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Endemic versus epidemic viral spreads display distinct patterns of HTLV-2b replication

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Abstract

As the replication pattern of leukemogenic PTLVs possesses a strong pathogenic impact, we investigated HTLV-2 replication in vivo in asymptomatic carriers belonging into 2 distinct populations infected by the same HTLV-2b subtype. They include epidemically infected American blood donors, in whom HTLV-2b has been present for only 30 years, and endemically infected Bakola Pygmies from Cameroon, characterized by a long viral endemicity (at least few generations). In blood donors, both the circulating proviral loads and the degree of infected cell proliferation were largely lower than those characterizing asymptomatic carriers infected with leukemogenic PTLVs (HTLV-1, STLV-1). This might contribute to explain the lack of known link between HTLV-2b infection and the development of malignancies in this population. In contrast, endemically infected individuals displayed high proviral loads resulting from the extensive proliferation of infected cells. The route and/or the duration of infection, viral genetic drift, host immune response, genetic background, co-infections or a combination thereof might have contributed to these differences between endemically and epidemically infected subjects. As the clonality pattern observed in endemically infected individuals is very reminiscent of that of leukemogenic PTLVs at the pre-leukemic stage, our results highlight the possible oncogenic effect of HTLV-2b infection in such population.

Keywords: Deltaretroviruses; HTLV; HTLV-2; HTLV-2b; Epidemiology; Genetic variability; Clonal expansion; Replication; Leukemogenesis

Introduction

The retrovirus HTLV-2 has not been clearly associated with the development of hematological malignancies. Only a few reports have described lymphomas in HTLV-2 carriers (Farias de Carvalho et al., 1997; Guitart, 2000; Poiesz et al., 2000). In contrast, the virus has been found to be etiologically linked with the development of neurological symptoms (Murphy et al., 1997) and with susceptibility to certain bacterial and fungal infections (Murphy et al., 1999). The predominant mode of replication of primate T cell lymphotropic viruses (PTLVs) in vivo is via the clonal expansion of the infected cell. Such a mode of replication has been demonstrated for HTLV-1 (Mortreux et al., 2003), HTLV-2 (Cimarelli et al., 1996), and STLV (Gabet et al., 2003a, 2003b). Such cell-associated proviral multiplication explains the low level of genetic drift of these viruses (Mortreux et al., 2003).

There are three main subtypes of HTLV-2. HTLV-2a and HTLV-2b are both present in intravenous drug users (IDUs) in Europe, the USA, Vietnam and in several native American Indian or African tribes (Slattery et al., 1999), whereas HTLV-2d was isolated from a Mbuti Efe Pygmy (Vandamme et al., 1998). The phylogenetic distinctiveness of an additional subtype, HTLV-2c, composed mainly of strains from Brazilian Kayapo Indians and IDU from Sao Paulo, is less clear (Slattery

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et al., 1999). Two clearly distinct populations of individuals are infected with HTLV-2, corresponding respectively to endemically and epidemically infected populations. HTLV-2 infection is endemic in culturally and geographically isolated ethnic tribes, American Indians in North America (Hjelle et al., 1993), Central America (Lairmore et al., 1990) and peripheral regions of South America (Biglione et al., 1993; Duenas-Barajas et al., 1993; Ferrer et al., 1993; Ijichi et al., 1993), as well as in Bakola and Efe Bambuti Pygmies in Central Africa (Gessain et al., 1995; Goubau et al., 1992; Vandamme et al., 1998). These populations are characterized by long viral endemicity time (at least few generations) with breast feeding and sexual contacts as the major routes of infection. Epidemically infected individuals mainly correspond to IDUs in the USA, Europe and Asia (Vandamme et al., 2000). This population is characterized by a short viral endemicity (~30 years) and by needle sharing and sexual contacts as the major routes of dissemination.

Recently, by using molecular phylogenies, Salemi et al. investigated the population dynamics of HTLV-2 in IDUs and endemically infected tribes (Salemi et al., 1999). Molecular clock analysis showed that HTLV-2 has two different evolutionary rates, with the molecular clock for the virus ticking 150–350 times faster in IDUs than in endemically infected tribes. The present study was conducted in order to compare HTLV-2 replication between individuals from both groups. To this end, both the proviral loads and the clonal distribution of circulating HTLV-2-infected cells were compared between endemically infected Bakola Pygmies from Cameroon and American blood donors with epidemic virus spread, both populations being infected by the same HTLV-2b subtype, in the absence of HIV co-infection.

