experiments owing to the saturating amounts of NSF used[2,3]. Its existence supports earlier findings that γ-SNAP doubles the assembly of α-SNAP into 20S fusion particles[8] and that α- and γ-SNAP are both needed for optimal NSF binding to Golgi membranes[9]. Although γ-SNAP is apparently not essential for cell-free transport[2,3] or for the assembly of 20S fusion particles[8] (S.W.W., unpublished results), it does appear to stabilize the interactions among other components of the 20S particle. Cross-linking studies indicate that α- and γ-SNAP interact directly when bound to membranes (S.W.W., unpublished results). Both proteins also interact directly with NSF, but only α-SNAP seems to contact an integral membrane receptor protein[9]. The recombinant proteins expressed in *E. coli* were used as antigens to produce polyclonal anti-SNAP antibodies. Immunofluorescent localization of α- and γ-SNAP using affinity-purified antibodies shows strong staining of intracellular membranes including, among others, the reticular endoplasmic reticulum (ER) and perinuclear Golgi (data not shown).

The role of NSF in intercisternal Golgi transport[10], ER–Golgi transport[11], endosome–endosome fusion[12] and transcytotic vesicle fusion with the plasma membrane[13] has clearly demonstrated its general function in the cell. The SNAPs mediating NSF binding to membranes appear to be equally widely distributed. Transport vesicles must therefore be directed to the appropriate target by some other mechanism, probably involving the SNAP receptor (SNARE) family described in the accompanying article[4].

β-SNAP may be the first example of a specialized SNAP. It is very similar in sequence to α-SNAP, and competes for the same binding site on SNAP receptors[8,9], but unlike α-SNAP cannot restore cell-free Golgi transport activity to cytosol from *sec17* mutant yeast. Even in brain, it is less abundant than α-SNAP. Its regionalized expression (with highest concentrations in areas of high neuron density[6]) suggests that it is specialized for certain types of neurosecretion. □

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HIV infection is active and progressive in lymphoid tissue during the clinically latent stage of disease

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PRIMARY infection with the human immunodeficiency virus (HIV) is generally followed by a burst of viraemia with or without clinical symptoms[1–3]. This in turn is followed by a prolonged period of clinical latency. During this period there is little, if any, detectable viraemia, the numbers of infected cells in the blood are very low, and it is extremely difficult to demonstrate virus expression in these cells[4]. We have analysed viral burden and levels of virus replication simultaneously in the blood and lymphoid organs of the same individuals at various stages of HIV disease. Here we report that in early-stage disease there is a dichotomy between the levels of viral burden and virus replication in peripheral blood versus lymphoid organs. HIV disease is active in the lymphoid tissue throughout the period of clinical latency, even at times when minimal viral activity is demonstrated in blood.

Viral burden was simultaneously analysed by DNA polymerase chain reaction (PCR) in mononuclear cells from peripheral blood and lymphoid tissues in 12 HIV-seropositive individuals who were divided into three groups according to the absolute count of $CD4^+$ T cells (Table 1). In agreement with our previous study[5], in two representative patients in early and intermediate stages of disease, there is between 5 and 10 times greater frequency of infected cells in the lymphoid tissue (Fig. 1*a*, *b*). In patient 10 (advanced-stage disease), viral burden was equal in both peripheral blood and lymphoid tissue (Fig. 1*c*).

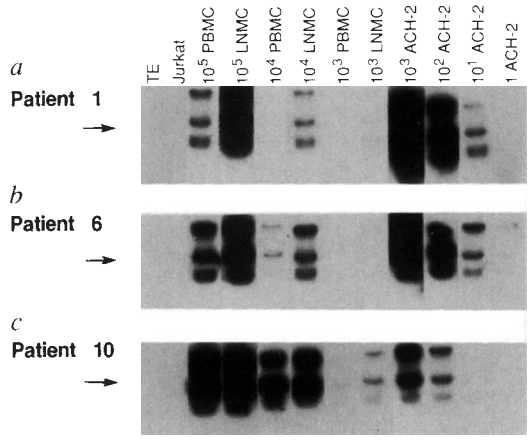
TABLE 1 Profiles of 12 patients in different stages of HIV infection who were analysed for viral burden

