basal core promoter double mutation (1762/1764).

Although HBV/Ac (A3) amino acid motifs in general were more similar to HBV/Aa (A1) than to HBV/Ae (A2) motifs, HBV/Ac (A3) strains had some specific sites: Thr^{84} in preS1, Ala^{146} in Pol/terminal protein, Ser^{239}, Trp^{246}, Ser^{257} in Pol/spacer, Asp^{356}, Arg^{501}, Ser^{607} in Pol/RT, and Thr^{47} in X proteins when compared with consensus sequences composed according to 60 % or higher incidence at the corresponding amino acid position (Table 3). Pre-core/core amino acid patterns had no specific substitutions among HBV/A subtypes (subgenotypes).

Table 3. Subtype (subgenotype) specific sites (bold) in amino acid sequences of HBV/Aa (A1), HBV/Ac (A3) and HBV/Ae (A2)

Consensus sequences were composed according to 60 % or higher incidence at the corresponding amino acid position.

ORF	Position (aa)	HBV/Aa (A1)	HBV/Ac (A3)				HBV/Ae (A2)
			CMR711	CMR82	CMR983	CMR151	
PreS1	54	Q	–	–	–	–	A
	67	F	–	–	–	–	L
	74	V	–	–	–	–	I
	84	I	T	T	T	–	–
	86	A	T	T	T	T	T
	89	P	–	–	–	–	S
	90	A	–	–	–	–	T
	91	V	–	–	–	–	I
PreS2	32	L	V	V	V	V	V
	47	S	–	–	–	–	A
S	209	L	–	–	–	–	V
Pol/terminal protein	17	E	G	G	G	G	G
	33	E	–	–	A	A	A
	74	P	Q	Q	–	–	–
	102	T	N	N	–	–	–
	120	N	–	–	–	–	T
	146	T	A	A	A	A	–
Pol/spacer	236	T	–	–	–	–	S
	239	P	S	S	S	S	–
	246	R	W	W	W	–	–
	257	F	S	S	S	S	–
	269	Y	–	–	–	–	H
	271	A	–	–	–	–	V
	273	S	–	–	–	–	N
	308	S	K	K	–	–	C
	334	Q	–	–	K	K	K
	338	K	E	E	E	E	E
	348	L	–	–	–	–	R
Pol/RT	356	E	D	–	D	D	–
	501	W	R	R	R	R	–
	607	T	S	S	A	S	–
	617	I	L	L	–	–	–
	619	H	–	–	D	D	–
	666	K	R	R	–	–	–
X region	11	S	–	–	–	–	P
	31	A	S	S	S	S	S
	34	L	V	V	–	F	–
	47	S	T	T	–	T	A
	146	S	F	–	–	A	A
	147	S	–	–	–	P	P

DISCUSSION

A previous study carried out in Cameroon among the Bantus and the Pygmies (Kowo *et al.*, 1995) demonstrated a high (18·6 %) overall seroprevalence of HCV, which was significantly higher in Bantus (31·7 %) than in Pygmies (11·1 %). The results of the present study also indicate the very high HCV seroprevalence (14·5 %), and support the difference between the two populations. However, in our study, HCV seroprevalence among the Pygmies was lower (2·3 %), which might be attributed to the younger age of examined subjects compared with the cohort previously studied (Kowo *et al.*, 1995). The difference in HCV seroprevalence between the two populations might be explained by exposure of the Bantus to transmission routes such as medical procedures and blood transfusion, to which the Pygmies are not exposed. However, HBV seroprevalence (HBsAg and anti-HBc) was equally high among the two populations and different regions of the country, which is concordant with previous data (Ndumbe *et al.*, 1993). Further epidemiological investigation is required to evaluate factors contributing to the difference in HBV and HCV transmission in the Pygmies, in contrast with neighbouring Bantus.

The only data available on HBV genotypes in Cameroon demonstrated the predominant prevalence of HBV/A in human immunodeficiency virus-positive cohort (Mulders *et al.*, 2004). The present study revealed that both HBV/A and HBV/E are distributed equally in both native populations in Cameroon. The phylogenetic analysis revealed a close relationship in the large S coding region among the Cameroonian strains sequenced in this study and those from the same country available from previous reports (Mulders *et al.*, 2004; Norder *et al.*, 1992). Based on phylogenetic analysis of the complete genome, including four sequences in this study, the presence of a third phylogenetic cluster was confirmed within HBV/A in this study. The cluster was distinct from known HBV/Aa (A1) and HBV/Ae (A2) subtypes (subgenotypes), and designated HBV/Ac (A3) (where 'c' stands for Cameroon and Central Africa). The inter-subtype (subgenotype) nucleotide divergence over the complete genome sequences falls within the 4–8 % range that justifies the classification of HBV/Ac (A3) into a distinct subtype (subgenotype) according to the recent proposals on HBV nomenclature (Kato *et al.*, 2005; Kramvis *et al.*, 2005). The high intra-subtype (subgenotype) nucleotide divergence of four HBV/Ac (A3) complete genomes suggests a long natural history of this subtype (subgenotype) within the native population of Cameroon, as has been reported for subtype (subgenotype) HBV/Aa (A1) in southern African Blacks (Kimbi *et al.*, 2004). On the other hand, HBV/E strains obtained from the Pygmies did not group together separately from the strains isolated in different geographical regions, even though the Pygmies represent an isolated population in Africa. The presence of low divergent HBV/E genotype among the Pygmies might not support the hypotheses proposed previously that HBV/E has a very short history in humans (Mulders *et al.*, 2004).

The newly described subtype (subgenotype) HBV/Ac (A3) possesses a combination of the sites specific for either HBV/Aa (A1) or HBV/Ae (A2) within the corresponding enhancer/promoter elements and amino acid motifs (Kimbi *et al.*, 2004; Sugauchi *et al.*, 2004; Tanaka *et al.*, 2004). Moreover, the subtype (subgenotype) also has HBV/Ac (A3) unique substitutions. The recombination affecting a short, non-overlapping segment of the polymerase RT domain found in one of the Cameroonian strains is the first event documented to have occurred between HBV/A and HBV/E. The sequencing data generated in the present study could be used to design assays that can discriminate between HBV/Ac (A3) and the other subtypes (subgenotypes) of HBV/A in order to characterize its clinical–virological features. Cohort studies are required to investigate a possible association of HBV/Ac (A3) infection with early HBeAg/anti-HBe seroconversion and low HBV DNA levels in carriers indicated by the tendencies observed on the small number investigated in present study.

At the present time, investigation of HBV molecular heterogeneity, global distribution of HBV genetic forms, including recombination and mutations as well as efficient implications of the data, is one of the major directions in the field of virus research (Kramvis *et al.*, 2005). In this respect, further standardization of the HBV nomenclature and, an efficient and logical classification should be based on a consensus of the accumulated data including recent studies.

In conclusion, the complete genome of the third subtype (subgenotype) of HBV/A, identified in Cameroon, has been analysed and unique nucleotide/amino acid substitutions have been identified within this subtype (subgenotype). The high intra-group divergence suggests that this subtype (subgenotype) represents an indigenous HBV strain with a long natural history. Recombination between this subtype (subgenotype) and genotype E is described.

