CD4 T cells remain the major source of HIV-1 during end stage disease

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Objective: To assess the source of HIV-1 production in lymphoid tissue biopsies from HIV-infected patients, with no prior anti-retroviral protease inhibitor treatment, with a CD4 cell count $> 150 \times 10^6$/l (group I) or $< 50 \times 10^6$/l (group II), co-infected with *Mycobacterium tuberculosis* or *Mycobacterium avium* complex.

Design and methods: Lymphoid tissue biopsies from 11 HIV-1-infected patients, taken for diagnostic purposes, were studied by HIV-1 RNA *in situ* hybridization and immunohistochemistry.

Results: Patients of group I showed well organized granulomas, in contrast with patients of group II, in which granuloma formation was absent. HIV-1 RNA-positive cells in group I patients were found mainly around the granulomas, whereas in group II HIV-1-producing cells were confined to areas with remaining intact lymphoid tissue. Despite the abundant presence of macrophages, the productively infected HIV-1-positive cells in both groups were almost exclusively CD4 T cells.

Conclusion: In contrast with previously published data, CD4 T cells appear to remain the major source of HIV-1 production in end-stage disease.

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AIDS 1999, **13**:1015–1019

Keywords: HIV-1, lymphoid tissue, pathogenesis, virus production, macrophages, opportunistic infections, tuberculosis

Introduction

Molecular histopathological studies of lymphoid and non-lymphoid tissues of HIV-1-infected individuals have contributed considerably to our knowledge of the mechanisms involved in HIV-1-induced disease. Soon after infection, when HIV-1-specific humoral and cellular immune responses can be demonstrated, virus production is confined largely to CD4 T cells located within lymphoid tissues such as lymph nodes, mucosa associated lymphoid tissue and spleen [1–4]. During the entire asymptomatic stage of the disease, CD4 T cells are the major source of HIV-1, whereas productively infected cells expressing macrophage or dendritic cell markers are observed rarely in lymphoid tissues. Productively infected macrophages and dendritic cells have been observed in nasopharyngeal lymphoid tissues, cervix and brain [5–10]; however, these cells are generally believed to contribute minimally to the high levels of virus produced throughout the course of the infection. During the asymptomatic and early symptomatic stages (CD4 cell count $> 200 \times 10^6$/l), follicular hyperplasia with irregularly shaped follicles are often observed [11,12]. Large numbers of virus particles within immune complexes are then captured onto the processes of follicular dendritic cells [1,2].

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Received: 4 December 1998; revised: 26 March 1999; accepted: 31 March 1999.

In the intermediate stages of the infection, the numbers of productively infected CD4 T cells within lymphoid tissue increase [1,2]. Ultimately however CD4 T cells almost disappear and the architecture of lymphoid tissue, especially with regard to the germinal centres and the follicular dendritic cells network, is disrupted and the ability to trap virus is lost. Also at this stage of the disease, virus production is relatively high [13]. The increase of cell-free plasma HIV RNA and the virtual absence of CD4 T cells, which are thought to be the major source of virus production, is until now an unresolved paradox. Recently it has been suggested by Orenstein *et al*., that a possible explanation may be a switch from CD4 T cells to another cell type as the major source of virus during end-stage disease [14]. An alternative explanation could be that due to the increased replication rate of HIV-1 at end-stage disease, as well as a higher percentage of infected cells, a small reservoir of CD4 T lymphocytes is sufficient to produce the observed increased levels of cell-free virus in plasma [15].

To address this issue we studied lymphoid tissue biopsies from 11 HIV-1-infected patients co-infected with *Mycobacterium tuberculosis* (MTB) or *M. avium complex* (MAC), by HIV-1 RNA in situ hybridization and immunohistochemistry. Patients co-infected with MTB or MAC were selected because their lymphoid tissue generally contains large numbers of macrophages. The hypothesis of Orensetin *et al*., that at end-stage disease the bulk of HIV-1 production shifts from CD4 T cells to macrophages may be tested by studying these patients.

