B cell activation in peripheral blood and lymph nodes during HIV infection

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Background: The spontaneous in-vitro antibody synthesis observed in unstimulated lymphocyte cultures from HIV-infected patients closely reflects the in-vivo activation of the B cell compartment; however, the mechanisms underlying this phenomenon are far from clear.

Methods: We compared the ability of peripheral blood mononuclear cells (PBMC) and lymph-node cells (LNC) from 10 HIV-infected patients to produce *in vitro* HIV-specific and total Ig spontaneously, and we correlated these parameters with tumour necrosis factor alpha (TNF-α) expression by CD4 T cells, viral dissemination in the organism, and the extent of HIV spread into lymph-node germinal centres, measured by in-situ hybridization (ISH).

Results: In-vitro spontaneous synthesis of both HIV-specific and total antibody was significantly higher in PBMC than in LNC; the two variables showed a good correlation in LNC, but not in PBMC. In both compartments, no correlation was found between B cell activation and the percentage of CD4 T cells expressing TNF-α, which was increased compared with seronegative donors. Furthermore, no correlation was found between in-vitro spontaneous antibody synthesis and the number of T cells containing proviral HIV in PBMC and LNC, or the plasma levels of HIV RNA. On the contrary, a good correlation was found between HIV-specific B cell activation and the extent of viral spread into lymph-node germinal centres, evaluated by ISH.

Conclusion: These data suggest that the adhesion of HIV virions to the follicular dendritic cell network in lymph-node germinal centres may primarily contribute to sustaining the steady B cell activation observed in HIV-infected patients.

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Introduction

An important B cell activation is a hallmark of HIV infection [1–5]; a general consensus exists on the fact that the spontaneous in-vitro antibody synthesis observed in non-stimulated lymphocyte cultures from these patients closely mirrors the in-vivo activation of the B cell compartment [1,2,4]. Most of the antibodies spontaneously produced in culture are specific for HIV determinants [1,6], although a polyclonal B cell activation also occurs, as shown by the synthesis of antibodies against other antigens [7]. On the other hand, B cells

from HIV-infected patients are relatively refractory to in-vitro stimulation, with eventual profound impairment in memory function [1,8]. This important B cell function derangement might be involved in several aspects of HIV-associated disorders [9], and in particular the increased prevalence of B cell lymphomas in seropositive patients [10,11]. Although it was shown that IL-6 may play a role in sustaining B cell activation [8,12], the mechanisms underlying this phenomenon are mostly unclear, in particular as far as the first activation steps are concerned. Whereas some workers suggested a direct B cell activating effect exerted by viral products [13], other data do not support this possibility [14]. It has also been suggested that tumour necrosis factor alpha (TNF-α) expression on CD4 T cell membranes could be responsible for B cell activation [15]. In fact, an increased number of CD4$^+$TNF-α^+ lymphocytes was found in the circulation of seropositive patients; moreover, HIV-infected T cell clones expressing surface TNF-α were able in vitro to induce strong antibody production in resting B cells, and this effect was abrogated by anti-TNF-α antibodies [15].

The behaviour of the B cell compartment has mostly been studied in peripheral blood, which constitutes a minor district of the entire immune system; indeed, it is now clear that peripheral lymphoid organs are a major HIV reservoir, where most of the immuno-pathological events that contribute to the natural history of HIV infection and AIDS pathogenesis take place [16,17]. So, we felt it appropriate to compare the status of the B cell compartment in circulating lymphocytes and in cells purified from the lymph nodes of HIV-infected patients, and to attempt to correlate these data with different immunological and virological parameters, such as the degree of TNF-α expression on CD4 lymphocytes, the extent of T cell infection by HIV, and the amount of virions bound to the follicular dendritic cell network in the lymph nodes. Our findings show that B cell activation is strictly related to the extent of HIV particle adhesion to the follicular dendritic cell network in lymph-node germinal centres, and stress the theory that peripheral blood is only a minor district of the immune system, which may not entirely reflect an antigen-driven response in the rest of the body.

Materials and methods

Study population
Informed consent was obtained from all the individuals participating in this study. Ten male HIV-infected intravenous drug abusers (mean age 31 years, range 25–41) underwent routine clinical and laboratory evaluation, and were staged according to the Walter Reed staging classification [18]. One patient was in stage WR2, six were in stage WR4, two were in stage WR5 and one was in stage WR6; the mean number of circulating CD4 lymphocytes/mm^3 was 262 ($\pm$ 109 SD; range 114–382). None of the patients was on highly active antiretroviral therapy (HAART) at the time of this study. All patients underwent a lymph-node biopsy for diagnostic purposes; the sample was divided into three parts, one for histological examination, one for lymphocyte isolation, and the third for in-situ hybridization (ISH) analysis. Blood samples were also obtained from 18 healthy laboratory staff members of comparable age, which served as seronegative controls. Control lymph-node samples were obtained from three seronegative individuals undergoing minor surgery; no gross histological abnormalities were recorded in these samples.

Cell preparation
Peripheral blood mononuclear cells (PBMC) were isolated from preservative-free heparin-anticoagulated venous samples by Ficoll-Paque (Pharmacia-LKB, Uppsala, Sweden) gradient centrifugation, as described elsewhere [19]. After washings with phosphate-buffered saline, the cells were centrifuged through a fetal calf serum (FCS; Gibco, Grand Island, NY, USA) gradient, to guarantee the complete removal of serum Ig [20]. Lymph-node cell (LNC) suspensions were obtained by mechanical dissociation of the tissue in RPMI 1640 medium supplemented with 10% FCS; the cells were recovered after three washes with RPMI and centrifugation through an FCS gradient as above.

