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The non-clonal and transitory nature of HIV *in vivo*

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Summary

From *a posteriori* analyses of genetic variation, recombination can only be identified when the parental genomes are distinct. For viruses like HIV-1, this requires the producer cell to be infected by more than one virus. Using fluorescence *in situ* hybridisation, the provirus copy numbers in splenocytes from two HIV-1 patients were determined. More than 75% of infected splenocytes harboured two or more proviruses, range 1–8, with a mean of ~3–4 per cell. Sequencing of amplified DNA from single laser micro-dissected cells showed an extraordinary degree of diversity while numerous recombinants were evident. Given the dynamics of HIV-1 turnover *in vivo* and a recombination rate of ~3 cross-overs per cycle, some genomes from a fifteen year old infection may have undergone as many cross-overs as bases in the genome. Thus, recombination profoundly influences HIV evolution and gives it a non-clonal and transitory nature *in vivo*.

Key words: HIV; recombination; replication

Retroviral recombination was described soon after the identification of reverse transcription [1–3] and follows from the diploid nature of the virion. Recombinants are generated via a copy choice mechanism which involves template switching during reverse transcription [4]. A precise estimation of the HIV-1 recombination rate was made, with an average result of ~3 crossovers/genome/round of replication with a range of 1–7 crossovers [5]. Thus, the recombination rate for HIV-1 is approximately 10 fold greater than the point substitution rate (~0.25/genome/round, [6]).

Recombination is found at many levels of HIV-1 genetics [7–9]. Some strains in widespread circulation are clearly composites of viruses from 2–3 clades [10–12]. Although relatively rare at the moment, recombinants between M and O group viruses have been described [13, 14] while phylogenetic analysis of N group isolates suggests that recombination involving large segments of the genome have probably occurred in the lineage [15, 16]. Within an infected individual, recombinant genomes show up in network analyses of HIV sequences [17–20]. In an experimental setting, wild type simian immunodeficiency (SIVmac) could be recovered from peripheral blood of macaques co-infected 15 days earlier by viruses carrying deletions in the *vif* and *nef* genes [15, 16, 21]. Even SIVcpz, the suspected founder of HIV-1 in humans, was recently shown to be a recombinant of 2 SIVs from different species [22].

Do HIV recombinants arise infrequently or are they constantly being spawned? Given the massive and rapid turnover of virus [23–25], relatively few are identified. As one virion gives rise to a single provirus, recombinants can be identified when a single cell is infected by two or more genetically distinct viruses. Fluorescence *in situ* hybridisation (FISH) can quantify the number of proviruses per cell while PCR and sequencing can address the question of whether the proviruses are divergent. Using a 7 kb HIV-1Δ*env* probe, the single provirus in ACH-2 cells [26], as well as the two copies in U1 cells [27] could be identified [28]. By way of internal control, a co-hybridised chromosome 12 specific α-satellite probe (D12Z1) identified the expected two copies of chromosome 12 in each cell (data not shown). When applied to experimentally infected human CD4+ cells from peripheral blood, FISH readily identified integrated HIV-1 genomes. However FISH failed to detect non-integrated HIV DNA.

Given that the proportion of infected cells is greater in lymphoid tissues, as opposed to the periphery, splenocytes from two HIV-1 infected patients, B and R were analysed [29]. Frozen cells were thawed and positively selected by anti-CD4 antibody coated magnetic beads which resulted in

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~94% CD4+ T cells as characterised by FACS analysis. For both patients more than 100 HIV-positive cells were studied. HIV-1 proviruses showed up as green spots in interphase nuclei or on chromosomes in metaphase spreads while the control region on chromosome 12 showed up in red (Figure 1). The proviral copy number ranged from 1 to 8 per cell with a mean around 3.2 [28]. 75–80% of the infected cells *in vivo* harboured on average 3–4 proviruses. The frequency distributions were remarkably similar for both patients despite different clinical presentations and viral load [29, and legend to figure 1].

