Primary HIV-1 infection sets the stage for important B lymphocyte dysfunctions

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Objectives: To investigate the effects of primary HIV-1 infection (PHI) and of two antiretroviral therapies [highly active antiretroviral therapy (HAART) or reverse transcriptase inhibitors (RTI)] on activation, differentiation and survival of B cells.

Methods: Naive and memory B cells from three groups [PHI (31), chronic infection (26) and healthy donors (12)] were studied for surface expression of Fas, LAIR-1, CD70, intracellular expression of Bcl-2 and spontaneous apoptosis. Fluorescence activated cell sorting (IgD+IgM+CD19+CD27+) and short-term cell culture to analyse induction of CD25 on B cells were performed in five patients with PHI. Patients with PHI were sampled at baseline, and after 1 and 6 months of therapy. Results were analysed by parametric and non-parametric tests and by mathematical modelling.

Results: In PHI, B cells were significantly decreased; naive and memory B lymphocytes showed a high degree of activation, manifested by hypergammaglobulinaemia, altered expression of Fas and LAIR-1, and high rate of spontaneous apoptosis. Antiretroviral treatment improved the activation/differentiation status of B cells, reduced apoptosis to levels comparable to those in healthy individuals and restored the ability of B cells to respond to T cell-dependent activation. B cells showed slightly better recovery in patients taking HAART than in those taking RTI. Decreased IgM-positive memory B cells and lower induction of CD25 expression on B cells upon T cell activation at diagnosis of PHI was shown in five patients tested. These parameters normalized after 6 months of therapy.

Conclusion: B cell dysfunctions found in chronic HIV-1 infection appear during PHI and initiation of antiretroviral therapy early during infection may help to preserve the B cell compartment.

Keywords: primary HIV infection, B cells, antiretroviral therapy, immune activation

Introduction

B cell abnormalities are an important feature of HIV-1 pathogenesis, resulting in polyclonal B cell activation and altered B cell function [1]. HIV-1 antigenic pressure and consequent virus-induced immune activation are key mechanisms leading to B lymphocyte dysfunctions [2,3], including hypergammaglobulinaemia [4–6], increased
expression of activation markers [7,8], loss of memory B cells and serological memory [3,9–11] and defective B cell costimulatory functions [2].

Primary HIV-1 infection (PHI) is a distinct stage of HIV infection characterized in a majority of patients by an acute retroviral syndrome. Symptoms usually resolve over a few weeks when plasma viral load decreases and HIV-1-specific antibodies and cellular immune responses appear [12–16]. This apparent control of viraemia during acute HIV-1 infection is only partial and is accompanied by a virus-specific cytotoxic T lymphocyte response. However, antibodies to HIV-1 may also contribute to early control of viraemia [17]. PHI may present the best opportunity for studying how the immune system may control HIV infection [18].

Since the host immune system is still relatively intact during PHI, early initiation of therapy is an issue of contention among physicians, because of drug toxicity and compliance problems and the potential emergence of drug-resistant viruses [19].

Studies on asymptomatic patients have reported that B cell abnormalities are the first leukocyte defects to occur in HIV-1 infection [5,20] and it was recently shown that a steady state of immune activation is achieved early during infection [21]. The present study of patients with PHI has looked to establish a timeline for specific B cell abnormalities and to examine the effects of antiretroviral therapy on these abnormalities. Activation/differentiation status and apoptosis of naive and memory B cells were analysed.

Methods

Study population
A group of 31 patients with PHI attending the San Raffaele Institute, Milan were studied. The eligible patients had to fulfil at least one clinical criterion (signs/symptoms of acute retroviral syndrome at first visit or during the previous 60 days; HIV exposure in the previous 3 months and a negative HIV test in the previous 6 months) and one laboratory criterion [detectable plasma HIV RNA; detectable HIV p24; gp120, gp160 and/or p24 bands at Western blotting; low positive result with enzyme-linked immunosorbent assay (ELISA) with increasing reactivity over time], according to international guidelines [22]. Patients were offered antiretroviral therapy at diagnosis, and the cohort consisted of patients who decided to start therapy: 22 patients were treated with highly active antiretroviral therapy (HAART) consisting of two nucleoside reverse transcriptase inhibitors (NRTI) and one or two protease inhibitors, and 11 patients were treated with two NRTIs.