Cytofluorimetric analysis
PBMC and LNC were analysed cytofluorographically by standard methods, using an Epics C cytofluorometer (Coulter Electronics, Hialeah, FL, USA) as previously reported [21]. Leukocyte populations were discriminated on the basis of forward and 90° light scatter; computer-generated windows were created to obtain the optimal discrimination of lymphocytes from monocytes. At least 10 000 cells were collected in each fluorescence histogram. The monoclonal antibodies (mAb) used were fluorescein isothiocyanate-labelled anti-CD19, anti-CD8, and anti-CD4 (all from Dakopatts, Glostrup, Denmark); in double-fluorescence experiments, a phycoerythrin-labelled anti-CD4 mAb (Coulter) was used in combination with an anti-TNF-α mAb (B154.2.1, kindly provided by Dr B. Perussia, Philadelphia, WA, USA), and a secondary fluorescent anti-mouse IgG antibody (Becton–Dickinson, Mountain View, CA, USA).

In-vitro antibody production
The ability of PBMC and LNC to produce antibody spontaneously in-vitro was assessed according to a standardized protocol [20], which allows optimal in-vitro antibody production, and enables the detection of

as few as 1 ng of newly synthesized HIV-specific Ig. Cell suspensions (2×10^6/ml) were cultured in RPMI medium supplemented with 10% FCS, 1% L-glutamine, 1% non-essential amino acids, and 2×10^{-5} M 2-mercaptoethanol (complete RPMI) in 48-well flat-bottom tissue culture plates (Falcon, Grenoble, France) at 37°C, with 5% carbon dioxide in air for 12 days. Cell-free supernatants were then recovered by low-speed centrifugation, and stored at −20°C until use.

Detection of anti-HIV antibody and total Ig in culture supernatants

HIV-specific and total Ig contents in culture supernatants were assessed by solid-phase radioimmunoassay, as previously reported [1,20]. Briefly, HIV proteins (HIV lysate, Sclavo-Dupont, Siena, Italy; 10 μg/ml in carbonate buffer, pH 9.6) or goat anti-Ig serum (Sera-Lab, Milan, Italy; 5 μg/ml in carbonate buffer) were coupled to the wells of 96-well polyvinyl plates (Falcon), and the wells were saturated with 3% bovine serum albumin (Sigma Chemicals, S. Louis, MO, USA). After three washes, supernatant aliquots (50 μl) were added to individual wells in triplicate; the plates were left to stand for 3 h at 37°C, washed, and then incubated for 4 h at room temperature with ^{125}I-labelled goat anti-human Ig F(ab)2 (Amersham, Buckinghamshire, UK; sp. act. 19–74 TBq/mM). Finally, the plates were washed thoroughly, and each well counted in a γ counter (Packard, Grove Hill, IL, USA).

As a result of the lack of a standard reagent, anti-HIV antibody contents in supernatants were evaluated against a reference HIV-positive serum, which was arbitrarily assigned an HIV-specific antibody content of 8×10^6 arbitrary units (AU)/ml [20]. Ig levels were evaluated against a reference curve obtained by doubling dilutions of a standard human serum containing known Ig amounts. Results were corrected for the actual number of B lymphocytes present in culture, as determined by cytofluorimetric analysis with anti-CD19 mAb, and expressed as AU/ml/10^6 B cells for anti-HIV antibody, and as μg/ml/10^6 B cells for total Ig.

Quantitative determination of HIV proviral copies

The amount of HIV proviral copies in PBMC and LNC was determined by polymerase chain reaction (PCR) analysis, as reported elsewhere [22]. A standard reference curve was prepared using 8E51 cells, which contain one proviral copy per cell [23]. Samples of standard reference cells and patients' cells were lysed as reported [24]; 25 μl of cell lysate (corresponding to 1×10^5 cells) were mixed with 75 μl of PCR buffer (10 mM Tris–HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$) containing a 200 μM concentration of each of four deoxynucleotide triphosphates, a 50 pM concen-

tration of each of primer SK29 and SK30 [25], and 2.5 U of Taq polymerase (Perkin–Elmer, Norwalk, CT, USA), and then amplified in a DNA thermal cycler (Perkin–Elmer). Thirty cycles were run at a denaturation temperature of 94°C, an annealing temperature of 50°C, and an extension temperature of 72°C. Each specimen was prepared and run in duplicate. To control the reaction and quality of the DNA samples to be amplified, we used PCO3/PCO4 primers specific for the β-globin gene [26]. Twenty microlitres of each amplified sample were electrophoresed and transferred to Nytran filters. Hybridization was achieved with a 5′ end ^{32}P-labelled SK31 probe, and filters were exposed to X-ray films for 12 h. The specific band intensity was determined by densitometer analysis (Ultrascan LX enhancer, Laser densitometer, LKB, Bromma, Sweden). In standard reference samples optical density values were linearly related up to 1000 HIV copies, with a coefficient of correlation ranging from 0.96 to 0.98. Optical density values in patient samples compared against the standard reference curve gave the number of proviral copies/10^5 PBMC or LNC. The results were corrected for the actual number of CD4 T cells present in each sample (determined by cytofluorometric analysis with anti-CD4 mAb) and were expressed as the number of HIV proviral copies/10^5 CD4 T cells.

Plasma RNA copy quantification

Ethylenediamine tetraacetic acid-anticoagulated venous samples were obtained from each patient. Plasma was immediately separated by centrifugation, and the samples were stored at −80°C until testing. The number of HIV-RNA copies was measured using reverse transcriptase PCR (Amplicor, Roche, Milan, Italy), according to the manufacturer's instructions.