With such a high frequency of multiple infected cells *in vivo*, the scene is set for rampant recombination which should be picked up by sequencing. Single HIV-positive interphase nuclei were laser micro-dissected and transferred to PCR tubes. The hypervariable V1V2 region of the HIV-1 *env* gene was chosen as it is one of the most variable regions of the HIV-1 genome and thus offered greatest resolution. This choice also meant there was no interference from the HIV-1Δ*env* probe. The nested PCR protocol used had a sensitivity of ~1 copy [29]. A collection of sequences derived from three cells from patients B and R all harbouring 3 or 4 proviruses is shown in Figure 2. The most striking features are the numbers of distinct sequences per cell and the extent of genetic variation within a single cell – up to 29% amino acid difference for cell R5 (compare sequences R5-2 and R5-3). Such a degree of variation is typical for inter-isolate comparisons. This supports the notion that patient R was infected by two distinct HIV-1 strains and that cell R5 harboured genomes of each.

A number of sequences are arguably recombinants. For example B9-3* could have arisen from recombination between B9-4 and a sequence akin to B9-3. B7-2*, B7-4 and B7-5* all have identical 3' sequences while their 5' halves are all different. For R5-3 and R5-4 there is a segment between residues 55 and 82 that differs by 9 base changes suggesting that one of the two might well be a recombinant. Comparing sequences between cells reveals further evidence of recombination, eg, B9-4* and B10-4. Most probably if more proviruses from other cells were sequenced it would be possible to identify more recombinants.

At the bottom of Figure 2 is a collection of four distinct sequences from cell R10 which contained a single provirus. It is known that for every provirus (integrated genome) there are numerous non-integrated covalently closed circular forms, some studies suggest a 2–10 fold excess [30, 31]. However, small circles are relatively inaccessible to probes since the strands readily re-anneal on themselves. Of course they will be amplified along with