Results

High proviral load in HTLV-2b endemically infected individuals

The lower limit of detection was 1 copy per 5000 PBMCs (0.02%). As shown in Table 1, we estimated the absolute copy number of HTLV-2b proviral DNA per 100 PBMCs. Each sample was analyzed in triplicate, and the values represented in the table correspond to the mean viral copy number obtained for each sample after 3 experiments. Although all analyzed samples had circulating HTLV-2b sequences, as evidenced by nested PCR (not shown), 24/43 (56%) were found to have proviral loads under the detection threshold of our quantitative PCR assay. These samples with very low HTLV-2b proviral loads derived from 8 of the 13 blood donors (62%), whereas all samples derived from the Bakola Pygmies displayed proviral loads >0.02% ($P = 0.003$, Fisher's Exact Test). Assuming a proviral load of 0.02% for samples under the quantitative PCR detection threshold, the copy numbers in US blood donors varied from 0.02% to 2.11%; those of Bakola Pygmies varied from 0.4% to 3.2%. The mean ± standard error of mean (SEM) and median of the copy numbers were 0.28% ± 0.09% and 0.02% for US blood donors, and 1.52% ± 0.29% and 1.51% for Bakola Pygmies. The median copy number of Bakola Pygmies was more than 75-fold higher than that of US blood donors. The difference between Bakola Pygmy and blood donor samples was statistically significant ($P = 0.004$, t test for unpaired samples). In contrast, when the comparison was restricted to samples with proviral loads ≥0.02%, there was no significant difference in the mean circulating HTLV-2b copy number between the 10 samples derived from endemically (mean ± SEM, 1.52% ± 0.29%) and the 9 samples derived from epidemically infected individuals (mean ± SEM, 0.96% ± 0.24%) ($P = 0.3$). DNA samples derived from 5 blood donors were tested 5 times for HTLV-2b proviral loads using PBMCs samples drawn at several years interval over a (~10-year period. Three were found repeatedly negative for HTLV-2b quantitative PCR (<0.02%). One was found repeatedly positive with low fluctuation of the proviral copy number between samples (0.2%, 0.7%, 1.6%, 1.01% and 0.80%). The remaining individual had 4 negative (<0.02%) and one positive samples; this one corresponded to the first sample harvested and displayed a low proviral load (0.04%).

Clonal expansion of HTLV-2b-infected cells in individuals with high or low proviral loads

The HTLV-2b LMPCR detection threshold was 20 copies in 150,000 cells, meaning that each detected signal after run-off analysis of quadruplicate LMPCR products corresponded to a cluster of at least 20 cells sharing the same HTLV-2b integration site and therefore belonging into the same clone. The stochastic nature of HTLV-2b LMPCR (Cavrois et al., 1995) was found to appear at HTLV-2b integration site frequencies ranging between 20 and 1000 copies of the HTLV-2b provirus per microgram of DNA (Fig. 1). Hence, a quadruplicate LMPCR analysis of *Nla*III digested plasmid bearing an integrated HTLV-2b LTR showed that above 1000 copies (diluted in 1 μg) detection was 4/4. At copy numbers ranging from 20 to 125, 125 to 500 and 500 to 1000, detection was 1/4, 2/4 and 3/4, respectively. Infected clones detected once or twice after quadruplicate analysis were defined as having a polyclonal pattern of replication, whereas those detected more frequently, i.e., 3 or 4 times, were defined as having oligoclonal distribution. Quadruplicate LMPCR (4 × 0.5 μg) was carried out with samples derived from 9 Bakola Pygmies (9 samples) and 12 blood donors (27 samples). Typical results from quadruplicate LMPCR analysis of HTLV-2b integration in the DNA from infected individuals are shown in Fig. 2. All 6 analyzed samples displayed signals each corresponding to a cluster of cells sharing the same integration sites, i.e., belonging in the same clone deriving from a unique infected progenitor. No signal was observed with negative controls (NC). Overall, the figure shows that there was a wide variation in the number of detected clones between samples (compare samples 670502 and PD303926), their distribution being detailed in Table 1. In addition, the figure clearly evidence that the frequency of detection varied from clone to clone as some were detected 1, 2, 3 or 4 times. As the frequency of detection represent the degree of clonal expan-

sion, it appears evident from Fig. 2 that there was a wide variation in the degree of clonal expansion between clones. In addition, as for the overall number of detected clones, their degree of expansion varied between sample. Hence, 0 and 2 oligoclonally expanded forms were respectively detected samples derived from patient PD501686 and PD303926, whereas 11 and 11 such clone were respectively present in samples from patient 670502 and 550602. In fact, circulating

Table 1
Initial characteristics, proviral loads and clonality of HTLV-2b infected cells in endemically and epidemically infected individuals

