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Human Herpesvirus 6 Chromosomal Integration in Immunocompetent Patients Results in High Levels of Viral DNA in Blood, Sera, and Hair Follicles

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Six immunocompetent patients with human herpesvirus 6 (HHV-6) chromosomal integration had HHV-6 and β-globin DNA quantified in various samples by PCR. The mean HHV-6 DNA concentration (log_{10} copies/milliliter) in blood was 7.0 (≥1 HHV-6 DNA copies/leukocyte), and in serum it was 5.3 (≥1 HHV-6 DNA copies/lysed cell). The mean HHV-6 DNA load (log_{10} copies)/hair follicle was 4.2 (≥1 copies/hair follicle cell), demonstrating that viral integration is not confined to blood cells. The characteristically high HHV-6 DNA levels in chromosomal integration may confound laboratory diagnosis of HHV-6 infection and should be given due consideration.

Human herpesvirus-6 (HHV-6) is classified into the closely related yet molecularly and biologically distinct variants A and B (HHV-6A and HHV-6B) (1). During a British Isles-wide survey in immunocompetent children up to 3 years old, we identified occasional individuals in whom HHV-6 DNA, variant A or B, persisted at high level in all sera tested, in some cases over several years (16). Some older immunocompetent children and adults were also shown to have persistent high serum HHV-6 DNA levels (16). Both HHV-6A and HHV-6B are, in rare cases, found integrated in leukocyte chromosomes (5, 12, 13), and it was suspected (14, 15, 16) that the persistently high serum levels originated from lysis of leukocytes with chromosomally integrated HHV-6.

For the present work, six immunocompetent individuals were investigated for evidence of viral chromosomal integration because of high levels of HHV-6 DNA, i.e., >6 log_{10} copies/ml, in whole blood. Such levels are typical of viral chromosomal integration (4) and are strikingly different from the situation in immunocompetent persons with latent but not integrated HHV-6, in whom viral DNA is detected at the much lower level of around 1 copy per 10^4 to 10^5 leukocytes (3), i.e., ~2 log_{10} copies/ml.

HHV-6 chromosomal integration was proven by fluorescent in situ hybridization using previously published methods (4).

For quantitative PCR, DNA was extracted from whole blood, serum, or plasma using the QIAmp DNA Mini kit (QIAGEN Ltd., Crawley, United Kingdom) and from hair follicles (a 0.5- to 1-cm length of a plucked hair with follicle) using the QuickExtract DNA Extraction Solution (Epicenter, Wisconsin). Extracts were tested using quantitative PCR for HHV-6 DNA (4) and human β-globin DNA (9). As regards variation between replicates, the mean difference between duplicates was 8%. The result was expressed as the concentration, i.e., log_{10} copies/milliliter, for whole blood, serum, or plasma or as load, i.e., log_{10} copies per hair follicle. The *t* distribution was used to calculate the 95% confidence limits for a sample mean. Restriction enzyme analysis was used to distinguish HHV-6A from HHV-6B (8).

To determine HHV-6 DNA copies/cell, 5 μl of DNA extract from whole blood, serum, plasma, or hair follicles was subjected to quantitative HHV-6 PCR, and the results were compared with that for β-globin. The HHV-6 PCR (4) amplified DNA from part of the HHV-6 U67 gene, of which there is only one copy per virus genome (7). Since there are two copies of β-globin/cell, the number of viral DNA copies/cell is twice the number of HHV-6 copies/the number of β-globin copies. The number of HHV-6 DNA copies in serum was reported per lysed cell.

HHV-6 chromosomal integration was demonstrated by fluorescent in situ hybridization in all six patients, either in phytohemagglutinin-stimulated peripheral blood leukocytes or in an Epstein-Barr virus-transformed lymphoblastoid cell line (LCL) derived from their peripheral blood lymphocytes (Table 1). In each case, the fluorescent signal came from only one homologue of the chromosome in question. Patient 5 was a stem cell donor, and after engraftment, viral chromosomal integration was detected in the recipient's bone marrow cells (4), confirm-

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TABLE 1. Identification of chromosomal HHV-6 integration together with numbers of viral DNA copies/cell or lysed cell in various samples from patients 1 to 6

Patient no.	Whole blood		LCL derived from patient's lymphocytes		HHV-6 DNA variant and no. of copies/ lysed cell[b] for serum	HHV-6 DNA variant and no. of copies/ cell[a] for hair follicle
	HHV-6 chromosomal integration identified in leukocytes	HHV-6 DNA variant and no. of copies/ leukocyte[a]	HHV-6 chromosomal integration identified	HHV-6 DNA variant and no. of copies/ cell[a]		
1	Yes	B, ≥1	NT	NT	B, ≥1[c]	B, ≥1
2	NT[d]	NT	Yes	B, ≥1	NT	B, ≥1
3	Yes	B, ≥1	NT	NT	B, ≥1	B, ≥1
4	NT	NT	Yes	B, ≥1	B, ≥1	B, ≥1
5	NT	NT	Yes	A, ≥1	A, ≥1	A, ≥1
6	NT	NT	Yes	B, ≥1	NT	B, ≥1