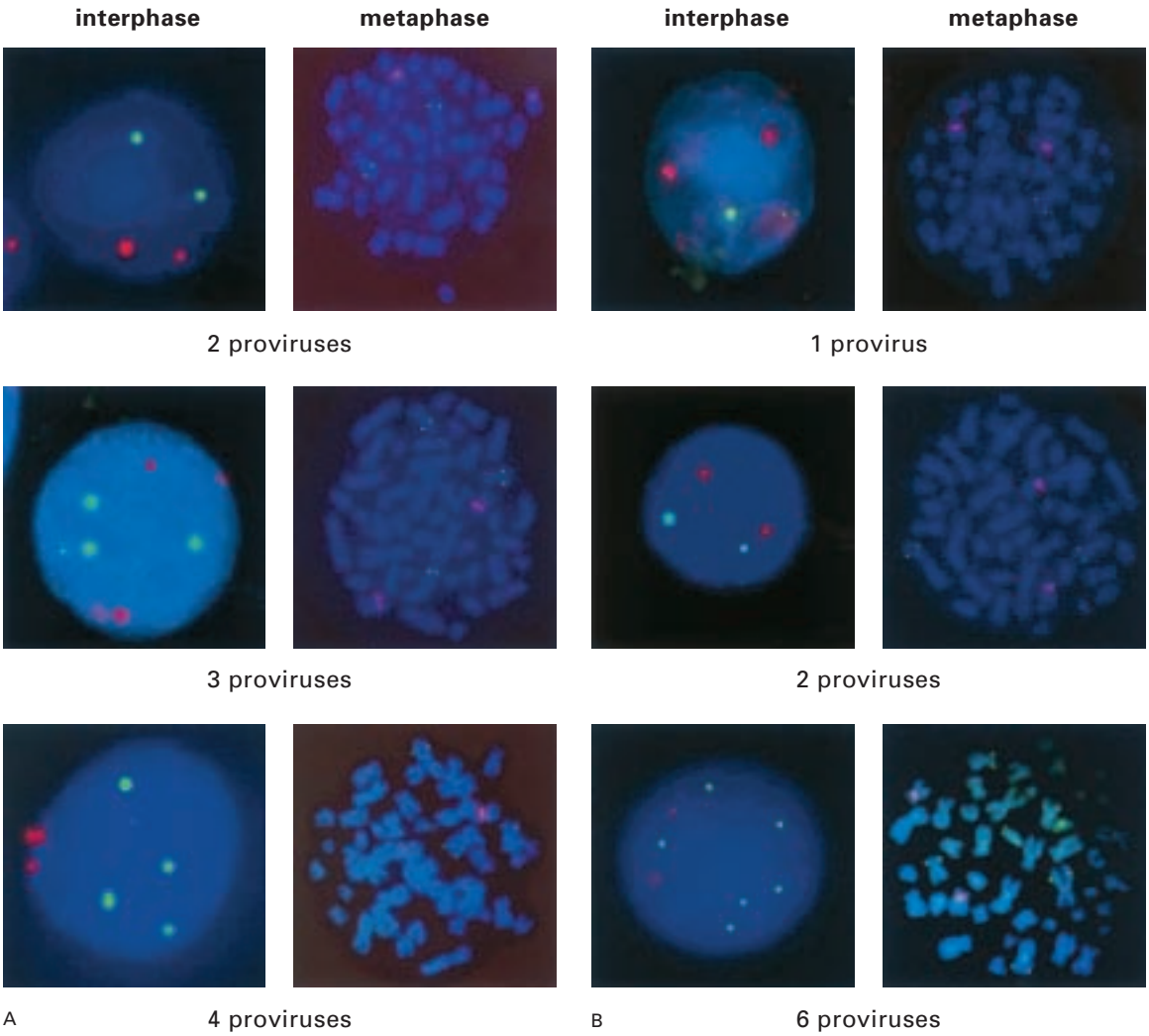


Figure 1

Multiply infected splenocytes from two HIV-1 positive patients. Green spots correspond to integrated HIV genomes, or proviruses (HIV-1Δ*env* probe), while red indicates the centromere region of chromosome 12 (D12Z1 probe).

A patient B had a peripheral CD4 cell count of 583/μl and a plasma viral load of 5,900 copies/ml.

B patient R had a peripheral CD4 cell count of 317/μl and a plasma viral load of 126,900 copies/ml [29].