Patient*	Sex	Age (years)	Transmission	Absolute CD4 count (cells per mm^3)	Tissue
1	F	24	Heterosexual	571	Axillary LN†
2	F	33	Heterosexual	620	Cervical LN
3	M	32	Homosexual	688	Axillary LN
4	M	26	IVDU‡	707	Axillary LN
5	M	28	Homosexual	423	Cervical LN
6	M	43	Homosexual	355	Cervical LN
7	M	30	Homosexual	365	Axillary LN
8	F	3	Perinatal	1,798§	Cervical LN, tonsils, adenoids
9	M	5	Perinatal	913§	Tonsils
10	M	43	Homosexual	42	Axillary LN
11	M	26	IVDU	194	Axillary LN
12	F	37	Heterosexual	128	Tonsils

* Lymph node biopsies were obtained under a protocol approved by the appropriate institutional review boards; no patients had an opportunistic infection at the time of the biopsy. Patients were divided into three groups on the basis of absolute count of $CD4^+$ T cells: early-stage disease (patients 1–4), more than 500 $CD4^+$ cells per mm^3; intermediate-stage disease (patients 5–9) 200 to 500 $CD4^+$ T cells per mm^3; late-stage disease (patients 10–12), less than 200 $CD4^+$ cells per mm^3.
† LN, lymph node.
‡ IVDU, intravenous drug user.
§ Patients 8 and 9 are paediatric patients; $CD4^+$ cell counts and clinical status are compatible with intermediate-stage disease.

These results indicate a preferential localization of HIV-infected cells in the lymphoid tissue early in the course of HIV infection. This is associated with follicular hyperplasia that may be related to an abnormal movement of $CD4^+$ T lymphocytes, including those infected with HIV, resulting in sequestration of HIV-infected cells in the lymphoid organs.

The relative degree of virus replication was compared in lymphoid tissue mononuclear cells (LTMC) and peripheral blood mononuclear cells (PBMC) by RNA PCR. The sensitivity of the PCR assay and the linear relationship between the relative intensity of the PCR signals and different amounts of HIV RNA were determined first on RNA prepared from 10-fold serial cell dilutions of 8E5, a chronically HIV-infected T-cell clone that constitutively expresses HIV[6] (Fig. 1*g*). Total RNA was prepared simultaneously from PBMC and LTMC, reverse-transcribed and amplified with primer pairs specific for *gag* and *tat/rev* gene segments. Striking differences in the levels of HIV RNA synthesis were consistently observed between PBMC and LTMC in all patients analysed, regardless of the stage of disease (Fig. 1*d–f*). In early and intermediate stages of disease (patients 1–9), expression of both structural and regulatory messages was barely detectable or undetectable in PBMC, whereas high levels of HIV-specific messages were detected in LTMC (Fig. 1*d*, *e*). In late-stage disease (patients 10–12), HIV RNA synthesis was