Venous samples were drawn at baseline and at 1 and 6 months after initiation of therapy. The comparison groups were 26 patients with chronic HIV infection (CHI: > 5 years; South Hospital, Stockholm) who had been taking HAART for at least 1 year and 12 healthy age- and sex-matched blood donors (controls). Informed consent was obtained from all subjects before enrolment and the ethical committees of San Raffaele Hospital and Karolinska Institute approved the study.

Clinical laboratory analysis
Serological confirmation of screening reactivity was obtained by ELISA (ORTHO Diagnostic Systems, Seattle, Washington State, USA) and Western blot (HIV 2.2 WB, Genelabs Diagnostics, Singapore). Plasma viraemia was monitored using the NASBA system (Organon Teknika, Boxtel, the Netherlands), which has a detection limit for HIV RNA of 80 copies/ml. Determination of plasma IgG levels was performed by nephelometry in 19 of 31 patients with PHI and in all controls.

Flow cytometric analysis
Peripheral blood mononuclear cells (PBMC) isolated from whole blood were frozen (10 × 10^6 cells/vial) in liquid nitrogen until analysis. The following mouse monoclonal antibodies labelled with fluorescein isothiocyanate, phycoerythrin, allophycocyanin or Cyochrome (BD Pharmingen, San Diego, California, USA) were used in three or four-colour analysis: anti-CD19, anti-CD25, anti-CD27, anti-CD70, anti-IgM, anti-IgD, anti-CD25, anti-CD27, anti-CD70, anti-IgM, anti-IgD, anti-LAIR-1, anti-CD95 and isotype controls. Cells (0.3 × 10^6) were incubated with monoclonal antibodies on ice for 30 min, washed with phosphate-buffered saline/2% fetal calf serum and fixed in phosphate-buffered saline/2% paraformaldehyde. Intracellular expression of Bcl-2 was analysed using the Intrastain kit (DakoCytomation, Glostrup, Denmark) and a phycoerythrin-conjugated anti-Bcl-2 monoclonal antibody (BD Pharmingen) after surface staining for CD19 and CD27. Cells were sorted on a Becton Dickinson FACScan machine and analysed using CellQuest software. An acquisition forward/side scatter dot plot was used to gate live lymphocytes and at least 30 000 cells/sample were collected. Analysis performed on paired frozen and fresh samples from six HIV-1-infected patients gave comparative results.

Culture of peripheral blood mononuclear cells
Spontaneous and Fas-mediated (anti-Fas monoclonal antibody clones UB-2 or CH-11; MBL, Tokyo, Japan) apoptosis of B lymphocytes was assessed by culturing PBMCs overnight, as described elsewhere [23]. Cells were then stained for CD19 and CD27 to gate naive and memory B cells and with annexin V–fluorescein isothiocyanate (BD Pharmingen) to detect apoptotic cells.

Expression of CD25 on B cells was analysed on PBMCs that had been cultured for 72 h at 1 × 10^6 cells/ml in the presence of agonistic anti-CD40 monoclonal antibody (1 μg/ml; DIACLONE Research, Lyon, France),
interleukin-2 (50 U/ml, Sigma-Aldrich; St Louis, Missouri, USA) and interleukin-4 (5 ng/ml; ImmunoTools, Friesoythe, Germany).

**Statistical analysis**

Graphical procedures and analytical tests were used to study the shape of the data. Parametric and non-parametric tests compared HIV-1 patients and controls. Box plots (median and the 25–75 percentiles) were drawn to describe temporal trends of parameters, stratified by treatment, using Stata Version 8.2 (Stata Corp, College Station, Texas, USA). In order to take proper account of the correlation among repeated measurements, random effects models for longitudinal data were fitted in Stata, using a user written program GLAMM [24], where the main effects of treatment and temporal trend were included, together with the interaction between treatment and time. Both main and interaction effects (time and treatment) were tested using a multivariate Wald $\chi^2$ test.

