

even have resulted from incomplete biological cloning of the virus stock before final amplification. In a second set of experiments (Fig. 2), viral DNA obtained from cultured versus uncultured tissue specimens, and from parallel co-cultures of patient P.B.L. with lymphocytes from different normal donors, were found to have identical genotypic patterns. These data suggest that virus populations isolated by short-term culture of patient tissues are generally representative of viral populations present *in vivo*, although it is well established that under conditions of selective pressure genetic changes in the HIV-1 genome can occur *in vitro*¹³.

Previously, we mapped and sequenced predominant viral clones representing three sequential HIV-1 isolates (WMJ1, WMJ2, WMJ3) from a child with AIDS and calculated the rate of viral evolution to be on the order of 10^{-3} nucleotide substitutions per site per year⁸. The current study extends these findings by demonstrating rapid, parallel evolution of large numbers of related but distinguishable HIV-1 genotypes during chronic viral infection. These data are similar to those reported for equine infectious anaemia virus (EIAV) in which parallel evolution of multiple genotypic and antigenic variants has been documented in experimentally infected animals^{14,15}. The potential molecular mechanisms underlying HIV-1 variation have been discussed⁴⁻⁹. It is of interest and potential clinical relevance that in none of the RJS, WMF or WMJ genomic libraries, nor in a large number of virus isolates from multiply exposed high-risk individuals^{1,2}, was there evidence for concomitant superinfection with HIV-1 strains that were genotypically unrelated to the predominant viral forms. Whether this was due to selective pressures of *in vitro* cultivation or to *in vivo* mechanisms that protect against superinfection is unknown.

The biological and immunological significance of HIV-1 variation in viral pathogenesis is currently uncertain, but there are indications that similar variation in FeLV¹⁶, EIAV^{14,15,17} and visna^{18,19} is important. There are also reports that some isolates of HIV-1 have a preferential tropism for mononuclear phagocytes^{20,21}, and, more recently, that more virulent forms of HIV-1 may become apparent as clinical immunodeficiency progresses²². Ten of 12 clones of RJS4, WMF1 and WMF3 that were tested for transfection competence gave virions that were morphologically indistinguishable from wild type, but which expressed biological phenotypes ranging from highly cytopathic lymphotropic viruses, to forms that replicated selectively in monocytes or not at all (A. Fisher, L. Kong and G. M. S., unpublished data). Future studies examining the genetic, biological and immunological properties of HIV-1 will need to account for the extensive variability of this virus.

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Biologically diverse molecular variants within a single HIV-1 isolate

Amanda G. Fisher*†, Barbara Ensoli†, David Looney†, Andrea Rose†, Robert C. Gallo†, Michael S. Saag§, George M. Shaw§, Beatrice H. Hahn§ & Flossie Wong-Staal†

† Laboratory of Tumour Cell Biology, NCI, NIH, Bethesda, Maryland 20892, USA

‡ Department of Viral Diseases, Walter Reed Army Institute of Research, Washington DC 20307, USA

§ Division of Haematology and Oncology, University of Alabama at Birmingham, University Station, Birmingham, Alabama 35295, USA

AIDS is a disorder characterized by a slow progressive impairment of immune function and by infection of human immunodeficiency viruses (HIV-1, HIV-2)¹⁻⁴. Our knowledge of how these viruses cause disease in man, or how the related lentiviruses (visna and equine infectious anaemia virus) cause disease in animals, is still fragmentary. In particular, the significance of genetic variation in HIV-1, occurring within populations, within individuals and over periods of time^{5,6}, and the mechanisms of viral persistence remain unclear. To address these issues we prepared a series of recombinant clones of HIV-1 originating from a single patient and compared their biological properties. Here we show that hybrid genomes (in which the envelope region of six viral clones were separately substituted into a prototype HIV-1 genome) generated viruses with widely differing capacity to grow in human T cells, cell lines and monocytoid cultures. These data suggest that extensive biological variation exists *in vivo* within an infected individual and is in part determined at the level of the viral envelope.