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REFERENCES

Abe, A., Inoue, K., Tanaka, T., Kato, J., Kajiyama, N., Kawaguchi, R., Tanaka, S., Yoshiba, M. & Kohara, M. (1999). Quantitation of hepatitis B virus genomic DNA by real-time detection PCR. *J Clin Microbiol* 37, 2899–2903.

Ahn, S. H., Kramvis, A., Kawai, S., Spangenberg, H. C., Li, J., Kimbi, G., Kew, M., Wands, J. & Tong, S. (2003). Sequence variation upstream of precore translation initiation codon reduces hepatitis B virus e antigen production. *Gastroenterology* 125, 1370–1378.

Arauz-Ruiz, P., Norder, H., Robertson, B. H. & Magnius, L. O. (2002). Genotype H: a new Amerindian genotype of hepatitis B virus revealed in Central America. *J Gen Virol* **83**, 2059–2073.

Bowyer, S. M., van Staden, L., Kew, M. C. & Sim, J. G. (1997). A unique segment of the hepatitis B virus group A genotype identified in isolates from South Africa. *J Gen Virol* **78**, 1719–1729.

Clewley, J. P. & Arnold, C. (1997). MEGALIGN. The multiple alignment module of LASERGENE. *Methods Mol Biol* **70**, 119–129.

Gojobori, T., Ishii, K. & Nei, M. (1982). Estimation of average number of nucleotide substitutions when the rate of substitution varies with nucleotide. *J Mol Evol* **18**, 414–423.

Kane, M. (1995). Global programme for control of hepatitis B infection. *Vaccine* **13**, S47–S49.

Kato, H., Fujiwara, K., Gish, R. G. & 9 other authors (2005). Classifying genotype F of hepatitis B virus into F1 and F2 subtypes. *World J Gastroenterol* (in press).

Kimbi, G. C., Kramvis, A. & Kew, M. C. (2004). Distinctive sequence characteristics of subgenotype A1 isolates of hepatitis B virus from South Africa. *J Gen Virol* **85**, 1211–1220.

Kowo, M. P., Goubau, P., Ndam, E. C., Njoya, O., Sasaki, S., Seghers, V. & Kesteloot, H. (1995). Prevalence of hepatitis C virus and other blood-borne viruses in Pygmies and neighboring Bantus in southern Cameroon. *Trans R Soc Trop Med Hyg* **89**, 484–486.

Kramvis, A. & Kew, M. C. (2005). Relationship of genotypes of hepatitis B virus to mutations, disease progression and response to antiviral therapy. *J Viral Hepat* (in press).

Kramvis, A., Weitzmann, L., Owiredu, W. K. & Kew, M. C. (2002). Analysis of the complete genome of subgroup A′ hepatitis B virus isolates from South Africa. *J Gen Virol* **83**, 835–839.

Kramvis, A., Kew, M. & Francois, G. (2005). Genotypes of hepatitis B virus. *Vaccine* **23**, 2407–2421.

Lole, K. S., Bollinger, R. C., Paranjape, R. S., Gadkari, D., Kulkarni, S. S., Novak, N. G., Ingersoll, R., Sheppard, H. W. & Ray, S. C. (1999). Full-length human immunodeficiency virus type 1 genomes from subtype C-infected seroconverters in India, with evidence of intersubtype recombination. *J Virol* **73**, 152–160.

Miyakawa, Y. & Mizokami, M. (2003). Classifying hepatitis B virus genotypes. *Intervirology* **46**, 329–338.

Mulders, M. N., Venard, V., Njayou, M. & 11 other authors (2004). Low genetic diversity despite hyperendemicity of hepatitis B virus genotype E throughout West Africa. *J Infect Dis* **190**, 400–408.

Ndumbe, P. M., Atchou, G., Biwole, M., Lobe, V. & Ayuk-Takem, J. (1993). Infections among pygmies in the Eastern Province of Cameroon. *Med Microbiol Immunol* **182**, 281–284.

Norder, H., Hammas, B., Lofdahl, S., Courouce, A. M. & Magnius, L. O. (1992). Comparison of the amino acid sequences of nine different serotypes of hepatitis B surface antigen and genomic classification of the corresponding hepatitis B virus strains. *J Gen Virol* **73**, 1201–1208.

Norder, H., Courouce, A. M. & Magnius, L. O. (1994). Complete genomes, phylogenetic relatedness, and structural proteins of six strains of the hepatitis B virus, four of which represent two new genotypes. *Virology* **198**, 489–503.

Okamoto, H., Tsuda, F., Sakugawa, H., Sastrosoewignjo, R. I., Imai, M., Miyakawa, Y. & Mayumi, M. (1988). Typing hepatitis B virus by homology in nucleotide sequence: comparison of surface antigen subtypes. *J Gen Virol* **69**, 2575–2583.

Robertson, D. L., Hahn, B. H. & Sharp, P. M. (1995). Recombination in AIDS viruses. *J Mol Evol* **40**, 249–259.

Stuyver, L., De Gendt, S., Van Geyt, C., Zoulim, F., Fried, M., Schinazi, R. F. & Rossau, R. (2000). A new genotype of hepatitis B virus: complete genome and phylogenetic relatedness. *J Gen Virol* **81**, 67–74.

Sugauchi, F., Mizokami, M., Orito, E. & 7 other authors (2001). A novel variant genotype C of hepatitis B virus identified in isolates from Australian Aborigines: complete genome sequence and phylogenetic relatedness. *J Gen Virol* **82**, 883–892.

Sugauchi, F., Orito, E., Ichida, T. & 10 other authors (2003). Epidemiologic and virologic characteristics of hepatitis B virus genotype B having the recombination with genotype C. *Gastroenterology* **124**, 925–932.

Sugauchi, F., Kumada, H., Acharya, S. A. & 10 other authors (2004). Epidemiological and sequence differences between two subtypes (Ae and Aa) of hepatitis B virus genotype A. *J Gen Virol* **85**, 811–820.

Tanaka, Y., Hasegawa, I., Kato, T. & 11 other authors (2004). A case-control study for differences among hepatitis B virus infections of genotypes A (subtypes Aa and Ae) and D. *Hepatology* **40**, 747–755.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**, 4876–4882.

Usuda, S., Okamoto, H., Iwanari, H., Baba, K., Tsuda, F., Miyakawa, Y. & Mayumi, M. (1999). Serological detection of hepatitis B virus genotypes by ELISA with monoclonal antibodies to type-specific epitopes in the preS2-region product. *J Virol Methods* **80**, 97–112.

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Short Communication

A novel simian immunodeficiency virus from black mangabey (*Lophocebus aterrimus*) in the Democratic Republic of Congo

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In order to understand primate lentivirus evolution, characterization of additional simian immunodeficiency virus (SIV) strains is essential. Here, an SIV from a black mangabey (*Lophocebus aterrimus*) originating from the Democratic Republic of Congo was analysed phylogenetically. The monkey had cross-reactive antibodies against human immunodeficiency virus type 1 (HIV-1) and HIV-2. The viral *pol* region sequence was amplified by nested PCR and sequence analysis confirmed that it was related to known SIV sequences. This is the first report to characterize genetically an SIV from the monkey genus *Lophocebus*. Phylogenetic analysis of the *pol* region revealed that this novel SIV, designated SIVbkm, fell into the SIVsyk and SIVgsn virus group, containing viruses isolated from the genus *Cercopithecus*, and suggests that cross-species transmission has occurred between species of the genera *Lophocebus* and *Cercopithecus*.