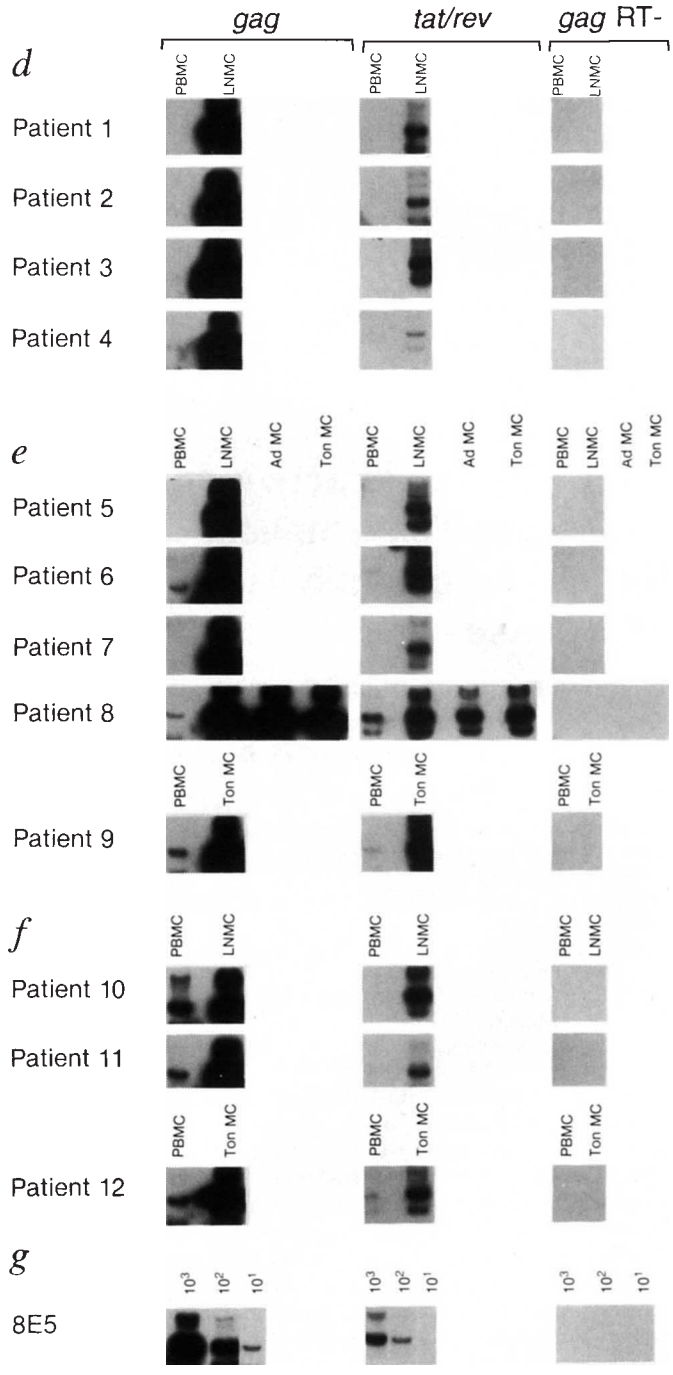


FIG. 1 *a–c*, PCR amplification of HIV-1 DNA in unfractionated cell populations isolated from peripheral blood (PB) and lymph node (LN) of three separate patients in different stages of disease. Intensities of the PCR signals in 1×10^5 PBMC and LNMC are compared with 10-fold serial dilutions of cell lysates from ACH-2, a chronically infected T-cell clone containing one proviral copy per cell[18]. *a*, In patient 1, the frequency of HIV-infected cells is 1/10,000 in the PB, and between 1/100 and 1/1,000 in the LN. *b*, In patient 6, the frequency is between 1/1,000 and 1/10,000 in the PB, and 1/1,000 in the LN. *c*, In patient 10, the frequency is between 1/10 and 1/100 in both PB and LN. *d–g*, Analysis of HIV expression in PBMC and LTMC from patients in different stages of disease by RNA-PCR. *d*, Comparative analysis of expression of structural (*gag*) and regulatory (*tat/rev*) HIV-specific messages in PBMC and LNMC isolated from four patients (patients 1–4) in early-stage disease. *e*, Patients 5–9 were in intermediate-stage disease. In patients 5–7, HIV expression was comparatively analysed in PBMC and LNMC; in patient 8, similar analysis was done in MC isolated from PB, LN, adenoids (Ad) and tonsils (Ton) from the same patient, and in patient 9 in PBMC and TonMC. *f*, Patients 10–12 were in late-stage disease. A negative control (that is, RNA treated with DNase and amplified with primer pair specific for *gag* in the absence of reverse transcriptase) is shown for each patient. *g*, Analysis of expression of *gag* and *tat/rev* HIV-specific messenger RNA in 10-fold serial cell dilutions of 8E5.