Virus was isolated from the peripheral blood of an HIV-1 infected individual (coded R.J.S., HIV-1 isolate 4), who was a promiscuous homosexual male, chronically infected with HIV-1 for 5 years. Previous analyses had shown that serial isolates from R.J.S. were highly related and comprised a mixture of similar but distinct viral substrains⁷. Molecular clones were made of 27 full length proviral forms from isolate 4 and these were found to represent at least 17 different prototypes⁸. Of these, six clones were selected for further study (numbers 6, 15, 16, 22, 24 and 26) as detailed in Fig. 1. Because these proviruses were cloned using *EcoRI* (an enzyme which severs the viral genome)⁸ and thus cloned in a permuted form, we isolated *env*-containing fragments (nucleotides 5,364-8,054) from each clone and placed these into the corresponding sites in the biologically active molecular clone pXHB2gpt⁹. This, we reasoned, would provide a panel of related viruses (designated JS4) in which the effects of envelope variation could be measured independently of the effects created by differences in other areas of the genome. It was also hoped that the constituents of such a panel would serve as useful reagents for neutralization studies. As shown in Fig. 1, each of the hybrid JS4 clones generated morphologically normal virus particles (Fig. 1c-1j) and high levels of reverse transcriptase upon transfection into the COS-1

* Present address: ICRF Human Tumour Immunology Unit, Courtauld Building, 91 Riding House Street, London W1P 8BT, UK.

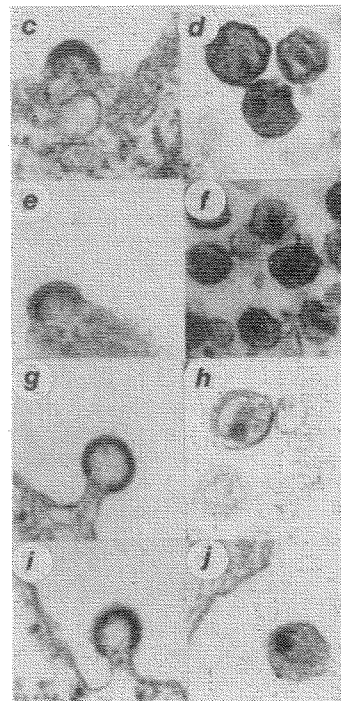
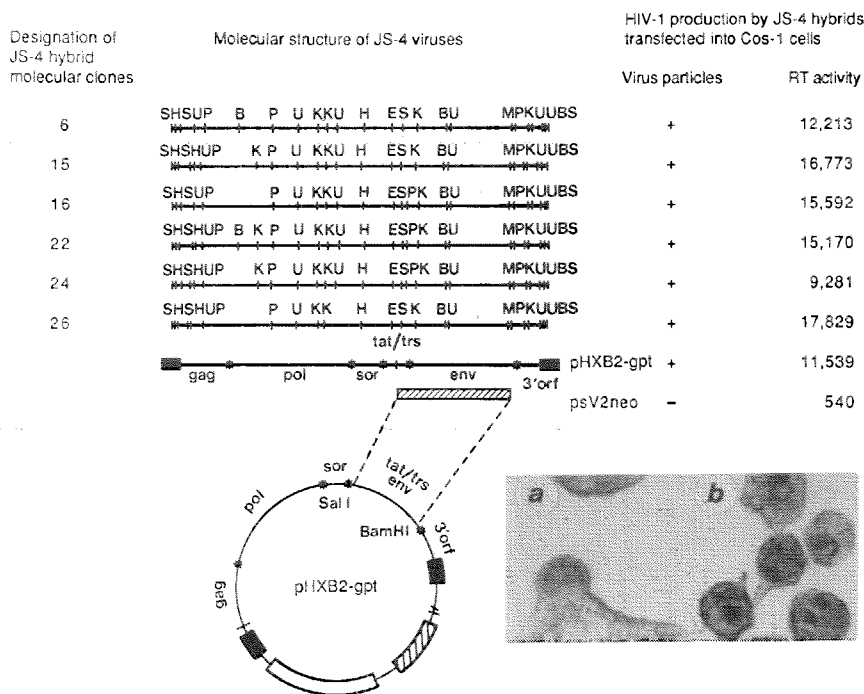