Status	UCN[a]	Age	Gender	Race/Ethnicity	Risk/Route of HTLV-2b infection	Time from contamination	Duration of follow-up (years)	Proviral loads[b]	Overall number of HTLV-2b circulating clones after quadruplicate LMPCR[c]	Number of clones detected 1 or 2 times[c]	Number of clones detected 3 or 4 times[c]
Bakola Pygmies											
	550602	30	F	Pygmy	Breast-feeding/ Sexual contacts	–		2.03	22	12	10
	590102	55	F	Pygmy	Breast-feeding/ Sexual contacts	–		1.87	15	6	9
	940701	36	M	Pygmy	Breast-feeding/ Sexual contacts	–		0.4	15	12	3
	590101	58	M	Pygmy	Breast-feeding/ Sexual contacts	–		2.64	26	14	12
	670502	60	F	Pygmy	Breast-feeding/ Sexual contacts	–		0.57	27	18	9
	670802	36	F	Pygmy	Breast-feeding/ Sexual contacts	–		0.84	40	29	11
	500102	50	F	Pygmy	Breast-feeding/ Sexual contacts	–		0.65	33	24	9
	590303	10	M	Pygmy	Breast-feeding	10		1.24	15	7	8
	670102	50	F	Pygmy	Breast-feeding/ Sexual contacts	–		1.77			
	330302	25	F	Pygmy	Breast-feeding/ Sexual contacts	–		3.2	28	16	12
Blood donors											
	301559	44	F	Vietnamese	No	–		<0.02	0	0	0
	302298	54	M	Hispanic	>7 sexual partners	18		1.75	26	25	1
	303926	31	F	Hispanic	No	–	0.00	0.22	21	19	2
							2.00	0.69	15	12	3
							4.25	1.56			
							7.00	1.01			
							9.20	0.8	6	4	2
	316023	32	F	White	>7 sexual partners	4		<0.02	0	0	0
	401243	32	F	White	>7 sexual partners	9	0.00	<0.02	0	0	0
							1.78	<0.02			
							4.47	<0.02	0	0	0
							6.99	<0.02	0	0	0
							9.88	<0.02	0	0	0
	501993	30	F	Black	>7 sexual partners	7		2.11	22	15	7
	100238	33	F	Black	Sex with IDU	8		<0.02	0	0	0
	302276	45	F	White	>7 sexual partners	14		<0.02	0	0	0
								<0.02	0	0	0
								<0.02	0	0	0
								<0.02	0	0	0
								<0.02	0	0	0
	304507	39	M	Black	IDU	18		<0.02	0	0	0
	304518	34	F	White	Transfusion	12		0.43			
	400322	36	F	White	Sex with IDU	4		<0.02	0	0	0

(continued on next page)

Table 1 (*continued*)

Status	UCN[a]	Age	Gender	Race/Ethnicity	Risk/Route of HTLV-2b infection	Time from contamination	Duration of follow-up (years)	Proviral loads[b]	Overall number of HTLV-2b circulating clones after quadruplicate LMPCR[c]	Number of clones detected 1 or 2 times[c]	Number of clones detected 3 or 4 times[c]
	490046	58	M	White	Transfusion	21	0.00	<0.02	2	2	0
							2.11	<0.02	6	6	0
							4.54	<0.02	1	1	0
							7.27	<0.02	0	0	0
							9.17	<0.02	0	0	0
	501686	51	F	Indian (North American)	Transfusion	11	0.00	0.04	3	3	0
							2.11	<0.02			
							4.53	<0.02	0	0	0
							6.61	<0.02	3	3	0
							8.82	<0.02	4	4	0

[a] UCN unique carrier number.
[b] per 100 PBMCs.
[c] per 150,000 PBMCs.

clones of HTLV-2b bearing cells could be detected in 19 of the 36 samples (53%) (Fig. 2, Table 1). All 17 samples with negative LMPCR results had a circulating proviral load <0.02%. Five samples with proviral loads below the quantitative PCR threshold were found to have a positive result after LMPCR (Table 1). Of these, 4 displayed only 1 to 6 one-time detected signals (mean ± SEM, 3.2 ± 0.97) after quadruplicate experiments, whereas one had a unique two-time detected clone. This result was consistent with the higher sensitivity of LMPCR, when compared to real-time quantitative PCR. The mean proviral load of samples having a negative LMPCR result was 0.02% (assuming a 0.02% value for samples below the detection threshold) versus 0.99% for those with a positive result. Although the ubiquitous glucose transporter GLUT-1, the receptor for HTLV-1/2 retroviruses, is expressed by both $CD4^+$ and $CD8^+$ T cells (Manel et al., 2003), the $CD8^+$ subset has been found to be the main reservoir for HTLV-2 in vivo (Casoli et al., 1995; Ijichi et al., 1992). We therefore enriched the cellular fraction in $CD8^+$ T cells by negative selection, as described in the Materials and methods section. This was performed with 2 samples derived from 2 individuals (UCN 60689 and 61006) with proviral loads <0.02% and no detectable clone of HTLV-2b positive cells after LMPCR. The same procedure was applied to a sample having a positive LMPCR result (UCN 60998) used as positive control. After negative selection, purity, contamination and percentage of