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Simian immunodeficiency viruses (SIVs) naturally infect a wide range of wild African non-human primates (Hahn *et al.*, 2000; Hayami *et al.*, 1994; Peeters *et al.*, 2002). On the basis of their genomic sequences, SIVs are currently classified into six major phylogenetic lineages: (i) SIVcpz in chimpanzees (*Pan troglodytes*), which clusters with human immunodeficiency virus type 1 (HIV-1) (Corbet *et al.*, 2000; Gao *et al.*, 1999; Huet *et al.*, 1990); (ii) SIVsm in sooty mangabeys (*Cercocebus atys*), which clusters with HIV-2 (Chen *et al.*, 1996; Hirsch *et al.*, 1989); (iii) SIVagm in four species of African green monkeys (*Chlorocebus aethiops*) (Allan *et al.*, 1991; Fukasawa *et al.*, 1988); (iv) SIVmnd-1 in mandrills (*Mandrillus sphinx*), which forms a cluster with SIVlhoest in l'Hoest monkeys (*Cercopithecus lhoesti*) and SIVsun in sun-tailed monkeys (*Cercopithecus solatus*) (Beer *et al.*, 1999; Hirsch *et al.*, 1999; Tsujimoto *et al.*, 1988, 1989); (v) SIVsyk in Sykes' monkeys (*Cercopithecus albogularis*) (Hirsch *et al.*, 1993); and (vi) SIVcol in guereza colobus monkeys (*Colobus guereza*) (Courgnaud *et al.*, 2002). SIVmus from moustached monkeys (*Cercopithecus cephus*) and SIVmon from mona monkeys (*Cercopithecus mona*) are relatives of SIVgsn from greater spot-nosed monkeys (*Cercopithecus nictitans*), which carry the *vpu* gene in their genome (Barlow *et al.*, 2003; Courgnaud *et al.*, 2002, 2003a). They have been designated as a new SIV lineage, although Biollet-Ruche *et al.* recently suggested that the clusters of SIVgsn and SIVsyk including SIVdeb should be considered as one ancestral SIV lineage that infected monkeys of the genus *Cercopithecus* (Bibollet-Ruche *et al.*, 2004; Courgnaud *et al.*, 2003a; Verschoor *et al.*, 2004). In addition, some novel SIV strains such as SIVtal from talapoins (*Miopithecus*

The GenBank/EMBL/DDBJ accession number for the nucleotide sequence determined in this work is AY518534.

talapoin), SIVwrc from western red colobus (*Piliocolobus badius*) and SIVolc from olive colobus monkeys (*Procolobus verus*) have been reported, although their sequences have only been partially characterized (Courgnaud *et al.*, 2003b; Peeters *et al.*, 2002).

It is now widely accepted that HIV-1 originated from SIVcpz (Gao *et al.*, 1999; Hahn *et al.*, 2000). Bailes *et al.* (2003) suggested that SIVcpz could be a recombinant virus between SIVrcm from red-capped mangabeys (*Cercocebus torquatus*) and SIVgsn. Furthermore, viruses of the above-mentioned six major lineages may also have complex mosaic genomes (Salemi *et al.*, 2003). To understand better the evolutionary relationships among primate lentiviruses, a search for additional SIVs from other non-human primate species is essential. Thus, our research efforts have focused on non-human primates whose habitats are adjacent to or overlap the habitat of common chimpanzees in the forests of central Africa. In the present study, we genetically characterized a new SIV strain from a black mangabey (*Lophocebus aterrimus*) originally isolated in the Democratic Republic of Congo (DRC).

The animal was caught in the wild in 2001 in the Bas-Congo region, in the south-eastern part of the DRC, and kept in a separate cage for 1 year at the Kinshasa Zoo. Peripheral blood was collected in 2002 using acid citrate glucose anticoagulant solution. A plasma sample was initially tested for antibodies using a commercial particle agglutination kit (Genedia HIV-1/2, Fujirebio) and showed positive reactivity, with an antibody titre of 1:64. We then conducted a Western blot assay using commercial Western blot kits (HIV-1 and -2 LAV blot; Bio-Rad) and demonstrated strong reactivity against p18 and p25 (HIV-1 core antigens), p26 (HIV-2 core antigens) and gp105 (HIV-2 glycoprotein) (data not shown). These data suggested that this monkey was infected with an SIV strain related to HIV-1 or -2.

We carried out nested PCR to amplify the viral *pol* region using chromosomal DNA extracted from peripheral blood mononuclear cells. DNA was extracted using the QIAamp Blood DNA mini kit (Qiagen) according to the manufacturer's instructions. We first amplified a fragment of 150 bp in the *pol* region using oligonucleotide primers DR1 (5′-TRCAYACAGGRGCWGAYGA-3′) and DR2 (5′-AIADRT-CATCCATRTAYTG-3′) for the first round and primers DR4 (5′-GGIATWCCICAYCCDGCAGG-3′) and DR5 (5′-GGIGAYCCYTTCCAYCCYTGHGG-3′) for the second round (Clewley *et al.*, 1998; Courgnaud *et al.*, 2002). Amplification was carried out with an initial denaturation at

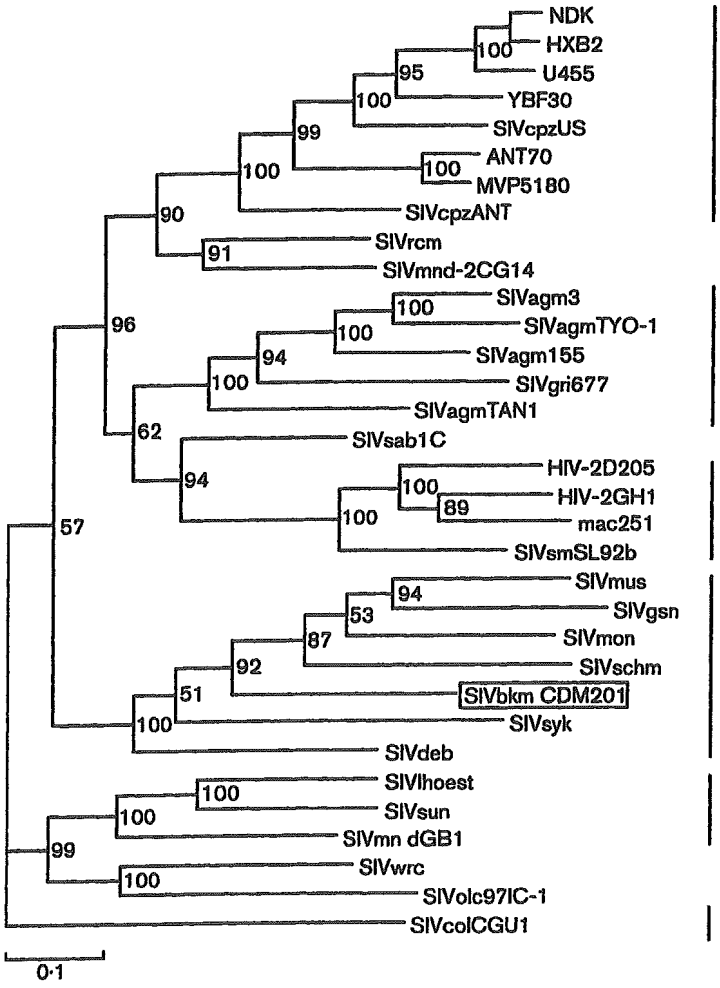

Fig. 1. Phylogenetic tree of HIV and SIV based on the RT–IN region, which is part of the *pol* gene. The new isolate, SIVbkm CDM201 (boxed), was composed of other strains representing each group. The numbers at the nodes indicate estimated posterior probabilities. Bar, number of nucleotide substitutions per site. A neighbour-joining tree (Saitou & Nei, 1987) gave almost identical results (not shown).