The final analysis was of the temporal trend of parameters measured in HIV-1-positive patients to see if this levelled off during the follow-up independent of treatment. A random effect model was fitted where the outcome for each patient was the deviation of each measurement from the mean value of controls. Data in the text are represented as median and the 25–75% percentile (25–75% CI) or as mean $\pm$ SD.

**Results**

The characteristics of the study population is given in Table 1.

**Phenotypic alterations in B lymphocytes during primary HIV infection**

The expression of activation and differentiation markers were analysed on naive and memory B lymphocyte in the PHI, CHI and control groups (Fig. 1). Total B cell percentages were significantly reduced in PHI compared with healthy controls [4.8% (25–75% CI, 3.1–6.9) and 9.5% (25–75% CI, 8.0–15.5), respectively; $P < 0.01$] and patients with CHI [9.4% (25–75% CI, 6.0–14.4); $P < 0.01$]. Interestingly, the percentage of circulating memory B cells in patients with PHI and healthy controls were similar [38.9% (25–75% CI, 26.7–44.6) and 42.4% (25–75% CI, 26.7–63.4), respectively; $P = 0.45$] and significantly higher than in CHI patients [16.9% (25–75% CI, 12.5–21.8); $P < 0.001$].

The percentage of CD70 naive B cells in PHI was comparable to that in healthy controls but over two-fold lower than in CHI ($P < 0.05$). The percentage of CD70 memory B cells from patients with PHI was comparable to levels in controls ($P = 0.30$) and patients with CHI ($P = 0.6$).

In patients with PHI, Fas-positive naive and memory B cells were significantly increased compared with controls ($P < 0.01$). Expression levels of Fas in patients with CHI were also significantly higher than in controls for both naive B cells ($P < 0.05$) and memory B cells ($P < 0.001$), suggesting that long-term HAART may not normalize Fas on B cells.

We have previously reported altered LAIR–1 expression on naive B cells during CHI [3]. The percentages of LAIR–1-positive naive and memory B cells from patients with PHI were significantly decreased compared with controls ($P < 0.001$ for both), indicating an advanced differentiation stage for memory B cells in early phase of infection. Interestingly, LAIR–1 expression in naive and memory B cells from patients with CHI and PHI was comparable.

Plasma IgG in patients with PHI (14 3.54 g/l) and CHI (15.3 0.7 g/l) were comparable and significantly higher than in healthy subjects (9.6 0.5 g/l) ($P < 0.001$), indicating dysregulated production of IgG already during PHI.

**Spontaneous apoptosis and Bcl-2 expression in B cells during primary HIV infection**

We previously reported that B cells from patients with CHI were primed for apoptosis [23,25]. Here we found

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**Table 1. Study population profile.**

| Characteristic                              | Primary HIV infection (baseline characteristics) | Chronic HIV infection |
|---------------------------------------------|-------------------------------------------------|-----------------------|
| Mean age [years (range)]                    | 32 (17–53)                                      | 43 (30–79)            |
| Male [No. (%)]                              | 24 (77)                                         | 19 (73)               |
| Female [No. (%)]                            | 7 (23)                                          | 7 (27)                |
| Mean CD4 cell count $\times 10^6$ cells/l (range) | 645 (204–1412)                                  | 591 (68–850)          |
| Mean HIV RNA [log$_{10}$ copies/ml (range)]  | 4.57 (2.69–5.46)                                | 2.28 (1.7–4.5)        |
| Mean time from acute retroviral syndrome [days (range)] | 32 (10–84)                                      | NA                    |
| Homosexual sex                              | 90                                              | 90                    |
| Heterosexual sex                            | 10                                              | 10                    |
| Antiretroviral therapy [No.]                | 20                                              | 26                    |
| Highly active antiretroviral therapy        | 11                                              | 0                     |