[a] HHV-6 DNA copies/cell is twice the number of HHV-6 DNA copies/the number of β-globin DNA copies.
[b] HHV-6 DNA copies/lysed cell is twice the number of HHV-6 DNA copies/the number of β-globin DNA copies.
[c] Plasma also was tested, with the same result.
[d] NT, not tested.

ing that chromosomal integration in LCL cells reflects similar integration in leukocytes in vivo.

The mean DNA load ($\log_{10}$ copies/hair follicle) from the six patients was 4.2 (95% confidence limits, 3.6 to 4.8). However, in 44 adult volunteers, HHV-6 DNA was not detected in hair follicles.

The viral DNA concentration in various blood and serum samples from five of the patients is shown in Table 2, together with clinical details. Patient 6 had previously been shown to have a persistently high viral load in peripheral blood mononuclear cells (3) and to have HHV-6 chromosomal integration (4), but no blood samples remained for further testing. The mean HHV-6 DNA concentration (in $\log_{10}$ copies/milliliter) for the eight different samples of whole blood from the five patients was 7.0 (95% confidence limits, 6.9 to 7.2), and for the four sera from each of patients 1, 3, 4, and 5, the mean HHV-6 DNA concentration was 5.3 (95% confidence limits, 3.7 to 6.0). In this connection it should be noted that patient 3 had already been shown to have a mean HHV-6 DNA concentration of 4.8, as measured by semiquantitative PCR in five previously tested serum samples (16).

Table 1 shows the number of HHV-6 DNA copies/cell or lysed cell (calculated as described above using the ratio of HHV-6 DNA to β-globin DNA) for various samples. Overall, the median copy number/cell or lysed cell in all samples tested was 4 (range, 1 to 9); this did not vary according to individual or type of sample tested (data not shown). Nevertheless, we chose to express our result as ≥1 copies/cell because the two TaqMan PCR measurements (i.e., of HHV-6 and β-globin DNA) used to derive the ratio have an inherent imprecision, and it was therefore not possible to be more accurate. Thus, every patient had ≥1 HHV-6 DNA copies/cell in either whole blood or LCL cells and also in hair follicles. Where serum or plasma was examined, ≥1 HHV-6 DNA copies/lysed cell were detected. In patients 3, 4, and 5, an alternative method to calculate the HHV-6 DNA copies/leukocyte was possible, since a full blood count had been carried out in parallel with the quantitation

TABLE 2. Concentration of HHV-6 DNA in whole blood and serum for patients 1 to 5[e]

Patient no./sex	Age at onset of illness	Days after onset-of-illness sample taken	HHV-6 DNA concn ($\log_{10}$ HHV-6 DNA copies/ml) in:		Clinical detail(s)
			Whole blood	Serum	
1/F	0 mo	4	NT	6.4	Neonatal convulsions
		21	7.0	NT	
		44	6.9	NT	
		178	6.8	NT	
2/F	0 mo	16	6.8	NT	Tachypnoeic, meconium aspiration
		248	7.2	NT	
3/M	23 yr	0	NT	+[a]	Epstein-Barr virus encephalitis
		6	NT	+[a]	
		12	NT	+[a]	
		25	NT	+[a]	
		108	NT	+[a]	
		1,701	7.0[b]	5.0	
4/F	58 yr	7	NT	4.6	Meningitis, Parkinson's disease
		10	7.3[c]	NT	
5/F	NA	NA	6.9[d]	5.0	Adult stem cell donor

[a] All five samples gave positive results when tested previously by semiquantitative PCR (16); mean DNA concentration, 4.8.
[b] White blood count, 5.5×10^6 leukocytes/ml.
[c] White blood count, 7.0×10^6 leukocytes/ml.
[d] White blood count, 6.0×10^6 leukocytes/ml.
[e] NT, not tested; NA, not applicable; F, female; M, male.

of viral DNA in whole blood, and the results were 2, 3, and 1, respectively. In the case of patients 4 and 5, these values served to confirm the estimates given in Table 1, which were based on LCL cells rather than leukocytes.