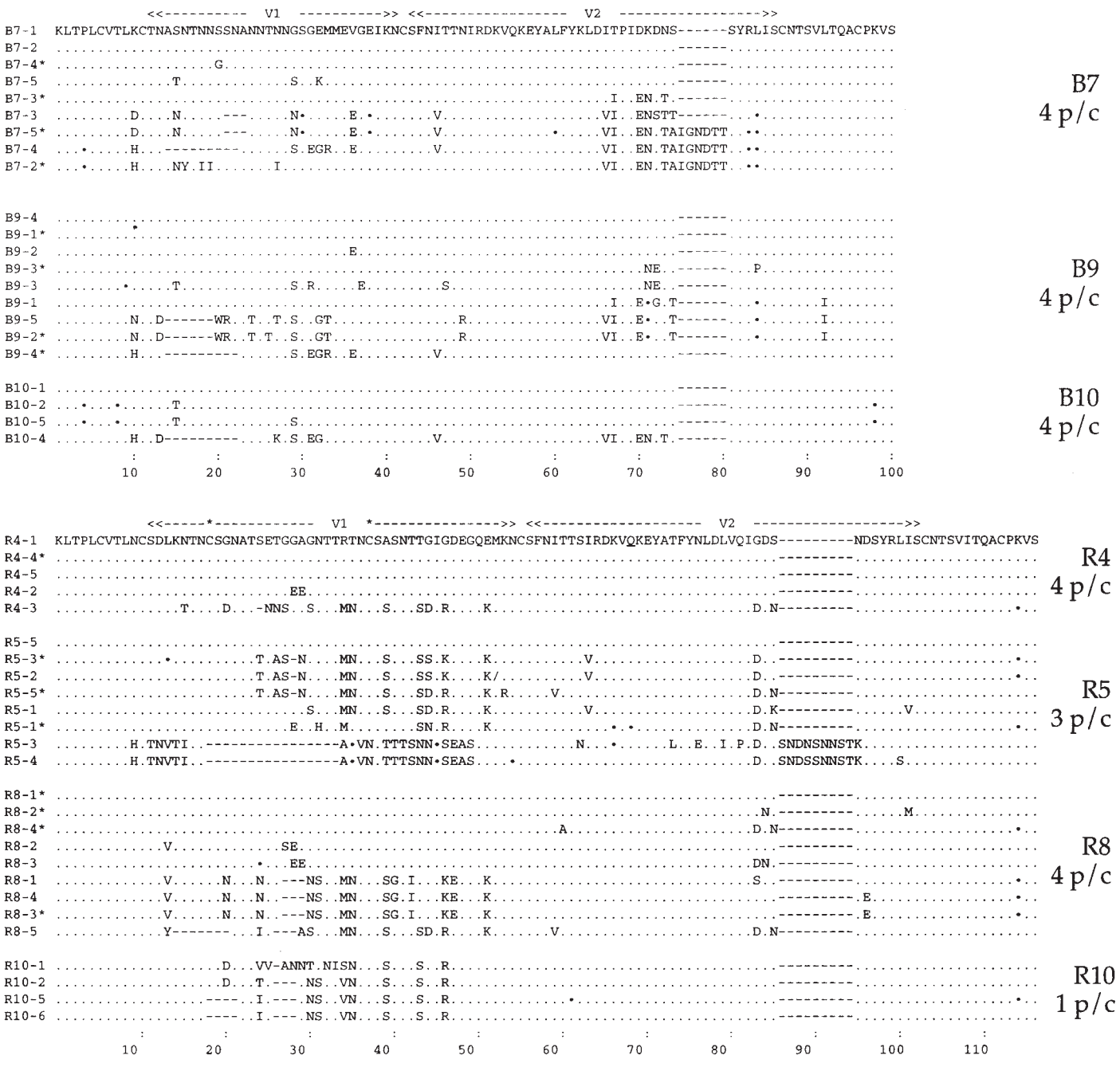
the proviruses by nested PCR. If occasionally say 10 proviruses may be found per cell, this would mean that it was infected by as many as 100 virions. Indeed, given the capacity of a cultured lymphoblast to produce hundreds of progeny as evidenced by electron microscopy, in the dense confines of lymphoid tissue, it is not difficult to imagine many virions infecting an adjacent lymphoblast.