In-situ hybridization

ISH analysis was performed as described elsewhere [16]. Briefly, lymph-node fragments were fixed in formaldehyde, embedded in paraffin and subjected to routine pathological examination. To identify lymph-node germinal centres (B cell areas), after digestion with proteolytic enzymes to remove viral envelope proteins, the slides were first subjected to staining with anti-CD21 mAb (clone F18, Dakopatts), which recognizes both B lymphocytes and follicular dendritic cells (FDC) in formaldehyde-fixed tissues, although at a different extent. The slides were then hybridized with sense or antisense HIV probes [27], and run in duplicate; RNA probes were synthesized from 5′ DNA templates that represent 90% of the HIV genome [27]. After hybridization, autoradiograms were made with NTB3 emulsion (Eastman Kodak, Rochester, NJ, USA), stained with haematoxilin-eosin, and examined by conventional and dark-field microscopy. The section area stained by anti-CD21 mAb, which corresponds to B cell areas, as well as the areas reacting with

antisense HIV probes after proteolytic digestion, were recorded by computer-assisted analysis; the results were calculated in square millimetres, and expressed as a percentage of the total lymph-node section area.

Statistical analysis
Data were managed by StatGraphics software (Stat-graphics Statistical Graphics System, version 2.6). The Mann–Whitney and the Spearman rank correlation tests were used when appropriate.

Results

In-vitro spontaneous antibody production in peripheral blood and lymph-node cells
We first compared the ability of PBMC and LNC from HIV-infected patients to produce HIV-specific and total Ig spontaneously in-vitro. The results are summarized in Fig. 1, and are also analytically presented in Table 1. The spontaneous production of HIV-specific antibody by both PBMC and LNC could be shown in all seropositive patients (Fig. 1, left panel), but not in cultures from seronegative controls (not shown in Fig. 1).

As we and others have previously reported [1,2,5], the spontaneous total Ig synthesis (Fig. 1, right panel) was significantly higher in PBMC from HIV-infected patients (mean 93 ± 64 µg/ml/10^6 B cells) than in PBMC cultures from non-infected individuals (mean 1.4 ± 1.0 µg/ml/10^6 B cells; shaded area in Fig. 1; $P < 0.01$ according to Mann–Whitney test). This was also true for lymph-nodes; in fact, the mean values of spontaneous Ig synthesis by LNC from HIV-infected patients (closed symbols) were 6 ± 4 µg/ml/10^6 B cells, compared with 0.2, 0.25 and 0.3 µg/ml/10^6 B cells in LNC from three seronegative controls (shown as open symbols in Fig. 1).

In seropositive patients, both HIV-specific and total Ig synthesis were higher in PBMC than in LNC (Fig. 1 and Table 1, $P < 0.01$ according to Mann–Whitney). In addition, HIV-specific and total Ig production were highly correlated in LNC cultures ($r = 0.73$, $P = 0.03$ according to the Spearman rank correlation test) but not in PBMC cultures ($r = 0.32$, P n.s.). A significant correlation was also seen in HIV-specific antibody production from PBMC and LNC ($r = 0.66$, $P = 0.04$); on the other hand, when we compared the spontaneous total Ig synthesis between LNC and PBMC, no correlation was found ($r = 0.21$, P n.s.).

Tumour necrosis factor alpha expression in peripheral blood lymphocytes and lymph-node cells
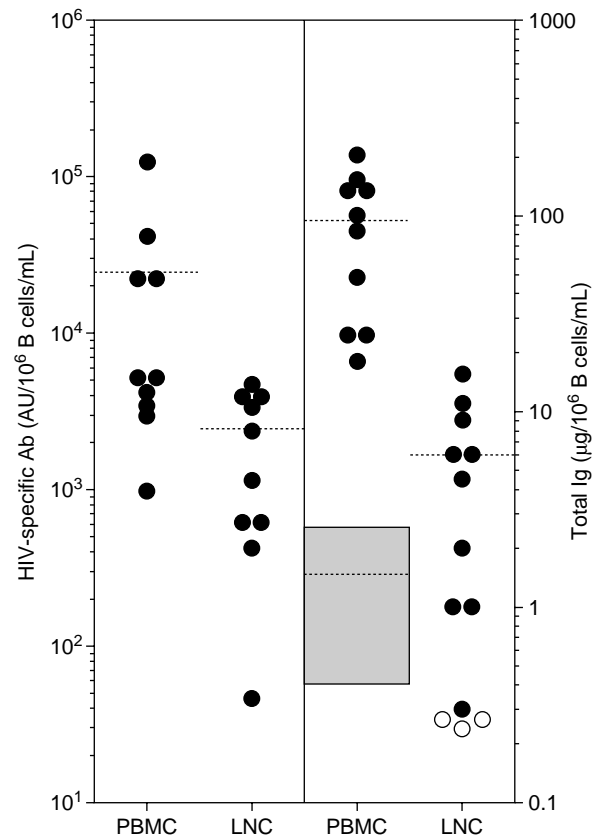We then analysed the expression of TNF-α on the membrane of CD4 T cells in PBMC and LNC. Two-

Fig. 1. In-vitro spontaneous production of HIV-specific antibody and total Ig in peripheral blood and lymph-node cells from HIV-infected patients. Non-stimulated cells were cultured *in vitro*, and antibody contents in culture supernatant were determined as detailed in the Materials and methods section. HIV-specific antibody contents were expressed as arbitrary units (AU)/10^6 B cells/ml, and total Ig as µg/10^6 B cells/ml. Individual patients are indicated by closed circles; the dashed lines denote mean values. The shaded area indicates figures obtained in peripheral blood mononuclear cells (PBMC) from 18 seronegative donors (mean value $\pm$ 1 SD), whereas open circles refer to values obtained in lymph-node cell (LNC) samples obtained from three seronegative controls.

