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CICLO XXV

DYNAMICS OF EPSTEIN-BARR VIRUS (EBV) IN HUMAN IMMUNODEFICIENCY VIRUS (HIV)-1 INFECTED ADULTS AND CHILDREN

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Sommario

Il virus di Epstein-Barr (EBV) è coinvolto nello sviluppo di un ampio spettro di malattie linfoproliferative, in particolare nei soggetti immunocompromessi. Oltre all'immunodepressione, l'attivazione immunitaria cronica può indurre la stimolazione della cellula B, promuovendo l'espansione delle cellule infettate da EBV. I fattori che possono contribuire all'attivazione e all'espansione delle cellule B infettate da EBV, anche nei soggetti con infezione da HIV-1, sono per lo più sconosciuti. In Africa l'infezione primaria da EBV insorge durante la prima infanzia e i linfomi associati ad EBV sono un'importante causa di morbidità e mortalità nei bambini. Elevati livelli di EBV costituiscono un fattore di rischio per l'insorgenza dei tumori associati al virus. Ad oggi, non sono disponibili dati circa l'infezione da EBV e le patologie associate nei bambini africani con infezione da HIV-1. La mancanza di questi dati è in parte dovuta alla difficoltà di analisi di laboratorio nei Paesi in via di sviluppo. L'utilizzo di Dried Blood Spot (DBS) potrebbe rappresentare un metodo semplice per raccogliere, conservare campioni di sangue, e consentire il loro trasporto in laboratori specializzati.

Gli scopi della ricerca svolta durante il corso di Dottorato sono stati i seguenti:

1) studiare la relazione tra viremia di EBV e marcatori di attivazione immunitaria nei soggetti con infezione da HIV-1.
2) studiare l'infezione da EBV nei bambini africani con infezione da HIV-1 e/o con tumori EBV-correlati.
3) sviluppare una nuova applicazione di DBS per la rilevazione della clonalità delle cellule B, per la diagnosi e il monitoraggio della patologia neoplastica.

1) Viremia di EBV e attivazione immunitaria in soggetti con infezione da HIV-1

Nello studio sono stati inclusi 156 pazienti con infezione da HIV-1. 85 pazienti erano in terapia antiretrovirale (ART). Il DNA di EBV è stato rilevato in 114 pazienti, e in tutti i casi tranne 3 era EBV tipo 1. I livelli mediani di EBV-DNA erano di 43[1-151] copie/10^5 cellule. I livelli di EBV

erano più elevati nei soggetti con plasmaviremia di HIV-1 rilevabile, nonostante un buono stato immunologico (CD4>500 cellule/µl), rispetto a pazienti con plasmaviremia di HIV-1 non rilevabile, indipendentemente dallo stato immunologico (46[5-136] *vs* 17[1-56] copie/10^5 cellule, p=0.008). Pazienti con alti livelli di EBV-DNA (> valore mediano) avevano livelli più alti di lipopolisaccaride batterico (LPS) e di citochine proinfiammatorie (IL-6, IL-10 e TNF-α) rispetto a pazienti con bassa viremia di EBV. Inoltre, la percentuale di cellule B attivate correlava con i livelli di EBV (r=0.754; p<0.001). Nell'insieme, questi dati indicano una significativa associazione tra plasmaviremia di HIV-1, marcatori di attivazione immunitaria e citoviremia di EBV e suggeriscono che la persistenza di HIV-1 e l'attivazione immunitaria, indipendentemente dalla ripopolazione dei linfociti CD4 periferici, possono favorire l'espansione delle cellule B infettate con EBV.

2) Associazione tra livelli di EBV e NHL nei bambini della Tanzania e parametri relativi a EBV nei bambini con infezione da HIV-1 in Uganda

E' stato condotto uno studio caso-controllo in bambini con linfomi non-Hodgkin (NHL) ammessi in tre centri clinici della Tanzania e controlli comparabili per età. I campioni di sangue sono stati raccolti in DBS. Ventuno di 35 (60%) pazienti con NHL e solo 21 di 70 (30%) controlli avevano EBV rilevabile nel sangue periferico (OR=4.77 [95% CI 1.71–13.33], p=0.003). Inoltre, i livelli di EBV erano più alti nei casi NHL rispetto ai controlli EBV-positivi (p=0.024). Nell'insieme, questi dati indicano che la viremia di EBV nel sangue periferico potrebbe avere rilevanza diagnostica.

In un secondo studio, i campioni di sangue di 213 bambini con infezione da HIV-1 sono stati raccolti su DBS presso l'ospedale Nsambya, in Kampala, Uganda. Novantadue di 140 (66%) bambini in ART e 57 di 73 (78%) naive sono risultati positivi per EBV. In un'analisi multivariata per CD4 Z-score, età e stadio WHO, bambini in ART avevano minori probabilità di avere EBV rilevabile rispetto ai bambini naive (OR=0.39 [95% CI 0.16-0.94], p=0.036). La viremia di EBV era significativamente più elevata nei bambini naive rispetto a quelli in ART (4.22 [3.87-4.58] vs 3.99

[3.55-4.33] log_{10} copie/ml; p=0.016). I livelli di 16S rDNA, utilizzato come marcatore di traslocazione microbica, erano significativamente più elevati nei bambini naive rispetto a quelli in ART (2.16 [2.11-2.28] vs 2.09 [2.02-2.22] log_{10} copie/µl; p=0.007) e correlavano con i livelli di EBV-DNA (r= 0.382, p=0.016), suggerendo che i prodotti microbici circolanti promuovono l'attivazione e l'espansione della cellula B infettata da EBV. Il trattamento con ART, probabilmente limitando la viremia di HIV-1 e la conseguente attivazione immunitaria, può ridurre la replicazione di EBV e l'espansione delle cellule infettate da EBV.

3) Rilevabilità delle popolazioni clonali B da campioni di DBS: uno strumento per la diagnosi e il monitoraggio dei tumori delle cellule B

In primo luogo, utilizzando campioni di sangue di diversa origine, è stato verificato che i DBS contenessero un numero sufficiente di linfociti per stimare la clonalità senza ottenere risultati falsi positivi. Mediante l'impiego di cellule Namalwa, che contengono 2 copie di EBV/cellula, è stata trovata una ottima correlazione tra i risultati attesi e le copie di EBV rilevate (r=0.987, p<0.0001); si è potuto quindi stabilire che una popolazione clonale B è rilevabile su DBS quando sono presenti almeno 200 cellule clonali nel campione analizzato. Inoltre, sono stati ottenuti risultati molto simili per la clonalità cellulare analizzando il DNA ottenuto da DBS e da sangue fresco di pazienti con leucemia linfatica cronica. Questo studio, dimostrando che è possibile valutare la clonalità su campioni di sangue conservato su DBS, allarga le opzioni diagnostiche e di monitoraggio.

Conclusioni

In questi studi abbiamo trovato una relazione significativa tra marcatori di traslocazione microbica, livelli di citochine pro-infiammatorie e livelli di EBV-DNA negli adulti e nei bambini con infezione da HIV-1. Di interesse e in accordo con studi precedenti, abbiamo anche dimostrato che i livelli di EBV sono più elevati nei pazienti con aumento di linfociti CD4 ma incompleta soppressione della viremia di HIV-1 rispetto a pazienti con plasmaviremia non rilevabile, indipendentemente dal

numero di linfociti CD4. Nell'insieme, questi dati suggeriscono che l'attivazione immunitaria indotta da HIV-1 può risultare nella stimolazione e nell'espansione delle cellule B infettate con EBV, un fattore di rischio per lo sviluppo di neoplasie EBV-associate.

La relazione tra attivazione immunitaria e livelli di EBV è risultata anche negli studi effettuati nei bambini con infezione da HIV-1, dove abbiamo trovato che i livelli di EBV-DNA e di 16S rDNA erano significativamente più elevati nei bambini in ART rispetto a quelli naive. Il trattamento con ART, probabilmente limitando l'attivazione immunitaria, potrebbe prevenire la stimolazione della cellula B e l'espansione della cellula B. Queste osservazioni possono essere di particolare interesse anche per pianificare strategie terapeutiche nei soggetti con infezione da HIV-1.

Inoltre, la dimostrazione che i linfomi NHL sono significativamente associati ad elevati livelli di EBV nel sangue periferico, suggerisce che la determinazione di EBV nel sangue potrebbe avere una rilevanza diagnostica e prognostica per le neoplasie delle cellule B associate ad EBV. In questo contesto abbiamo potuto stimare che i campioni di DBS sono anche idonei ad identificare la presenza di una popolazione clonale B. Questo è importante perché estende le opzioni di diagnosi e monitoraggio dei linfomi B anche dove non sono di facile accesso queste analisi.

Summary

Epstein-Barr Virus (EBV) is involved in a wide range of malignancies, particularly in immunocompromised subjects. Besides immunodepression, chronic immune activation may induce B-cell stimulation leading to an expansion of EBV-infected cells, thus increasing the risk of EBV-related malignancies. The factors that may contribute to HIV-1-induced B cell activation and expansion of EBV-infected cells are largely unknown. In Africa EBV primary infection occurs during infancy and early childhood and EBV-associated lymphomas represent an important cause of morbidity and mortality in children. High levels of EBV represent a risk factor for the onset of EBV-related malignancies. To date, no data are available about EBV-infection and related malignancies in the context of HIV-1 infection in African children; this may be partly due to lack of access of laboratory analyses in developing countries. The use of Dried Blood Spot (DBS) may represent an easy method to collect and store blood samples and allow their reliable transport to specialized laboratories.

My PhD research focused on the following themes:

1) the relationship between markers of immune activation and EBV load in HIV-1 infected patients.

2) EBV infection in HIV-1 infected and uninfected children in African countries.

3) a new application of Dried Blood Spot to type and quantify EBV and to identify B cell clonality in order to diagnose and monitor B-cell malignancies.

1) EBV load and immune activation in HIV-1-infected patients

A total of 156 HIV-1-infected patients were included in this study, 85 of which were under antiretroviral therapy (ART). EBV-DNA was detected in 114 patients, and in all but 3 it was EBV type 1. The median [interquartile] EBV-DNA load was 43[1-151] copies/10^5 cells and it was higher in patients with detectable HIV-1 plasma viremia, despite good immunological status (CD4>500 cells/µl), than in patients with undetectable HIV-1 plasma viremia regardless of immunological status (46[5-136] vs 17[1-56] copies/10^5 cells, p=0.008). Patients with high EBV-DNA load

(>median value) presented higher levels of LPS and proinflammatory cytokines (IL-6, IL-10 and TNF-α) than patients with low EBV load. Furthermore, percentages of activated B-cells correlated with EBV-DNA load (r=0.754; p<0.001). Overall, these findings indicate a strong association between HIV-1 viremia, markers of immune activation and EBV load, and suggest that persistence of HIV-1 viremia and immune activation, regardless of peripheral CD4 cell depletion/repopulation, may favour the expansion of EBV-infected cells.

2) Association between NHL and blood levels of EBV in children in Tanzania, and dynamics of EBV in HIV-1-infected children in Uganda

A matched case-control study was performed in children with Non-Hodgkin's Lymphoma (NHL) admitted to three clinical centers in Tanzania, and their age-matched controls. Blood samples were collected on DBS. 21 out of 35 (60%) NHL patients and only 21 out of 70 (30%) controls presented EBV detectable in peripheral blood, thus showing a significant association between NHL and EBV in blood (OR=4.77 [95% CI 1.71–13.33], p=0.003). Furthermore, EBV-DNA levels were higher in cases compared to EBV-positive controls (p=0.024). Overall, these findings indicate that EBV-DNA load in peripheral blood might have diagnostic relevance.

In a second study, blood samples from 213 HIV-1-infected children were collected on DBS at the Nsambya Home Care, Kampala, Uganda. 92 out of 140 (66%) children on ART and 57 out of 73 (78%) ART-naive children were found to be EBV-positive. After adjusting for CD4 Z-score, age and WHO stage, children on ART presented less odds of having detectable EBV than ART-naive children (OR=0.39 [95% CI 0.16-0.94], p=0.036). EBV load was significantly higher in ART-naive children than those on ART (4.22 [3.87-4.58] vs 3.99 [3.55-4.33] $\log_{10}$ copies/ml; p=0.016). Levels of 16S rDNA, a marker of microbial translocation, were significantly higher in ART-naive children than those on ART (2.16 [2.11-2.28] vs 2.09 [2.02-2.22] $\log_{10}$ copies/μl; p=0.007) and correlated with EBV-DNA levels (r=0.382, p=0.016), suggesting that circulating microbial products lead to B

cell activation and expansion of EBV-infected B cells. Treatment with ART, likely by limiting HIV-1 load and thus the HIV-1-driven immune activation, may restrict EBV replication and expansion of EBV-infected cells.

3) Detection of clonal B-cell populations from DBS sampling: a tool for the diagnosis and monitoring of B-cell malignancies

Firstly, through blood samples from donors, we ensured that DBS contains sufficient lymphocytes to perform a clonality assay without yielding false positive results. Using Namalwa cells that contain 2 EBV copies/cell, we found a good relationship between the expected and detected EBV-DNA copies ($r=0.987$, $p<0.0001$) and we established that a clonal B-cell population on DBS was detected when there were at least 200 clonal cells in the analysed sample. Moreover, very similar clonal results were obtained between DNA from DBS and fresh whole blood from patients with chronic lymphocytic leukemia. This study demonstrated the possibility to perform clonality testing on DBS sampling, thus improving the diagnostic and monitoring options.

Conclusions

In these studies, we found a significant relationship between markers of microbial translocation, levels of pro-inflammatory cytokines and EBV-DNA levels in both HIV-1 infected adults and children, suggesting that cell activation driven by HIV-1 antigens may result in chronic B cell stimulation and expansion of EBV-infected B cells, a risk factor for the development of EBV-related malignancies. Of interest, we found that EBV-DNA levels were higher in patients with a gain in CD4 lymphocytes, but incomplete suppression of HIV-1 viremia than in patients with undetectable plasma viremia, regardless their CD4 cell number.

The relationship between immune activation and EBV levels was also supported by the findings in HIV-1 infected children in Uganda. Moreover, we found that EBV-DNA and 16S rDNA levels were significantly lower in children on ART than in those ART-naive, suggesting that treatment

with ART, likely by limiting immune activation, may prevent B cell stimulation and expansion of EBV-infected B cells. These observations may become particularly interesting to plan future therapeutic strategies in HIV-1 infected patients.

We also found that NHLs are strongly associated with EBV load in peripheral blood, suggesting that high levels of EBV in blood might have diagnostic and prognostic relevance for the diagnosis and monitoring of EBV-related B cell malignancies. In this context we assessed that DBS sampling was also suitable to identify the presence of a B cell clonal population. Our results showed it is possible to perform clonality testing on DBS sampling, thereby improving the diagnostic and monitoring capability of B cell lymphomas in resource-limited settings.