Our results have important implications. We describe six immunocompetent British individuals, all of whom had HHV-6 chromosomal integration (one of variant A and five of variant B). This phenomenon has also been reported from Italy and Japan (5, 6, 11–13), and as with our cases, integration was with either HHV-6A or HHV-6B but not both. Although the site of HHV-6 chromosomal integration was identified by us only in peripheral blood leukocytes (or LCL cells derived from lymphocytes), HHV-6 DNA was also detected in the hair follicles of each patient, supporting an earlier finding (12). Moreover, we found for the first time that there are ≥1 HHV-6 copies/hair follicle cell. It can therefore be presumed that the virus is inherited in the germ line and found in all cells in the body, as suggested by the previous reports of vertical transmission of chromosomally integrated HHV-6 from parent to child (5, 6, 12, 13).

We also demonstrate that in the immunocompetent, persistently high HHV-6 DNA levels in whole blood are characteristic of chromosomal integration, confirming previous reports (5, 12, 13). In addition, we have shown that there are ≥1 HHV-6 copies/leukocyte in patients with viral chromosomal integration, a finding which is consistent with that of Tanaka-Taya et al. (12), who estimated there was "nearly a viral genome equivalent" per peripheral blood leukocyte in such persons.

Notably, we also found persistently high levels of HHV-6 DNA in the serum of our patients; indeed, one showed this phenomenon when tested over a period of more than 4 years. This is unlikely to be due to free virions in serum, since there is no evidence to date of HHV-6 replication in individuals with viral chromosomal integration (10, 13). The explanation lies in the fact that human chromosomal β-globin DNA is found in serum presumably originating from cells damaged before or during separation from clotted blood (9). Thus, in HHV-6 integration, fragments of chromosomal viral DNA will inevitably similarly be present. Indeed, just this hypothesis has already been raised by Ward et al. (16). As predicted then, we now confirm that high levels of HHV-6 DNA are found in the serum of individuals with chromosomal integration. Moreover, the number of HHV-6 DNA copies/lysed cell was ≥1, as expected if cellular chromosomal DNA contained integrated virus. The fact that the serum HHV-6 DNA level was about 50-fold lower than that in whole blood also validates this conclusion, since if virus replication were occurring many free virions would be released from each infected cell and the amount of viral DNA in serum and whole blood should be approximately the same.

In one of the cases of integration, two plasma samples were also available for testing and viral DNA was detected at ≥1 copy/lysed cell, suggesting that, as for serum, fragments of chromosomal DNA containing HHV-6 are released into plasma. However, the HHV-6 DNA concentration in the plasma sample separated from whole blood only a few hours after venesection was 100-fold lower than that in serum from the same patient but only 10-fold lower in the other plasma sample separated after at least 24 h (data not shown). These results imply that cell damage increases with the time elapsed before separation of plasma. They also suggest that extra HHV-6 DNA is released from cells during the clotting process, a view supported by the prior observation of raised levels of β-globin DNA in serum compared to plasma (9). Tanaka-Taya et al. (12) did not detect HHV-6 DNA in plasma in individuals with viral integration, probably because the small amount of viral DNA present was below the limit of detection for their PCR.

The finding of HHV-6 DNA in serum is usually interpreted as indicating virus replication due to primary infection, reactivation, or reinfection. However, it is now clear that, aside from primary infection, viral chromosomal integration provides the most likely explanation for serum HHV-6 DNA in the immunocompetent. Thus, in young children, serum viral DNA was due to either primary infection with HHV-6 (variant B, not A) or persistent high-level HHV-6A or HHV-6B DNA (16) now proven to result from chromosomal integration. Notably, there was no evidence in this group for viral reinfection or reactivation, suggesting a low frequency of either (<0.5%). This confirms the recent prospective study of HHV-6 in healthy immunocompetent children (2) which showed a very low frequency of intermittent reactivation or reinfection. As regards normal adults, primary infection is extremely unlikely, leaving chromosomal viral integration as the obvious alternative.

In summary, the present investigation shows that very high persisting HHV-6 DNA levels in whole blood and serum are found in immunocompetent individuals with viral chromosomal integration. Such high levels may have been misinterpreted in the past as active infection, and diagnosis must consider integration in the future; indeed, four of the patients described here were actually diagnosed initially as having HHV-6 meningitis or encephalitis presumed to be due to active virus replication. Further observations on this topic will be published elsewhere. As demonstrated in this paper, identification of patients with viral chromosomal integration is easily done for the immunocompetent by comparing the HHV-6 DNA load in whole blood and serum; in both cases the result will be characteristically high in viral integration, but the concentration will be about 50-fold lower in serum. A further distinguishing feature is the presence not only of variant B but also, in some cases, A instead. In contrast, HHV-6B is found exclusively in primary infection, where viral DNA is only seen transiently in acute-phase sera (16), and at a 100-fold lower level than that in chromosomal integration. Further studies of the natural history of HHV-6 infection must take due note of these points and include quantification of HHV-6 DNA to identify cases of viral chromosomal integration.

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