NA, not applicable.
that, compared with controls, patients with PHI had significantly higher percentages of apoptotic naive [16.8% (25–75% CI, 7.2–32.9) and 5.9% (25–75% CI, 4.5–25.5), respectively; \( P = 0.09 \)] and memory [20.9% (25–75% CI, 11.2–43.3) and 3.3% (25–75% CI, 0.7–18.2), respectively; \( P < 0.01 \)] B cells. It was possible, therefore, that alterations in the anti-apoptotic molecule Bcl-2 could be a contributing factor. Interestingly, Bcl-2 expression in naive B cells from patients with PHI was significantly increased compared with controls [87% (25–75% CI, 70.5–96.4) and 69.8% (25–75% CI, 57.8–83.4), respectively; \( P < 0.01 \)] but the percentage of Bcl-2-positive memory B cells was comparable to that in controls [89.1% (25–75% CI, 73.5–97.1) and 80.4% (25–75% CI, 75.2–93.6), respectively; \( P = 0.55 \)]. Bcl-2 expression expressed as fluorescence intensity gave similar results (not shown).

**Effects of antiretroviral therapy on B cell dysfunctions during primary HIV infection**

The effects of 6 months of antiretroviral therapy on the expression of the dysregulated B cell markers was analysed. CD70 expression on naive and memory B cells was not affected by therapy [from 1.1% (25–75% CI, 0.4–2.2) to 1.1% (25–75% CI, 0.7–4) and from 10.1% (25–75% CI, 5.8–22.6) to 7.7% (25–75% CI, 4–18.1), respectively]. In both naive and memory B cells, Fas expression after therapy was significantly decreased compared with baseline levels [from 66.7% (25–75% CI, 33.6–78.1) to 26.3% (25–75% CI, 12–43.9) and from 94.5% (25–75% CI, 83.6–100) to 71.6% (25–75% CI, 54.8–91.2), respectively], although it remained elevated on memory B cells (\( P < 0.001 \)). The expression of LAIR-1 on naive B cells was unaffected by treatment [from 88% (25–75% CI, 83–94.3) to 89.7% (25–75% CI, 81–94.9), respectively].

**Fig. 1. Effects of HIV-1 infection on B cell markers and spontaneous B cell apoptosis.** Expression of CD70, Fas and LAIR-1 and spontaneous apoptosis was examined in naive and memory B cells. Box plots represent comparisons between expression levels for HIV-negative controls (controls, \( n = 12 \)), patients at baseline of primary HIV-1 infection before initiation of therapy (PHI, \( n = 31 \)) and patients with chronic HIV-1 infection (CHI, \( n = 26 \)).
Antiretroviral therapy reduced spontaneous apoptosis in naive [10.2% (25–75% CI, 5.1–18.1)] and memory [13.5% (25–75% CI, 4–22.8)] B cells but normalization was only achieved in naive B cells. Moreover, agonistic anti-Fas triggering did not increase apoptosis of B cells compared with that in control cultures (not shown). Plasma IgG levels after 6 months of therapy were reduced compared with baseline levels [to 12.6 g/l (25–75% CI, 10.8–15.2)] from 13.2 g/l (25–75% CI, 11.34–15.1; P < 0.05] but remained elevated compared with controls [9.0 g/l (25–75% CI, 8.0–10.0)].

The study also examined the evolution associated with use of HAART or NRTI therapy over time to see if any observed differences in therapy outcome occurred because of therapy alone or therapy plus duration of treatment (summarised in Fig. 2). At follow-up, patients taking HAART and NRTI had increased CD4 T cell counts and reduced viral loads. However, the effect of therapy was statistically significant only in patients taking HAART (not shown); 10 out of 20 patients undergoing HAART showed at least a two-fold increase in B cell percentage after therapy [from 5.1% (25–75% CI, 4.3–8.4) to 13.5% (25–75% CI, 10.9–16.3); P = 0.008]). This increase was more modest in patients taking NRTI [from 4.4% (25–75% CI, 2.8–5.7) to 5.1% (25–75% CI, 5–6.2); P = 0.16].