Fig. 1 Construction and properties of JS4 hybrid viruses. Molecular clones of R.J.S.⁸ were mapped by restriction enzyme digests using *Sst*I (S), *Eco*RI (E), *Bgl*II (B), *Pvu*II (U), *Hind*III (H), *Pst*I (P), *Kpn*I (K) and *Bam*HI (M) and used to prepare hybrid genomes in which the *env*-containing, *Sal*I-*Bam*HI fragment (2.7 kb) of each clone was substituted into corresponding sites in the proviral clone pHXB2gpt⁹. Solid boxes, crossed-hatched boxes and open boxes depict the HIV-1 long terminal repeats (LTRs), the bacterial gene for xanthanine guanine phosphoribosyltransferase and a region containing the genes for ampicillin, SP6 and SV40, respectively. Electron micrographs of COS-1 cells following transfection (day 3) with hybrid clones show budding and mature HIV-1 virus in pHXB2gpt (a and b, respectively), JS4-15 (c and d, respectively), JS4-22 (e and f, respectively), JS4-16 (g and h, respectively), JS4-26 (i and j, respectively), JS4-6 and JS4-24 (not shown)-derived samples. Culture supernatants removed 5 days after transfection were concentrated 20-fold before performing assays for reverse transcriptase (RT). The results shown are the mean of three to seven independent experiments, except for a single value obtained for JS4-24. Values shown represent disintegrations per minutes (d.p.m.) of ³H incorporated with the use of dT.rA as a primer template (values of < 1,500 were obtained using dT.dA as a template).

Methods. Molecular analyses and subcloning were performed using conventional approaches¹⁶. Transfection was carried out using 1 × 10⁶ COS-1 cells and 10 μg of plasmid DNA¹⁷ and reverse transcriptase assays were performed using standard protocols¹⁸.

cell line. These data suggest that elements such as *gag*, *pol*, *tat* and *tr*s/*art*, which are critical for virus production, were functional in each of the JS4 clones.

The biological properties of these viruses were investigated in transmission studies in which transfected COS-1 cells were cocultured with various target cells and the incidence of HIV-1 expressing cells in the recipient populations was monitored with time (see Fig. 2). JS4 viruses proved extremely difficult to propagate in culture. In the early period of cocultivation (3-5 days) with transfected COS-1 cells, recipient H9 (Fig. 2a), MOLT-3 (Fig. 2b) and CEM cell cultures contained syncytial cells and rare HIV-1 *gag*-expressing cells (data not shown), but the frequency of expressing cells decreased with time until none were detected at 20-24 days in JS4-6, 15, 24 and 26 infected cultures. Productive infection of these target cell lines was achieved with JS4-22, but it required more than 30 and 45 days for 10% of H9 and MOLT-3 cultures respectively to be judged HIV-1 *gag*-p24 positive, whereas the parental HXB2 strain achieved this level within 7 days. Similar results were obtained using Jurkat target cells (Fig. 2c), with 10% infection being achieved by HXB2, JS4-22 and JS4-6 after <7, 15 and 37 days respectively, while JS4-15, 16 and 26 did not maintain productive infection. Using phytohaenagglutinin-activated cord blood T cells as targets (CB) (Fig. 2d) clones JS4-6 and 22 did establish productive infection, but at a lower and slower level than the parental clone HXB2. No HIV-1 expressing cells were seen in

cultures derived from clones JS4-15, 16, 24 and 26, and Southern blot analysis failed to detect viral DNA in these cell populations.