CHAPTER 1

Introduction

1. Introduction

1.1 Epstein-Barr Virus

1.1.1 General characteristics

Epstein-Barr Virus (EBV), also defined as *Human Herpesvirus 4* (HHV4), belong**s** to *herpesviridae* family, *gammaherpesviridae* subfamily, *lymphocryptovirus* gender. In 1961 Burkitt described a lymphoma, which will be later named Burkitt's lymphoma (BL) in his honour, that affected many young children in some area of Africa and New Guinea. In 1964, the two researchers, Epstein AM and Barr Y, isolated a virus from BL cells, namely Epstein-Barr.

EBV presents a ubiquitary behaviour and its primary infection is usually asymptomatic, but it occasionally may induce a benign lymphoproliferative disorder, known as infectious mononucleosis. EBV starts the infection in the oropharyngeal cells permissive for viral replication. B lymphocytes that migrate through the oropharyngeal epithelial get infected after contact with them. As all herpesviruses, EBV presents a latent and a lytic cycle. In individuals that go beyond primary infection, the virus remains stable in B cells (about 1 infected cell per 10^5-10^6 B cells) and in some epithelial cells in the oropharynx. The reactivation of EBV determines the expression of lytic proteins, the viral replication and lysis of infected cells (Figure 1).

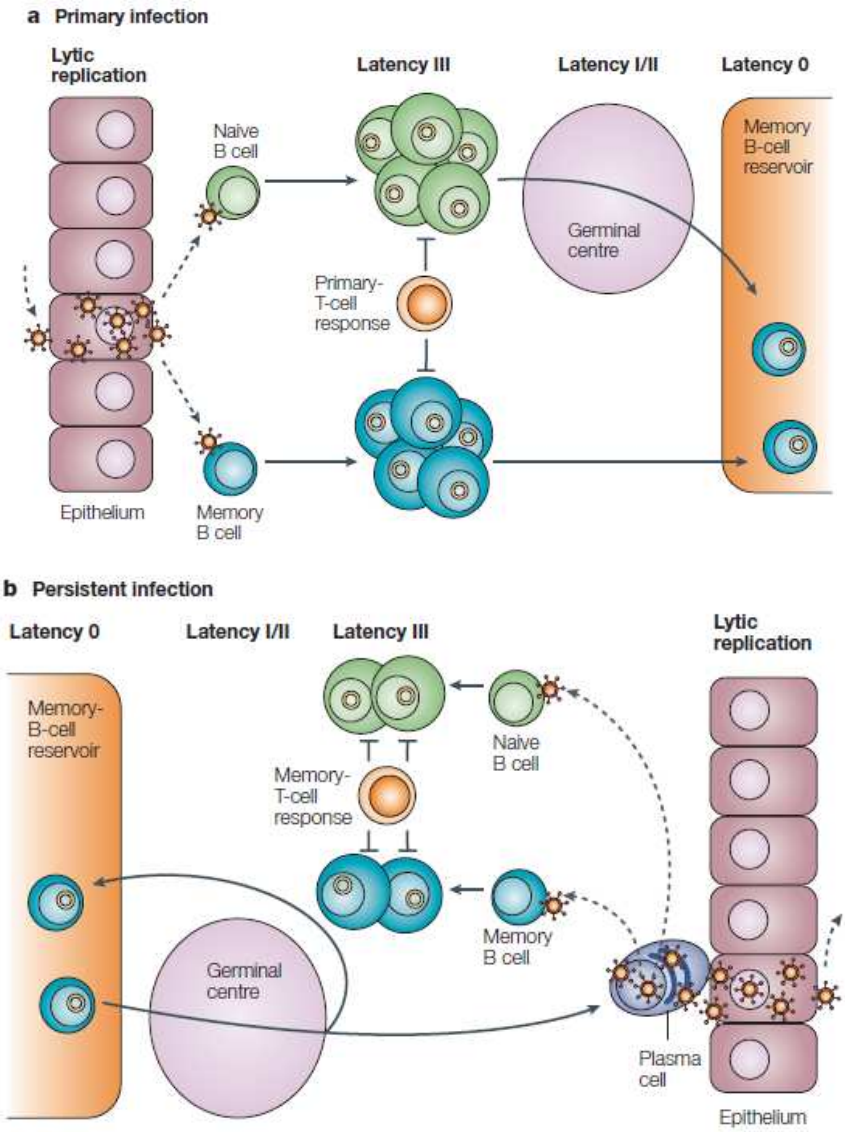


Figure 1. Interaction between Epstein-Barr virus and host cells. a. primary infection; **b**. persistent infection (from Young LS et al, 2004 modified).

EBV is associated to a wide range of human tumours *in vivo*, nasopharyngeal carcinoma (NPC), endemic BL, Hodgkin Lymphoma (HL), X-linked Lymphoprolipherative Disease (XLPD), post-transplanted lymphoprolipherative disease (PTLD), immunoblastic lymphoma (IBL) and diffuse large B cell lymphoma (DLBCL), arising in immunocompromised patients. *In vitro*, EBV shows a potent transforming ability, being able to efficiently induce uncontrolled proliferation of infected B

lymphocytes, leading to the genesis of lymphoblastic cell lines (LCLs). In tumours and in LCLs only latent viral proteins are usually expressed.

1.1.2 Structure and genome

EBV particles diameter measures approximately 120-200 nm. The nucleocapsid, composed of 162 capsomeres, presents a icosahedral structure with a diameter of 100-110 nm and is surrounded by the tegument. The core is formed by proteins anchored to the capsid on which EBV genome is rolled. The EBV genome consists of a linear double stranded DNA molecule of approximately 172 kilobase pairs (kb) and is composed by 60% of guanine and cytosine bases. EBV is composed of a series of 0.5 kb terminal repeats (TRs) and 3 kb internal repeat sequences (IRs). The EBV genome encodes for 9 genes that are expressed in latently infected cells, and for many genes that are expressed during the lytic phase for the production of new virions. The switch from the latent to the lytic cycle is determined by the activation of the BZLF1 gene, encoding for the ZEBRA protein.

Before the virus enters the cell, the major envelope glycoprotein, gp350/220, binds to the CD21 molecule (i.e. C3d complement receptor) on the surface of the cells. This triggers the fusion of viral envelope with cell membrane, allowing EBV to enter cell. After 12-16 hours of infection, the linear EBV genome becomes circular, and remains latent in nucleus in the form of an episome. The transcription programme starts beginning to the promoter Wp (Figure 2). EBV use the DNA polymerase II of the host cell to transcribe its genes. The first proteins encoded are the EBV nuclear antigen (EBNA)-LP and EBNA-2. EBNA-2 up-regulates the expression of latent membrane protein (LMP)-1 and LMP-2, as well as cellular proteins that contribute to the growth and transformation of B cells, such as CD23, CD21 and oncogenes *c-frg* and *c-myc* (Kaiser C et al, 1999). EBNA-LP interacts with EBNA2 and is required for the efficient outgrowth of virus-transformed B cells *in vitro*. The transcriptional activation, mediated by EBNA-2 in conjunction with EBNA-LP, is modulated by the EBNA-3 (-3A, -3B, -3C) family of proteins. The EBNA-1 is expressed in all virus infected cells and it achieves its role in the maintenance and replication of the episomal EBV

genome through sequence-specific binding to the origin of replication, OriP (Young LS et al, 2004). LMP-1 is the main transforming protein of EBV and it is essential for EBV-induced B-cell transformation *in vitro*. LMP-1 functions as a constitutively activated member of the tumour necrosis factor receptor (TNFR) superfamily, and activates several signalling pathways in a ligand-independent manner. Functionally, LMP-1 mimics CD40 - a member of the TNFR superfamily - thus providing both growth and differentiation signals to B cells. The LMP-1 activates several downstream signalling pathways that contribute to the induction of various genes encoding anti-apoptotic proteins (for example, BCL2 and A20) and cytokines, such as interleukin (IL)-1 and CD40L (Eliopoulos AG et al, 2001). The LMP-2 proteins, LMP-2A and LMP-2B, are not essential for EBV-induced B-cell transformation *in vitro* (Young LS et al, 2004). However, LMP-2A prevents reactivation of EBV from latently infected cells by blocking tyrosine kinase phosphorylation. Furthermore, expression of LMP-2A can drive the proliferation and survival of B cells in the absence of signalling through the B-cell receptor. In addition to these effects, LMP-2A was found to induce expression of several genes involved in cell-cycle induction, inhibition of apoptosis and suppression of cell-mediated immunity. The two small nonpolyadenylated (non-coding) RNAs, EBER-1 and EBER-2, are expressed in all forms of latency. The EBERs do not encode proteins and are not essential for the EBV induced transformation of primary B lymphocytes; however, it has been suggested that EBERs might be important for viral persistence (Nanbo A et al, 2002), and their expression in BL cell lines might increase tumorigenicity, promote cell survival and induce IL-10 expression (Ruf I et al, 2000; Nanbo A et al, 2002).

Latent viral proteins are expressed according to three patterns of latency, characteristic of different EBV-associated malignancies. In the latency type I only EBNA-1 is expressed, whereas in the latency type II, EBNA-1, LMP-1, LMP-2, and EBER are expressed. In the latency type III all the latency genes are expressed (Table 1).

Table 1. Expression of EBV latent genes in diseases

Pattern of latency	EBNA 1	EBNA 2	EBNA 3A	EBNA 3B	EBNA 3C	EBNA LP	LMP 1	LMP 2A	LMP 2B	EBERs	Disease
Type I	+	-	-	-	-	-	-	-	-	+	Burkitt's Lymphoma
Type II	+	-	-	-	-	-	+	+	+	+	Nasopharyngeal Carcinoma, Hodgkin's Disease, Peripheral T-cell Lymphoma
Type III	+	+	+	+	+	+	+	+	+	+	Lymphoproliferative Disease, X-linked Lymphoproliferative Disease, Immunoblastic Lymphoma

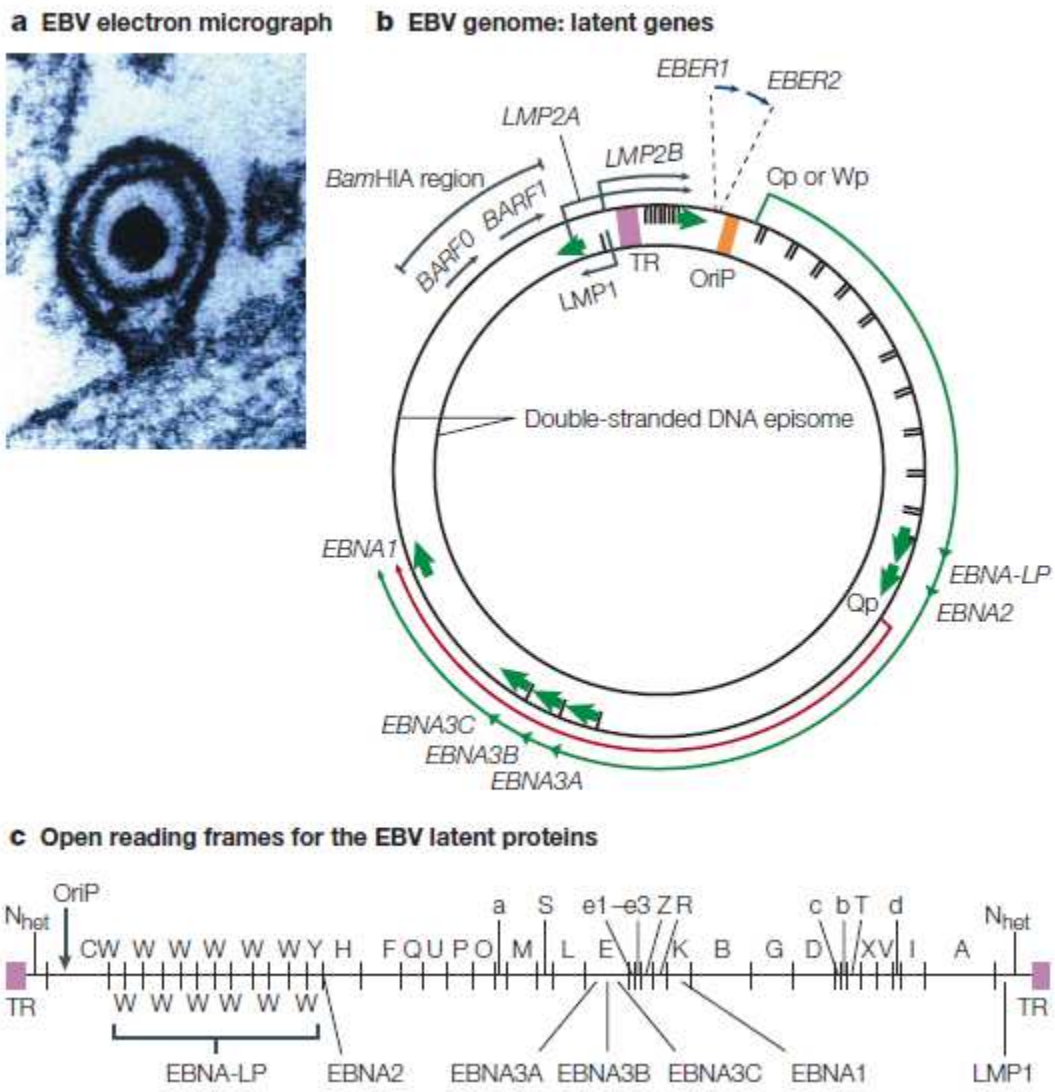


Figure 2. The Epstein–Barr virus genome. **a.** Electron micrograph of the Epstein–Barr virus (EBV) virion. **b.** Diagram showing the location and transcription of the EBV latent genes on the double-stranded viral DNA episome. The orange segment represents the origin of replication (OriP). The large green solid arrows represent exons encoding each of the latent proteins, and the arrows indicate the direction in which the genes encoding these proteins are transcribed. The latent proteins include the six nuclear antigens (EBNA-1, 2, 3A, 3B and 3C, and EBNA-LP) and the three latent membrane proteins (LMP-1, 2A and 2B). EBNA-LP is transcribed from a variable number of repetitive exons. LMP-2A and LMP-2B are composed of multiple exons, located on either side of the terminal repeat (TR) region, which is formed during the circularization of the linear DNA to produce the viral episome. The blue arrows at the top represent the highly transcribed

nonpolyadenylated RNAs EBER-1 and EBER-2; their transcription is a consistent feature of latent EBV infection. The long outer green arrow represents EBV transcription during the latency III, in which all the EBNAs are transcribed from either the Cp or Wp promoter; the different EBNA are encoded by individual mRNA that are generated by differential splicing of the same long primary transcript. The inner, shorter red arrow represents the EBNA-1 transcript, which originates from the Qp promoter during Lat I and Lat II. **c.** Location of open reading frames for the EBV latent proteins on the BamHI restriction-endonuclease map of the prototype B95.8 genome (from Young LS et al, 2004 modified).