No significant difference was observed between the effects of HAART and NRTI on CD70 expression on naive (P = 0.4) and memory (P = 0.5) B cells (Fig. 2, top panel). HAART induced a larger reduction of Fas expression on naive and memory B cells, although the difference was not statistically significant (Fig. 2, second panel). LAIR-1 expression on naive B cells was increased in patients taking HAART while it decreased in patients taking NRTI. However, the treatment effect was not statistically significant (P = 0.3). As for LAIR-1 expression on memory B cells, HAART induced a more substantial increase compared with NRTI (Fig. 2, third panel). Interestingly, NRTI and HAART had similar effects on Bcl-2 expression in naive B cells (P = 0.5) but only HAART induced a significant increase in Bcl-2 expression in memory B cells (P < 0.05) (Fig. 2, last panel). The levels of spontaneous apoptosis in naive and memory B lymphocytes were significantly and similarly reduced by both NRTI and HAART (not shown).

Discussion

Disturbances in activation/differentiation of B lymphocytes characterize CHI [1] but few data are available on B cells in PHI. We characterized naive and memory B cells during PHI by analysing activation and differentiation status, apoptosis and functional response to activation by T cells. We found that peripheral B cells were decreased...
during PHI compared with healthy individuals and with patients having treated CHI. Circulating B cells increased to normal levels in patients receiving HAART but not NRTI therapy, suggesting a beneficial effect of HAART. This effect was not a result of changes in apoptosis, since the two treatments were similarly effective in reducing B cell apoptosis.

It is known that memory B cells are reduced in CHI [9,10]. Though patients with PHI have normal frequency of memory B cells, we detected an abnormal activation/differentiation state, shown by altered Fas and LAIR-1 expression and priming for apoptosis. The memory B cell pool at diagnosis of PHI also showed a lower frequency of IgM-positive cells and an increased IgM−IgD+ fraction. These alterations were also detected in patients with CHI (not shown). Interestingly, in all patients studied, the pool of memory B cells completely normalized after 6 months of HAART. Considering that IgM-positive memory B cells are believed to control Streptococcus pneumoniae and other encapsulated bacteria infections [27], our preliminary data suggest that early treatment may be important to revert B cell dysfunctions. Interestingly, antiretroviral therapy during PHI significantly reduced the frequency

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**Fig. 2. Effects of highly active antiretroviral therapy and nucleoside reverse transcriptase inhibitors on naive and memory B cells.** Patients with primary HIV-1 infection received highly active antiretroviral therapy (shaded columns) or reverse transcriptase inhibitors (open columns) and were sampled at baseline (0), 1 month (1) and 6 months (6) after therapy. CD70, Fas, LAIR-1 and Bcl-2 expression on naive and memory B cells were determined and statistical significance of differences in therapy effects assessed by Wald χ² test. Differences at baseline (0) were not significant.
of opportunistic infections [28]. Moreover, in patients with PHI we found an increase in the fraction of IgM–IgD+ memory B cells, normally very low in healthy controls. This is in line with previous studies reporting abnormally increased serum IgD levels in asymptomatic patients [29]. The relevance of this preliminary observation on IgM–IgD+ memory B cells merits further investigation.

Recent studies showed that HAART treatment of CHI does not result in improved memory B cell numbers [9,30]. Indeed, despite the reduction of spontaneous memory B cell apoptosis after therapy, Fas and LAIR–1 expression remained significantly altered. Chong and colleagues [30] reported that memory B cells maintained high Fas expression in treated CHI. Fas is a pro-apoptotic molecule but also a marker of activated lymphocytes [31] and it plays an important role in regulating lymphocyte homeostasis during immune responses. Since Fas plays a key role in regulation of B cells, particularly autoreactive cells [32,33] and B cells are able to modulate their intrinsic Fas sensitivity in dynamic fashion [34], Fas may have an activating rather than an apoptosis-inducing role during PHI. B cells from patients with PHI were not susceptible to Fas-mediated apoptosis, confirming previous data [23] and the fact that high expression of Fas does not necessarily translate into susceptibility to Fas-mediated apoptosis since other molecules are likely involved in regulation of B cell apoptosis and Fas can transduce non-apoptotic signals [35]. Moir and colleagues showed that expression of tumour necrosis factor family receptors on B cells was altered in HIV viraemic patients, making these cells more susceptible to cell death mediated via these receptors [36]. The difference between those results and the ones described here may reflect that Moir et al. used Fas ligand in 2 h cultures to induce apoptosis while we used anti-Fas monoclonal antibodies in 18 h cultures. We found that HAART (but not NRTI) induced a significant increase of Bcl-2 expression in memory B cells. The possible role of Bcl-2 in regulation of memory B cell apoptosis in HIV-1 infection and the contribution of protease inhibitors merits further investigation.