colour immunofluorescence with anti-CD4 and anti-TNF-α mAb (Fig. 2) disclosed that the percentage of circulating CD4 T cells that also expressed TNF-α was significantly higher in HIV-infected patients (mean 2.86 ± 2.0; median 2.23; range 1.25–8.0) than in seronegative controls (mean 0.80 ± 0.63; median 0.53; range 0.1–2.5; shaded area in Fig. 2; $P < 0.01$ according to Mann–Whitney test). Our figures confirmed previous data by Macchia et al. [15], who showed a similar increase in circulating CD4+TNF-α+ lymphocytes in HIV-infected patients. A notable membrane TNF-α expression was also seen in the LNC from patients (Fig. 2, closed symbols); CD4+TNF-α+ cells ranged from 1.0 to 8.7% (mean 3.65 ± 2.2; median

Table 1. Immunological and virological parameters in peripheral blood and lymph nodes of HIV-infected patients

Patient code	PBMC				LNC				Viraemia[e]
	% CD4 TNF-α[+a]	HIV-specific antibodies[b]	Total Ig[c]	HIV proviral copies[d]	% CD4 TNF-α[+a]	HIV-specific antibodies[b]	Total Ig[c]	HIV proviral copies[d]	
23	1.7	974	100	74 214	3.4	1138	15.5	11 684	252 696
64	2.86	2960	48	702	4.16	46	0.3	1033	199 763
65	2.30	41 345	134	537	2.09	3918	9	668	124 104
66	1.61	22 245	83.5	622	4.00	609	4.5	1180	114 187
67	1.61	123 319	204	282	1.00	4681	6	871	2016
68	2.80	5172	18	1783	1.69	3866	11	75	3248
69	2.16	21 680	139	1408	2.61	3368	6	1546	4542
70	8.00	4750	26	8218	5.77	421	1	4531	58 062
71	4.31	4184	24.5	2048	8.69	603	1	5400	55 872
72	1.25	3458	153	44 198	3.07	2342	2	19 737	208 908
Mean	2.86	23 009	93	13 401	3.6	2099	6	4672	102 340
SD	2	37 530	64	25 262	2.2	1738	5	6355	92 878
Median	2.23	4961	91.75	1595.5	3.235	1740	5.25	1363	8612.5

LNC, Lymph-node cells; PBMC, peripheral blood mononuclear cells.
[a]Values represent the percentage of CD4 cells also expressing membrane tumour necrosis factor alpha (TNF-α), as detailed in the Materials and methods section.
[b]In-vitro spontaneous production of HIV-specific antibody, expressed as arbitrary units (AU)/ml/10^6 B cells.
[c]In-vitro spontaneous production of total Ig, expressed as μg/ml/10^6 B cells.
[d]Number of HIV proviral copies/10^5 CD4 T cells.
[e]Number of HIV-RNA copies per ml of plasma.

3.23), whereas in the LNC from three seronegative donors TNF-α expression on CD4 lymphocytes was much lower, and accounted for 0.5, 0.7 and 1.1% of the cells (open symbols in Fig. 2). However, probably because of the small number of cases studied, no significant correlation between TNF-α expression in PBMC and LNC from seropositive patients was observed (P = 0.06 according to the Spearman rank test). No correlation was also found between TNF-α expression on T cell surfaces and the spontaneous in-vitro synthesis of HIV-specific and total Ig in either compartment (P = 0.23 in both cases, according to the Spearman rank correlation test; see Table 1).

Evaluation of HIV spread and correlation with the different parameters

The extent of virus spread into the organism was evaluated by both an assessment of the number of HIV proviral copies in the circulating and lymph-node compartments and by plasma RNA copy enumeration; results are summarized in Table 1. The proviral copy number/10^5 CD4 cells, which may closely reflect the actual viral burden in the organism, showed a wide variation in the patients studied, ranging from 282 to 74 214 (mean 13 401 ± 25 262; median 1595) in PBMC and between 75 and 19 737 (mean 4672 ± 6355; median 1363) in LNC; a significant correlation was observed between the two values (r = 0.73, P = 0.02 according to the Spearman rank test). However, the extent of viral burden in lymphoid organs was not uniformly higher than in peripheral blood, and in some cases the number of proviral copies/10^5 CD4 cells was higher in the latter; this may be conceivable,

as a single lymph node may not entirely reflect viral spread in peripheral lymphoid tissues.

On the other hand, when we measured the number of HIV copies in patients' plasma, which may be considered to be a reliable index of viral activation and replication, a wide range of variability was observed, and in the different patients the number of HIV copies/ml of plasma ranged from 2016 to 252 696 (Table 1). No correlation was found between the number of proviral copies in PBMC or LNC and the plasma RNA copy number; this is not surprising, as proviral load and virus activation are two independent variables that probably reflect different stages of the HIV biological cycle.