1.1.3 EBV type 1 and 2

Two EBV types are recognized and usually known as 1 and 2, although A and B were widely used in the past. They were classified according to the differences on EBNA-2; EBV isolated from a North American case, B95-8, classified as type 1, was found to have a slightly longer EBNA-2 reading frame than that of AG876, a type 2 virus isolated from a BL case in Central Africa. Additional differences between the genomes of type 1 and 2 appear in genes that encode for the EBNA-LP, 3A, 3B and 3C proteins.

EBV type 1 is more common in most populations; 80% to 90% of EBV isolates from Caucasian and Southeast-Asian population are type 1. However these observations, largely based on *in vitro* virus isolation, still have to be fully verified by direct analyses *ex vivo*, thereby avoiding potential bias introduced by the more efficient *in vitro* transforming function of EBV type 1 strain (Rickinson and Kieff, 2007). EBV type 2 show an almost equal prevalence of EBV type 1 in the populations of Africa and New Guinea. EBV type 2 is also frequently detected among people infected with HIV-1 (Rickinson and Kieff, 2004). Another question unsolved is whether particular EBV strains carry greater disease risk; to date, no clear evidence has been found of strain-specific disease association (Rickinson and Kieff, 2004).

1.1.4 EBV-associated lymphomas in HIV-1 infected individuals

In healthy individuals, EBV infection is tightly controlled by both humoral and T cell-mediated immune responses. Immunodepression was long considered to play pivotal role in the etiology of cancer. Immunosurveillance was argued to be the central mechanism for checking tumour development and predictions were risen about immunosuppressed individuals being at increased risk of developing cancer (Beral V et al, 1998). Patients with HIV-1 infection present an high risk of developing EBV-related diseases, ranging from lymphoprolipherative disorders to B-cell non-Hodgkin's lymphomas (NHL), including BL, DLBCL, IBL and primary central nervous system lymphoma (PCNSL) (Carbone A et al, 2009).

Besides immunodepression, chronic immune activation, a hallmark of HIV-1 pathogenesis (Douek DC et al, 2009), may play a critical role in the genesis of B-cell lymphomas (Grulich AE et al, 2002; Moir S et al, 2009). Polyclonal B-cell activation was one of the first described immunological abnormalities in HIV-1 infected individuals (Lane HC et al, 1983; Brenchley JM et al, 2006). Cell activation driven by HIV-1 antigens, and impaired immunosurveillance against EBV may result in chronic B-cell stimulation and expansion of EBV-infected B-cells (Gaidano G et al, 1995; Righetti E et al, 2002; Burighel N et al, 2006), thus increasing the risk of developing EBV-related malignancies. The factors that may contribute to HIV-1 induced B-cell activation and expansion of-EBV-infected B-cells are largely unknown. Several cytokines and growth factors have been suggested to directly or indirectly trigger the activation of B cell in HIV-1 infected individuals. The expression of IL-2 receptor is greatly enhanced in B lymphocytes of HIV-1-infected subjects, and IL-2 may directly stimulate *in vitro* proliferation of B-cells (David D et al, 1998). Several cytokines, such as tumor necrosis factor (TNF)-α, IL-10 and IL-6, may stimulate proliferation and activation of B-cells (Macchia D et al, 1993; Mauray S et al, 2000). Furthermore, the bulk of CD4 T-cell depletion induced by HIV-1 infection occurs rapidly within the first few week of infection and is predominantly localized to the gastrointestinal tract (Brenchley JM et al, 2006), causing an early breach in the integrity of the mucosal immune system. Notably, the extent of mucosal CD4 T-

cell depletion in pathogenic Simian Immunodeficiency Virus (SIV) infection of *Rhesus Macaques* determine the rate of progression to AIDS (Picker LJ et al, 2004). This has led to the concept that injury to immune component of gastrointestinal mucosal surface, along with damage to the intestinal epithelial microenvironment with its antimicrobial functions, may affect systemic immune activation during the chronic phase of HIV-1 infection through the increase translocation of luminal microbial products (Brenchley JM et al, 2006), a phenomenon defined as microbial translocation. Damage-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs) translocate into circulation after HIV-1 induced cellular/tissue damage. DAMPs are endogenous molecules released by damaged, apoptotic, or secondary necrotic cells that trigger a potent innate immune response and cause inflammation through the engagement of the Toll-Like Receptor (TLRs) (Zhang Q et al, 2010; Cossarizza A et al, 2011). PAMPs are exogenous molecules, produced and released by several microorganisms, such as bacterial lipopolysaccharide (LPS) and 16S ribosomal DNA (16S rDNA). LPS is a major component of Gram-negative bacterial cell walls and a potent immunostimulatory product; it can be quantitatively assessed in plasma and is directly associated with the degree of intestinal permeability following invasive gastrointestinal surgery (Brenchley JM et al, 2006). 16S rDNA is a DNA sequence encoding the well-conserved 16S rRNA subunit common to most bacteria (Jiang W et al, 2009). PAMPs trigger a potent innate immune response, activate several signalling pathways, through the engagement of several TLRs, in particular TLR-4 (Medzhitov R, 2001), induce production of pro-inflammatory cytokines and activate B-cells.

The introduction of antiretroviral therapy (ART) into clinical practice has dramatically changed the development of HIV-1 related diseases. Deaths from AIDS-related diseases have been reduced by 75% (Tirelli U et al, 2001) and people diagnosed with HIV-1 can expect to live for 30 or 40 years after infection, equivalent to about a one-third reduction in lifespan (Grulich AE, 2009). The use of protease inhibitor (PI) treatment alone or used with non-nucleoside-analogue reverse-transcriptase inhibitors (NNRTI) as part of combination drug regimens, has profoundly modified the natural

course of HIV-1 infection, resulting in a significant and sustained decrease in HIV-1 RNA plasmaviremia, an increase in CD4 T cells, and a decrease in HIV-1-associated opportunistic infections, thus indicating restoration of immune function (Palella FJ Jr et al, 1998). After ART introduction, the incidence of Kaposi's Sarcoma (KS) is declining whereas the situation for EBV-related malignancies seems to be less favourable (Simard EP et al, 2011). It can be advanced that improved immune function and reduced B cell stimulation in patients receiving ART may reduce the risk of developing lymphomas. However, it is possible that patients treated with ART may survive longer, with continued B cell stimulation resulting in an increased risk of lymphoma over time. Discordant response to ART with persisting HIV-1 plasma viremia and chronic immune activation may contribute to sustaining B-cell activation and increasing EBV levels.

1.1.5 EBV in Africa

The relationship between EBV, immunodeficiency and immune activation is of great interest also in African countries, where EBV seroprevalence is nearly universal, with acquisition in early childhood (Orem J et al, 2007). EBV-associated lymphomas represent an important cause of morbidity and mortality in children (Cader FZ et al, 2010). NHL commonly occurs in African children, with endemic BL being the most common subtype (Walusansa et al, 2012). The endemic form of BL was first described in 1958 in Africa by Denis Burkitt, who noted its high prevalence in young children and its geographic distribution around the equatorial belt of Africa, apparently determined by climate and other factors, such as holoendemic malarial infection, EBV and HIV-1.

EBV plays a pivotal role in the pathogenesis of BL and in African countries, such as Uganda, the association of BL with EBV is very strong (Orem J et al, 2007). BL accounts for up to 75% of all childhood malignancies (Cader FZ et al, 2010), withs an annual incidence of 5 to 10 cases per 100.000 and a peak incidence at 6/7 years of age (Magrath IT, 2006).

Malaria represents an additional risk factor for endemic BL (Carpenter LM et al, 2008; Mutalima N et al, 2008). Malaria engages TLR-9 and induces the EBV latently infected memory B cells to

proliferate, thus leading the stimulation of EBV infected B cells and increasing the risk of emergence of a malignant B-cell clone (Rochford R et al, 2005).

High levels of EBV-DNA represent a risk factor for the onset of EBV-related malignancies. To date, no data are available about EBV load and related malignancies in the context of HIV-1 infection in African children. The absence of these data may be partly due to lack of access of equipped laboratories. One possible strategy to improve diagnosis is to simplify the collaboration between institutions in developed and developing countries, and/or between small laboratories and central facilities of the same country. Dried Blood Spot (DBS) may represent an easy method to collect and store blood samples and allow their reliable transport to specialized laboratories. DBS cards have successfully been used for metabolic disease screening in neonates (Guthrie et al, 1963). More recently, DBS filter papers are used to diagnose and monitor different DNA/RNA viral loads (Snijdewind IJ et al, 2012). However, to date no studies investigate the quantification of EBV-DNA levels from DBS. Therefore, DBS may represent an easy method to collect and store blood samples at remote sites where the laboratory equipment, personnel, or infrastructures may not be available.

Aims

My PhD research focused on the following themes:

1) the relationship between marker of immune activation and EBV load in HIV-1 infected patients;
2) the EBV infection in HIV-1 infected and uninfected children in African countries.
3) a new application of Dried Blood Spot to type and quantify EBV, and to identify B cell clonality in order to expand possibilities for diagnosis and monitoring of EBV-associated B cell malignancies.

CHAPTER 2

Epstein-Barr Virus load and immune activation in Human Immunodeficiency Virus type 1- infected patients

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Epstein-Barr Virus load and immune activation in Human Immunodeficiency Virus type 1-infected patients

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ABSTRACT

Background: Patients infected with HIV-1 are at high risk of developing Epstein-Barr Virus (EBV)-related diseases. Chronic immune activation is a hallmark of HIV-1 pathogenesis and may play a role in B-cell stimulation and expansion of EBV-infected cells.
Objectives: The aim of the study was to define the relationship between parameters of immune activation and EBV load in HIV-1-infected subjects.
Study design: A total of 156 HIV-1-infected patients were studied. EBV types 1 and 2 were quantified on peripheral blood mononuclear cells by multiplex real-time PCR. Plasma levels of cytokines and lipopolysaccharide (LPS) were determined by immunoenzymatic assays. B-cell activation was analyzed by flow cytometry.
Results: EBV-DNA was detected in 114 patients, and in all but 3 was EBV type 1. The median [interquartile] EBV-DNA load was 43[1–151] copies/10^5 PBMC. EBV-DNA load was higher in patients with detectable HIV-1 plasma viremia, despite good immunological status (CD4 > 500 cells/μl), than in patients with undetectable HIV-1 plasma viremia regardless of immunological status (46[5–136] copies/10^5 cells *vs* 17[1–56] copies/10^5 cells, $p = 0.008$). Patients with high EBV-DNA load (>median value) had higher levels of LPS and proinflammatory cytokines (IL-6, IL-10 and TNF-α) than patients with low EBV load. Furthermore, percentages of activated B-cells correlated with EBV-DNA load ($r_s = 0.754$; $p < 0.001$).
Conclusions: Overall, these findings indicate a strong association between HIV-1 viremia, markers of immune activation and EBV load and suggest that persistence of HIV-1 viremia and immune activation, regardless of peripheral CD4 cell depletion/repopulation, may favor expansion of EBV-infected cells and onset of EBV-related malignancies.

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1. Background

EBV infects B lymphocytes and has a potent transforming ability, capable of inducing uncontrolled proliferation and transformation of infected cells.[1] Patients with HIV-1 infection are at high risk of developing EBV-related diseases, ranging from lymphoproliferatives disorders to B-cell non-Hodgkin's lymphomas (NHL).[2,3]

Besides immunodepression, chronic immune activation, a hallmark of HIV-1 pathogenesis,[4] may play a critical role in the genesis of B-cell lymphomas.[5,6] Cell activation driven by HIV-1 antigens, and impaired immunosurveillance against EBV may result in chronic B-cell stimulation and expansion of EBV-infected B-cells,[7–9] thus increasing the risk of developing EBV-related malignancies. The factors that may contribute to HIV-1-induced B-cell activation and expansion of EBV-infected cells are largely unknown. The expression of interleukin (IL)-2 receptor is greatly enhanced in B lymphocytes of HIV-1-infected subjects, and IL-2 may directly stimulate *in vitro* proliferation of B-cells.[10] Furthermore, several IL-2-induced cytokines, such as tumor necrosis factor (TNF)-α and IL-6, may stimulate proliferation and activation of B-cells.[11,12] Microbial products, such as bacterial lipopolysaccharide (LPS), released into circulation following damage to intestinal mucosa due to massive HIV-1-induced T cell depletion in the gut, may also activate B-cells.[13]

Abbreviations: HIV-1, Human Immunodeficiency Virus type-1; EBV, Epstein-Barr Virus; NHL, non Hodgkin's lymphoma; IL, interleukin; TNF-α, tumor necrosis factor-alpha; LPS, lipopolysaccharide; HAART, highly active antiretroviral therapy; ADC, AIDS-defining illness; OT, out of therapy; TI, therapy interruption; PBMC, peripheral blood mononuclear cells.

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Highly active antiretroviral therapy (HAART) has greatly modified the natural course of HIV-1 infection, resulting in decreased HIV-1 RNA plasma viremia, increased CD4 T cells, and decreased HIV-1-associated opportunistic infections, indicating restoration of immune function.[14] However, the impact of HAART seems to be less favorable on EBV-related diseases than on other AIDS-defining illness (ADC), including Kaposi sarcoma. Notably, NHL was the most common ADC during the HAART era.[15] Discordant response to HAART with persisting HIV-1 plasma viremia and chronic immune activation[16] may contribute to sustaining B-cell activation and increasing EBV levels.

2. Objectives

The aim of this study was to investigate the relationship between HIV-1 viremia, markers of immune activation and EBV load in HIV-1 infected HAART-naïve and HAART-experienced patients.

3. Study design

3.1. Patients and samples

A total of 156 (117 male and 39 female) HIV-1-infected patients who attended the Infectious Diseases Division of Rovigo Hospital consecutively from July 2007 to December 2009 were included in this study. Their median age was 41 (range 18–68) years. At the time of sample collection for the study, 71 (45%) were HAART-naïve, and 85 (55%) HAART-experienced with a median (range) of 6 (1–10) years of HAART. Patients were treated according to current guidelines for HIV-1 infection.[17,18] Among HAART-experienced patients, 64 were under HAART and 21 were out of therapy, either due to CD4-guided therapy interruption (TI) ($n = 10$), development of adverse events with a previous regimen ($n = 7$) or non-adherence to therapy ($n = 4$).