When cells, or virus-containing supernatants, were removed from cultures at the end of these experiments (day 31) and used to infect fresh CB cultures, the kinetics of infection in the secondary cultures paralleled those seen previously, suggesting that the infection rates are intrinsic properties of the viral strains. As all clones carry the same genetic information as HXB2, except for sequences contained within nucleotides 5,364-8,054, this region (which includes portions of *env*, *tat*, *tr*s/*art* and *orf*-1 from the positive strand) must house the elements responsible for the altered phenotypes. We confirmed that the *tat* and *tr*s functions are preserved in the JS4 viruses by showing that each of the JS4 clones transactivated at levels comparable with that seen for HXB2 and that the level and pattern of viral mRNA expression in COS-1 cells transfected with JS4 clones were indistinguishable from those of the wild-type clone (data not shown). These data do not, of course, exclude subtle alterations in these genes that might contribute to the biological properties of the viruses. It is also conceivable that JS4 viruses have altered phenotypes because regulatory elements (such as TAR) present in the native R.J.S. proviruses have been removed in forming the hybrid genomes, and so we performed additional transfections using concatamerized DNA from the parental, full-length R.J.S. clones as controls. Preliminary results (data not shown) indicate that the biological properties of matched parental and

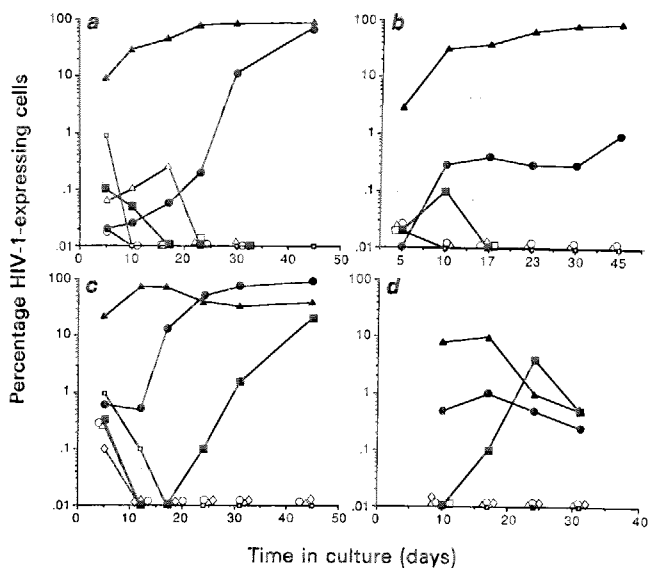


Fig. 2 Propagation of JS4 viruses in T cells. COS-1 cells (5×10^5 to 1×10^6) were transfected with plasmid DNA (5–10 μg) and after 24 h combined with 1×10^6 polybrene-treated ($2 \mu\text{g ml}^{-1}$ for 30 min) H9 cells (a), MOLT-3 cells (b), Jurkat cells (c) or cord blood mononuclear cells from normal donors (d). After 5 days non-adherent cells were removed and maintained independently in suspension culture in RPMI 1640 media containing 10% fetal calf serum. Cultures were examined for the expression of HIV-1 *gag* (p24) using the monoclonal antibody BT3¹⁹. The results shown in each of panels (a), (b), (c) and (d) summarize at least three independent experiments, for clones JS4-6 (■), JS4-15 (△), JS4-16 (○), JS4-22 (●), JS4-26 (□) and pHXB2gpt (▲) and duplicate experiments for clone JS4-24 (◇). Values between zero and <0.01% are shown as 0.01% to represent the lowest accurate level of detection.

Methods. Transfection and establishment of cocultures was carried out as described previously^{17,20}. Indirect immunofluorescence assays were performed on acetone-methanol fixed cells (50%, 10 min). Cord blood mononuclear cells were stimulated with phytohaemagglutinin for 5 days and maintained in media supplemented with IL-2 (10%) thereafter.

hybrid viruses were similar, making such an interpretation unlikely.