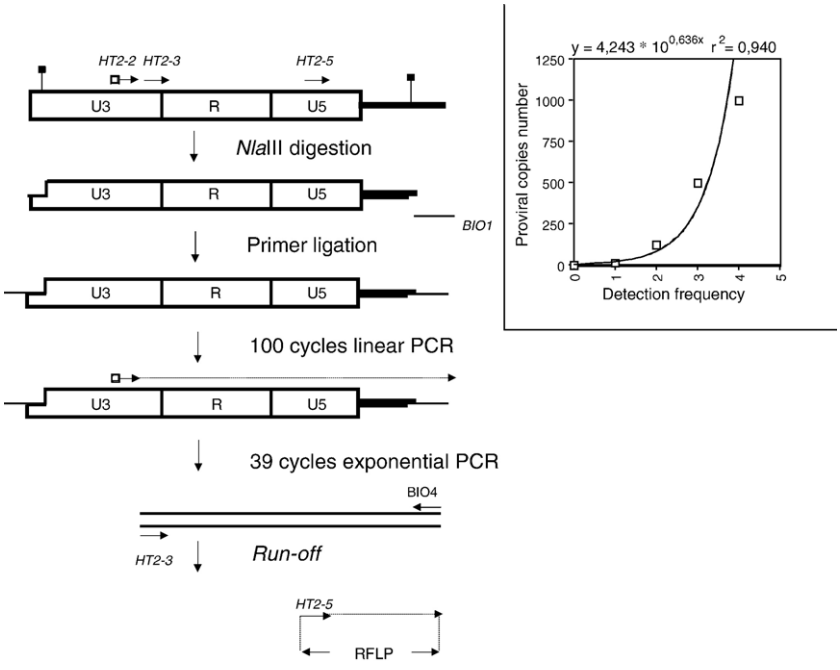

Fig. 1. Ligation-mediated PCR protocol used for amplifying 3′ HTLV-2b integration sites. The inset represents the stochastic nature of HTLV-2b 3′ integration site detection by quadruplicate IPCR.

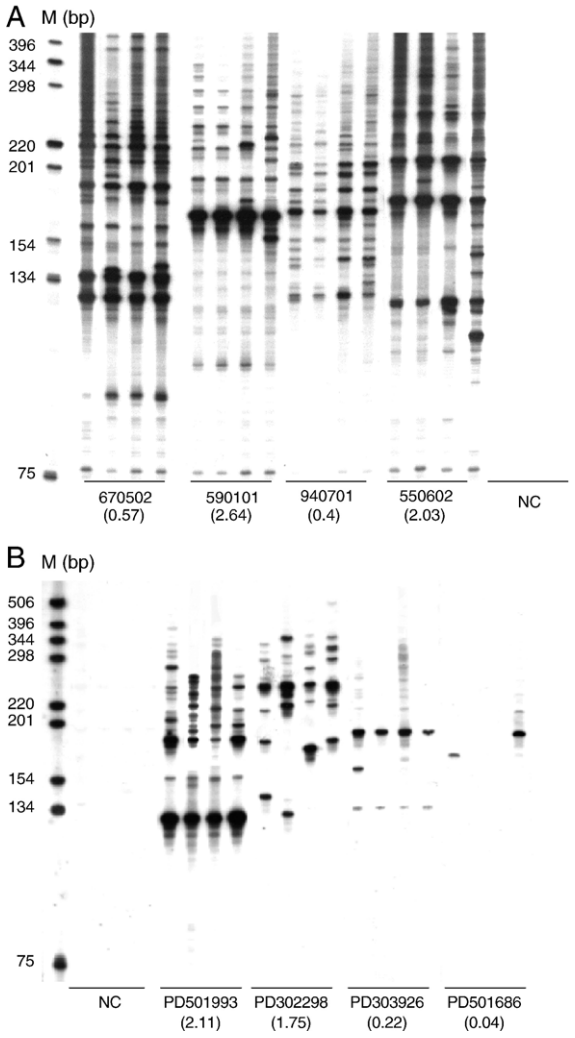

Fig. 2. Ligation-mediated PCR analysis of HTLV-2b replication in peripheral blood cells from endemically (A) and epidemically (B) infected individuals. Each sample was analyzed in quadruplicate by LMPCR as detailed in the Material and methods section. The molecular weight marker (M) was the MWM × DNA. Carriers are identified by their unique carrier number (UCN). NC, negative control. For each sample, the percentage of infected peripheral blood mononuclear cells (PBMCs), as estimated by quantitative PCR, is given between brackets.