Peripheral blood mononuclear cells (PBMC) were obtained by Ficoll Hypaque gradient separation. PBMC and plasma samples were cryopreserved and stored in liquid nitrogen and at −80 °C until use. For real-time polymerase chain reaction (PCR) analyses, two million PBMC were lysed in 100 μl TE buffer, as previously described.[8]

For correlation of EBV load with HIV-1 viremia and immune activation, samples were studied for EBV-DNA levels, HIV-1 plasma viremia, HIV-1 proviral DNA levels, and for several markers of immune activation (*i.e.* microbial translocation and circulating cytokines), as detailed below. When the sample size was compatible, search for B-cell activation by flow cytometry was also performed.

3.2. EBV-DNA quantification

EBV types 1 and 2 were quantified by multiplex real-time PCR. The standard reference curve was performed using serial five-fold dilutions of two amplicons, one for EBV type 1 and one for EBV type 2. Amplicons were constructed as follows: DNA from B95.8 cells was amplified using forward (5′-GGGACTTAGTATCCGTTCTA-3′) and reverse (5′-CAAATATCTACACGTCATG-3′) primers (nucleotide sequence (nt) 1083–1102 and nt1277–1295; Genebank accession number GI330443), and DNA from AG876 was amplified using forward (5′-GGGACCTAGT ATCCGTTCTA-3′) and reverse (5′-CAAACATCTACACGTCGCG-3′) primers (nt1080–1099 and nt1291–1309; Genebank accession number GI330441). The amplicon EBV type 1 (210 bp) and EBV type 2 (230 bp) were run on agarose gel, purified by using Nucleospin Exstract Kit (M-Medical), and quantified by spectrophotometry. Probes were designed to discriminate EBV type 1 (5′-FAM-AATCCTCCTACCCTCTCTTTATGCCATGTGTGT-TAMRA-3′; nt1191–1215, Genebank GI330443) and EBV type 2 (5′-Cy5-TGG TGGGCTGTTAGTAGGGT-BBQ-3′; nt1194–1209, Genebank GI330441). The multiplex PCR assay had a detection limit of 5 copies/10^5 cells and a dynamic range from 5 to 2×10^5 copies (Fig. 1).

Each PCR was performed in a 25 μl reaction mix containing 5 μl of sample, 12.5 μl LightCycler 480 Probes Master, 300 nM each EBNA2 primer,[8,19] and 100 nM of each EBV type 1 and type 2 probe. Amplification was carried out in a thermal cycler (LightCycler 480, Roche Diagnostics). Appropriate negative controls (DNA from EBV-negative A301 cells, and distilled water) were included in each

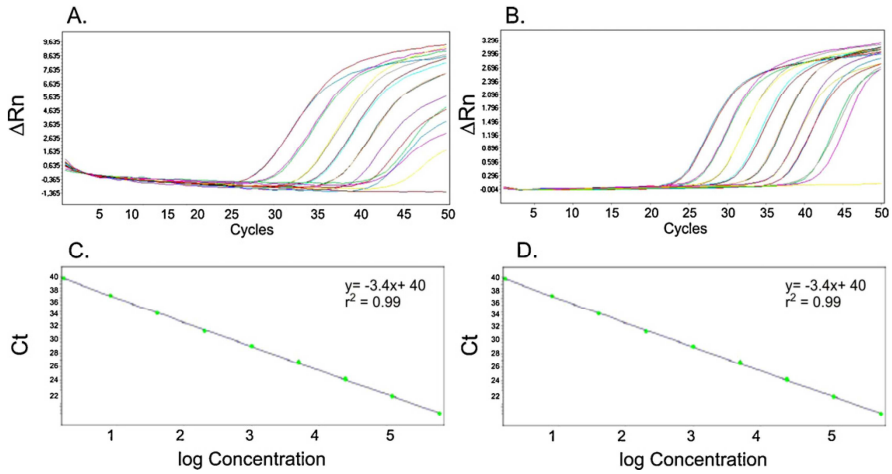


Fig. 1. Quantification of EBV type 1 and type 2 by multiplex real-time PCR. Five-fold serial dilutions of EBV type 1 (A) and type 2 (B) amplicons were amplified for EBNA2 gene. For each dilution, fluorescent emission (ΔRn) was plotted against the cycle number of PCR. Standard curves were constructed for EBV type 1 (C) and type 2 (D) by plotting the DNA concentration against the threshold cycle (Ct). The efficiency of standard curve was 98.4% for EBV type 1 (C) and 95.4% for EBV type 2 (D). Each dilution was run in duplicate; mean Ct values are shown.

Table 1
EBV load, CD4 cell count and HIV-1 plasma viremia in patients classified according to therapy.

Therapy	No. pts	EBV DNA copies/10^5 PBMC Median [IQR]	CD4 cells/μl Median [IQR]	HIV-1 RNA copies/ml Median [IQR]
Treatment naïve	71	49[12–187]	362[201–513]	38,469[11,886–150,326]
HAART	64	17[1–60]	636[384–822]	–
HIV-1 RNA undetectable[a]	56	16[1–55]	666[446–825]	–
HIV-1 RNA detectable[b]	8	80[5–1140]	516[203–808]	1411[565–1878]
Out of therapy	21	125[22–377]	364[289–591]	47.560[8453–77.853]
CD4-guided therapy interruption (TI)	10	148[1–692]	591[389–690]	42,717[7619–54,755]
Others	11	125[22–323]	302[216–339]	71,971[8265–157,216]

[a] HIV-1 RNA < 50 copies/ml.
[b] Low levels of plasma viremia (50 copies/ml < HIV-1 RNA < 2000 copies/ml).

set of reactions. The EBV copy number was normalized against the β-actin copy number and results were expressed as EBV-DNA copies/10^5 cells.[19]

3.3. HIV-1 quantification

Plasma HIV-1 RNA levels were determined using the COBAS *Taq*man HIV-1 test (Roche). The lower limit of detection was 50 HIV-1 RNA copies/ml. HIV-1 proviral DNA levels were quantified by real-time PCR.[20] The HIV-1 copy number was normalized against the β-actin copy number, and results were expressed as HIV-1 DNA copies/10^6 PBMC.

3.4. Microbial translocation and quantification of circulating cytokines

Lipopolysaccharide (LPS) was determined in plasma samples diluted five-fold with endotoxin-free water and then heated to 70 °C for 10 min to inactivate plasma proteins. Plasma LPS was quantified using a chromogenic assay (Limolus Amebocyte Lysate QCL-1000), as previously reported.[16]

Levels of IL-6, IL-10, and TNF-alpha were determined in the sera of 94 patients. Cytokines were quantified with Fluorokine MAP Human IL-6 kit, Fluorokine MAP Human TNF-α/TNFSF2 kit and Fluorokine MAP Human IL-10 kit (R&D Systems) designed for using the Luminex 200™, according to manufacturer's instructions.

3.5. B-cell activation

Approximately 250,000 PBMC were stained with anti-CD19 [peridinin chlorophyll protein (PerCP)], anti-CD69 [phycoerythrin (PE)] and anti-CD86 [allophycocyanin (APC)] monoclonal antibodies (Becton–Dickinson) and analyzed by four-color flow cytometry using a fluorescence activated cell sorter (FACS) Calibur (Becton–Dickinson). A total of 50,000 events were collected in the lymphocyte gate using morphological parameters (forward and side scatter). Data were processed using CellQuest Pro Software (Becton–Dickinson) and analyzed using the Summit Software version 4.3 (Dako).

3.6. Statistical analyses

EBV-DNA levels and virological and immunological parameters were compared by non-parametric tests, the Mann–Whitney *U*-test or Kruskal–Wallis test, as appropriate. Samples in which EBV-DNA was not detected were assigned the value of 1 copy/10^5 cells to include EBV-DNA load in statistical analysis. Samples in which plasma HIV-1 RNA or HIV-1 proviral DNA was not detected were assigned the value of 10 copies/ml, and 1 copy/10^6 cells, respectively, in order to include these data in statistical analysis. The association between EBV with CD4 and HIV-1 RNA variables was evaluated by a two-way analysis of variance. All statistical analyses were performed with SPSS, software version 18 (SPSS Inc.).

4. Results

4.1. EBV-DNA levels in relationship to CD4 cell count and HIV-1 load

EBV-DNA was detected in 114 patients; in all but 3 cases EBV was type 1. The median [interquartile, IQR] EBV-DNA load was 43[1–151] copies/10^5 PBMC. EBV-DNA levels were higher in naïve and out of therapy patients than in those in HAART (overall, $p = 0.001$) (Table 1).

CD4 cell number was lower in naïve patients and in those out of therapy than in HAART patients (overall, $p < 0.0001$) (Table 1). Only 17 naïve patients had severe immunodepression (CD4 < 200 cells/μl), whereas 52 (73%) naïve, 21 (33%) HAART and 15 (71%) patients out of therapy had CD4 < 500 cells/μl, a cut-off that defined mild immunodepression.[21] Overall, EBV-DNA levels were significantly higher in patients with CD4 < 500 cells/μl than in those with CD4 > 500 cells/μl (72[15–322] copies/10^5 PBMC *vs* 18[1–74] copies/10^5 PBMC; $p < 0.0001$) (Fig. 2A).

HIV-1 plasma viremia in TI patients did not differ from that of naïve patients ($p = 0.567$) (Table 1); 56 (87.5%) HAART patients and only one naïve patient had undetectable HIV-1 plasma viremia. EBV-DNA load was significantly higher in patients with detectable HIV-1 RNA than in those with undetectable plasma viremia (74[12–323] copies/10^5 PBMC *vs* 17[1–56] copies/10^5 PBMC; $p = 0.001$) (Fig. 2B). Of interest, in 8 HAART patients with persistently low HIV-1 plasma viremia and in TI patients the levels of EBV-DNA were similar and even higher than those observed in naïve patients, despite less pronounced CD4 cell depletion (Table 1). A search in the entire cohort of patients indicated that EBV-DNA load was higher in those with detectable HIV-1 plasma viremia, despite their good immunological status (CD4 > 500 cells/μl) than in patients with undetectable plasma viremia, regardless of CD4 cell count (46[5–136] copies/10^5 PBMC *vs* 17[1–56] copies/10^5 PBMC; $p = 0.008$) (Fig. 2C). Analysis of variance confirmed that HIV-1 plasma viremia was associated with EBV-DNA levels, independent of CD4 cell count ($p = 0.004$). This suggested that HIV-1 stimulated immune activation, leading to expansion of EBV-positive B-cells, regardless of CD4 depletion and/or immunoreconstitution.[13,16] Since immune activation may have ultimately led to an expansion of HIV-1 infected cells, we analyzed the relationship between EBV-DNA load and levels of HIV-1 proviral DNA, a marker of infected cells. Patients with EBV-DNA levels above median value had higher levels of HIV-1 DNA than patients with EBV-DNA levels below median value (50[1–154] HIV-1 DNA copies/10^6 PBMC *vs* 10[1–78] HIV-1 DNA copies/10^6 PBMC; $p = 0.042$).

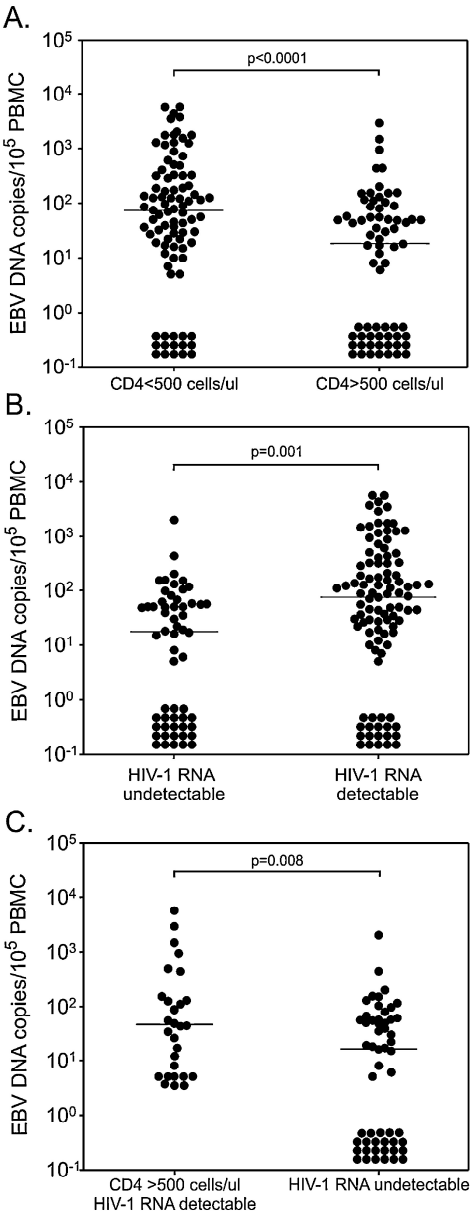


Fig. 2. EBV-DNA load according to HIV-1 plasma viremia and CD4 cell count. (A) EBV-DNA levels in patients classified according to CD4 cell count. (B) EBV-DNA levels in patients classified according to HIV-1 RNA plasma viremia (HIV-1 RNA undetectable: <50 copies/ml; HIV-1 RNA detectable: >50 copies/ml). (C) EBV-DNA levels in patients with CD4 > 500 cells/μl and detectable HIV-1 and in patients with undetectable HIV-1 RNA (<50 copies/ml), regardless of CD4 cell count. Each plot represents one patient. The lines indicate the median values.

4.2. EBV-DNA levels in relation to markers of immune activation

Together with HIV-1 antigens, chronic immune activation may be driven by microbial translocation products, such as LPS. Median [IQR] values of plasma LPS were higher in patients with detectable HIV-1 plasma viremia than in virologically well suppressed patients (106[86–172] pg/ml *vs* 92[83–119] pg/ml; $p = 0.046$) (Fig. 3A). EBV-DNA load was defined as high or low, according to the value above or below the median. Overall, LPS levels were significantly higher in patients with high *vs* low EBV-DNA load (109[88–153] pg/ml *vs* 94[78–123] pg/ml; $p = 0.037$) (Fig. 3B).

TNF-α, IL-6 and IL-10 were higher in patients with detectable HIV-1 RNA than in those with undetectable plasma viremia (5.96[4.75–8.75] pg/ml *vs* 5.25[4.65–5.86] pg/ml, $p = 0.056$, for TNF-α; 2.11[1.44–2.41] pg/ml *vs* 1.77[1.52–2.02] pg/ml, $p = 0.049$, for IL-6; 1.61[1.20–2.43] pg/ml *vs* 0.95[0.83–1.08] pg/ml, $p = 0.001$, for IL-10) (Fig. 3). Furthermore, all three cytokines were higher in patients with high *vs* low EBV-DNA load (5.86[4.69–8.28] pg/ml *vs* 5.19[4.65–5.85] pg/ml, $p = 0.053$, for TNF-α; 2.06[1.65–2.52] pg/ml *vs* 1.77[1.52–2.11] pg/ml, $p = 0.054$, for IL-6; 1.58[1.05–2.35] pg/ml *vs* 0.99[0.83–1.39] pg/ml, $p = 0.001$, for IL-10) (Fig. 3).