When we attempted to verify the correlations between the virological parameters examined above and the in-vitro total or HIV-specific antibody synthesis (summarized for each patient in Table 1), no correlation was found in either PBMC and LNC. Unfortunately, the technique used for lymph-node sample processing did not permit the evaluation of HIV-RNA levels in LNC, which would have allowed a correlation between B cell activation and HIV replication in a restricted area. In addition, no correlation was observed between the number of proviral copies in PBMC or LNC and the percentage of CD4+TNF-α+ lymphocytes in the corresponding district (Table 1); no correlation was found either between this latter variable and the plasma RNA copy number. Because of the small number of cases studied, multivariate analysis could not be performed.
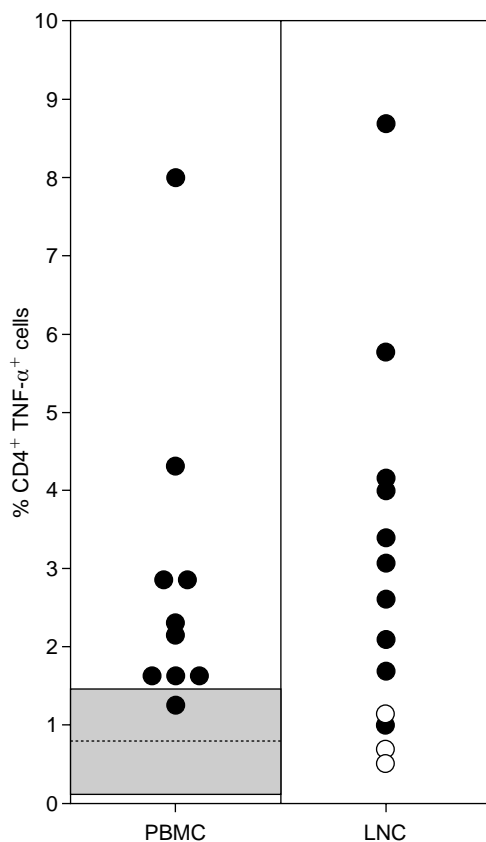
Fig. 2. Tumour necrosis factor alpha expression in peripheral blood and lymph-node cells from HIV-infected patients. The percentage of CD4 T cells also expressing tumour necrosis factor alpha (TNF-α) was evaluated as detailed in the Materials and methods section. The shaded area indicates figures recorded in peripheral blood mononuclear cells (PBMC) from 18 seronegative donors (mean value ± 1 SD). The open circles refer to values obtained in three lymph-node cell (LNC) samples obtained from seronegative controls.

Evaluation of HIV spread into lymph-nodes by in-situ hybridization

In view of these results, which did not help to understand the factor(s) driving B cell activation, we finally attempted to correlate the extent of in-vitro spontaneous antibody production in LNC with the adhesion of HIV particles to FDC within lymph-node germinal centres. Indeed, that the FDC network could contribute to B cell activation in seropositive patients was conceivable, as it is known that antigen–antibody complexes, in combination with complement components, may adhere to FDC in lymph-nodes, where they can persist for a very long time, thus maintaining long-term B cell memory [28]. To this end, we performed a semi-quantitative analysis of the extent of HIV presence at this location by means of an ISH technique. The ISH pattern in a representative seropositive (panels a, b and c) and seronegative (panels d, e and f) individual is shown in Fig. 3, and the results obtained

in the five HIV-infected patients in whom such an analysis was feasible are summarized in Table 2.

In seropositive patients, lymph-node architecture was altered, and in most cases a general follicular involution was observed, jeopardized by local hyperplastic figures, with progressive vascular or fibrotic substitution of the germinal centres (Fig. 3, panel a). The histological appearance of lymph nodes from seronegative donors was normal, and several lymphoid follicles could be identified by FDC staining (Fig. 3, panels d and e). In seropositive patients, the presence of HIV RNA was mostly recorded in B cell areas (identified by anti-CD21 mAb staining), as shown by the presence of silver grains in the germinal centres (Fig. 3, panel a, and panel b at higher magnification); in seronegative individuals no HIV RNA could be observed (Fig. 3, panels e and f). When we measured the HIV-targeted area by computer-assisted analysis of dark-field images (panel c in Fig. 3), in the different patients examined the percentage of HIV areas ranged from 1.2 to 18.7% of the total lymph-node section (Table 2).

When these figures were related to B cell areas, a notable percentage of the CD21+ area, corresponding to the lymphoid follicle germinal centres, was covered by HIV RNA (Table 2). In one case (patient no. 65 in Table 2), the HIV-related area was greater than that identified by the anti-CD21 mAb; this finding may be related to lymph-node architecture and FDC network disruption, with the eventual binding of HIV virions to scattered FDC, poorly stained by anti-CD21 mAb. No correlation was found between the extent of HIV adhesion to FDC and the number of proviral HIV copies in lymph-nodes and PBMC or viraemia (see Table 1). Instead, a significant correlation was observed between the degree of spontaneous in-vitro HIV-specific antibody or total Ig production by LNC and the percentage of the lymph-node B cell areas reacting with the RNA-specific HIV probe (P < 0.01 according to the Spearman rank correlation test). On the other hand, a significant correlation was not observed with the spontaneous HIV-specific or total antibody synthesis in PBMC (see Table 1 and Table 2).