94 °C for 3 min, followed by 40 cycles of 94 °C for 30 s, 45 °C for 30 s and 72 °C for 1 min, with a final extension at 72 °C for 5 min. We succeeded in amplifying a 150 bp fragment from this seropositive sample. The PCR product purified from an agarose gel was subcloned into the pUC119 (Takara Bio) vector and sequenced using a cycle sequencing kit and automated sequencers (BigDye Terminator Cycle Sequencing Ready Reaction, ABI 373 and 3100; Applied Biosystems). We then performed a semi-nested PCR to amplify a 1800 bp fragment using the primers DR1 and Unipol2 (5′-CCCCTATTCCTCCCCTTCTTTTAAAA-3′) for the first-round PCR and bkmpol1 (5′-GGATAT-GAAAGATGCTTACTATTCAG-3′) and Unipol2 for the second-round PCR (Miura *et al.*, 1990). The primer bkmpol1 was specifically designed on the basis of the sequence of the 150 bp fragment. PCR was carried out as described above but with cycle conditions of 94 °C for 1 min, 45 °C for 1 min and 72 °C for 3 min. The sequence of the obtained fragment was determined as described above.

The SIV derived from the black mangabey was designated SIVbkm strain CDM201. We constructed a phylogenetic tree to compare the sequence of SIVbkm CDM201 with sequences of representative isolates of SIVs and HIVs. Sequences were aligned using CLUSTAL W with minor manual modifications and a phylogenetic tree was constructed using the maximum-likelihood method with the MOLPHY program (Higgins & Sharp, 1989; Adachi & Hasegawa, 1996; Yamaguchi-Kabata & Gojobori, 2000; Yamaguchi-Kabata *et al.*, 2004). A phylogenetic tree based on 1930 bp fragments of the *pol* region showed that SIVbkm was divergent from other SIV strains, but relatively close to SIVgsn and SIVsyk group isolates (Fig. 1). Subsequently, we carried out a distance plot analysis to investigate the extent of sequence differences (Takehisa *et al.*, 1999). The reference strains used were HIV-1 groups M (strain HXB2), O (ANT70), SIVcpz (US), SIVsm (SL92L), SIVagm (TYO-1), SIVmnd-1 (GB1), SIVsyk (syk173), SIVcol (CGU-1), SIVgsn (99CM71), SIVrcm (Ngm), SIVmon (NG1) and SIVmus (01CM1085). The genetic distance (estimated using Kimura's two-parameter method) between each selected pair of sequences was determined by moving a window of 300 bp along the genome alignment in 10 bp increments and the distance was plotted at the midpoint. SIVmon exhibited a relatively close distance to SIVbkm throughout the *pol* reverse transcriptase (RT) to integrase (IN) region and, interestingly, HIV-1 group O strain ANT70 showed a close relationship to SIVbkm, with the shortest distance in the RNase H region (Fig. 2). At the amino acid level, SIVbkm CDM201 showed moderate similarities with three other primate lentiviruses, SIVgsn (62·5 %), SIVmon (62·0 %) and SIVsyk (59·3 %) (Table 1). However, in the RNase H region, SIVbkm CDM201 showed relatively high similarities to HIV-1 group O (ANT70) (68·6 %), SIVrcm

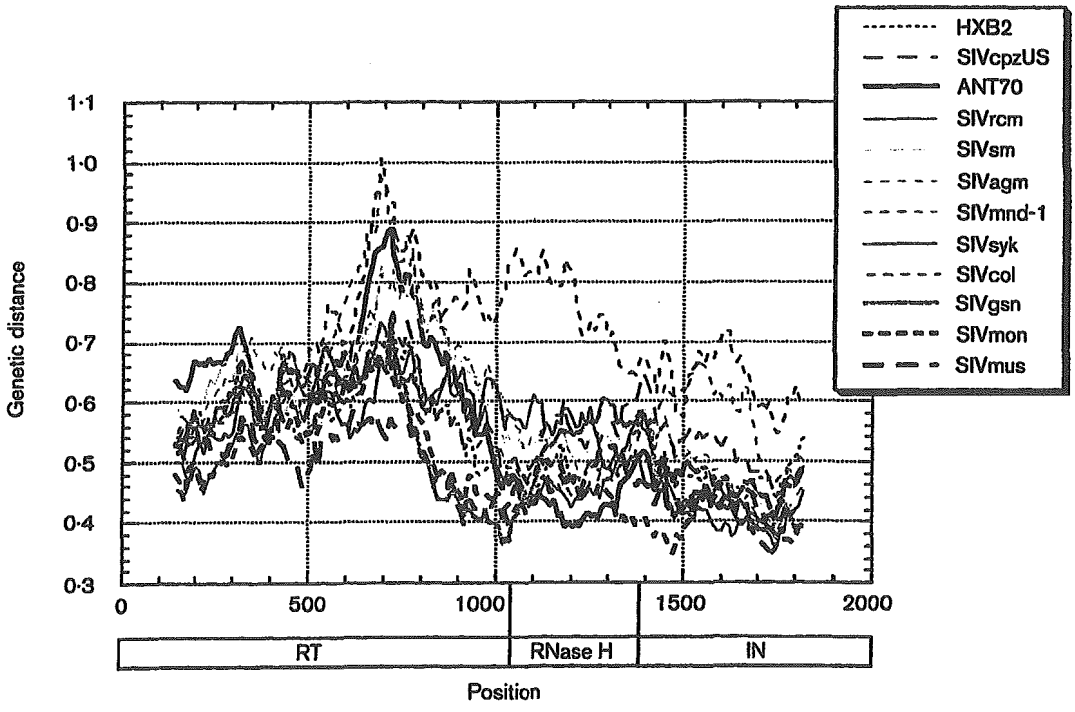

Fig. 2. Distance plots comparing SIVbkm CDM201 with representative primate lentiviruses. The sequences used were HIV-1 groups M (HXB2) and O (ANT70), SIVcpzUS, SIVrcm, SIVsm, SIVagm, SIVmnd-1, SIVsyk, SIVcol, SIVgsn, SIVmon and SIVmus. The genetic distance was estimated using Kimura's two-parameter method (Kimura, 1980). The alignment was sectioned into 300 bp fragments, which were moved along the genome in 10 bp increments. The distance value for each segment was plotted at the midpoint.

Table 1. Percentage amino acid identity in the *pol* region between SIVbkm CDM201 and representatives of other HIV/SIV lineages

Numbers in bold indicate the three highest identities in each respective region.