All these findings suggested a link between immune activation and expansion of EBV-infected cells. Flow cytometric analysis of PBMC from 16 subjects disclosed wide variability in B-cell activation, ranging from 0.89% to 24.77%. A significant relationship was observed between percentages of activated B-cells and level of EBV-DNA ($r_s = 0.754$; $p < 0.001$) (Fig. 4).

5. Discussion

Following the introduction of HAART, the incidence of opportunistic infections and AIDS-related diseases, such as Kaposi sarcoma, dramatically decreased.[22,23] However, the impact of HAART in preventing EBV-associated lymphomas seems to be less effective.[15] Chronic immune activation is a hallmark of HIV-1 infection and may contribute to the expansion of EBV-infected cells. Persistence of plasma viremia during HAART, despite immunoreconstitution, is not an infrequent observation,[16,20,24–26] and HIV-1 is known to stimulate the immune system, regardless of the status of peripheral CD4 depletion/repopulation.[13,16]

In this study, we described for the first time the relationship between markers of chronic immune activation and EBV load in HIV-1 infected subjects. HIV-1 viremic patients, including those with low levels of plasma viremia under HAART and those with CD4-guided therapy interruption, had significantly higher levels of LPS and inflammatory cytokines than patients with undetectable plasma viremia. These low viremic and TI patients, despite a gain in CD4 lymphocytes, had high levels of EBV-DNA. The significant relationship between activated B-cells and EBV-DNA load observed in a subgroup of patients supports the concept that immune activation plays a pivotal role in the expansion of EBV-infected B-cells. Notably, immune activation may promote B cell expansion, regardless of EBV infection.[5] B cell proliferation may be driven by ligands of Toll-like receptors (TLR), such as DNA unmethylated CpG dinucleotides which trigger TLR9. However, while CpG DNA alone stimulates only memory B cells, ligands of TLR9 synergistically increase EBV-mediated activation and proliferation of both memory and naïve B cells.[27] It is likely that immune activation preferentially promotes the expansion of EBV-infected B-cells.

HIV-1 may act as a direct or indirect activator of B-cells. Binding of HIV-1 envelope protein to B-cell membranes may trigger a polyclonal immmunoglobulin class switch recombination,[28] and viral protein Nef can promote B-cell activation.[29] However, most of the mechanisms by which HIV-1 activates B-cells probably involve proinflammmatory cytokines and microbial products generated after cellular/tissue damage. Since B-cells do not express LPS receptor,[30] the mechanism of B-cell activation may be mediated through TNF-α.[13] Notably, in our study LPS levels tended to correlate with TNF-α levels ($r = 0.423$, $p = 0.0532$).

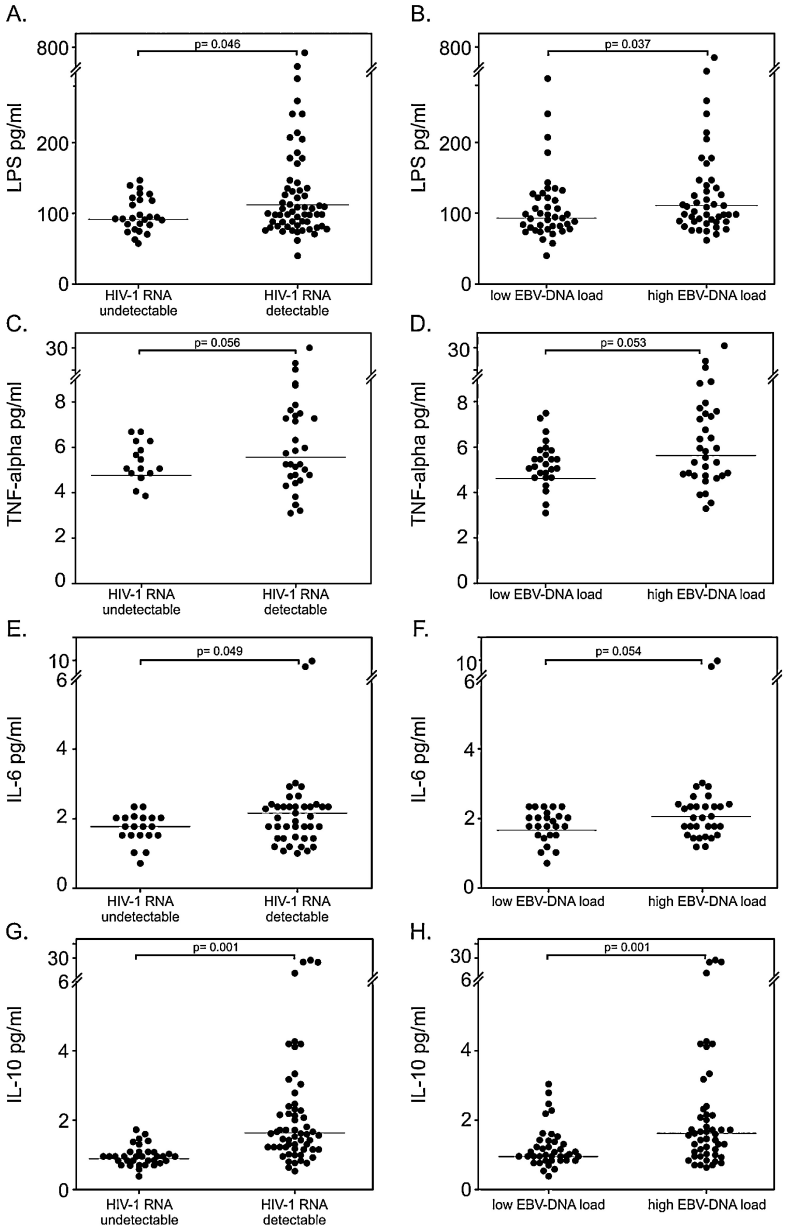


Fig. 3. EBV-DNA load and markers of immune activation. LPS (A), TNF-α (C), IL-6 (E), and IL-10 (G) levels in patients with detectable HIV-1 plasma viremia (HIV-1 RNA > 50 copies/ml) and in patients with undetectable HIV-1 plasma viremia (<50 copies/ml). LPS (B), TNF-α (D), IL-6 (F), and IL-10 (H) levels in patients with high EBV load (values above the median) and low EBV-load (values below the median). Each plot represents one patient. The lines indicate the median values.

It has been demonstrated that HAART may reduce, but not normalize, most of the biomarkers associated with B-cell hyperactivation, including TNF-α.[31,32] Furthermore, increased IL-6 levels have been reported in immune reconstitution inflammatory syndrome in patients starting HAART,[33,34] and in association with increased HIV-1 plasma viremia during HAART interruption.[35] Along with TNF-α, we found that the pro-inflammatory cytokines IL-6 and IL-10, which can directly stimulate B-cells, were high in viremic patients. The observation that circulating high levels of IL-6, and IL-10 were repeatedly detected prior to AIDS-NHL diagnosis implies a role of these cytokines in driving B-cell stimulation in the development of lymphoma.[36,37]

Overall, these findings indicate that HIV-1 viremia, circulating microbial products and pro-inflammatory cytokines all play

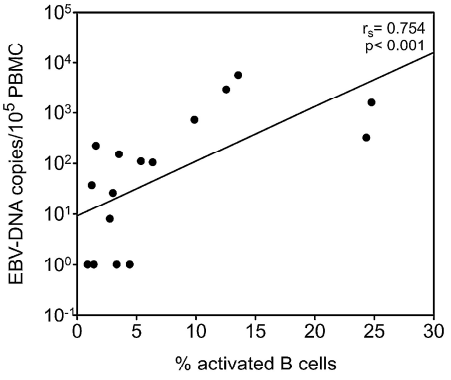


Fig. 4. Relationship between EBV load and activated B-cells. Values of EBV-DNA copies/10^5 PBMC were plotted against percentages of activated B-cells estimated by flow cytometry.

important roles in B-cell stimulation and expansion of EBV-infected cells. Despite partial immunoreconstitution, B-cell stimulation driven by persistent immune activation poses a risk factor for the development of EBV-related malignancies.

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CHAPTER 3

Relationship between Non-Hodgkin's lymphoma and blood levels of Epstein -Barr Virus in children in north-western Tanzania: a case control study

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Relationship between Non-Hodgkin's lymphoma and blood levels of Epstein-Barr Virus in children in north-western Tanzania: a case control study

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Abstract

Background: Non-Hodgkin's Lymphomas (NHL) are common in African children, with endemic Burkitt's lymphoma (BL) being the most common subtype. While the role of Epstein-Barr Virus (EBV) in endemic BL is known, no data are available about clinical presentations of NHL subtypes and their relationship to Human Immunodeficiency Virus (HIV) infection and Epstein Barr Virus (EBV) load in peripheral blood of children in north-western, Tanzania.

Methods: A matched case control study of NHL subtypes was performed in children under 15 years of age and their respective controls admitted to Bugando Medical Centre, Sengerema and Shirati district designated hospitals in north-western, Tanzania, between September 2010 and April 2011. Peripheral blood samples were collected on Whatman 903 filter papers and EBV DNA levels were estimated by multiplex real-time PCR. Clinical and laboratory data were collected using a structured data collection tool and analysed using chi-square, Fisher and Wilcoxon rank sum tests where appropriate. The association between NHL and detection of EBV in peripheral blood was assessed using conditional logistic regression model and presented as odds ratios (OR) and 95% confidence intervals (CI).

Results: A total of 35 NHL cases and 70 controls matched for age and sex were enrolled. Of NHLs, 32 had BL with equal distribution between jaw and abdominal tumour, 2 had large B cell lymphoma (DLBCL) and 1 had NHL-not otherwise specified (NHL-NOS). Central nervous system (CNS) presentation occurred only in 1 BL patient; 19 NHLs had stage I and II of disease. Only 1 NHL was found to be HIV-seropositive. Twenty-one of 35 (60%) NHL and 21 of 70 (30%) controls had detectable EBV in peripheral blood (OR = 4.77, 95% CI 1.71 – 13.33, p = 0.003). In addition, levels of EBV in blood were significantly higher in NHL cases than in controls (p = 0.024).

Conclusions: BL is the most common childhood NHL subtype in north-western Tanzania. NHLs are not associated with HIV infection, but are strongly associated with EBV load in peripheral blood. The findings suggest that high levels of EBV in blood might have diagnostic and prognostic relevance in African children.

Keywords: Non-Hodgkin's Lymphoma, Children, HIV, EBV

Background

Non-Hodgkin's Lymphomas (NHLs) are common in African population and several histological subtypes with different clinical presentations and treatment options have been described based on the WHO classification of the mature B-cell neoplasm [1]. Worldwide, reports on the incidence of lymphomas are variable, with 60% of all childhood lymphomas being classified as NHL, representing 8% of all childhood malignancies [2-4].

In Equatorial African countries, the most common subtype of NHL in children is endemic Burkitt's lymphoma (BL) [5-7], possibly due to the high prevalence of malaria, Human Immunodeficiency Virus (HIV) and Epstein Barr Virus (EBV) infections in this region [5]. The magnitude of NHL in Tanzania is unknown because of the lack of a national cancer registry. However, a recent clinical study in north western Tanzania reported an incidence of endemic BL of 4.2 per 100,000 [8].

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The clinical presentation of childhood NHL depends primarily on the subtype and the site of involvement. Approximately 70% of children who present with NHL have advanced disease (i.e., stage III or IV) and/or have metastatic involvement including bone marrow and central nervous system (CNS) [9-11].

Endemic BL is commonly seen in children aged 4–9 years [12] and presents with a painful jaw tumour, loose or disarranged teeth [13] and less commonly with abdominal mass or paraspinal disease. However, few African studies reported a predominance of abdomen over jaw manifestation [14,15]. Bone and bone marrow involvement is unusual. Sporadic childhood BL is more evident in white, male children and commonly presents with enlarged lymph nodes and abdominal tumours [16].

Childhood lymphoblastic lymphoma is commonly manifested by either intrathoracic and/or mediastinal mass, with various forms of respiratory distress, bilateral pleural effusions and hepatosplenomegaly [17] and rarely involve both the CNS and bone marrow [18]. Large B-cell lymphoma presents with an abdominal or mediastinal mass, enlarged lymph nodes and rarely involve both the CNS and bone marrow [2].

In African children, the association between NHL and HIV is uncertain. Two studies from Uganda and Rwanda described a relationship between NHL and HIV infection [19,20] but most studies, including those with cases confirmed by histology, reported no association between HIV and BL [21-23].

In paediatric patients, studies have reported an association between EBV and NHL in 21-25% of cases [24,25] and even 100% in children with primary and acquired immunodeficiency [26]. Moreover, 95% of endemic BL have been reported to be EBV positive compared to 15 to 20% of the sporadic BL cases [4,7,8]. Both EBV type 1 and EBV type 2 have been associated with BL, with a predominance of EBV type 1 [17,27-30]. However, very little is known about EBV load in peripheral blood of African children with NHL. A study in samples from Malawi reported higher EBV DNA loads in peripheral blood of children with BL compared to their controls [31].

The aim of this study was to describe the subtypes and clinical presentations of NHL in children in north-western Tanzania and their relationship with HIV infection and EBV load in peripheral blood.

Methods

Study area and population

This was a case control study, done in paediatric and oncology wards of the three clinical centres in north-western Tanzania, namely Bugando Medical Centre (BMC), Sengerema and Shirati designated district hospitals in Mwanza and Mara regions, between September 2010 and April 2011. BMC is the zonal consultant and teaching hospital located in Mwanza city; it has a 900 bed capacity and it serves around 14 million people from 6 regions of the Lake Zone, namely Mwanza, Kagera, Shinyanga, Tabora, Mara and Kigoma. About five children with tumours are admitted monthly for investigation and treatment. Sengerema and Shirati DDH have approximately 300 and 200 bed capacities, respectively, and they each serve around 30 and 15 cases of childhood lymphomas every year.

Cases were enrolled if they were ≤15 years of age with a diagnosis of NHL and if consent from caretakers to participate in the study was obtained. Controls were included if they were admitted to the general paediatric wards with non-malignant conditions and were matched for age and sex with NHL cases. Children with malignancies that overlapped NHL, e.g. acute lymphoblastic leukaemia, and children with relapse of NHL were excluded from the study.

Sample size estimation

Sample size was calculated using OpenEpi software, version 2 [32], in which Fleiss formulae for case control studies was applied to determine the appropriate sample size for a power of 0.8 and significance level of 0.05. The minimum sample size for cases was 1/3 (33) and for controls was 2/3 (66) making the minimum sample size of 99.