To analyse HIV-1-specific protein expression by JS4 clones, we performed immunoprecipitation studies using lysates from COS-1 transfected cells. Expression of HIV-1 *gag* proteins by JS4 genomes was similar to that seen for the parental strain. As illustrated in Fig. 3, lysates from COS-1 cells transfected with JS4-6, 16, 22 and 26 (lanes 5, 7, 9, 11) showed prominent virus-specific bands corresponding to the HIV-1 *gag*-related products p24, p26 and p39, when precipitated with a high titre human sera obtained from an AIDS patient. Similar results were seen with clones JS4-15 and 24 (data not shown) confirming that the HIV-1 *gag* expression by the hybrid clones was comparable to that of the wild type (pHXB2gpt, lane 1). Expression of HIV-1 *env*-encoded products (gp160 precursor and gp120) appeared altered in three of the five JS4 clones analysed. JS4-6 and JS4-22 lysates showed prominent bands of relative molecular mass (M_r) 120,000 (120K) and 160K (lanes 6 and 10) when precipitated with goat antisera raised to HIV-1 recombinant envelope, which were absent from control samples (lane 4). Comparable species could not be routinely visualized in lysates prepared from JS4-15, 16 and 26 although occasionally a faster migrating (< 120K) species, which appeared to be HIV-1 specific, was detected using high titre patient sera (see lanes 16, 15 and 17 respectively). Failure to detect normal-sized *env*-derived products may result

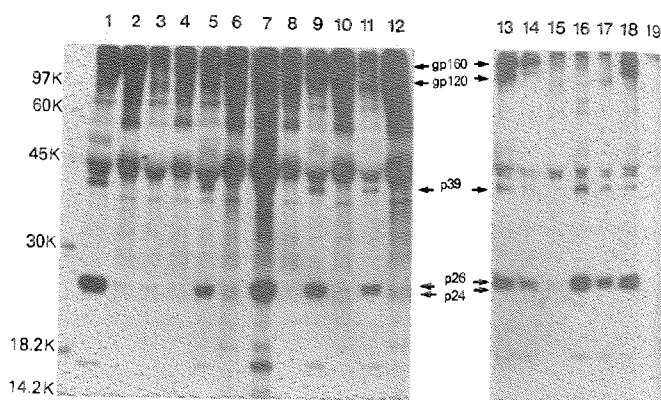


Fig. 3 Radioimmunoprecipitation analysis of HIV-1 specific proteins produced by COS-1 cells transfected with JS4 genomes. Lysates of COS-1 cells following transfection with pHXB2gpt (lanes 1, 2 and 18), pSP65gpt (lanes 3, 4 and 19), JS4-6 (lanes 5, 6 and 14), JS4-15 (lane 16), JS4-16 (lanes 7, 8 and 15), JS4-22 (lanes 9, 10 and 13) and JS4-26 (lanes 11, 12 and 17) were precipitated with human sera obtained from an AIDS patient (lanes 1, 3, 5, 7, 9, 11 and 13–19), or with goat antisera raised against HIV-1 recombinant envelope protein (*env*9). Virus-specific *gag*- (p24, p26, p39) and *env*-derived products (gp120, gp160) are shown.

Methods. COS-1 cells (1×10^6) were transfected with 10 μg of plasmid DNA and 48 h later labelled with [³⁵S]methionine and [³⁵S]cysteine (200 $\mu\text{Ci ml}^{-1}$) for 6 h. Cell lysates were prepared, processed and separated by SDS-PAGE (10%) as described elsewhere²¹.

from *env* truncation or modification, or from the envelope proteins having a reduced stability. At present, without the nucleotide sequence of these clones, it is not possible to distinguish between these possibilities but we note that clones which had previously failed to establish stable infection of T cells, (JS4-15, 16 and 26) were also those for which *env* products were unusual or could not be visualized.