HIV/SIV strains	Amino acid identity (%)			
	RT–IN	RT	RNase H	IN
HXB2	55·7	52·2	65·3	55·7
YBF30	55·4	52·2	62·8	56·3
ANT70	56·0	49·6	**68·6**	59·6
SIVcpz	55·4	51·0	63·6	57·9
SIVrcm	57·4	51·3	**70·3**	60·1
SIVmnd-2	57·1	50·7	68·6	61·2
HIV-2 D205	53·6	46·8	62·8	60·1
SIVsm	55·0	48·4	66·9	59·6
SIVagm	55·0	47·8	63·6	62·8
SIVmnd-1	53·8	48·1	62·0	59·0
SIVsyk	**59·3**	**55·6**	62·0	64·5
SIVcol	46·5	47·2	39·7	49·7
SIVolc	47·1	44·0	53·7	48·6
SIVgsn	**62·5**	**58·2**	63·6	**70·0**
SIVmon	**62·0**	**58·4**	67·8	**65·0**

(70·3 %) and SIVmnd-2 (68·6 %). In the RT and IN regions, the similarities between SIVbkm CDM201 and these three viruses were relatively low.

In the present study, we have described a novel primate lentivirus, SIVbkm, from a black mangabey in the DRC. This is the first report to characterize genetically an SIV from monkeys of the genus *Lophocebus*. SIVbkm was found to be related to the SIVgsn and SIVsyk lineages, all of which were isolated from monkeys of the genus *Cercopithecus* (Barlow *et al.*, 2003; Courgnaud *et al.*, 2002, 2003a; Bibollet-Ruche *et al.*, 2004). These relationships were supported by high bootstrap values. Bibollet-Ruche *et al.* (2004) stated that the SIVsyk and SIVgsn lineages have the same ancestor and evolved with host-species specialization. The phylogenetic relationships between SIVbkm and the SIVsyk/SIVgsn cluster suggest that cross-species transmission occurred between species of the genus *Cerocopithecus* and a species of the genus *Lophocebus*. Clarifying the cross-species transmission between black mangabeys and *Cercopithecus* monkeys and the spread of the ancestral virus of this cluster may lead to an understanding of the origin of the *vpu* gene, since the SIVgsn lineage carries *vpu*, whereas the SIVsyk lineage does not. A better understanding of these events may also help to understand the origin and evolution of HIV-1.

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References

Adachi, J. & Hasegawa, M. (1996). MOLPHY version 2.3: programs for molecular phylogenetics based on maximum likelihood. *Computer Science Monographs*, vol. 28. Tokyo: Institute of Statistical Mathematics.

Allan, J. S., Short, M., Taylor, M. E., Su, S., Hirsch, V. M., Johnson, P. R., Shaw, G. M. & Hahn, B. H. (1991). Species-specific diversity among simian immunodeficiency viruses from African green monkeys. *J Virol* 65, 2816–2828.

Bailes, E., Gao, F., Bibollet-Ruche, F., Courgnaud, V., Peeters, M., Marx, P. A., Hahn, B. H. & Sharp, P. M. (2003). Hybrid origin of SIV in chimpanzees. *Science* 300, 1713.

Barlow, K. L., Ajao, A. O. & Clewley, J. P. (2003). Characterization of a novel simian immunodeficiency virus (SIVmonNG1) genome sequence from a mona monkey (*Cercopithecus mona*). *J Virol* 77, 6879–6888.

Beer, B. E., Bailes, E., Goeken, R. & 9 other authors (1999). Simian immunodeficiency virus (SIV) from sun-tailed monkeys (*Cercopithecus solatus*): evidence for host-dependent evolution of SIV within the *C. lhoesti* superspecies. *J Virol* 73, 7734–7744.

Bibollet-Ruche, F., Bailes, E., Gao, F. & 13 other authors (2004). New simian immunodeficiency virus infecting De Brazza's monkeys (*Cercopithecus neglectus*): evidence for a *Cercopithecus* monkey virus clade. *J Virol* 78, 7748–7762.

Chen, Z., Telfier, P., Gettie, A., Reed, P., Zhang, L., Ho, D. D. & Marx, P. A. (1996). Genetic characterization of new West African simian immunodeficiency virus SIVsm: geographic clustering of household-derived SIV strains with human immunodeficiency virus type 2 subtypes and genetically diverse viruses from a single feral sooty mangabey troop. *J Virol* 70, 3617–3627.

Clewley, J. P., Lewis, J. C., Brown, D. W. & Gadsby, E. L. (1998). A novel simian immunodeficiency virus (SIVdrl) *pol* sequence from the drill monkey, *Mandrillus leucophaeus*. *J Virol* 72, 10305–10309.

Corbet, S., Muller-Trutwin, M. C., Versmisse, P. & 9 other authors (2000). *env* sequences of simian immunodeficiency viruses from chimpanzees in Cameroon are strongly related to those of human immunodeficiency virus group N from the same geographic area. *J Virol* 74, 529–534.

Courgnaud, V., Salemi, M., Pourrut, X. & 8 other authors (2002). Characterization of a novel simian immunodeficiency virus with a *vpu* gene from greater spot-nosed monkeys (*Cercopithecus nictitans*) provides new insights into simian/human immunodeficiency virus phylogeny. *J Virol* 76, 8298–8309.

Courgnaud, V., Abela, B., Pourrut, X., Mpoudi-Ngole, E., Loul, S., Delaporte, E. & Peeters, M. (2003a). Identification of a new simian immunodeficiency virus lineage with a *vpu* gene present among different cercopithecus monkeys (*C. mona, C. cephus, and C. nictitans*) from Cameroon. *J Virol* 77, 12523–12534.

Courgnaud, V., Formenty, P., Akoua-Koffi, C., Noe, R., Boesch, C., Delaporte, E. & Peeters, M. (2003b). Partial molecular characterization of two simian immunodeficiency viruses (SIV) from African colobids: SIVwrc from Western red colobus (*Piliocolobus badius*) and SIVolc from olive colobus (*Procolobus verus*). *J Virol* 77, 744–748.

Fukasawa, M., Miura, T., Hasegawa, A., Morikawa, S., Tsujimoto, H., Miki, K., Kitamura, T. & Hayami, M. (1988). Sequence of simian immunodeficiency virus from African green monkey, a new member of the HIV/SIV group. *Nature* 333, 457–461.

Gao, F., Bailes, E., Robertson, D. L. & 9 other authors (1999). Origin of HIV-1 in the chimpanzee *Pan troglodytes troglodytes*. *Nature* **397**, 436–441.

Hahn, B. H., Shaw, G. M., De Cock, K. M. & Sharp, P. M. (2000). AIDS as a zoonosis: scientific and public health implications. *Science* **287**, 607–614.

Hayami, M., Ido, E. & Miura, T. (1994). Survey of simian immunodeficiency virus among nonhuman primate populations. *Curr Top Microbiol Immunol* **188**, 1–20.

Higgins, D. G. & Sharp, P. M. (1989). Fast and sensitive multiple sequence alignments on a microcomputer. *Comput Appl Biosci* **5**, 151–153.

Hirsch, V. M., Dapolito, G. A., Goldstein, S. & 7 other authors (1993). A distinct African lentivirus from Sykes' monkeys. *J Virol* **67**, 1517–1528.