Study clinical procedures

All consecutive NHL cases were recruited until the required sample size was attained, whereas simple randomization was done to select two controls per each case from all eligible patients. The investigator or trained research assistant either admitted the patient or reviewed the files of all eligible children within 2 days of admission and using a structured data collection tool, demographic data, clinical data and required investigation results were recorded.

To collect samples for cytological or histological diagnosis of NHL subtypes, fine needle aspiration of the tumour was carried out, using a needle of 25 or 27 gauge and a 20 ml disposable plastic syringe, under sedation with midazolam (0.1 mg/kg) and ketamine (1 mg/kg), or tissue biopsy of the tumour was carried out under general anaesthesia. In the case of an abdominal mass, an ultrasound scan was used to guide the needle into the tumour. Fine needle aspirate was smeared between two standard microscope slides with one slide immediately fixed with 95% alcohol and the other air dried and then transported in a slide carrier to the histopathology laboratory. Alcohol fixed smears were stained by Papanicolaus stain and air dried slides were prepared and stained with Giemsa.

Tissue biopsies were taken in the operating theatre, fixed immediately with 10% formalin and then transported to the

pathology laboratory. Histology slides were stained with haematoxylin and eosin. At least two pathologists examined the slides under light microscopy and children with confirmed NHL diagnosis were enrolled in this study.

Lumbar puncture was performed in order to obtain cerebral spinal fluid (CSF) for cytological evaluation. A sterile 22, 38 mm gauge lumbar puncture needle (BD spinal needle) was inserted between the fourth and fifth lumbar vertebrae and the stylet withdrawn to allow about 5 ml of CSF to freely flow through the needle into the sterile tube which was then sent to the pathology laboratory to detect the presence or absence of cancer cells. Presence of cancer cells in the CSF indicated involvement of the CNS.

Bone marrow aspiration was performed, using a 13,5 cm gauge bone marrow needle (Jamshidi bone marrow aspiration needle), under sedation with midazolam and ketamine; 1, 2 ml of bone marrow were aspirated into the syringe and then smeared on 8 slides (4 fixed with alcohol and 4 without fixation) that were air dried and sent to the pathology laboratory for cytological analysis. Presence of lymphoma cells indicated bone marrow involvement.

NHL staging was done using the St. Jude's staging of childhood NHL [33].

Blood was drawn from the median cubital vein and collected in plain and EDTA bottles and then sent to the haematology and biochemistry laboratories for analysis of complete blood count and erythrocyte sedimentation rate, serum creatinine, alanine amino transferase and aspartate amino transferase.

The Tanzania national algorithm for HIV testing was followed to determine the HIV status of both cases and controls, using two antibody tests, the SD Bioline (SD Standard Diagnostics, Inc) and the Determine (Determine HIV 1/2 Serum/Plasma Assays); the second test was carried out in samples reactive to the first test. Those samples that were reactive to the first and second tests were considered to be HIV positive. Discordant results were subjected to a third test, Unigold (The Trinity Biotech PLC) [34]. Counselling on HIV testing was done and consent was sought prior to testing. Among HIV positive children, CD4 cell count was measured using Facscount (BD, San Jose, California, USA).

Blood collection and DNA elution

Approximately 50 μl of blood was spotted onto each circle of Whatman 903 filter paper and then dried at room temperature overnight. Dried Blood Spots (DBS) were stored in individual ziplock bags containing a desiccant, and sent to the laboratory of the Department of Surgery, Oncology and Gastroenterology, Section of Oncology and Immunology, University of Padova, Unit of Viral Oncology-IOV IRCCS, Italy. From each 50 μl DBS, three 3 mm-diameter circles, equivalent to 5 μl of whole blood each for a total of 15 μl whole blood, were used to extract DNA with the DNA Micro Kit (Qiagen, Hilden, Germany) and resuspended in 50 μl final volume.

EBV-DNA quantification

To control the ability of the eluted DNA from DBS to be amplified, 5 μl of DNA from each sample were amplified for the human telomerase reverse transcriptase (hTERT) located in the 5p15.33 (Gene Bank accession: AF128893), employed as housekeeping gene. Amplification was carried out as previously described [35]. A quantitative method based on Multiplex Real-Time PCR assay was performed to quantify EBV type 1 and EBV type 2, using a primer pair for the EBNA2 gene [36,37] and the probes designed to discriminate between EBV type 1 and EBV type 2 (EBV type 1: 5'-FAM-AAT CCT CCT ACC CTC TCT TTA TGC CAT GTG TGT-TAMRA-3'; EBV type 2: 5'-Cy5 TGG GCT GTT AGT AGG GT-BBQ-3') [38]. A standard reference curve was obtained by five-fold serial dilution of two amplicons, one for EBV type 1 and the other for EBV type 2, and amplification was carried out, as already detailed [38]. The multiplex assay showed a dynamic range from 5 to 2×105 copies. The results were expressed as EBV-DNA copies/ml. In those who had both EBV 1 and 2, viral levels were added and their total viral loads were analysed to investigate the association between NHL and EBV viral load.

Statistical analysis

Data were screened and edit checks were done to minimize data entry errors. Data were analysed with the STATA 11 software (College Station, Texas, USA). Categorical variables were summarized as percentages and were analysed by Chi-square or Fisher's exact tests where appropriate. Continuous variables were summarized as mean (standard deviation) or median (range) where appropriate. EBV-DNA levels of cases and controls were compared using Wilcoxon rank sum (Mann–Whitney) test. The association between NHL and EBV was investigated using conditional logistic regression model. Odds ratios (OR) and 95% confidence intervals (CI) were determined by maximum likelihood estimation. The data were considered significant if the p-value was < 0.05.

Ethical considerations

Ethical approval was obtained from the Joint Bugando Medical Centre/Bugando University College of Health Sciences research and publication committee and guidelines on ethical requirement for conducting research and sample transportation outside the country were adhered to. Confidentiality was assured and the study did not interfere with the decision of the attending physician in case of absence of the investigator.

Results

Patient characteristics of NHL cases and controls

35 NHL cases and 70 controls matched for age and sex were enrolled in this study. Among the NHL patients, 21 (60%) were females and 14 (40%) were males ($x^2 = 0.92$, p-value = 0.337). Age at presentation ranged from 3 to 14 years with a peak age of 5 to 9 years (68.57%) and a mean age of 7.48 years (SD +/− 2.77).

Of the 35 NHL cases, 32 patients had a diagnosis of BL, 2 had diffuse large B cell lymphoma (DLBCL) and 1 had NHL-not otherwise specified (NHL-NOS). The controls were diagnosed with sickle cell anaemia (10, 14.3%), severe pneumonia (8, 11.4%), gastroenteritis (5, 7.1%), congenital heart diseases (5, 7.1%), urinary tract infections (5, 7.1%), rheumatic heart diseases (5, 7.1%), malnutrition (5, 7.1%), tuberculosis (4, 5.7%), nephrotic syndrome (3, 4.3%) and others infections or bleeding conditions (20, 28.8%).

Clinical presentations of NHL cases

The primary tumour site was recorded in all cases. Of the 32 BL cases, jaw and abdomen had an equal distribution; 16 cases (50%) in each tumour site. One BL patient had both abdominal and jaw involvement. Lymph nodes were enlarged in all non-BL NHL cases as the primary tumour site and these involved submandibular, axillary, cervical and inguinal lymph nodes. Axillary and inguinal lymph nodes were enlarged in two BL cases. Fever was reported in 11 cases (31.4%) and anaemia in 28 cases (80%), of which 3 (10.7%) had severe anaemia (Hb ≤ 5 g/dl).

Bone marrow was involved in the patient with NHL-NOS, whereas CNS presentation occurred in one BL patient who was a 7 years old boy and presented with a jaw tumor involving the left eye and weakness of the lower limbs. He was HIV negative but EBV positive and had cancer cells in the CSF. The majority of BL cases (19 of 32) had an early presentation (stage I or II), while the DLBCL and NHL-NOS had a late presentation (stage III and IV) (Table 1). Analysis of fever, anaemia and lymph node enlargement showed a significant association between fever and late stage presentation (p = 0.035).

Association of NHL with HIV infection

Only 1 NHL case and two controls were found to be HIV-seropositive. No significant association was found between NHL and HIV infection.

Association of NHL with EBV infection, EBV subtypes and viral load

Of the 105 DBS samples, 42 (40.0%) were found to be positive for EBV-DNA by PCR; therefore 42 subjects had detectable levels of EBV in their peripheral blood. Of this subgroup of EBV positive subjects, 21 cases and 21 controls (Table 2), 27 (64.3%) had EBV type 1, while 8 (19.0%) had both subtypes (EBV type 1 and 2). There was no

Table 1 Clinical and demographic features of NHL patients

	NHL subtypes			Total
	BL	DLBCL	NHL-NOS	
Number of cases	32/35 (91.4%)	2/35 (5.7%)	1/35 (2.9%)	35/35 (100%)
Age				
1-4	5/32 (15.6%)	0/2 (0%)	0/1 (0%)	5/35 (14.3%)
5-9	21/32 (65.6%)	2/2 (100%)	1/1 (100%)	24/35 (68.6%)
10-15	6/32 (18.8%)	0/2 (0%)	0/1 (0%)	6/35 (17.1%)
Sex				
Male	14/32 (43.8%)	0/2 (0%)	0/1 (0%)	14/35 (40%)
Female	18/32 (56.2%)	2/2 (100%)	1/1 (100%)	21/35 (60%)
Primary tumour site				
Jaw	16/32 (50%)	0/2 (0%)	0/1 (0%)	16/35 (45.7%)
Abdomen	16/32 (50%)	0/2 (0%)	0/1 (0%)	16/35 (45.7%)
Lymph Node	0/32 (0%)	2/2 (100%)	1/1 (100%)	3/35 (8.6%)
CNS involvement	1/32 (3.1%)	0/2 (0%)	0/1 (0%)	1/35 (2.9%)
[a]Bone marrow involvement	0/11 (0%)	0/11 (0%)	1/11 (9.1%)	1/11 (9.1%)
Fever	9/32 (28.1%)	1/2 (50%)	1/1 (100%)	11/35 (31.4%)
Anaemia	26/32 (81.3%)	1/2 (50%)	1/1 (100)	28/35 (80%)
Clinical stage				
Early (I/II)	19/32 (59.3%)	0/2 (0%)	0/1 (0%)	19/35 (54.3%)
Late (III/IV)	13/32 (40.7%)	2/2 (100%)	1/1 (100%)	16/35 (45.6%)

[a] Bone marrow sampling was done in 11 only cases because of unavailability of facilities in two study sites.

significant difference between EBV subtypes and either age (Fisher's exact test = 1) or sex (fisher exact test = 0.213). twenty one of 35 (60%) NHL patients had EBV detectable in peripheral blood compared to 21 of 70 (30%) controls; a significant association was found between NHL and EBV (OR = 4.77, 95% CI = 1.71 – 13.33, p -value = 0.003) (Table 2).

EBV levels were not normally distributed. Hence, a two sample Wilcoxon rank sum test (Mann–Whitney test) was used to analyse the median viral load distribution between cases and controls. This analysis showed a significantly higher median viral load in cases compared to controls (p-value = 0.024) (Table 2).

Analysis of cases revealed no significant association between EBV status and demographic characteristics or clinical presentation (Table 3).

Discussion

In this study, the most common subtype of NHL was found to be BL, a finding similar to previously reported studies [7]. This frequency might be due to the causal relationship between BL and co-infections with malaria and EBV in the north-western regions of Tanzania, which are considered part of the "lymphoma belt" of Africa. BL should therefore be highly suspected in cases of childhood NHL.

The common age at presentation in this study was similar to previous studies, with the majority of patients ranging from 5 to 9 years [12,13]. However, conversely to other studies [2,3] females were more affected than males; this might have been due to the small number of cases reported in this study. Nevertheless, there was no statistically significant relationship between NHL and gender.

The majority of patients in this study were in an early stage of disease at presentation, possibly due to ongoing sensitization programmes and an increase in the number of hospitals that provide free cancer treatment in the Lake zone regions in recent years, compared to the past treatment modalities for NHL cases [2,9-11]. The increased awareness of cancer management, easier accessibility and better healthcare provided by these hospitals today may have sensitized more patients to present early for treatment.

Jaw and abdomen were the most common primary tumour sites in patients with BL. While other studies reported more jaw BL in African children [14], we found no predominance of jaw over abdominal presentation. One reason for more jaw BL cases might be an easier access to jaw tumours for diagnostic purposes compared to abdominal tumours which require more expertise and diagnostic facilities than were not available in the past.

Lymph nodes were involved in all non-BL NHL subtypes. Only 1 patient presented with CNS disease, which is more commonly observed in sporadic BL [11,14]. As previously reported [14], bone marrow involvement was rare. However, a Brazilian study reported 23% of cases with primary bone marrow disease [11]. We could not rule out a possible underestimation since bone marrow sampling was not done in all cases because of unavailability of facilities in two sites.

HIV is believed to act as a trigger in the causation or progression of some cancers because of its ability to cause immune depression. Only one case and two controls were found to be HIV-seropositive and, in agreement with previous observations [21-23], this study did not find any significant association between childhood NHL and HIV infection.

A statistically significant association was found between NHL and EBV detection in peripheral blood, with a

Table 2 Frequency of EBV detection and EBV load in NHL patients and controls

	NHL		p-value
	Cases	Controls	
EBV-positive detection in blood	21/35 (60%)	21/70 (30%)	0.003
EBV sub-type			
EBV-1	11/21 (52%)	16/21 (76%)	
EBV-2	5/21 (24%)	2/21 (10%)	
EBV type 1 & 2	5/21 (24%)	3/21 (14%)	
EBV viral load count Median (range) copies/ml	4720 (988–6250164)	2525 (1290–19452)	0.024

Table 3 EBV status and demographic characteristics and clinical presentations in NHL cases

	EBV-DNA positive	EBV-DNA negative	p-value
Age			
1-4 years	2 (40%)	3 (60%)	0.813
5-9 years	17 (70.8%)	7 (29.2%)	
10-14 years	2 (33.3%)	4 (66.7%)	
Sex			
Male	10 (71.4%)	4 (28.6%)	0.22
Female	11 (52.4%)	10 (47.6%)	
NHL subtype			
BL	19 (59.4%)	13 (40.6%)	0.652
Non-BL	2 (66.7%)	1 (33.3%)	
Primary anatomical site			
Jaw	7 (43.8%)	9 (56.2%)	0.247
Abdomen	12 (75%)	4 (25%)	
Lymph nodes	2 (66.7%)	1 (33.3%)	
Clinical stage			
Early	10 (52.6%)	9 (47.4%)	0.332
Late	11 (68.6%)	5 (31.4%)	

predominance of EBV type 1. Furthermore, children with NHL had higher viral load in their peripheral blood than EBV positive controls. The high level of EBV in blood may be relevant in predicting tumour burden, prognosis and possibly the outcome of chemotherapy. Moreover, the strong association between NHL and EBV may suggest that control subjects with high detectable levels of EBV in blood might be at higher risk of developing lymphoma.