Discussion

In an attempt to gain a better understanding of the mechanisms underlying B cell activation during HIV infection, we addressed the spontaneous in-vitro synthesis of HIV-specific and total Ig in the peripheral blood and lymph-node compartments from HIV-infected patients. When we compared the spontaneous in-vitro production of HIV-specific and total antibody in peripheral blood and lymph-nodes, we found that B cell activation was much higher in the circulating
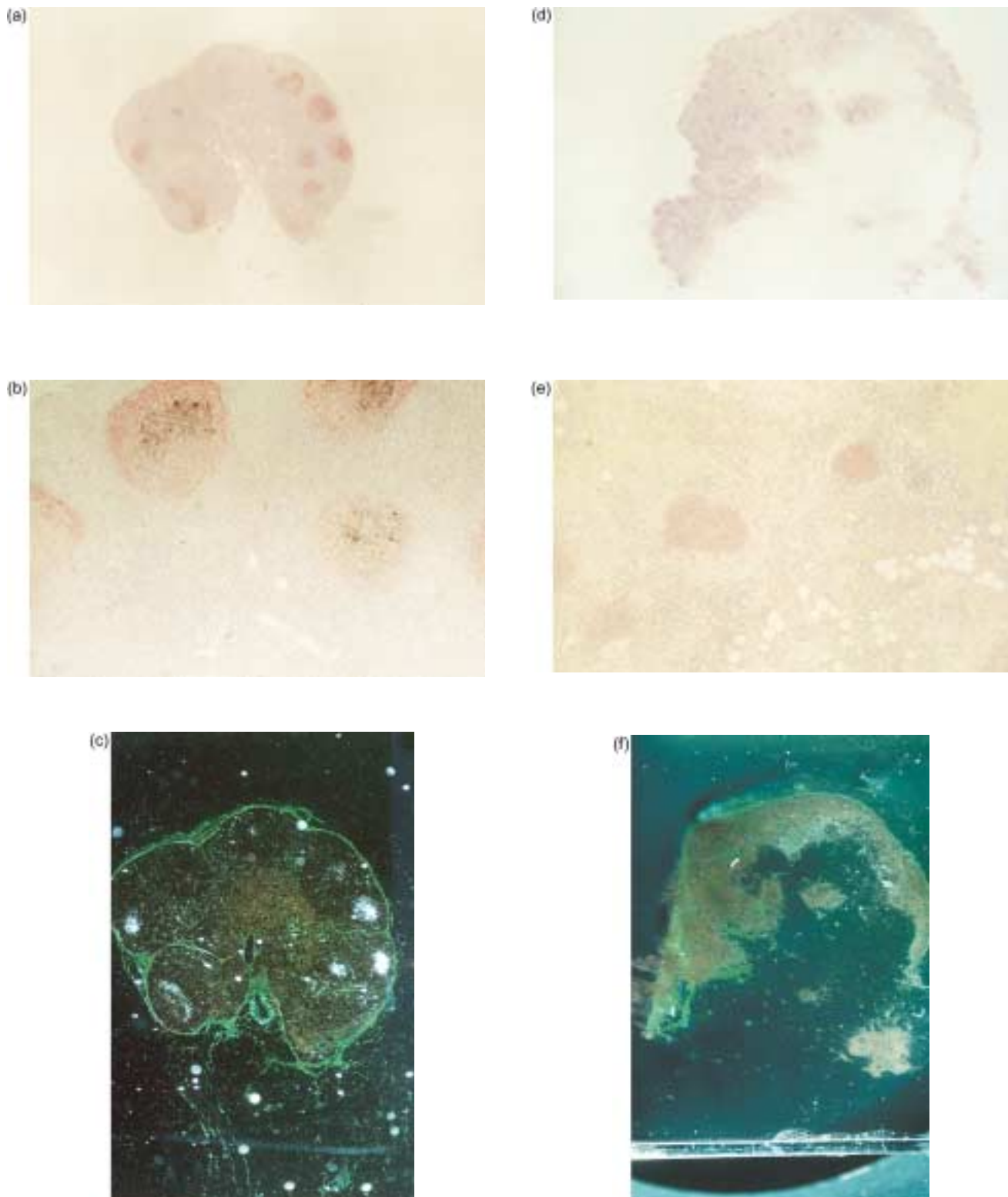
Fig. 3. In-situ hybridization for HIV RNA in lymph-node sections from HIV-infected patients and seronegative controls. Results obtained in a representative HIV-infected patient (panels a, b and c, corresponding to patient no. 68 in the Tables) and a seronegative individual (panels d, e and f) are presented. Sections were immunohistochemically stained with anti-CD21 monoclonal antibody, which identifies the follicular germinal centres. After protease digestion and anti-sense hybridization, in the sample from the HIV-infected patient the location of HIV RNA is indicated by silver grains, which appear as brown (panels a, and b at higher magnification) or white spots in the dark-field image (panel c), mostly confined to areas corresponding to follicular germinal centres. No brown (panel e) or white spots (panel f) are visible in germinal centres of the control lymph node.

Table 2. Correlation between HIV adhesion to follicular dendritic cells and spontaneous in-vitro antibody production in lymph nodes

Patient code	Lymph-node section area[a]	CD21+ area[b]	HIV+ area[c]	% HIV+ area/ CD21+ area	HIV-specific antibodies[d]	Total Ig[e]
65	27.48	2.97 (10.8)	5.15 (18.74)	173.40	3918	9
68	34.85	2.9 (8.32)	1.73 (4.96)	59.66	3866	11
69	37.09	4.58 (12.3)	2.7 (7.28)	58.95	3368	6
70	97.63	6.16 (6.31)	1.17 (1.2)	19	421	1
71	38.96	2.53 (6.5)	0.96 (2.46)	37.95	603	1
Mean	47.2	3.8 (8.85)	2.3 (6.93)	69.8	2435	6
SD	28.5	1.5 (2.64)	1.7 (7)	60.3	1770	4.6
Median	37.09	2.97 (8.32)	1.73 (4.96)	58.95	3368	6

[a]Lymph-node sections from HIV-infected patients were stained with anti-CD21 monoclonal antibody to identify B cell areas, and subjected to in-situ hybridization with HIV RNA-specific probes, as detailed in the Materials and methods section. The total lymph-node section area is expressed in mm^2.
[b]The lymph-node section area stained by the anti-CD21 monoclonal antibody is expressed in mm^2; the percentage of the total area is indicated in parentheses.
[c]The lymph-node area reacting with HIV probes is expressed in mm^2; the percentage of the total area is indicated in parentheses.
[d]Results are expressed as arbitrary units (AU)/ml of HIV-specific antibody produced spontaneously *in vitro* by 10^6 lymph-node B cells.
[e]Results are expressed as g/ml of total Ig spontaneously produced *in vitro* by 10^6 lymph-node B cells.

compartment. In addition, although a significant correlation was observed between the amounts of HIV-specific antibody spontaneously produced by LNC and PBMC, this correlation was not found when the spontaneous synthesis of total Ig by these compartments was compared. These data seem to indicate that virus-specific antibody production more closely reflects the overall B cell activation in lymphoid organs, which are a major HIV reservoir [16,17], and that LNC are mainly engaged in anti-HIV antibody synthesis. On the contrary, unlike figures obtained in a single lymph node, PBMC data may reflect B cell activation within the entire organism, which includes lymphoid and non-lymphoid districts, possibly affected by B cell-activating events such as opportunistic infections. Indeed, the observed lack of correlation between HIV spread into lymph-node germinal centres, as judged by ISH analysis (Table 2), and the spontaneous synthesis of total Ig by PBMC reinforces the notion that B cells may also be activated within lymphoid organs by antibody-complexed antigens of different origin (such as opportunistic microbe components), thus explaining the polyclonal nature of the humoral response.