$CD8^+$ T cell enrichment were, for samples derived from carriers 60689, 61006 and 60998, 82.28%, 0.01% and 514%; 89.77%, 0.03% and 504%; and 86.51, 0.09% and 474%, respectively. DNA was extracted from enriched cellular fractions, and LMPCR was performed as detailed in the Materials and methods section. Typical results of the run-off analysis of amplified products deriving from the DNA of $CD8^+$ T cells are shown in Fig. 3. After enrichment, 3 clones of HTLV-2b positive cells could be evidenced for $CD8^+$ cells derived from blood donor 401243. One clone was detected once and two were detected twice, with a clonal frequency of 2.1 10^{-3} and 1.3 10^{-2} per 100 PBMCs, respectively. Similarly, 4 HTLV-2b positive clones could be evidenced for $CD8^+$ cells deriving from blood donor 501686. Two clones were detected once, and one was detected twice, with a clonal frequency of 2.3 10^{-3} and 1.4 10^{-2} per 100 PBMCs, respectively. These values are consistent with negative LMPCR results obtained with the DNA deriving from whole PBMCs. Together, these results suggest that circulating clones of HTLV-2b positive cells can be found in all HTLV-2b-infected individuals, if $CD8^+$ enrichment is done for those having very low proviral loads.

High level of infected T cell oligoclonal proliferation in endemically infected HTLV-2b carriers

Having found a significantly higher circulating proviral load in endemically than in epidemically HTLV-2b-infected individuals, we subsequently compared the clonal distribution of integrated proviruses within circulating lymphocytes between the 2 populations. Typical data concerning HTLV-2b integration sites in samples derived from 4 Bakola Pygmies and 4 blood donors are presented in Fig. 2 while Table 1 summarizes the results obtained for the 21 HTLV-2b carriers studied. Ten of the 27 samples derived from blood donors had positive LMPCR results, whereas all 9 samples derived from Bakola Pygmies displayed circulating clones of HTLV-2b positive cells. Indeed, circulating clones of HTLV-2b positive cells could be evidenced in 5/12 (42%) blood donors versus 9/9 (100%) Bakola Pygmies. This difference was statistically significant ($P = 0.007$, Fisher's Exact Test). It is evident from Fig. 2 and Table 1 that, as for proviral loads, the degree of clonal expansion of HTLV2b positive cells was significantly higher in endemically infected individuals. After quadruplicate LMPCR, the overall number of HTLV-2b positive clones was significantly higher in Bakola Pygmies than in blood donors (Fig. 2, Table 1). It ranged from 15 to 40 for the former (mean ± SEM, 24.56 ± 2.91; median 26), and from 0 to 26 for the latter (mean ± SEM, 3.85 ± 1.40; median 0.00, $P < 10^{-4}$, Mann–Whitney test). As the overall number of HTLV-2b positive

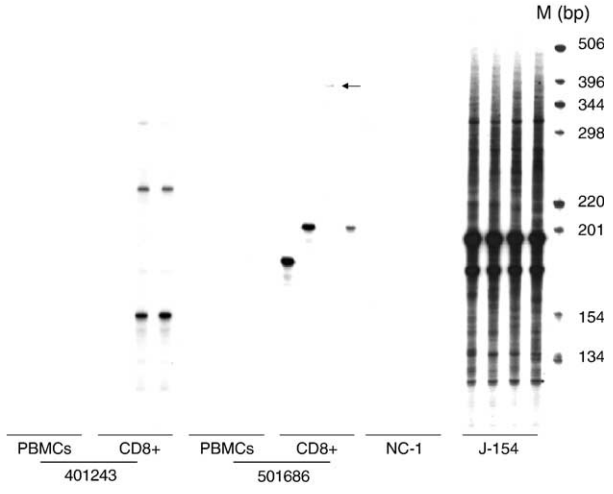

Fig. 3. Ligation-mediated PCR analysis of HTLV-2b integration within the DNA extracted from $CD8^+$ T cells. PBMCs were enriched in $CD8^+$ T cells by negative selection, as described in the Materials and methods section. Carriers are identified by their UCN. For each carrier, HTLV-2b integration is analyzed in quadruplicate for both the PBMCs and $CD8^+$ T cells. J-154 corresponds to a positive control (DNA from an HTLV-2b cell line established from the PBMCs of a Bakola Pygmy). And horizontal arrow identifies a faint signal obtained with sample from carrier 501686.

clones, the numbers of polyclonally and oligoclonally expanded cells were significantly higher in Bakola Pygmies: 15.33 versus 3.33, and 9.22 versus 0.52, respectively ($P < 10^{-4}$ for both, Mann–Whitney test). We next restricted the comparison to the 19 samples having at least one detected clone after LMPCR. The overall number of clones remained significantly higher in Bakola Pygmies than in blood donors (24.56 versus 10.4, $P = 0.004$, Mann–Whitney test). However, the mean number of polyclonally expanded cells was not significantly different between Bakola Pygmies and blood donors (12.5 versus 7.8, $P = 0.65$, Mann–Whitney test), whereas that of oligoclonally expanded cells was significantly higher in endemically infected individuals (14.8 versus 5.7, $P < 10^{-4}$, Mann–Whitney test). Therefore, these results demonstrated that the higher circulating proviral loads that characterized endemically infected individuals, when compared to epidemically infected subjects, resulted from a significantly higher degree of clonal expansion of circulating HTLV-2b-infected cells.