Additional community-based studies should be done in order to identify the common EBV subtypes circulating in Tanzanian children and to predict their causal relationship with NHLs in Tanzania.

Conclusions

BL is the most common childhood NHL subtype in north-western regions of Tanzania with rare CNS and bone marrow involvement. NHLs are not associated with HIV infection, but are strongly associated with EBV load in blood. Children with NHL at presentation had significantly higher frequency and higher levels of EBV in the peripheral blood than age matched controls. Overall, these findings suggest that EBV load in blood might be a diagnostic and prognostic marker for the onset and monitoring of NHL in African children. EBV detection in blood is less invasive and expensive than EBV detection in histological samples. Additional studies in larger populations are required to validate the diagnostic and prognostic value of EBV load in blood.

Competing interests

The authors declare that they have no competing interest and this work was supported in part by Programma Integrato Oncologia (RO 4/2007).

Authors' contributions

RK; Designed the study, collected and analyzed data, interpreted the results and drafted the manuscript. NM; Designed the study, analyzed data, interpreted the results and reviewed the manuscript. PR; Designed the study, carried out pathological analysis of samples and reviewed the manuscript. EK; Designed the study, analyzed data, interpreted the results and reviewed the manuscript. BK; Analyzed data, interpreted the results and reviewed the manuscript. AD; Carried out molecular studies, interpreted the results and reviewed the manuscript. RP; Carried out molecular studies. DM; Designed the study, analyzed data, interpreted the results and reviewed the manuscript. All authors read and approved the final manuscript.

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CHAPTER 4

Epstein-Barr Virus (EBV) load in Human Immunodeficiency Virus (HIV)-1-infected children in Uganda

Epstein-Barr Virus (EBV) load in Human Immunodeficiency Virus (HIV)-1-infected children in Uganda

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Running head: EBV load in HIV-1 infected children in Africa

Manuscript to be submitted

Abstract

Background: Epstein-Barr Virus (EBV) is involved in a wide range of malignancies, particularly in immunocompromised subjects. Besides immunodepression, chronic immune activation may induce B-cell stimulation leading to an expansion of EBV-infected cells, thus increasing the risk of EBV-related malignancies. In Africa, EBV primary infection occurs during early childhood, but little is known about EBV load in HIV-1-infected children.

Methods: Dried Blood Spot (DBS) samples from 213 HIV-1-infected children (0-18 years), 140 of whom were on antiretroviral therapy (ART), were collected at Nsambya Home Care. DNA was extracted and tested to quantify both EBV type 1 and EBV type 2 by multiplex real-time PCR. A real-time PCR assay was set up to quantify 16S ribosomial DNA (16S rDNA). Statistical analyses were performed using STATA software.

Results: 92 of 140 (66%) children on ART and 57 of 73 (78%) ART-naive children were found to be EBV-positive. EBV type 1 and EBV type 2 were detected in 31 (22%) and 21 (15%) children on ART, and in 15 (21%) and 11 (15%) ART-naive children, respectively; 40 (29%) children on ART and 31 (42%) ART-naive children were co-infected with both EBV types. After adjusting for CD4Z score, age and WHO stage, children on ART had less odds of having detectable EBV (OR 0.39 [95% CI 0.16-0.94], p=0.036). Mean [interquartile-IQR] levels of EBV-DNA were similar in children positive for EBV type 1 (3.80 [3.45-3.88] $\log_{10}$ copies/ml) or EBV type 2 (3.92 [3.52-4.29] $\log_{10}$ copies/ml), but higher in children co-infected with both viruses (4.34 [3.95-4.61] $\log_{10}$ copies/ml; overall p=0.001). EBV load was significantly higher in ART-naive children than those on ART (4.22 [3.87-4.58] vs 3.99 [3.55-4.33] $\log_{10}$ copies/ml; p= 0.016). Levels of 16S rDNA were significantly higher in ART-naive children than those on ART (2.16 [2.11-2.28] vs 2.09 [2.02-2.22] $\log_{10}$ copies/µl; p=0.007) and correlated with EBV-DNA levels (r= 0.382, p=0.016).

Conclusions: These finding suggest that chronic immune activation, induced by HIV-1 and circulating microbial products, leads to an increase of EBV load, a risk factor for the development of EBV-related malignancies. Treatment with ART, likely by limiting immune activation, may prevent B cell activation and expansion of EBV-infected B cells.

1. Introduction

Epstein-Barr Virus (EBV) is a gammaherpes virus involved in a wide range of malignancies, particularly in immunocompromised subjects, ranging from lymphoproliferatives disorders to B cell non-Hodgkin's lymphomas (NHL) (1,2).

Besides immunodepression, chronic immune activation, a hallmark of HIV-1 pathogenesis (3), may play a critical role in the genesis of B cell lymphomas (4,5). Cell activation driven by HIV-1 antigens, together with impaired immunosurveillance against EBV, may result in chronic B cell stimulation and expansion of EBV-infected B cells (6-8), thus increasing the risk of developing EBV-related malignancies. The factors that may contribute to HIV-1-induced B cell activation and expansion of EBV-infected cells are largely unknown. Massive HIV-1-induced T cell depletion causes a damage to intestinal mucosa, thus promoting a translocation of pathogen-associated molecular patterns (PAMPs) into circulation. PAMPs are exogenous molecules, produced and released by several microorganisms, such as 16S ribosomal DNA (16S rDNA). PAMPs trigger a potent innate immune response, causing immune activation through the engagement of several Toll-like receptors (TLRs), and may also activate B cells (9).

Antiretroviral therapy (ART) has greatly modified the natural course of HIV-1 infection, resulting in decreased HIV-1 load, increased CD4 T cells, and decreased HIV-1-associated opportunistic infections, thus indicating restoration of immune function (10). While the incidence of HIV-1 related malignancies, such as Kaposi sarcoma (KS), has declined markedly as access to ART has expanded throughout Europe and North America, the incidence of NHL still remains elevated (11). The trend of incidence of NHL in HIV-1 infected African patients has not been estimated (12).

In Africa, EBV primary infection occurs during infancy and early childhood, and EBV-associated lymphomas represent an important cause of morbidity and mortality in children. NHL commonly occur in African children, with endemic Burkitt’s Lymphoma (BL) being the most common subtype (13). Endemic BL is strongly associated with EBV and accounts for up to 75% of all childhood malignancies (14). BL, in the central part of Africa, counts an annual incidence of 5

to 10 cases per 100 000, and a peak incidence at 6/7 years of age (15). Malaria may represent an additional risk factor for BL (16,17); malaria induce B cell activation, thus leading to the stimulation of EBV-latent infected B cells (18).

High levels of EBV are a risk factor for the onset of EBV-related malignancies. To date, no data are available about EBV-infection and related malignancies in the context of HIV-1 infection in African children. The absence of these data may be partly due to the lack of access of laboratory analyses in developing countries. One possible strategy to improve diagnosis is to simplify the collaboration between institutions in developed and developing countries, and/or between small laboratories and central facilities of the same country. Dried Blood Spot (DBS) may represent an easy method to collect and store blood samples and allow their reliable transport to specialized laboratories.

2. Material and Methods

2.1 Patients and sample collection

This is a cross-sectional study, conducted in the Home-Based Care department of the St. Raphael of St. Francis Hospital at Nsambya in Kampala, Uganda. Blood samples from 213 HIV-1-infected children (0-18 years) were collected. At the time of sampling for this study, 140 (66%) children were on ART and 73 (34%) were under no therapy (ART-naive).

Blood from each child was collected in EDTA-containing tubes by trained nurses. 50 µl of this blood-EDTA were spotted onto each circle of Protein Saver TM 903 Card (Whatman GmbH, Hahnestra, Germany) and 5 blood spots were prepared for each card with one blood sample. DBS were dried at room temperature overnight, stored in individual ziplock bags containing a desiccant, and sent to the laboratory of the Department of Surgery, Oncology and Gastroenterology, Section of Oncology and Immunology, University of Padova, Viral Oncology Unit and AIDS Reference Centre, Italy. This study was approved by the Ethical Committee of the Uganda.

2.2 DNA elution

From each 50 µl DBS, three 3 mm-diameter circles, equivalent to 5 µl of whole blood each (15 µl total), were used to extract DNA with the Qiagen DNA MicroKit (Qiagen, Hilden, Germany) and were resuspended in a final volume of 50 µl. To control the ability of eluted DNA to be amplified, 5 µl of final elution were amplified for the human telomerase reverse transcriptase (*hTERT*) located in the 5p15.33 (Gen Bank accession: AF128893), and employed as housekeeping gene. Amplification was carried out as previously described (19).

2.3 EBV-DNA quantification.

A quantitative method, based on Multiplex Real-Time PCR assay, was performed to quantify EBV type 1 and EBV type 2, exactly as described (20). A standard reference curve was obtained by five-fold serial dilution of two amplicons, one for EBV type 1 and the other for EBV type 2, and amplification was performed, as already detailed (20). The multiplex assay showed a dynamic range from 2 to $2x10^5$ copies. The results were expressed as EBV-DNA copies/ml.

2.4 16S rDNA quantification

A quantitative method based on a Real-Time PCR assay was performed to quantify 16S rDNA, using primer pair and probe as described (21). A standard curve was generated from five-fold serial dilution of plasmid DNA containing known copy numbers of the template. The assay showed a dynamic range from 3 to $2.5x10^5$ copies. Results were expressed as 16S rDNA copies/µl.

2.5 Definition of clinical malaria

Clinical malaria was defined by clinicians as a state in which a child presents symptoms that are suggestive of malaria. The symptoms range from mild headaches, high or mild fever, fatigue and joint pains in mild cases to malaise, marked weakness, loss of appetite, chills and rigors, nausea, vomiting, diarrhoea, convulsions, lethargy or drowsiness, and dark urine from hemolysis.

2.6 Statistical analyses

Firstly, missing data and small subject numbers in sub-groups were identified, the distribution for all variables was established and EBV data were $\log_{10}$ transformed. EBV outcomes were cross-tabulated against all interesting variables to strengthen the understanding of their potential for confounding or interaction. Odds ratios (OR) were calculated for each with 95% confidence intervals (CI) and chi-square p-values.

Logistical regression analysis was undertaken to allow the adjustment of multiple confounders. Considering a model containing main exposure, outcome of interest and all potential and *apriori* confounders, an iterative Likelihood Ratio Tests (LRTs) was conducted using alternative models which removed these potential confounders according to perceived 'strength' or importance. The fit of the model was assessed by the magnitude of the observed change in OR supported by the outcome of LRTs.

Analyses were also performed looking at the mean difference in the quantitative levels of EBV; univariate analysis was undertaken using t-tests to compare the $\log_{10}$ mean viral loads.

3. Results

3.1 EBV-DNA levels in HIV-1 infected children

The baseline characteristics at time of sample collection of the entire cohort of HIV-1 infected children enrolled were described in Table 1. Of the total 213 HIV-1-infected children, 149 (70%) children were found to have EBV detectable, 92 out of 140 (66%) on ART and 57 out of 73 (78%) ART-naive. EBV type 1 and EBV type 2 were detected in 31 (22%) and 21 (15%) children on ART, and in 15 (21%) and 11 (15%) ART-naive children, respectively. Co-infection with both EBV types was observed in 40 (29%) children on ART and 31 (42%) ART-naive children (Figure 1).

Univariate analysis identified differences in EBV-DNA detection between ART and ART-naive children (OR= 0.41 [95% CI: 0.45-1.05] p= 0.0793). This tendency was supported by a multivariate model. Indeed, after adjusting for CD4-Z score, age and WHO stage, children on ART presented

less odds of EBV-DNA detection in peripheral blood than ART-naïve children (OR= 0.39 [95% CI 0.16-0.94], p= 0.036) (Table 2).

Mean [interquartile-IQR] levels of EBV-DNA were similar in children infected with EBV type 1 (3.80 [3.45-3.88] $\log_{10}$ copies/ml) or EBV type 2 (3.92 [3.52-4.29] $\log_{10}$ copies/ml), but higher in children co-infected with both viruses (4.34 [3.95-4.61] $\log_{10}$ copies/ml; overall, p= 0.001) (Figure 2A). Overall, EBV load was significantly higher in children ART-naive than in those on ART (4.22 [3.87-4.58] vs 3.99 [3.55-4.33] $\log_{10}$ copies/ml; p= 0.016) (Figure 2B).

3.2 EBV-DNA levels in relation to markers of immune activation

Levels of 16S rDNA were significantly higher in children ART-naive compared to those on ART (2.16 [2.11-2.28] vs 2.09 [2.02-2.22] $\log_{10}$ copies/µl; p=0.007) (Figure 3A). Furthermore, EBV-DNA levels significantly correlated with levels of 16S rDNA in ART-naive children (r_p= 0.382; p= 0.016) (Figure 3B).

B cell activation may also be driven by malaria (Rochford et al 2005); thus, EBV-DNA load was evaluated in children with or without clinical malaria. Percentages of infection with EBV type 1, type 2 or co-infection reached 30%, 4%, and 44% respectively, in children with clinical malaria; 28%, 17%, and 29% respectively, in children without clinical malaria. Levels of EBV-DNA tended to be higher in children with clinical malaria (3.82 [3.45-4.33] vs 3.37 [2.00-4.17] $\log_{10}$ copies/ml; p= 0.093) (Figure 4).

Discussion

DBS filter papers are already commonly used for the diagnosis and monitoring of different DNA/RNA viral loads (22). However, to date very few studies (23) investigate the quantification of EBV-DNA levels from DBS. Previously, EBV antibodies (IgA and IgG) were tested successfully in eluates from DBS cards to define EBV serostatus in neuroimmunology studies (24) and in

nasopharyngeal carcinoma screening (25). While serological tests confirm primary infection and document remote infection, quantitative EBV-DNA assessments are increasingly emerging as an important laboratory tool in supporting the diagnosis and monitoring of EBV-associated diseases (19). In some regions of Africa, such as Uganda, EBV seroprevalence is nearly universal, with acquisition in early childhood, and there is a striking association of EBV with BL (26). Therefore, EBV viral load assessment in peripheral blood compartments may represent an important instrument in clinical practice for the prevention, diagnosis and monitoring of EBV-associated lymphoproliferative diseases, even more in African children whereas EBV-associated lymphomas are an important cause of mortality and morbidity. DBS sampling allows us to extend EBV load assays not only to cases from the developing world, but it also gives us the opportunity to collect specimens from young children in whom venipuncture is difficult to perform. Our results show that it is possible to perform EBV-DNA and, for the first time, 16S rDNA load assessment on DBS sampling, thereby improving the diagnostic and monitoring capabilities in resource-limited settings.