Hirsch, V. M., Olmsted, R. A., Murphey-Corb, M., Purcell, R. H. & Johnson, P. R. (1989). An African primate lentivirus (SIVsm) closely related to HIV-2. *Nature* **339**, 389–392.

Hirsch, V. M., Campbell, B. J., Bailes, E., Goeken, R., Brown, C., Elkins, W. R., Axthelm, M., Murphey-Corb, M. & Sharp, P. M. (1999). Characterization of a novel simian immunodeficiency virus (SIV) from L'Hoest monkeys (*Cercopithecus l'hoesti*): implications for the origins of SIVmnd and other primate lentiviruses. *J Virol* **73**, 1036–1045.

Huet, T., Cheynier, R., Meyerhans, A., Roelants, G. & Wain-Hobson, S. (1990). Genetic organization of a chimpanzee lentivirus related to HIV-1. *Nature* **345**, 356–359.

Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* **16**, 111–120.

Miura, T., Sakuragi, J., Kawamura, M. & 10 other authors (1990). Establishment of a phylogenetic survey system for AIDS-related lentiviruses and demonstration of a new HIV-2 subgroup. *AIDS* **4**, 1257–1261.

Peeters, M., Courgnaud, V., Abela, B. & 11 other authors (2002). Risk to human health from a plethora of simian immunodeficiency viruses in primate bushmeat. *Emerg Infect Dis* **8**, 451–457.

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

Salemi, M., De Oliveira, T., Courgnaud, V., Moulton, V., Holland, B., Cassol, S., Switzer, W. M. & Vandamme, A. M. (2003). Mosaic genomes of the six major primate lentivirus lineages revealed by phylogenetic analyses. *J Virol* **77**, 7202–7213.

Takehisa, J., Zekeng, L., Ido, E., Yamaguchi-Kabata, Y., Mboudjeka, I., Harada, Y., Miura, T., Kaptue, L. & Hayami, M. (1999). Human immunodeficiency virus type 1 intergroup (M/O) recombination in Cameroon. *J Virol* **73**, 6810–6820.

Tsujimoto, H., Cooper, R. W., Kodama, T. & 9 other authors (1988). Isolation and characterization of simian immunodeficiency virus from mandrills in Africa and its relationship to other human and simian immunodeficiency viruses. *J Virol* **62**, 4044–4050.

Tsujimoto, H., Hasegawa, A., Maki, N. & 7 other authors (1989). Sequence of a novel simian immunodeficiency virus from a wild-caught African mandrill. *Nature* **341**, 539–541.

Verschoor, E., Fagrouch, Z., Bontjer, I., Niphuis, H. & Heeney, J. (2004). A novel simian immunodeficiency virus isolated from a Schmidt's guenon (*Cercopithecus ascanius schmidti*). *J Gen Virol* **85**, 21–24.

Yamaguchi-Kabata, Y. & Gojobori, T. (2000). Reevaluation of amino acid variability of the human immunodeficiency virus type 1 gp120 envelope glycoprotein and prediction of new discontinuous epitopes. *J Virol* **74**, 4335–4350.

Yamaguchi-Kabata, Y., Yamashita, M., Ohkura, S., Hayami, M. & Miura, T. (2004). Linkage of amino acid variation and evolution of human immunodeficiency virus type 1 gp120 envelope glycoprotein (subtype B) with usage of the second receptor. *J Mol Evol* **58**, 333–340.

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Novel method of inactivation of human immunodeficiency virus type 1 by the freeze pressure generation method

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Abstract It has been reported that high-pressure (over 600 MPa) treatment at room temperature inactivates human immunodeficiency virus type 1 (HIV-1), and it has recently been shown that the high pressure generated by the expansion of water due to freezing (freeze pressure generation method, or FPGM) has an inactivating effect on bacteria and fungi. In this study, we examined the effects of treatment by FPGM on HIV-1. A sturdy vessel filled with water and securely closed with a lid was kept at 0°C to −30°C. High pressures of 200 MPa and 250 MPa were generated at −20°C and −30°C, respectively. When T-cell-tropic and macrophage-tropic laboratory strains of HIV-1 were kept at −10°C, the virus infectivity decreased to approximately 1/100, and was completely lost at −20°C and −30°C. Four T-cell-tropic and four macrophage-tropic laboratory strains and clinical isolates of HIV-1 became completely inactivated at −30°C. Treatment by FPGM at −20°C to −30°C reduced HIV-1 reverse transcriptase activity to approximately one tenth. In addition, treatment by FPGM at −20°C was found to destroy the ability of HIV-1 to bind to CD4+ cells. In conclusion, this study showed that treatment by FPGM at −20°C to −30°C destroyed the infectivity of a wide range of HIV-1 strains, and suggested that the mechanisms of HIV-1 inactivation were the reduction in viral enzyme activity and the loss of the cell-binding ability of a viral envelope protein.

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Introduction

High pressure is known to inactivate bacteria (Hite 1899; Timson and Short 1965). Pasteurization and processing of food products by high pressure has been in practical use for a long time (Cheftel 1995; Dumoulin and Hayashi 1998; Messens et al. 1997). High pressure has also been reported to inactivate several enveloped viruses such as influenza and herpes (Nakagami et al. 1992; Overman and Lewis 1959; Silva et al. 1992). We previously discovered the phenomenon that treatment at high hydrostatic pressures at room temperature of more than 400 MPa inactivates human immunodeficiency virus type 1 (HIV-1) (Nakagami et al. 1996; Otake et al. 2000), and proposed the application of this technique to the production of blood preparations presenting a low risk of infection. However, high hydrostatic pressure treatment requires a large-scale high-pressure generator. Recently, the phenomenon was reported that high pressure is generated when water, filling a sealed vessel and cooled below 0°C, expands as it freezes (freeze pressure generation method, or FPGM). FPGM has an inactivating effect on yeast, bacteria, and fungi (Hayakawa et al. 1998) and enables the generation of high pressures, using a simpler apparatus than a high-hydrostatic pressure generator. We investigated the effects of FPGM on HIV-1 and its action mechanisms.

Materials and methods

FPGM in the chamber

To obtain the theoretical pressure, we developed a pressure vessel that could withstand the high pressure generated by freezing water. The vessels were sealed with a screw cap with water leakage-preventing packing, and their walls were sufficiently thick enough to withstand a pressure of 300 MPa. A chamber was equipped with a pressure gauge on the cap. The chamber volume was 1,000 ml. The chamber was tightly sealed and kept under sub-zero temperatures

in a temperature-controlled freezer for 24 h. The pressure generated was measured by a pressure gauge.

To test virus inactivation, a 100-ml pressure vessel with no pressure gauge was used. A 3-ml plastic tube containing a virus suspension was placed in a pure-water-filled vessel, which was left in a freezer for 24 h, and then immersed in tap water.

Preparation of virus samples

HIV-1 strains used in this study were four T-cell-tropic strains (two laboratory strains, LAI and RF, and two clinical isolates, KK-1 from an AIDS patient and KK-2 from an asymptomatic carrier) and four macrophage tropic strains (a laboratory strain, HTLV-IIIBa-L; two clinical isolates, KK-20, KK-23; and a drug-resistant strain to reverse transcriptase inhibitors and protease inhibitors, KK-86-4).