Material and methods

Patient materials

Lymph node specimens taken from eleven HIV-1-seropositive patients for diagnostic purposes, were selected for this study. The selection criteria were: HIV-1 laboratory diagnosis; MTB or MAC laboratory diagnosis; peripheral blood CD4 T cell count $< 500 \times 10^6$/l or AIDS-defining disease; no prior anti-retroviral therapy with protease inhibitors. The peripheral blood CD4 cell counts and co-infections are listed in Table 1.

Immunohistochemistry

Tissues were fixed overnight with 4% buffered formalin (pH 7) and subsequently embedded in paraffin. The tissues were cut for routine histology (Giemsa, Ziehl–Neelsen, haematoxylin & eosin and Gomori's silver impregnation). For immunohistochemistry, dewaxed 5 μm paraffin sections were placed in a domestic pressure cooker containing 0.01 M sodium citrate solution (pH 6), boiled for 2 min, and chilled to room temperature [16]. Primary antibodies against CD4 (Novocastra, Newcastle upon Tyne, UK), CD68 (KP1), lysozyme and CD45RO (UCHL-1) (Dakopatts, Copenhagen, Denmark) were used as described previously [17]. The sections were subsequently either counter-stained with haematoxylin & eosin and mounted, or they were dehydrated and subjected to HIV RNA *in situ* hybridization.

Table 1. Clinical characteristics of 11 HIV-1-seropositive patients.

Patient	CD4 T cells $\times 10^6$/l	Co-infection
Group I[a]		
1	427	MTB
2	440	MTB
3	300	MTB
4	200	MTB
5	170	MTB
Group II[a]		
6	< 50	MTB
7	< 50	MTB
8	< 50	MAC
9	< 50	MAC
Undefined[a]		
10	ND	MTB
11	ND	MTB

[a]Group I, Patients with CD4 cell count 50–500 $\times 10^6$/l; Group II, patients with CD4 T cell count $< 50 \times 10^6$/l. ND, Not done; MTB, *Mycobacterium tuberculosis*. MAC, *M. avium* complex.

In situ hybridization

A ^{35}S-labelled, single stranded, anti-sense HIV-1 RNA probe (Lofstrand Labs., Gaithersburg, Maryland, USA), which contains 1.4–2.7 kb fragments collectively representing approximately 90% of the HIV-1 genome, was used as previously described [3]. In short, paraffin sections were either treated with proteinase K (0.01 mg/ml) for 8 min. at room temperature or heat denatured in citrate buffer (pH 6). The sections were incubated with prehybridization mixture (50% formamide, 0.5 M NaCl, 10 mM Tris–HCl (pH 7.4), 1 mM EDTA, 0.02% Ficoll–polyvinylpyrrolidone–BSA and 7 mg tRNA/ml) for 2 h at 37°C and then covered with hybridization mixture (prehybridization mixture supplemented with 10% dextran sulphate and 2×10^6 d.p.m. of probe/ml) overnight at 45°C. The sections were washed, RNAse treated (Boehringer Mannheim GmbH, Mannheim, Germany) for 40 min at 37°C, rewashed and then dipped into emulsion (NTB2; Kodak, Rochester, New York, USA). After exposure for 3–7 days the slides were developed, counter-stained with haemalaun and mounted as described previously. As negative controls, sections were hybridized with a radiolabelled sense probe. The sections were examined with a microscope equipped with epiluminescent illumination (Axiophot, Carl Zeiss Inc., Jena, Germany). Cells were considered positive for viral gene expression if the number of grains counted was more than six times the number in the background.

Results

Lymph nodes from patients with 150–500 × 10^6 peripheral CD4 T cells/l, hereafter referred to as group I patients, contained multiple well organized granulomas occupying 50–90% of the cutting surface. In parts of the tissue that were not involved in the granuloma formation, the nodal architecture with T cell dependent zone and several small inactive germinal centres was preserved. The granulomas were composed of epitheloid cells, multinucleated cells of the Langhans'-type and CD4 as well as CD8 T lymphocytes. The central part of the granulomas often showed caseation (data not shown). In contrast, in lymph nodes from patients with peripheral CD4 T cell counts < 50 × 10^6/l, hereafter referred to as group II patients, well organized granulomas were absent. The nodal architecture was obscured by a heavy confluent infiltrate consisting of macrophages, intermingled with reactive plasma cells. The macrophages contained many acid-fast bacilli. The number of CD4 T lymphocytes was decreased; they were present only in the remnants of lymphoid tissue seen as small islets at the periphery of the lymph nodes or between the bands of macrophages (Fig. 1a).