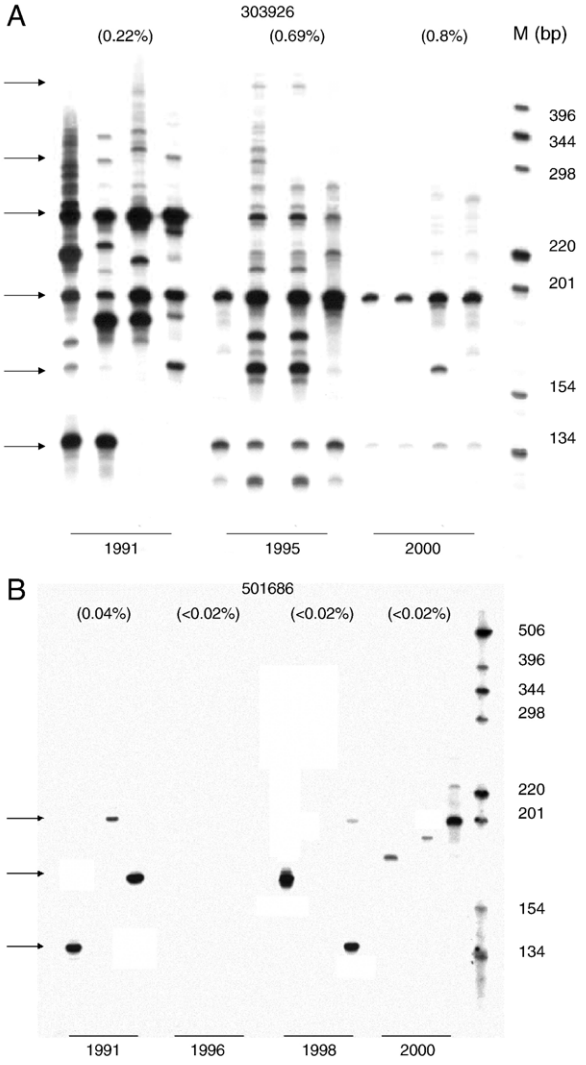

Fig. 4. Persistent clonal expansion of HTLV-2b positive cells in vivo. Quadruplicate LMPCR was carried out over time with samples derived from carriers 303926 (A) and 501686 (B), as described in the Materials and methods section. Persistent clones are identified by horizontal arrows.

Persistent clonal expansion of HTLV-2b-infected cells over time

DNA samples from PBMCs derived from the 5 blood donors monitored for proviral loads over time were also tested 2–5 times (20 samples tested) for the clonality of HTLV-2b-infected cells using samples drawn at several years interval over a ~10-year period. Typical results of LMPCR analysis of samples harvested over time are shown in Fig. 4. Of the 3 blood donors with very low proviral loads over time (<0.02%), 2 (UCN 401243 and 302276), tested 4 and 5 times over a ~10-year period, were found repeatedly negative for HTLV-2b quadruplicate LMPCR. In the remaining blood donor with repeatedly negative quantitative PCR results (UCN 490046), clones of HTLV-2b positive cells could be detected in the first analyzed sample as well as in those harvested 2.1 and 4.5 years later, whereas the last 2 samples, harvested at 7.3 and 9 years, gave negative results. For this blood donor, the first, second and third samples with positive LMPCR results displayed 2, 6 and 1 clones of HTLV-2-infected cells, respectively, each of these being detected only once after quadruplicate experiments and therefore having a clonal frequency of 20–125/150,000 (Table 1). The blood donor with repeatedly positive quantitative PCR results (UCN303926, Fig. 4A) was tested for clonality on three occasions, at enrollment then 2 and 8.2 years later. All 3 samples displayed positive LMPCR results with a pattern of oligoclonal expansion of HTLV-2b-infected cells. It is clear from Fig. 4A that some of these HTLV-2b positive clones were persistently detected over a 2- to 8-year period of time. The remaining blood donor tested over time (UCN 501686) had a proviral load of 0.04% in the first harvested sample, whereas the remaining 4 samples, harvested over a 9-year period, had very low proviral loads (<0.02%) (UCN501686, Fig. 4B). Three clones of HTLV-2b positive cells were detected in the first samples, all being detected only once. Three additional samples with very low proviral loads harvested over time were also tested by LMPCR. No signal was observed at 5.5 years, whereas 3 and 4 clones were detected at 7 and 9 years, respectively, all being detected only once after quadruplicate experiments. Therefore, as for other PTLV, the pattern of HTLV-2b replication in vivo involves the persistent clonal expansion of infected cells. As estimated from their frequency of detection, the degree of clonal expansion of persistently detected clones could vary over time. However, as for proviral loads, there was no significant correlation between the pattern of clonal expansion and the duration of follow-up in these 5 blood donors.