With ~100–200 consecutive rounds of replication per year [23, 25], most of which involve multiply infected cells as shown here, after fifteen years some descendants of the initial genome should have experienced up to 9000 crossovers (200 rounds × 3 crossovers/round × 15 years). Assuming that there are few recombination hot spots then a 9200 nucleotide genome should be marked by approximately as many recombination sites as nucleotides in the genome. The effects of such rampant recombination on phylogenic analyses of HIV need to be addressed. For example, simulations of sequence evolution allowing recombination have indicated that the molecular clock breaks down, while branch lengths appear to be overestimated [32]. However, the degree of recombination postulated was low compared to that indicated by the present study.

With the majority of infected cells harbouring ≥2 genetically different proviruses, the number of distinct antigenic epitopes that are presented by a single cell is increased. Furthermore, a mutation in an epitope encoded by one provirus would still

Figure 2

Sequence diversity within single infected CD4 T cells. The segment corresponds to the hypervariable V1V2 region of the gp120 surface envelope. Only differences are scored with respect to an arbitrarily defined reference sequence. Dashes indicate gaps, solid circles synonymous substitutions, slash (/) single base frame-shifts. On the left are the sequence codes. The prefixes B7, R4 etc. refer to the individual cell, the proviral copy number is given as p/c. The asterisks above two cysteine (C) residues for R4-1 indicate the additional amino acid pair which is exceptional.



leave the cell vulnerable to recognition of the same epitope encoded by the other proviruses. Therefore, in order to escape recognition by a cytotoxic T lymphocyte clone, mutations in the epitope encoded by all proviruses are necessary which is improbable given a mutation rate of only 0.3/base/cycle [6]. While recombination would allow HIV to recover from deleterious mutations and the effects of relentless bottlenecking inherent to the chronic phase of HIV infection [21, 33], the price to pay for multiple-infection may be a broadening of immune recognition.