In the lymphoid tissues from the group I patients, the number of HIV-1 RNA-positive cells was unevenly distributed. Significant numbers of cells with a heavy *in situ* hybridization overlay were observed mainly around the granulomas and occasionally within the granulomas (Fig. 1b). The numbers of HIV RNA-positive cells within the parts of the nodes where granulomas were observed, were approximately 10 fold higher than those in lymphoid tissue areas that did not contain granulomas (data not shown). In lymphoid tissues from end stage disease patients (group II), the numbers of HIV-1 RNA-positive cells were not significantly increased as compared to lymphoid tissue areas where no infiltrate of macrophages was observed. HIV-1-producing cells were confined to areas with remaining lymphoid tissue (Fig. 1a).

The phenotype of the productively HIV-1-infected cells was subsequently determined by double-labelling of HIV RNA by *in situ* hybridization and immunohistochemical staining for CD4, CD45RO, CD68 or lysozyme. Despite the abundant presence of macrophages and Langhans'-type giant cells, the HIV RNA-positive cells in the lymphoid tissues from patients of group I proved to be almost exclusively CD45RO+CD68–lysozyme– (Fig. 1b, insert I, d), identifying them as CD4 T cells. The HIV-1 RNA-positive cells in lymphoid tissues from end stage disease patients (group II) also proved to be CD4 T cells. Occasionally an HIV-1 RNA+CD45+CD68+ lyzosyme+ cell was observed (Fig. 1d), excluding the possibility that productively HIV-1-infected