Discussion

The study shows that the pattern of HTLV-2b replication in vivo is strongly different between endemically infected Bakola Pygmies and epidemically infected blood donors. The former regularly display high proviral loads resulting from the extensive proliferation of infected cells, whereas the latter have low or very low proviral loads associated with a weak degree of clonal expansion.

An enrichment of PBMCs in $CD8^+$ T cells, the preferential reservoir for HTLV-2 in vivo (Casoli et al., 1995; Ijichi et al., 1992), permitted to detect clones of infected cells in individuals with very low proviral loads. Accordingly, rather than being restricted to carriers with high proviral loads (Cimarelli et al., 1996), clones of HTLV-2b positive cells seem to be present in all infected individuals. Therefore, HTLV-2b replicates as other PTLV, i.e., by a combination of reverse transcription with clonal expansion. However, 62% of HTLV-2b-infected blood donors displayed very low proviral loads (<0.02%) and the clonal frequency of circulating HTLV-2b positive cells was less than 1.3 10^{-2}% in 33.3% of the population studied. These values clearly distinguish HTLV-2b carriers from other PTLV-infected individuals or animals. Hence, for HTLV-1 (Gabet et al., 2000; Nagai et al., 1998; Wattel et al., 1992), and STLV-1 (Gabet et al., 2003a, 2003b), samples with less than 0.02% of infected cells are exceptional (Nagai et al., 1998; Shinzato et al., 1991; Wattel et al., 1992), and expanded cells with a clonal frequency lower than 1.3% have never been observed to date, even in asymptomatically infected organisms (Cavrois et al., 1996a, 1996b; Cavrois et al., 1998; Gabet et al., 2000, 2003a, 2003b; Leclercq et al., 1998; Mortreux et al., 2001a, 2001b, 2003; Wattel et al., 1995). Therefore, it appears that the replication of HTLV-2b in vivo involves a significantly lesser degree of clonal expansion than that of other PTLV, resulting in significantly lower proviral loads. In vivo, HTLV-1 (Mortreux et al., 2001b, 2003) infected cells display a mutator phenotype with a level of cellular genetic instability proportionate to their level of clonal expansion. Accordingly, HTLV-1 carriers harboring a high level of clonal expansion are more at risk to develop ATLL, as demonstrated in the case of *Strongyloides stercoralis* infection (Gabet et al., 2000) or infective dermatitis (Gabet et al., 2003a, 2003b). Accordingly, the low degree of clonal expansion of HTLV-2b-infected cells in vivo might contribute to the lack of HTLV-2b-associated malignancy, at least in the group of US blood donors with very low circulating proviral loads. In fact, the two present populations of HTLV-2b-infected individuals could be easily distinguished on the basis of HTLV-2b replication pattern in vivo. Endemically infected Bakola Pygmies displayed a pattern of replication reminiscent of that of HTLV-1-infected individuals at the pre-ATLL stage (Fig. 2).

A longer duration of intraindividual HTLV-2b infection in endemically infected individuals could have accounted for the different patterns of HTLV-2b replication between the two populations. However, in the 5 blood donors followed over a 10-year period, there was no significant correlation between time and proviral loads or clonal expansion. A more prolonged period of infection might be necessary to influence HTLV-2b replication in vivo. Alternatively, infection during infancy as via breast feeding might constitute a pre-requisite for further extensive replication of the virus. Other factors than the route of transmission or the duration of the infection could have accounted for the differences observed between endemically and epidemically infected individuals. They might include the evolution rate of the virus itself, host immune response, the genetic background, co-infections, such as in the case of strongyloidiasis with HTLV-1 or a combination thereof.

HTLV-2b was only discovered 20 years ago, and hematological malignancy has not been found among infected individuals in the United States as in Europe. However, prospective studies on the possible higher incidence of hematological diseases in isolated HTLV-2b-infected individuals such as Bakola Pygmies have not been performed. For HTLV-1, early infection via breast feeding is a pre-requisite for the subsequent development of ATLL, which typically occurs after a 50- to 60-year period of latency (Blattner, 1989). As the clonality pattern observed in endemically infected individuals is reminiscent of that of HTLV-1-infected individuals at the pre-ATLL stage (Fig. 2), our results highlight the possible oncogenic effect of prolonged HTLV-2b infection in early infected individuals.