It was reported that the in-vitro infection of T lymphocytes by HIV translates into the expression of TNF-α molecules on the CD4 cell surface, and these lymphocytes acquire a tremendous ability to induce antibody production by B cells [15]. Our findings confirm and extend the data of Macchia et al. [15]; however, we could not observe any link between B cell activation and TNF-α expression on CD4 T cells, nor this latter variable correlated with viral load or replication into the organism. It is thus improbable that

HIV infection and eventual TNF-α expression could be a major factor underlying the huge in-vivo B cell activation observed in seropositive patients. On the other hand, the lack of correlation between the virological parameters examined here and the extent of TNF-α expression on CD4 cell membranes may also cast some doubt on the HIV specificity of such a phenomenon.

A most obvious idea would be that B cell activation could be strictly related to the extent of proviral load or virus replication. However, several lines of evidence in this paper seem to suggest that, in these terms, this notion could be simplistic. In fact, in both the circulating and lymph-node compartments we did not find any correlation between spontaneous in-vitro antibody production and the viral burden, measured as both the number of T cells harbouring the proviral genome and the number of RNA viral copies in plasma. The only virological parameter able to correlate with B cell activation was the extent of HIV adhesion to FDC in lymph-node germinal centres, as measured by an ISH technique. Previous data demonstrated that lymphoid organs are a major HIV reservoir, and that billions of virions stick to the FDC network even in apparently asymptomatic individuals [16,17,29]. In this paper we provided for the first time a semi-quantitative analysis of this phenomenon, and we showed that its magnitude correlated with the ability of lymph-node B cells to produce antibody spontaneously in-vitro.

We are perfectly aware that figures obtained in a few lymph-node sections could not reflect the situation in other areas of the same lymph node or in other lymph

nodes; indeed, in some cases the proviral load was higher in PBMC than LNC (Table 1), thus confirming that a single lymph node could not closely mirror the overall picture, and suggesting that it could be misleading to correlate antibody synthesis in LNC suspensions with the pattern of HIV trapping by FDC observed in a relatively restricted area of the same lymph node. Nevertheless, in the small case series studied here, the results of a semi-quantitative analysis of FDC-associated HIV virions in the germinal centres indicated a strict correlation with the extent of spontaneous B cell activation, and patients with more extended HIV-loaded lymph-node areas also showed a greater ability of LNC to secrete HIV-specific antibody spontaneously in vitro. In this regard, as previously stated, it is particularly significant that such a correlation was only found for antibody production by LNC, and not by PBMC, which likewise mirror the entire lymphoid compartment.

HIV spread in follicular germinal centres showed no correlation with other parameters that can reflect viral burden, such as the number of HIV proviral copies in lymphocytes or the number of RNA copies in plasma. This was not surprising, as it is conceivable that these two virological parameters are much more dynamic and subject to rapid modifications [30], compared with the FDC network, in which the lack of FDC phagocytic capacity and the eventual low virion turnover render this district much more inert in modifying its profile. In this sense, FDC-trapped virions may depict a relatively recent history of HIV replication. It is therefore reasonable to assume that B cells continuously recirculating through lymph nodes could interact with FDC-associated virions, thus receiving activatory signals to proliferation and antibody production. In this regard, our findings extend previous data obtained in experimentally SIV-infected macaques, in which FDC-associated virus increased FDC–B lymphocyte interactions and thus B cell activation [31]. To what extent this steady B cell activation contributes to the increased prevalence of non-Hodgkin's lymphomas (NHL) among seropositive patients is a key question [9–11]. Unpublished observations (Zamarchi R. et al.) in a limited series of patients tested before and after starting HAART indicated that in most individuals spontaneous in-vitro antibody synthesis by PBMC decreases dramatically approximately 3–6 months after treatment initiation. It would thus be intriguing to attempt prospectively to correlate the behaviour of spontaneous in-vitro antibody synthesis and HIV adhesion to the FDC network before and after HAART with NHL development. Although the incidence of NHL has greatly decreased in the HAART era [32], a considerable proportion of patients still develop this type of malignancy, and a surrogate marker to identify patients at higher risk of undergoing lymphomas is urgently needed.