METHODS. *a–c*, Isolation of MC from PB and LT and quantitative DNA PCR were done as previously described[5]. Briefly, for DNA PCR unfractionated cell populations isolated from PB and LN from the same patients were lysed, and dilutions were obtained by mixing different amounts of the stock lysate (corresponding to 10^6 cells) with Jurkat (leukaemia T-cell line) cell lysates (corresponding to 10^5 cells) in a total volume of 50 μl. DNA corresponding to 10^5, 10^4 and 10^3 cells was amplified using a primer pair specific for the *gag* (SK145/101) gene segment, and amplified products were hybridized to the specific probe (SK102 for *gag*) that had been end-labelled using [γ-^{32}P]ATP. Products of hybridization were analysed by electrophoresis in 10% polyacrylamide gels and visualized by autoradiography. As a positive control, 10-fold serial dilutions of ACH-2 were used; as a negative control lysis buffer (LB) and cell lysates from 10^5 Jurkat cells were used. *d–g*, RNA-PCR was done as previously described[19]. To determine the levels of HIV RNA synthesis in PB and LT, PBMC and LTMC were isolated simultaneously from the PB and LT from the same patients, and pelleted in aliquots of 3×10^6 cells per vial and stored at −70 °C until used. Total RNA was extracted by the RNAzol (Cinna/Biotecx Laboratories, Friendswood, Texas) method[20]. RNA was treated with an excess of RNase-free DNase. Two aliquots of each sample were reverse-transcribed and amplified with primer pairs specific for *gag* (SK 38/39)[21,22] and *tat/rev* (TR-5/TR-3)[23]. A third aliquot was amplified with a primer pair specific for *gag*, but not reverse-transcribed (negative control). Amplified products were hybridized to the specific probes (SK 19 for *gag* and TR-4 for *tat/rev*) that had been end-labelled using [γ-^{32}P]ATP, analysed by electrophoresis in 10% polyacrylamide gels and visualized by autoradiography.