Materials and methods

Samples studied

The materials consisted in 10 and 33 PBMC samples deriving respectively from 10 endemically infected Bakola Pygmies from Cameroon and 13 US blood donors with epidemic virus spread. The Bakola Pygmies are an ethnic group living in the area of Akok (Camo district), near Kribi in the Ocean Department of southern Cameroon (Gessain et al., 1995). Blood donors were recruited from five major US blood donation centers (Baltimore/Washington, Detroit, Oklahoma City, San Francisco and Los Angeles) participating in the HTLV Outcomes Study (Orland et al., 2003). All participating individuals were HIV-seronegative. Serological diagnosis of HTLV-2 infection was carried out as previously described (Gessain et al., 1995; Liu et al., 2001). For the HTLV-2 blood donors, duration of infection was estimated by a weighted analysis of risk factors and dates of these risk factors (Nass et al., 1999) (Table 1). Restriction fragment length polymorphism analysis of PCR product was performed to classify the virus into the HTLV-2b subtype (Switzer et al., 1995), as previously published (Liu et al., 2001). Five US blood donors were bled on different occasions, including 3 donors from whom 40 ml of blood was harvested for negative selection of $CD4^-CD8^+$ T lymphocytes. $CD8^+$ T cell enrichment was achieved by negative selection with the StemSep system.

Quantification of circulating HTLV-2b proviral loads

Circulating HTLV-2b proviral loads were measured by quantitative LightCycler real-time PCR using 0.3 μg of DNA from blood diluted to a final volume of 20 μl. The reaction mixture included polymerase (LightCycler Kit Fast Start DNA Master Hybridization Probes; Roche), 2 mM $MgCl_2$, 500 nM primer HT2BQF (5′-AATAGCAGTGTGGCTTG-3′), positioned at 6116–6132, 500 nM primer HT2BQR (5′-CCTGGGTAAGGTGGGA-3′), positioned at 6234–6249, 100 nM donor probe HT2BQP1 3′ end labeled with fluorescein (5′-GCCGGGACAGGTATCGC-FL), positioned at 6147–6163, and 200 nM acceptor probe HT2BQP2 5′ end labeled with LC Red640 (5′-LC Red640-GCGGAGTAA-

CAGGCTCCC-p) positioned at 6166–6183 (nucleotide coordinates are numbered according to the HTLV-2 sequence HTLV-II-Gab (Genbank reference: Y13051). Amplification conditions were 95 °C for 8 min to activate the polymerase, followed by 55 cycles at 95 °C for 10 s, 60 °C for 10 s and 72 °C for 10 s. In order to standardize the amount of DNA subjected to quantification, we used the albumin gene as an internal standard, as previously described (Gabet et al., 2000).

Semiquantitative linker-mediated PCR (LMPCR) amplification of HTLV-2b extremities together with their 3′ flanking sequences

Two micrograms of DNA was digested with 20 U of *Nla*III in 1x*Nla*III buffer for 3 h at 37 °C. One microgram of digested DNA was ligated with 10 pmol of BIO1 primer (Wattel et al., 1995) using 20 U of T4 DNA ligase in 40 µl for 16 h at 16 °C. This step was followed by phenol/chloroform extraction and precipitation. Ligated DNA was amplified for 100 cycles using the HT2-2 primer alone (5′-TTACCCCCTGCCCATAAAAT-3′), positioned at 8425–8445. Conditions were 1 × Stoffel fragment of the Taq DNA polymerase buffer, 1.5 mM $MgCl_2$, 10 pmol HT2-2, 200 µM of each dNTP and 10 U of Stoffel fragment of the Taq DNA polymerase in a final volume of 100 µl. Thermal cycling parameters were 95 °C, 10 min; 100 × (95 °C, 1 min; 58 °C, 1 min; 72 °C, 3 min) followed by a final elongation step of 10 min at 72 °C. Ten microliters of this linear PCR reaction was used in a classical PCR amplification using the HT2-3 (5′-GCAAGGACAGTTCAGGAGGT-3′), positioned at 8486–8505, and BIO4 (Wattel et al., 1995) primer pair. Amplification conditions were as before with 40 pmol of each primer, again in a final volume of 100 µl. Thermal cycling parameters were 95 °C, 10 min; 39 × (95 °C, 1 min; 58 °C, 1 min; 72 °C, 3 min) followed by a final elongation step of 10 min at 72 °C. The length polymorphism of HTLV-2 flanking sequences was studied by run-off analysis, as described for HTLV-1 with an HTLV-2b specific primer (5′-GGAGCGCAGCAAGGGCTAGGGC-3′) (position 8857–8879).

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