We reported that naive B cells with low LAIR–1 expression and upregulated CD70 may contribute to hypergammaglobulinaemia in HIV-1 infection [3]. We found downregulated LAIR–1 expression but normal CD70 expression on naive B cells in PHI. Surprisingly, neither HAART nor NRTI restored the number of LAIR–1–expressing naive B cells during PHI, suggesting that these cells still receive differentiation signals. Decreased LAIR–1 expression may represent a host response to remove the inhibitory effect of LAIR–1 on B cell signalling. To counter improper signalling induced by the infection, B cells may overcompensate by downregulating LAIR–1 to promote proper BCR signalling.

Fas expression on naive B cells was completely normalized by early antiretroviral therapy. We also found that naive B cells from patients with PHI expressed high levels of Bcl-2 protein that remained stable during treatment. The observation that naive B cells maintain high degree of activation (loss of LAIR–1 and increased Bcl–2) correlates with sustained hypergammaglobulinaemia at follow-up. A higher survival capacity of naive B cells might lead to a less-stringent process of clonal deletion and aid the maintenance of polyclonal clones, eventually contributing to hypergammaglobulinaemia.

Our analysis suggests that antiretroviral therapy might also improve B cell function. Induction of CD25 expression, a parameter of functional T–B cell interaction, is impaired in HIV-infected patients [26] and we observed this alteration at diagnosis of PHI. Following antiretroviral therapy, B cells recovered the ability to upregulate CD25 in vitro, suggesting that early treatment may help to maintain functional B–T cell communication.

Perturbations of B cells, including the appearance of CD21bright B cells, has been associated with viraemia [8]. We could not observe any significant influence of viraemia on B cell markers; however, considering the low numbers of viraemic patients at follow-up, it is still possible that the beneficial effects of HAART on B cells may be associated with a better control of viral replication.

Although the initial adaptive immune response to HIV-1 infection is characterized by a strong antibody response, the burst of HIV-1 replication is contained before the development of neutralizing antibodies [37,38]. However, an intact humoral response is important in mediating optimal control of early infection [17] and is an essential correlate of vaccine efficacy. An immunological activation set-point is established early in HIV-1 infection and determines the rate at which CD4 T cells are lost over time [21]. Therefore, recognition of incident HIV-1 infection presents unique opportunities for treatment and prevention of HIV-1 disease [39].

Initiation of therapy in patients with PHI is still debated. Arguing for early therapy, some studies describe PHI as a window of opportunity for immune rescue that would allow patients to maintain high levels of HIV-specific T helper cell responses, result in a narrower cytotoxic T cell response, and a less-diverse virus population [40–43]. Early HAART during PHI moderates progression to AIDS, preserves/restores virus-specific T cell responses and may limit the extent of viral replication, thus restricting damage to the immune system [13,22,28,44]. In addition, early treatment may in some patients induce the development of strong neutralizing antibody response [45,46]. In this study, we observed that antiretroviral therapy during PHI led to improved B cell phenotype,
significant reduction of apoptosis, functional recovery of B cells and restoration of memory B cell pools. Larger studies on acutely infected patients under diverse therapeutic regimens may elucidate whether HAART could have a better effect on B cell function than other therapies.

Our results on the course of B cell dysfunctions in PHI have emphasized the importance of acute infection in establishing B cell abnormalities. In light of recent renewed interest in humoral immunity and neutralizing antibodies in vaccine development, this study may contribute valuable information on various aspects relevant to pathogenesis and therapeutic strategies.

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