Acknowledgements

References

1. Amadori A, Zamarchi R, Ciminale V, *et al.* **HIV-1-specific B cell activation. A major constituent of spontaneous B cell activation during HIV-1 infection.** *J Immunol* 1989, **143**:2146–2152.
2. Pahwa S, Chirmule N, Leombruno C, *et al.* **In vitro synthesis of human immunodeficiency virus-specific antibodies in peripheral blood lymphocytes of infants.** *Proc Natl Acad Sci U S A* 1989, **86**:7532–7536.
3. Amadori A, De Rossi A, Gallo P, Tavolato B, Chieco-Bianchi L. **Cerebrospinal fluid lymphocytes from HIV-infected patients synthesize HIV-specific antibody *in vitro*.** *J Neuroimmunol* 1988, **18**:181–186.
4. Ng VL, Chen KH, Hwang KM, Khayam-Bashi H, McGrath MS. **The clinical significance of human immunodeficiency virus type 1-associated paraproteins.** *Blood* 1989, **74**:2471–2475.
5. Amadori A, Zamarchi R, Veronese ML, *et al.* **B-cell activation during HIV-1 infection. III. Down-regulating effect of mitogens.** *AIDS* 1991, **5**:821–828.
6. Zubler RH, Perrin LH, Doucet A, Zhang X, Huang YP, Miescher PA. **Frequencies of HIV-reactive B cells in seropositive and seronegative individuals.** *Clin Exp Immunol* 1992, **87**:31–36.
7. Shirai A, Cosentino M, Leitman-Klinman SF, Klinman DM. **Human immunodeficiency virus infection induces both polyclonal and virus-specific B cell activation.** *J Clin Invest* 1992, **89**: 561–566.
8. Amadori A, Zamarchi R, Veronese ML, *et al.* **B-cell activation during HIV-1 infection. II. Cell-to-cell interactions and cytokine requirement.** *J Immunol* 1991, **146**:57–62.
9. Amadori A, Chieco-Bianchi L. **B cell activation and HIV-1 infection: deeds and misdeeds.** *Immunol Today* 1990, **11**: 374–379.
10. Levine AM. **AIDS-related malignancies: the emerging epidemics.** *J Natl Cancer Inst* 1993, **85**:1382–1397.
11. Grulich AE, Wan X, Law MG, *et al.* **B-cell stimulation and prolonged immune deficiency are risk factors for non-Hodgkin's lymphoma in people with AIDS.** *AIDS* 2000, **14**:133–140.
12. Breen EC, Rezai AR, Nakajima K, *et al.* **Infection with HIV is associated with elevated IL-6 levels and production.** *J Immunol* 1990, **144**:480–484.
13. Pahwa S, Pahwa R, Good RA, Gallo RC, Saxinger C. **Stimulatory and inhibitory influences of human immunodeficiency virus on normal B lymphocytes.** *Proc Natl Acad Sci U S A* 1986, **83**: 9124–9128.
14. Spickett G, Beattie R, Farrant J, Briant A, Dalgleish A, Webster D. **Assessment of responses of normal human B lymphocytes to different isolates of human immunodeficiency virus: role of normal donor and of cell line used to prepare viral isolate.** *AIDS Res Hum Retroviruses* 1989, **5**:355–366.
15. Macchia G, Almerigogna F, Parronchi P, Ravina A, Maggi E, Romagnani S. **Membrane tumor necrosis factor-alpha is involved in the polyclonal B-cell activation induced by HIV-infected human T cells.** *Nature* 1993, **363**:464–466.
16. Pantaleo G, Graziosi C, Demarest J-F, *et al.* **HIV infection is active and progressive in lymphoid tissue during the clinically latent stage of disease.** *Nature* 1993, **362**:355–358.
17. Graziosi C, Pantaleo G, Demarest JF, *et al.* **HIV-1 infection in lymphoid organs.** *AIDS* 1993, **7**:S53–S58.
18. Redfield RR, Wright DC, Tramont EC. **The Walter Reed staging**

classification for HTLV-III/LAV infection. *N Engl J Med* 1986, **314**:131–132.

19. Zamarchi R, Panozzo M, Del Mistro A, *et al.* **B and T cell function parameters during zidovudine treatment of human immunodeficiency virus-infected patients.** *J Infect Dis* 1994, **170**:1148–1156.

20. Indraccolo S, Zamarchi R, Veronese ML, *et al.* **Standardization of in vitro synthesis and detection of HIV-1-specific antibodies.** *J Immunol Methods* 1993, **157**:105–115.

21. Amadori A, Zamarchi R, De Silvestro G, *et al.* **Genetic control of the CD4/CD8 T-cell ratio in humans.** *Nat Med* 1995, **1**: 1279–1283.

22. Amadori A, De Silvestro G, Zamarchi R, *et al.* **CD4 epitope masking by gp120/anti-gp120 antibody complexes. A potential mechanism for CD4$^+$ cell function down-regulation in AIDS patients.** *J Immunol* 1992, **148**:2709–2716.

23. Folks TM, Powell D, Lightfoote M, *et al.* **Biological and biochemical characterization of a clone of leu-3$^-$ cell surviving infection with the acquired immune deficiency retrovirus.** *J Exp Med* 1986, **164**:280–290.

24. Schnittman SM, Psallidopoulos MC, Lane HC, *et al.* **The reservoir for HIV-1 in human peripheral blood is a T cell that maintains expression of CD4.** *Science* 1989, **245**:305–308.

25. Ou C-Y, Kwok S, Mitchell SW, *et al.* **DNA amplification for direct detection of HIV-1 in DNA of peripheral blood mononuclear cells.** *Science* 1988, **239**:295–297.

26. Saiki RK, Scharf S, Faloona F, *et al.* **Enzymatic amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia.** *Science* 1985, **230**:1350–1354.

27. Fox CH, Tenner-Racz K, Racz P, Firpo A, Pizzo PA, Fauci AS. **Lymphoid germinal centers are reservoirs of human immunodeficiency virus type 1 RNA.** *J Infect Dis* 1991, **164**:1051–1057.

28. Barton GF, Masuda A, Heath SL, Smith BA, Tew JG, Szakal AK. **Follicular dendritic cells (FDC) in retroviral infections: host/pathogen perspectives.** *Immunol Rev* 1997, **156**:185–197.

29. Heath SL, Tew JG, Szakal AK, Burton GF. **Follicular dendritic cells and human immunodeficiency virus infectivity.** *Nature* 1995, **377**:740–744.

30. 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**:123–126.

31. Rosenberg YJ, Lewis MG, Greenhouse JJ, *et al.* **Enhanced follicular dendritic cell function in lymph nodes of simian immunodeficiency virus-infected macaques: consequences for pathogenesis.** *Eur J Immunol* 1997, **27**:3214–3222.

32. Kirk O, Pedersen C, Cozzi-Lepri A, *et al.* **Non-Hodgkin lymphoma in HIV-infected patients in the era of highly active antiretroviral therapy.** *Blood* 2001, **98**:3406–3412.