T-cell tropic strains were inoculated into the CD4+ cell line (MT-4 cells). The cells were maintained in RPMI-1640 medium, containing 10% fetal calf serum (FCS), and incubated at 37°C in the presence of 5% CO_2. After 5 days, the supernatant of the cell culture was collected as a viral solution.

Macrophage tropic strains inoculated into PHA-stimulated peripheral blood mononuclear (PBM) cells from healthy donors. Then PBM cells were incubated in RPMI-1640 medium containing 20% FCS and 200 U/ml of interleukin-2 (Shionogi Laboratories). After 10 days, the supernatant of the cell culture was collected as a viral solution.

Measurement of viral infectivity

The infectivity of T-cell tropic strains was determined based on the measurement of the 50% tissue culture-infectious doses ($TCID_{50}$) in MT-4 cells. Serial tenfold dilutions of virus samples were allowed to infect MT-4 cells in 96-well microtiter plates. Five days later, the cytopathic effects of HIV-1 were observed under a microscope, and the $TCID_{50}$ was calculated.

The infectivity of macrophage-tropic strains was measured using MAGIC-5 cells. MAGIC-5 (MAGI-CCR5 cell line) was developed from MAGI (HeLa-CD4-LTR-β-gal) cells and was kindly supplied by Dr. M. Tatsumi. MAGIC-5 cells were plated in 96-well microtiter plates in Dulbecco's modified Eagle medium (DMEM), supplemented with 2.5% FCS the day before infection. The cells were infected with serial dilutions of viral samples for 2 h at 37°C. Then, the virus solution was removed and 200 μl DMEM containing 2.5% FCS was added. After 2 days of culture, the medium was removed, and the cells were fixed at room temperature with 50 μl of a solution of 1% formaldehyde and 0.2% glutaraldehyde in phosphate-buffered saline (PBS), pH 7.2, for 5 min. The cells were washed twice with PBS and incubated for 1 h at 37°C in 100 μl of the staining solution, consisting of 4 mM potassium ferrocyanide, 4 mM potassium ferricyanide, 2 mM $MgCl_2$ and 40 mg/ml X-gal. Cells stained blue were counted under a microscope and the infectivity was indicated as blue focus units.

Reverse transcriptase activity

The reverse transcriptase (RT) activity in the samples was measured using an RT assay kit (Asahi Chemical Industry, Japan). This kit was based on a highly sensitive RT assay using primer-templates that were immobilized on microplates.

Measurement of the HIV core protein p24

An HIV-1 p24 antigen capture EIA kit (RetroTec, ZeptoMetrics, Buffalo, N.Y., USA) was used for the measurement of virus antigenicity.

Measurement of the cell-binding ability of viral envelope protein

FPGM-treated or untreated HIV-1 LAI strain samples were concentrated 100-fold by ultracentrifugation. The resulting samples were allowed to react with MT-4 cells at 37°C for 30 min, and then washed with PBS to remove unreacted viruses. A pair of MT-4 cells and the anti-HIV-1 gp120 (V3) monoclonal antibody (0.5β) were reacted at 37°C for 30 min. The cells were washed and then reacted with anti-mouse IgG1 antibody labeled with FITC (Research Diagnostics, Flanders, N.J., USA) at 37°C for 30 min. The cells were washed and then re-suspended in PBS containing 3% formaldehyde. The fluorescence intensity of the suspension was measured using a flow cytometry (Epics XL, Beckman Coulter, Fullerton, Calif., USA.), and the percentage fluorescence intensity of the cells was calculated.

Results

Generation of high pressures in the vessels

The chamber was filled with water, tightly sealed, and stored at sub-zero temperatures in a temperature-controlled freezer for 24 h.

The relationship between the pressure generated in the vessel and the temperature of the freezer is shown in Fig. 1. The pressure in the vessel decreased approximately linearly in the range of 0°C to −30°C, reaching 250 MPa at −30°C. The curve precisely fitted the equilibrium curve of water and type I ice in the range of 0°C to −22°C. However, the pressure generated increased with decreasing temperature in the range of −22°C to −30°C, reaching 250 MPa at −30°C, indicating that super-cooling had occurred in the range of −22°C to −30°C.

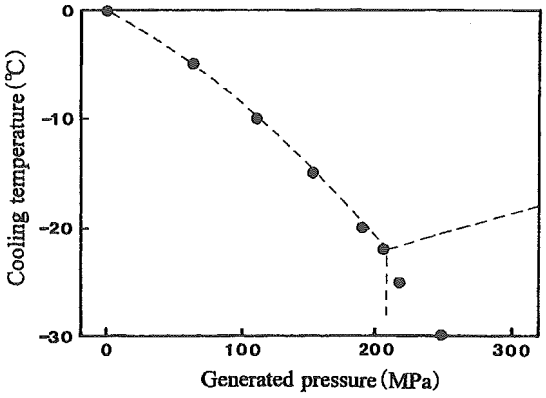

Fig. 1 Relationship between pressure generated in the pressure chamber and temperature of the cooling bath. The pressure chamber was kept in the cooling bath at different temperatures for 2 h, and the pressure generated in the chamber was read on the pressure gauge. • Observed pressure, - - - equilibrium curve of water and type I or type III ice (from Bridgman 1912)

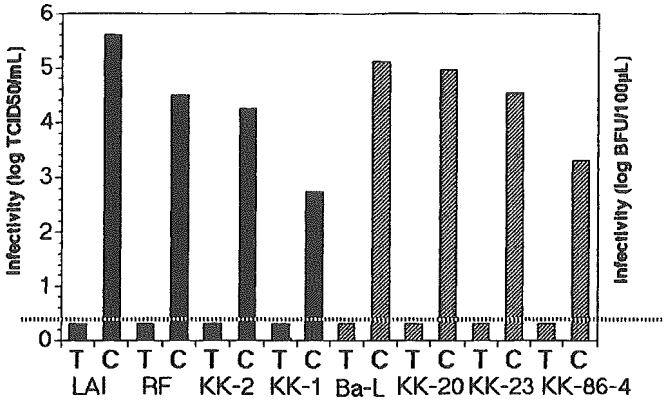

Fig. 2 Effects of the freeze pressure generation method (FPGM) on human immunodeficiency virus type 1 (HIV-1) infectivity. Four T-cell-tropic strains and four macrophage-tropic strains of HIV-1 were kept in an open or sealed vessel at −30°C for 24 h, then warmed to 4°C, and viral infectivity was measured. *T* Sample was kept in the sealed vessel, *C* sample was kept in the open vessel, *BFU* blue focus units, *dotted line* is limitation of detection. Data are mean values for two separate experiments

Effect of the treatment by FPGM on viral infectivity

The relationship between cold treatment temperature and virus inactivation was examined. The infectivities of the T-cell-tropic HIV-1 strain, LAI, and the macrophage-tropic HIV-1 strain, Ba-L, decreased to approximately 1/100 at −10°C, and became inactivated below the lower limit of detection at −20°C and −30°C (Table 1).

To examine the effects of treatment by FPGM in a wide range of virus strains, we used four T-cell-tropic and four macrophage-tropic strains. Treatment by FPGM at −30°C destroyed the infectivity of all HIV-1 strains, including clinical isolates and strains resistant to anti-AIDS drugs (Fig. 2).