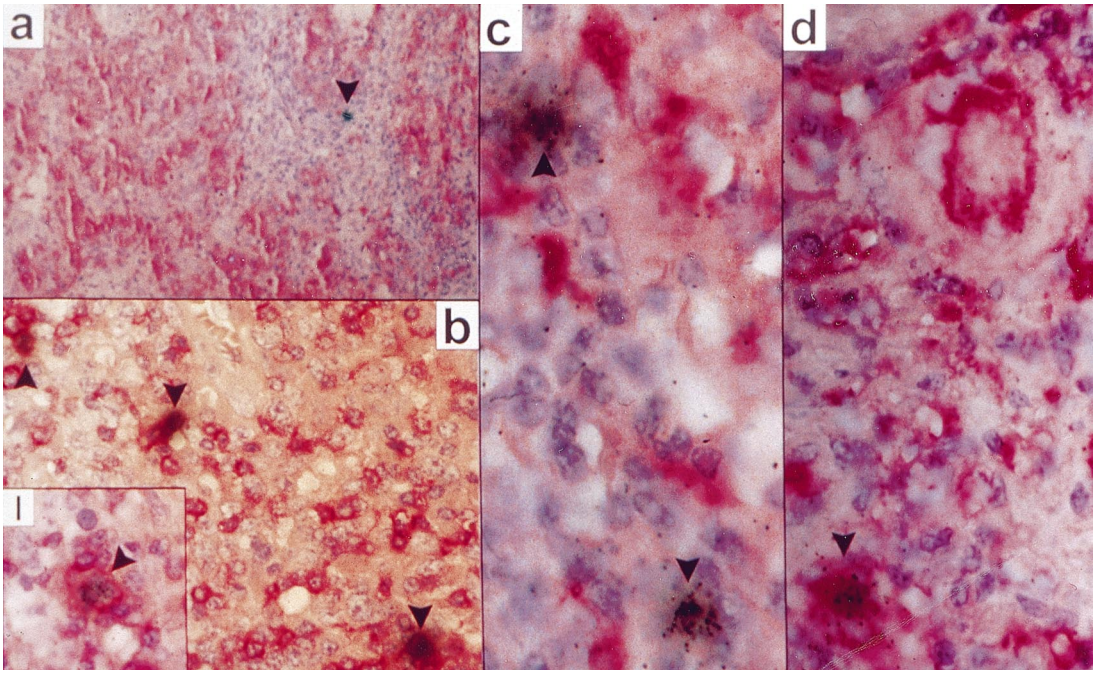

Fig.1. HIV-1 *in situ* hybridization on lymph nodes co-infected with Mycobacteria. (a) One MAC-infected lymph node from a HIV-1-infected patient No.8. The HIV-1 RNA signal (arrow) is present only in areas with some intact lymphoid architecture (original magnification × 40). (b) A *M. tuberculosis* infected lymph node (patient No.1) with considerable numbers of CD4 T cells (red), and a significantly higher number of HIV-1 RNA-positive cells (arrows); the inset (I) shows double-staining of HIV-1 RNA and CD45RO-positive cells (original magnification × 400). (c) Abundant numbers of macrophages (red) did not correspond with HIV-1 RNA-positive cells (arrows). (d) Occasionally an HIV RNA+CD68+lysozyme+ cell was observed (arrow).

macrophages were not identified due to down-regulation of CD68 and lysozyme expression by HIV-1 infection. Furthermore, the *in situ* hybridization grain counts of HIV-1 RNA-positive CD4 T cells were generally much heavier than those on HIV-1 RNA-positive macrophages, suggesting a lower level of virus production in the latter (Fig. 1c,d).

Discussion

We have shown that in contrast to previously published data, CD4 T cells remained the major source of HIV-1 production at end stage infection. The histological appearance and cellular composition of granulomas in lymphoid tissue from group I patients did not differ from those seen in immunocompetent patients [18]. Granuloma formation is characteristic in the cell mediated immune response to MTB, and it is well established that CD4 T cells play a pivotal role in host defence against intracellular microorganisms [18]. Around the granulomas massive HIV-1 production was mainly confined to CD4 T cells. This finding is in agreement with the observed increase of the non-cell associated plasma viral load during opportunistic infections [19,20] and in studies on lymphoid tissue from HIV-1-infected patients co-infected with opportunistic organisms [14]. Treatment of opportunistic or bacterial infections in HIV-1-infected patients resulted again in a decrease of plasma viral load levels to the levels observed before the onset of the opportunistic infection [19–21]. The abundant presence of macrophages in lymphoid tissue from both groups did not apparently contribute to the viral load. Not only was the number of HIV-1 RNA-positive infected macrophages low, but also the level of *in vivo* virus production by macrophages, assessed by in situ hybridization grain counting, was lower than in CD4 T cells. In addition, in *in situ* double labelling analyses of biopsies collected from 50 lymphatic tissues of more than 50 individuals at different stages of HIV-1 infection, productively infected macrophages have been observed only scarcely (unpublished observations). Considering the observation that at end-stage disease, in approximately 50% of patients so-called X4/syncitium inducing/rapid-high HIV-1 strains arise, which have shown to be less macrophage-tropic as compared with HIV-1 strains isolated from asymptomatic individuals [22,23], we postulate that at end stage disease CD4 T cells, rather than macrophages, remain the major source of HIV-1 production. Whether these clearly conflicting observations with previously published data [14] may attribute to differences in patient selection or laboratory techniques remains to be determined. The paradox of increasing viral loads with progressively declining circulating CD4 T cells therefore remains. We and others [24–26] have, however, recently shown that in patients with low peripheral CD4 T cells the decrease of tissue CD4 T cell count is slower. It may be assumed that patients with CD4 T cell counts $< 50 \times 10^6$/l, still have sufficient numbers of CD4 T cells to allow the production of the high viral loads generally observed in such patients. Recent studies in AIDS patients have shown a sharp increase of the peripheral blood CD4 T cell count after starting highly active antiretroviral therapy [26,27], probably reflecting redistribution of CD4 T cells from epitheloid organs such as the lung or the gut. Taking into account the increased percentage of productively infected cells and the increased virus replication rate of HIV-1 strains isolated, it seems likely that also at the end stage of the disease sufficient CD4 T cells are present in the individual to explain the high viral load observed.

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