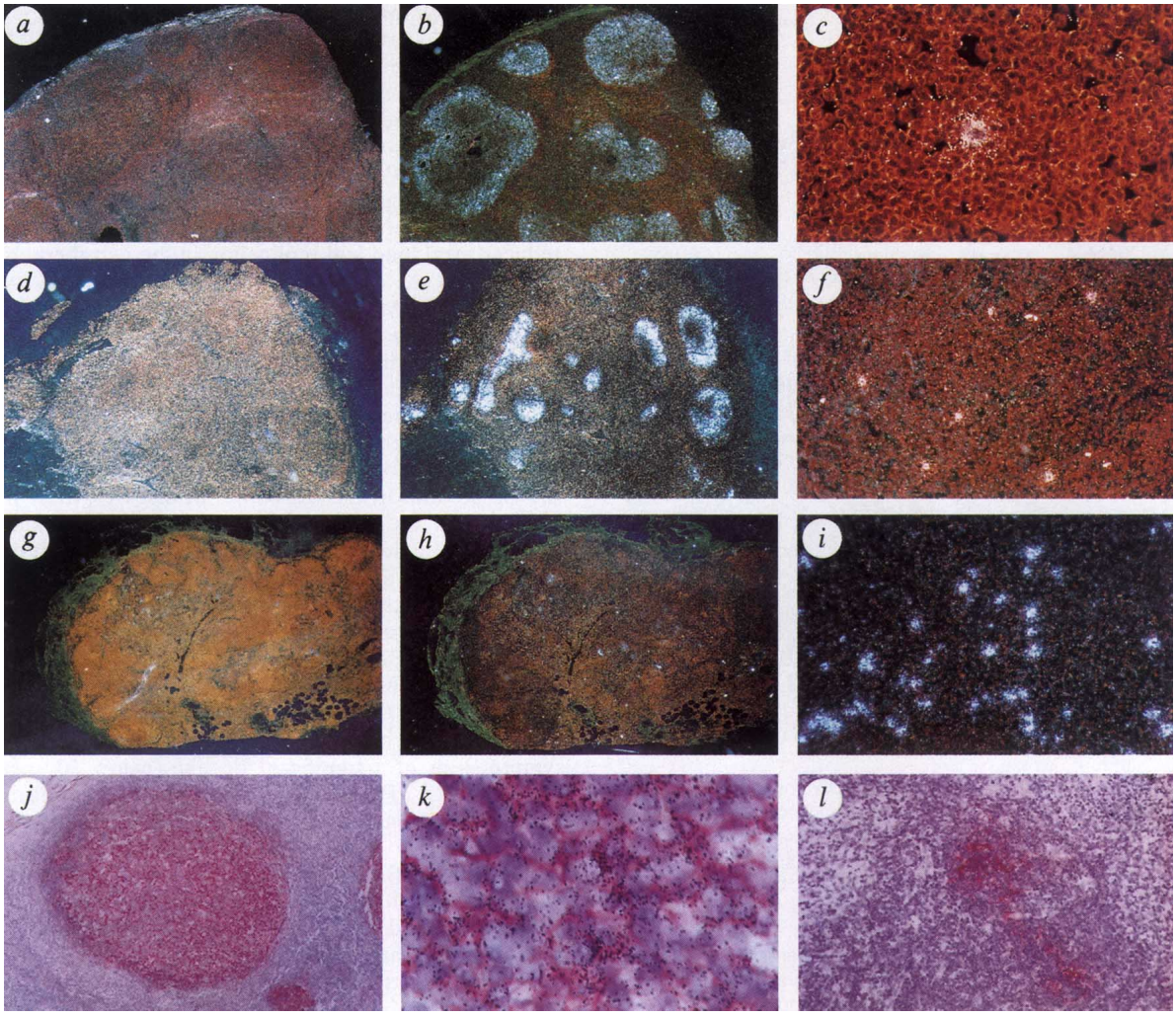


FIG. 2 *In situ* hybridization of lymph node sections from HIV-infected patients in different stages of disease. *a–c*, Early-stage disease. *a*, Darkfield image of lymph node section not digested with protease. *b*, After protease digestion. Location of HIV RNA is indicated by the silver grains, which appear as white dots. An intense, granular, diffuse signal is predominantly restricted to the area of the germinal centres. *c*, Darkfield image without protease digestion. Localization of HIV RNA signal over an individual cell in the paracortical area. *d–f*, Patient with intermediate-stage disease. *d*, Darkfield image without protease digestion. *e*, After protease digestion. Intense and diffuse signal is present over some germinal centres, whereas in the same section the signal is significantly decreased in other germinal centres. *f*, HIV RNA signal over individual cells in the paracortical area. *g–i*, Patient with late-stage disease. *g*, Darkfield image without protease digestion. Lymph node architecture is completely disrupted, and germinal centres are virtually absent. *h*, After protease digestion. A moderate increase in *in situ* hybridization signal is seen. *i*, Higher magnification of a protease-digested section showing distribution of silver grains over several isolated cells expressing HIV RNA in copious amounts. *j*, Brightfield image of a protease-digested lymph node section subjected to immunohistochemistry plus *in situ* hybridization showing the staining (New Fuchsin Red) of the FDC network with anti-CD21 antibody. *k*, Localization of HIV RNA (that is, with silver grains which appear as black dots) coincides with the staining of the FDC network. *l*, Presumed already involuted germinal centre with a remnant of FDC network. Data are representative of the qualitative changes as well as the degrees of changes among the different stages of disease seen in the 7 of 12 patients in the present study in whose lymph nodes *in situ* hybridization was done. These data were consistent with data obtained from an additional 30 patients in whose lymph nodes similar *in situ* hybridization studies were done.