The ability of JS4 viruses to replicate in monocytoic cells was investigated by inoculating human peripheral-blood derived monocyte-macrophage cultures (containing >99.99% peroxidase positive, non-specific esterase positive cells) with concentrated virus. Cultures were maintained in media supplemented with interleukin 3 (IL-3) and colony stimulating factor 1 (CSF-1) and stained for HIV-1 *gag* antigen expression 5 weeks after inoculation. As shown in Fig. 4, cultures receiving HXB2-derived virus failed to show evidence of HIV-1 *gag* expression in four separate experiments. This result is consistent with HXB2 being a component of the HTLV-IIIb isolate, one passaged extensively in T cells which appears to be very poorly infectious in monocytes¹⁰. In contrast, HIV-1 *gag*-expressing cells were detected in parallel cultures inoculated with virus derived from JS4-16 (Fig. 4d) and JS4-15 (Fig. 4e), and rare positive cells were observed in cultures derived from JS4-6 and JS4-22 (in three of four experiments). JS4-26 virus failed to establish infection under the conditions used and JS4-24 was not studied. Attempts to detect reverse transcriptase activity in the culture media removed at weekly intervals, showed low activity (at most 3 \times background) which peaked at 5–6 weeks.

The finding that four out of the five JS4 viruses can be maintained in monocytoic cultures, whereas only one of the viruses (JS4-22) grows well in H9 cells, might suggest that the JS4 viruses show a tropism for monocytes. However, it could reflect the interplay between virus infection and cell division: if a virus such as JS4-15 were capable of replicating slowly in culture, infection would tend to be lost in rapidly dividing cultures (such as H9, where infected cells would be quickly diluted out), but would be more easily retained in cultures

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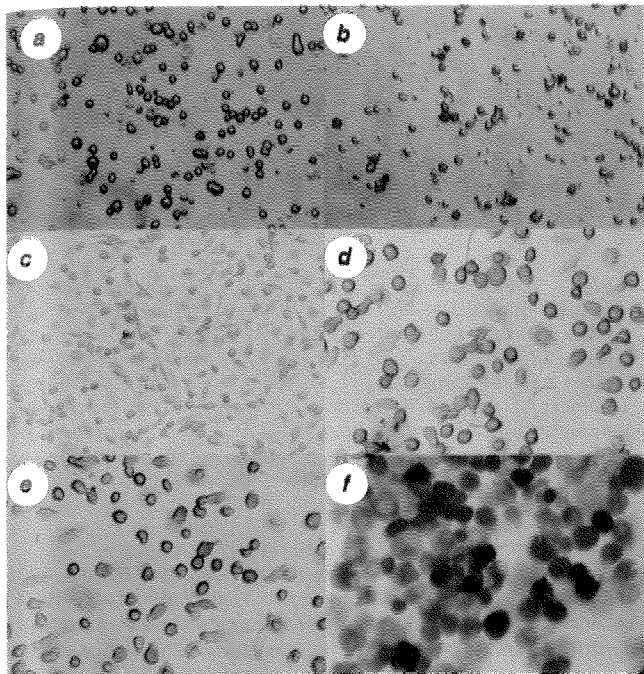


Fig. 4 Expression of HIV-1 by monocytoid cultures inoculated with JS4 viruses. Indirect labelling, using a monoclonal antibody to HIV-1 p24 (BT3)¹⁹ and an alkaline phosphatase coupled anti-mouse Ig, was used to visualize HIV-1 infected cells in H9/HTLV-III_B cultures (b), and monocytoid cultures which had been inoculated with JS4-16 (d) or JS4-15 (e) derived virus, five weeks previously. Parallel uninfected H9 cultures (a) and monocytoid cultures which had been inoculated with HXB2gpt derived virus (but failed to show evidence of HIV-1 expression) (c) are also shown. Uninfected cultures of monocytes (week 5) were stained for non-specific esterase (f) and peroxidase production to check that the populations were monocytoid.