The high HIV recombination rate together with the high frequency of multiple infected cells shows that vast numbers of HIV recombinants are being generated *in vivo* all the time. As the pandemic spreads novel circulating recombinant forms will arise as will recombinants of recombinants, their emergence probably reflecting the singularities of transmission dynamics. The ramifications of such widespread and intense recombination on phylogeny, vaccination and the evolution of multidrug resistance need to be explored.

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References

1 Vogt PK. Genetically stable reassortment of markers during mixed infection with avian tumor viruses. Virology 1971;46: 947–52.
2 Kawai S, Hanafusa H. Genetic recombination with avian tumor virus. Virology 1972;49:37–44.
3 Weiss RA, Mason WS, Vogt PK. Genetic recombinants and heterozygotes derived from endogenous and exogenous avian RNA tumor viruses. Virology 1973;52:535–52.
4 Coffin JM. Structure, replication, and recombination of retrovirus genomes: Some unifying hypotheses. J Gen Virol 1979;42: 1–26.
5 Jetzt AE, Yu H, Klarmann GJ, Ron Y, Preston BD, Dougherty JP. High rate of recombination throughout the human immunodeficiency virus type 1 genome. J Virol 2000;74:1234–40.
6 Mansky LM, Temin HM. Lower in vivo mutation rate of human immunodeficiency virus type 1 than that predicted from the fidelity of purified reverse transcriptase. J Virol 1995;69:5087–94.
7 Li WH, Tanimura M, Sharp PM. Rates and dates of divergence between AIDS virus nucleotide sequences. Mol Biol Evol 1988; 5:313–30.
8 Robertson DL, Sharp PM, McCutchan FE, Hahn BH. Recombination in HIV-1. Nature 1995;374:124–6.
9 McCutchan FE. Understanding the genetic diversity of HIV-1. AIDS 2000;14:S31–S44.
10 Carr JK, Salminen MO, Albert J, Sanders-Buell E, Gotte D, Birx DL, et al. Full genome sequences of human immunodeficiency virus type 1 subtypes G and A/G intersubtype recombinants. Virology 1998;247:22–31.
11 Robertson DL, Anderson JP, Bradac JA, Carr JK, Foley B, Funkhouser RK, et al. HIV-1 nomenclature proposal. Science 2000;288:55–6.
12 Hoelscher M, Kim B, Maboko L, Mhalu F, von Sonnenburg F, Birx DL, et al. High proportion of unrelated HIV-1 intersubtype recombinants in the Mbeya region of southwest Tanzania. AIDS 2001;15:1461–70.
13 Peeters M, Liegeois F, Torimiro N, Bourgeois A, Mpoudi E, Vergne L, et al. Characterization of a highly replicative intergroup M/O human immunodeficiency virus type 1 recombinant isolated from a Cameroonian patient. J Virol 1999;73:7368–75.
14 Takehisa J, Zekeng L, Ido E, Yamaguchi-Kabata Y, Mboudjeka I, Harada Y, et al. Human immunodeficiency virus type 1 intergroup (M/O) recombination in cameroon. J Virol 1999;73: 6810–20.
15 Simon F, Mauclere P, Roques P, Loussert-Ajaka I, Muller-Trutwin MC, Saragosti S, et al. Identification of a new human immunodeficiency virus type 1 distinct from group M and group O. Nat Med 1998;4:1032–7.
16 Gao F, Bailes E, Robertson DL, Chen Y, Rodenburg CM, Michael SF, et al. Origin of HIV-1 in the chimpanzee Pan troglodytes troglodytes. Nature 1999;397:436–41.
17 Plikat U, Nieselt-Struwe K, Meyerhans A. Genetic drift can dominate short-term human immunodeficiency virus type 1 nef quasispecies evolution in vivo. J Virol 1997;71:4233–40.
18 Kils-Hutten L, Cheynier R, Wain-Hobson S, Meyerhans A. Phylogenetic reconstruction of intrapatient evolution of human immunodeficiency virus type 1: predominance of drift and purifying selection. J Gen Virol 2001;82:1621–7.
19 Cheynier R, Kils-Hutten L, Meyerhans A, Wain-Hobson S. Insertion/deletion frequencies match those of point mutations in the hypervariable regions of the simian immunodeficiency virus surface envelope gene. J Gen Virol 2001;82:1613–9.
20 Wain-Hobson S, Renoux-Elbe C, Vartanian JP, Meyerhans A. Network analysis of human and simian immunodeficiency virus sequence sets reveals massive recombination resulting in shorter pathways. J Gen Virol 2003;84:885–95.
21 Wooley DP, Smith RA, Czajak S, Desrosiers RC. Direct demonstration of retroviral recombination in a rhesus monkey. J Virol 1997;71:9650–3.
22 Bailes E, Gao F, Bibollet-Ruche F, Courgnaud V, Peeters M, Marx PA, et al. Hybrid origin of SIV in chimpanzees. Science 2003;300:1713.
23 Ho DD, Neumann AU, Perelson AS, Chen W, Leonard JM, Markowitz M. Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection. Nature 1995;373:123–6.
24 Wei X, Ghosh SK, Taylor ME, Johnson VA, Emini EA, Deutsch P, et al. Viral dynamics in human immunodeficiency virus type 1 infection. Nature 1995;373:117–22.
25 Haase AT. Population biology of HIV-1 infection: viral and CD4+ T cell demographics and dynamics in lymphatic tissues. Ann Rev Immunol 1999;17:625–56.
26 Clouse KA, Powell D, Washington I, Poli G, Strebel K, Farrar W, et al. Monokine regulation of human immunodeficiency virus-1 expression in a chronically infected human T cell clone. J Immunol 1989;142:431–8.
27 Folks TM, Justement J, Kinter A, Dinarello CA, Fauci AS. Cytokine-induced expression of HIV-1 in a chronically infected promonocyte cell line. Science 1987;238:800–2.
28 Jung A, Maier R, Vartanian JP, Bocharov G, Jung V, Fischer U, et al. Multiply infected spleen cells in HIV patients. Nature 2002;418:144.
29 Gratton S, Cheynier R, Dumaurier MJ, Oksenhendler E, Wain-Hobson S. Highly restricted spread of HIV-1 and multiply infected cells within splenic germinal centers. Proc Natl Acad Sci USA 2000;97:14566–71.
30 Bell P, Montaner LJ, Maul GG. Accumulation and intranuclear distribution of unintegrated human immunodeficiency virus type 1 DNA. J Virol 2001;75:7683–91.
31 Vandegraaff N, Kumar R, Burrell CJ, Li P. Kinetics of human immunodeficiency virus type 1 (HIV) DNA integration in acutely infected cells as determined using a novel assay for detection of integrated HIV DNA. J Virol 2001;75:11253–60.
32 Schierup MH, Hein J. Consequences of recombination on traditional phylogenetic analysis. Genetics 2000;156:879–91.
33 Wain-Hobson S. Viral burden in AIDS. Nature 1993;366:22.

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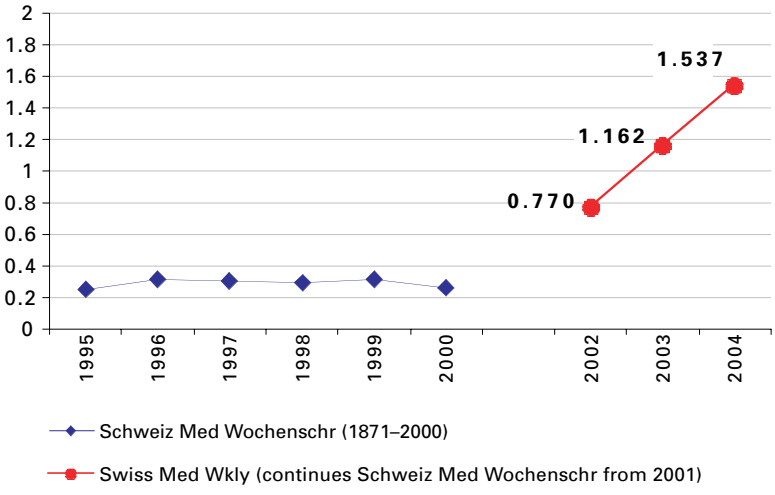

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