METHODS. *In situ* hybridization was performed as previously described[8]. RNA probes were synthesized from five DNA templates that represent 90% of the HIV-1 genome[8]. Tissues were fixed in formaldehyde, embedded in paraffin and digested with proteolytic enzymes. Slides were hybridized with sense or antisense probes and run in duplicate. After hybridization, autoradiograms were made with NTB3 emulsion (Eastman Kodak), stained with haematoxylin–eosin, and examined by darkfield microscopy. In double-labelling (immunohistochemistry plus *in situ* hybridization) experiments, slides were subjected first to staining with anti-CD21 antibody. Anti-CD21 antibody (clone 1F8) that specifically stains FDC in formaldehyde-fixed tissues was purchased from Dako Corporation, Carpinteria, CA.

increased in PBMC compared to that in earlier stages of disease (Fig. 1*f*), although it still remained clearly lower than in LTMC. Similar patterns of HIV expression in PBMC of patients at different stages of disease have been previously observed[7]. But this is the first demonstration of a striking dichotomy between peripheral blood and lymphoid tissue from the same individuals in viral burden and replication. Of note, high levels of HIV expression were observed in lymphoid tissue other than lymph nodes, such as adenoids and tonsils (Fig. 1*e*, *f*), indicating a systemic dissemination of HIV among lymphoid tissue and not a localization to lymph nodes.

In order to delineate the pathogenic mechanisms responsible for the dichotomy between the levels of viral burden and expression in peripheral blood compared to lymphoid tissue, we analysed the latter by *in situ* hybridization and transmission electron microscopy. All lymph nodes obtained from patients in early-stage disease had some degree of follicular hyperplasia. In these lymph nodes, after protease treatment, most of the hybridization signal, which corresponded to extracellular viral particles[8], was restricted to the germinal centres (Fig. 2*b*). The

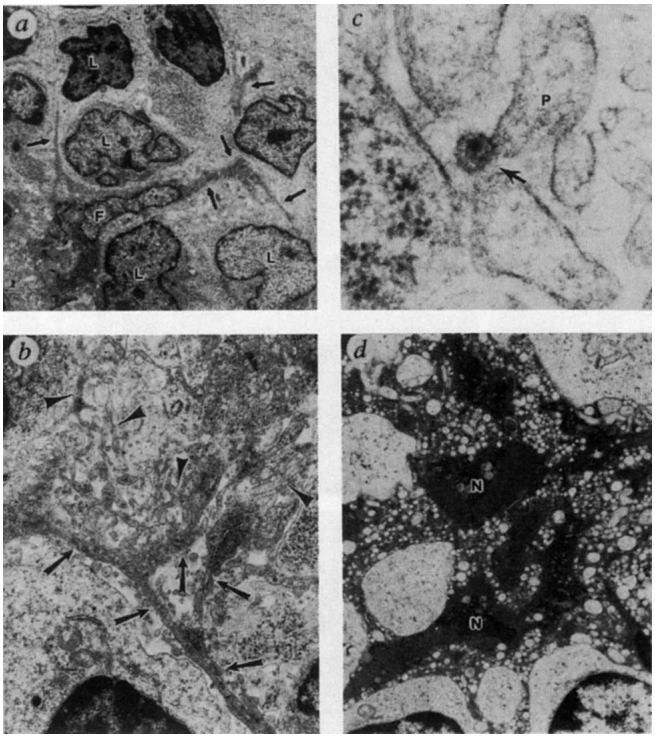


FIG. 3 *a*, Electron-dense FDC (for example, F) extends processes (for example, arrows) that contact several adjacent lymphocytes (for example, L). Magnification, ×1,920. *b*, Moderately dense extensions (for example, arrows) of the FDC branch several times before ending in numerous villus processes (for example, arrowheads). Magnification, ×6,720. *c*, A mature HIV particle (for example, arrow), with its typical conical nucleoid, located in the intercellular space of a hyperplastic germinal centre. It is closely associated with FDC processes (for example, P) and is partially obscured by filamentous material. Magnification, ×55,200. *d*, Several tightly clustered FDC have irregular, markedly condensed nuclei (for example, N) and electron-dense, vesiculated cytoplasm which are signs of degeneration and necrosis. Magnification, ×3,744. Micrographs were obtained from LN specimens of the patients listed in Table 1. Data are representative of the findings in the 5 of 12 patients in this study on whose lymph nodes electron microscopy was done. These data are consistent with an additional eight patients whose lymph nodes were similarly examined by electron microscopy.