Effect of treatment by FPGM on RTactivity

The effect of treatment by FPGM on RT activity of HIV-1 samples was measured (Fig. 3). Treatment by FPGM at

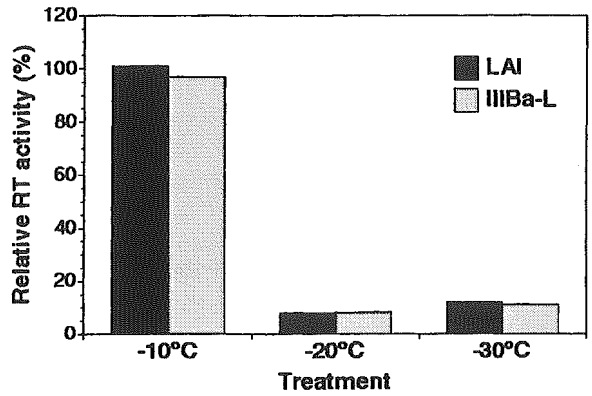

Fig. 3 Effects of FPGM on the reverse transcriptase (*RT*) activity of HIV-1. Relative RT activity is the ratio between the RT activity of the sample in the open vessel and the sealed vessel. Data are mean values for two separate experiments

Table 1 Effects of freeze pressure generation on human immunodeficiency virus type 1 (*HIV-1*) infectivity. T-cell tropic strain (*LAI*) and macrophage-tropic strain (*HTLV-IIIBa-L*) of HIV-1 were kept in open vessel or sealed vessel at −10, −20 and −30°C for 24 h, then warmed to 4°C, and viral infectivity was measured. Data are mean values for two separate experiments

Strain of HIV-1	−10°C		−20°C		−30°C	
	Open vessel	Sealed vessel	Open vessel	Sealed vessel	Open vessel	Sealed vessel
LAI (log $TCID_{50}$/ml)	5.25	3.5	5.25	<0.5	5.5	<0.5
HTLV-IIIBa-L (log BFU[a]/ml)	6.69	4.45	6.72	<1.0	6.11	<1.0

[a] *BFU* Blue focus units

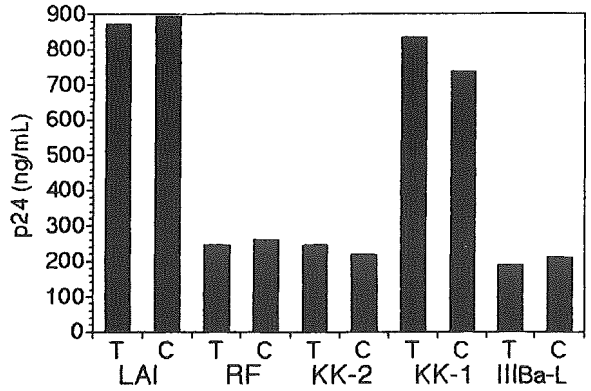

Fig. 4 Effects of FPGM on viral core values. The level of the HIV-1 core protein p24 in viral samples was measured using antigen-detecting EIA methods. *T* Sample was kept in the sealed vessel, *C* sample was kept in the open vessel. Data are mean values for two separate experiments

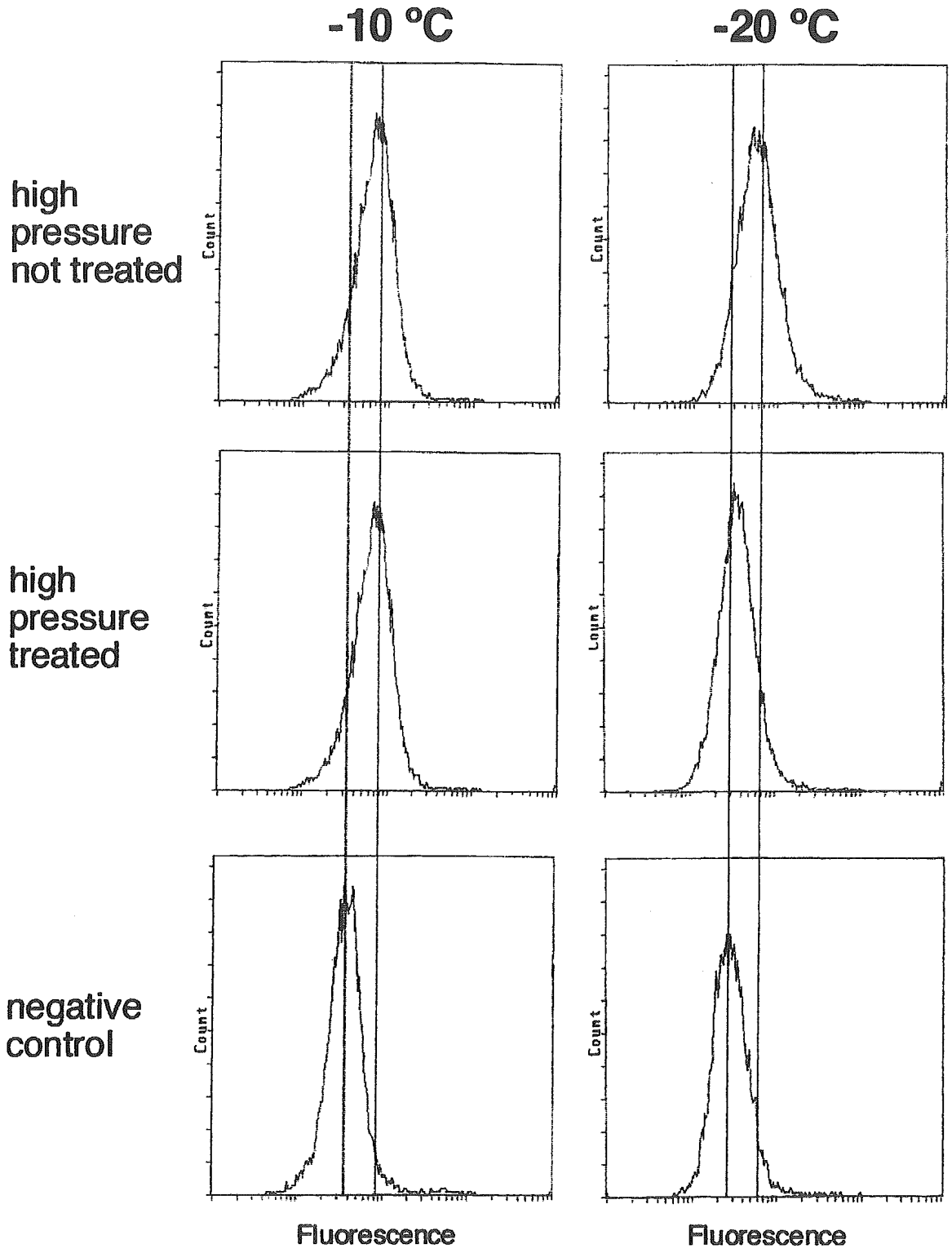

Fig. 5 Effects of FPGM on the cell-binding ability of HIV-1. FPGM treated or untreated HIV-1 samples were reacted with MT-4 cells. The amount of virus that combined with the MT-4 cells was measured using the anti-HIV-1 envelope monoclonal antibody and flow cytometry. The *left line* is the peak of the untreated MT-4 cells, and *right line* is the peak of the MT-4 cells that reacted with virus sample in the open vessel at −10°C or −20°C