Methods. Mononuclear blood cells were obtained from single normal donors and applied to a continuous percoll gradient to yield a cell population enriched for monocytes, as described elsewhere²². Cells were washed and plated at subconfluence ($3-6 \times 10^5$ per well) in 24-well plates, and maintained in RPMI 1640 media containing 5% human AB serum, 10% fetal calf serum and antibiotics. After 6-24 h, nonadherent cells were removed by washing and fresh media containing lipopolysaccharide (10 ng ml^{-1}) was added to the cultures. To stimulate the monocyte/macrophages, recombinant CSF-1 (15 units per ml), IL-3 (5 units per ml) or conditioned media removed (day 16-21) from primary cultures of human cord blood endothelial cells (CM-ENDO) (10%) was also included in the culture medium. After 5 days the cultures were treated with polybrene ($2 \mu\text{g ml}^{-1}$, 30 min), rinsed with phosphate buffer saline (PBS) and $100 \mu\text{l}$ of concentrated virus was applied to the cells for 1 h at 37°C . The cultures were fed with 2 ml fresh media (supplemented with IL-3+CSF-1 or 10% CM-ENDO) and maintained for 5 weeks (with weekly media changes) before being fixed *in situ* with 0.5% glutaraldehyde (in PBS) (5 min, 20°C), rinsed, incubated with a glycine solution (100 mM with 0.1% bovine serum albumin, 30 min, 20°C), rinsed and dried and stained. Virus used for inoculation was prepared by transfecting 6×10^7 COS-1 cells with $300 \mu\text{g}$ of plasmid DNA as described previously²⁰, harvesting the culture supernatant after 7 days and concentrating virus particles by direct centrifugation ($100,000\text{g}$ for 1 h at 4°C). The amount of virus contained in each preparation was standardized by performing RT assays and direct particle counts (by electron microscopy). In a typical experiment the values for RT and particle counts for virus stocks HXB2, JS4-6, JS4-15, JS4-16, JS4-22 and JS4-26 were 2.1×10^{10} particles $\text{ml}^{-1}/11,000$ d.p.m.; 3.1×10^{10} particles $\text{ml}^{-1}/16,000$ d.p.m.; 2.4×10^{10} particles $\text{ml}^{-1}/19,000$ d.p.m.; 2.2×10^{10} particles $\text{ml}^{-1}/24,000$ d.p.m.; 2.4×10^{10} particles $\text{ml}^{-1}/44,000$ d.p.m.; and 2.3×10^{10} particles $\text{ml}^{-1}/49,000$ d.p.m., respectively.

showing only limited proliferation (such as CB cultures) and would be maintained in cultures which do not divide (such as monocytes). Alternatively, the failure of JS4-15, JS4-16 and JS4-26 to be efficiently transmitted in culture could reflect the rapidity with which they kill an infected cell. It is conceivable that poorly replicating or defective HIV-1 substrains (such as JS4-26) do exist *in vivo* and are extremely cytopathic, but require the assistance of other strains (such as JS4-22) to help in transmission. A recent report¹¹ in which replication-defective variants of feline leukaemia virus (FeLV) were found to initiate rapid fatal immunodeficiency in cats when co-inoculated with a non-pathogenic replication-competent helper virus¹¹, adds credence to such a hypothesis. The finding that four of six clones analysed could not be maintained in Jurkat or CB cells (cells from which the viruses were originally cloned) is paradoxical. A possible explanation is that infected cells can accommodate more than a single viral prototype, and the co-existence of replication competent and defective clones with a single cell permits the transmission and survival of the defective form. Further experiments including the molecular analyses of DNA isolated from single cell clones will be needed to clarify this point.

In conclusion, we provide evidence that extensive biological variation exists *in vivo* in HIV-1 infected individuals and in isolates not subjected to extensive passage. It is worth contrasting the variation seen in the R.J.S. isolate with the close biological similarity of molecular clones derived from HTLV-III_B (an isolate adapted to long term growth in H9 cells including clones HXB2, HX10, HXB3; ref. 12). The biological diversity, present *in vivo*, is presumably lost *in vitro* by the selective outgrowth of HIV-1 strains capable of rapid replication. This notion is supported by extensive studies on influenza virus (in which isolation of virus from fertilized hens' eggs and from canine kidney cultures, results in the generation of two antigenically distinct populations)^{13,14} and by reports of host restriction in visna virus¹⁵. Our data also suggest that monocytoid cultures may be extremely useful for the future identification and propagation of slow-replicating HIV-1 strains. In addition, it will be of considerable importance to clarify how accurately *in vitro* adapted HIV-1 strains reflect the properties of *in vivo* strains and whether defective or slow replicating viruses have significance in disease progression.

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