paucity of the signal in the absence of protease treatment (Fig. 2*a*) was consistent with extracellular virions coated with proteins, forming immune complexes[8]. According to previous studies[8–15], and as confirmed by ultrastructural analysis, HIV particles are trapped on the villus processes of follicular dendritic cells (FDC) (Fig. 3*c*) that surround and are intimately associated with lymphocytes (Fig. 3*a*, *b*). As disease progresses, important changes in the distribution pattern of HIV in the lymph nodes occur. During intermediate-stage disease, within a given lymph node, virus is trapped in some germinal centres and not in others (Fig. 2*e*). In late-stage disease, the architecture of the lymph node is disrupted and most of the germinal centres are involuted concomitant with a loss of virus-trapping capability of the node (Fig. 2*g*, *h*). Degeneration and death of FDC, as indicated by ultrastructural analysis (Fig. 3*d*), is generally associated with disease progression. But varying degrees of histopathology can be seen focally in the lymph nodes of patients even in early stages of disease. Furthermore, isolated cells expressing HIV RNA are detected in the paracortical area regardless of the stage of disease, and the proportion of these cells seems to increase with disease progression (Fig. 2*c*, *f*, *i*).

The mechanism responsible for the trapping of virus was directly addressed by combining *in situ* hybridization for HIV with immunohistochemistry for FDC using anti-CD21 antibody. Figure 2*j* illustrates an intact dense FDC network in the germinal centre of a lymph node from a patient in early-stage disease. It is notable that the hybridization signal for HIV RNA, which corresponds to extracellular HIV particles, completely overlaps with the FDC network (Fig. 2*k*). In lymph nodes of patients in late-stage disease, only isolated FDC are detected (Fig. 2*l*), and virus trapping is virtually absent although the residual FDC can still retain virus (data not shown). These results indicate that efficient trapping of virus corresponds directly with the integrity of the FDC network. Thus, the mechanisms responsible for reduced viral load and replication in the peripheral blood compartment in the early stages of disease involve a combination of the mechanical filtering and trapping of virions by the FDC network and the sequestration of infected $CD4^+$ T cells in the hyperplastic lymph node. Furthermore, the formation of immune complexes (virus plus immunoglobulin or complement) would contribute to the attachment of HIV to the FDC. In this regard, it is likely that an initially adequate immune response itself also contributes substantially to the clearance of virus from the circulation. In the late stages of disease[4], these mechanical mechanisms are altered and an effective immune response against HIV is lost[16], leading to an increase in viral burden in PBMC and an acceleration of plasma viraemia, explained at least in part by the spillover of virus from the lymphoid organs.

The observations of low to absent levels of viraemia and virus replication in PBMC have led to the impression that HIV disease is not active during the period of clinical latency. This has contributed to the general policy of initiating antiretroviral therapy only at the time of the appearance of constitutional symptoms or of a significant decline (below 500 per mm^3) of $CD4^+$ T cells[17]. Here we have demonstrated that during clinical latency, HIV accumulates in the lymphoid organs and replicates actively despite a low viral burden and low-to-absent viral replication in PBMC. Therefore, a state of true microbiological latency does not exist during the course of HIV infection. The peripheral blood does not accurately reflect the actual state of HIV disease, particularly early in the clinical course of HIV infection. In fact, HIV disease is active and progressive even when there is little evidence of disease activity by readily measured viral parameters in the peripheral blood, and the patient is experiencing clinical latency. These findings not only advance our understanding of the immunopathogenic mechanisms of HIV infection, but may have important implications in the design of therapeutic strategies. □

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