Chronic immune activation, a hallmark of HIV-1 infection, may contribute to the expansion of EBV-infected B cells. One of the mechanisms by which HIV-1 activates B cells probably involves PAMPs. They are released into circulation after HIV-1-induced cellular/tissue damage, they engage TLRs and induce a potent immune response, involving B cells. In this study we found that EBV-DNA and 16S rDNA levels were significantly lower in children on ART than in those ART-naive, suggesting that treatment with ART lowered microbial translocation. Furthermore, malaria may induce B cell activation, leading to the stimulation of EBV-infected B cells via TLR-9 engagement (18). In this study, we found that 16S rDNA levels significantly correlate with levels of EBV-DNA in ART-naive children, and EBV-DNA levels tend to be higher in children with clinical malaria. These findings indicate that chronic immune activation induced by HIV-1, circulating microbial products and co-infections, such as malaria, may lead to the expansion of EBV-infected B cells, thus increasing EBV load. Findings that children on ART had lower EBV-DNA levels than

ART-naive children, suggest that treatment with ART may prevent B cell stimulation and expansion of EBV-infected B cells.

Conflict of interest

None.

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Table 1: Baseline descriptive characteristics of HIV-1 infected children

Characteristic		**n° children (%)**
On ART (n=213)	No	73 (34)
	Yes	140 (66)
WHO Stage (n=209)	I	60 (29)
	II	120 (57)
	>= III	29 (14)
WHO Stage of children on ART (n=140)	I	23 (16)
	II	97 (70)
	>= III	20 (14)
ART Type (n=140)	NVP Based	97 (69)
	EFV Based	34 (24)
	LPV Based	8 (6)
	3-NRTI	1 (1)
CD4 Category (n=177)	<350	18 (10)
	350-750	68 (38)
	750-1000	41 (23)
	>1000	50 (29)

All characteristics refer to the time of sample collection

Table 2. Multivariate models and adjusted odds ratios of EBV detection among HIV-1 infected children on ART vs ART-naive

Outcome	**Exposure**	**Covariates**	**Adjusted OR**	**95% CI**	**p-value**
EBV Total	ART	CD4 Z-Score; Age; WHO Stage	0.39	0.16-0.94	0.036
EBV1	ART	CD4 Z-Score; Age; WHO Stage	0.56	0.28-1.14	0.111
EBV2	ART	CD4 Z-Score; Age; WHO Stage	0.51	0.24-1.11	0.090
EBV co-infection	ART	CD4 Z-Score; Age; WHO Stage	0.45	0.20-1.00	0.050

Legend to figure

Figure 1. EBV infection in HIV-1 infected children. Percentage of detectable EBV-infection in all HIV-1 infected children and in children sub-grouped according to therapy: on ART or ART-naive.

Figure 2. EBV-DNA load in HIV-1 infected children. EBV-DNA levels in HIV-1-infected children A) sub-grouped according to infection with EBV type 1, EBV type 2 or co-infection with both EBV types; B) sub-grouped according to therapy: on ART or ART-naive. Each plot represents a child. The lines indicate the mean values.

Figure 3. 16S rDNA levels in HIV-1-infected children. 16S rDNA levels in HIV-1-infected children A) sub-grouped according to therapy: on ART or ART-naive; B) correlation with EBV-DNA levels in ART-naive children. Each plot represents a child.

Figure 4. EBV-DNA load and clinical malaria. EBV-DNA levels in HIV-1-infected children with or without clinical malaria. Each plot represents a child. The lines indicate the mean values. White dots represent patients on ART, black dots represent ART-naive patients.

Figure 1

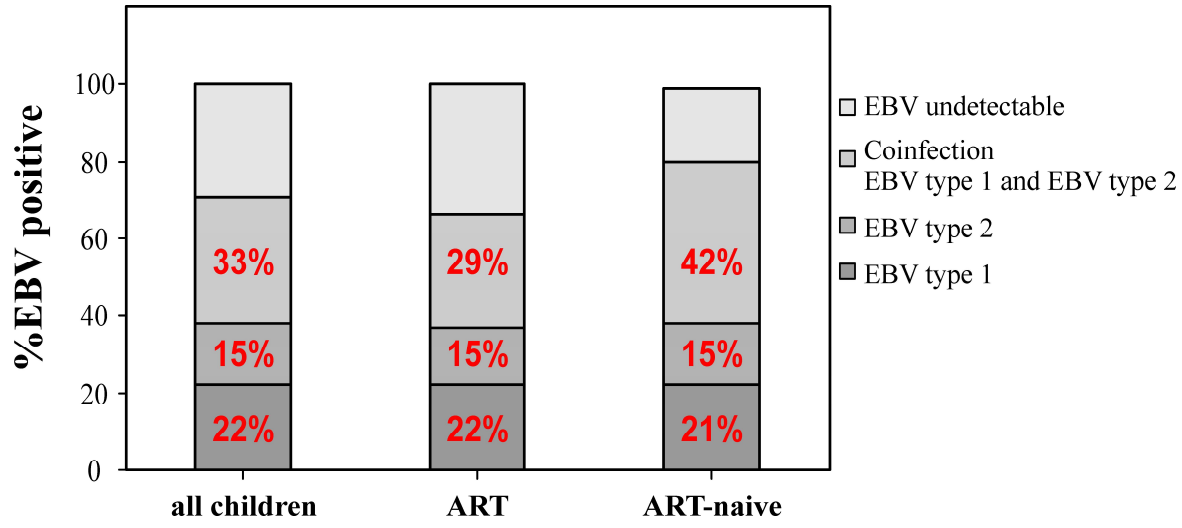

%EBV positive
100
80
60
40
20
0
33%
15%
22%
29%
15%
22%
42%
15%
21%
all children
ART
ART-naive
EBV undetectable
Coinfection
EBV type 1 and EBV type 2
EBV type 2
EBV type 1

Figure 2

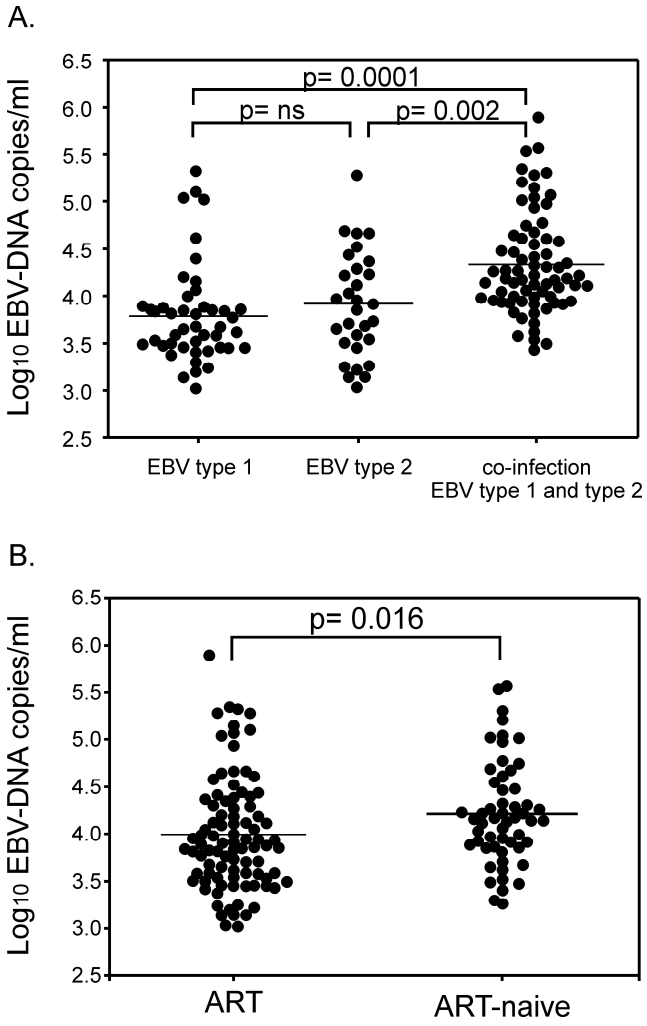

A.
Log10 EBV-DNA copies/ml
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
p= 0.0001
p= ns
p= 0.002
EBV type 1
EBV type 2
co-infection
EBV type 1 and type 2
B.
Log10 EBV-DNA copies/ml
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
p= 0.016
ART
ART-naive

Figure 3

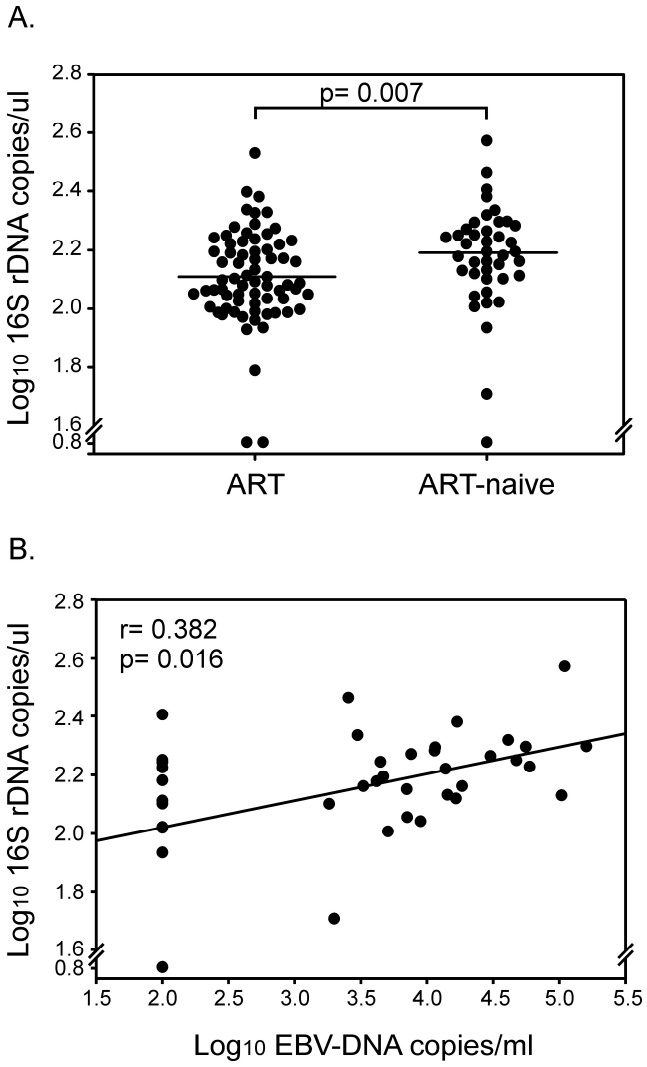
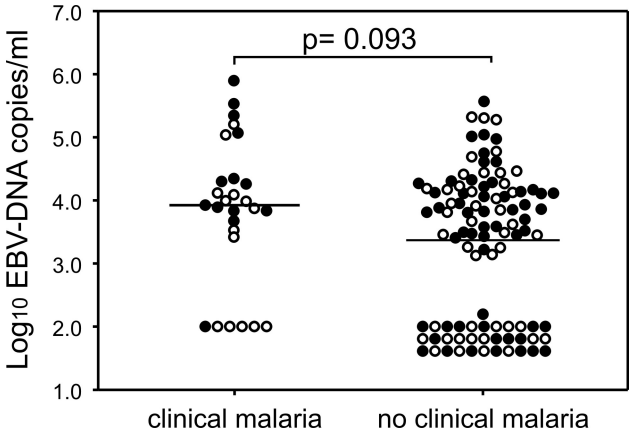

A.
p= 0.007
Log10 16S rDNA copies/ul
2.8
2.6
2.4
2.2
2.0
1.8
1.6
0.8
ART
ART-naive
B.
r= 0.382
p= 0.016
Log10 16S rDNA copies/ul
Log10 EBV-DNA copies/ml
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
5.5

Figure 4


p= 0.093
Log10 EBV-DNA copies/ml
7.0
6.0
5.0
4.0
3.0
2.0
1.0
clinical malaria
no clinical malaria

CHAPTER 5

Dried Blood Spot (DBS) Sampling for Detection of Monoclonal IGH Gene Rearrangement and EBV Load: a Tool for Improving the Diagnosis of Lymphoproliferative Diseases in Resource-Limited Settings

Dried Blood Spot (DBS) Sampling for Detection of Monoclonal IGH Gene Rearrangement and EBV Load: a Tool for Improving the Diagnosis of Lymphoproliferative Diseases in Resource-Limited Settings.

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Running head: DBS for clonality of lymphocytes and EBV load.

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Abstract

In many lymphoproliferative disorders complementary molecular methods are required in order to provide a conclusive diagnosis. The reliability of lymphoma diagnosis is strikingly different between developed and developing countries, partly due to lack of access to advanced molecular analyses. To overcome these hurdles, a new application of dried blood spots (DBS) is proposed for detecting clonal B-cell populations in peripheral blood (PB), and for the quantification of Epstein Barr Virus (EBV)-DNA levels.

We first ensured that DBS contain sufficient lymphocytes to perform a clonality assay without yielding false positive results. Using Namalwa cells, we found a good relationship between the expected and detected EBV-DNA copies ($r=0.987$, $p< 0.0001$) and we established that a clonal B-cell population on DBS was detected when there were at least 200 clonal cells in the analysed sample. EBV-DNA was detected even in the sample corresponding to 10 Namalwa cells. Moreover, very similar clonal results were obtained between DNA from DBS and fresh whole blood from patients with B-cell chronic lymphocytic leukemia (B-CLL).

This is the first study demonstrating that it is possible to perform clonality testing and EBV load assessment on DBS sampling, thus improving the diagnostic and monitoring options for lymphoproliferative diseases in resource-limited settings.

Keywords: Dried blood; clonality; IGH rearrangement; EBV; lymphoproliferative disease

Introduction

In many lymphoproliferative disorders, despite well-established histomorphological and immunophenotypical criteria, complementary molecular methods are required to provide a conclusive diagnosis. Unfortunately, striking differences still exist in the reliability of lymphoma diagnosis between developed and developing countries, partly due to lack of access to these advanced molecular analyses. This is especially true in many African countries where lymphoma diagnosis is based on morphology alone and does not include most of the entities recognized in recent years. One possible strategy to improve diagnoses is to simplify the collaboration between institutions in developed and developing countries, and/or between small laboratories and central facilities of the same country. According to published data, in about 10% of patients with suspected lymphoproliferative disorders, PCR-based clonality testing can be useful in discriminating between a reactive lymphoproliferation with polyclonally rearranged immunoglobulin or T-cell receptor genes, and a malignant proliferation with clonal rearrangements.[1-3] Although lymph nodes and bone marrow (BM) are the tissues of choice for the detection of monoclonal population in leukaemias and lymphomas, peripheral blood (PB) has been shown to be a reliable and safe tissue for detecting malignant clones.[4] The demonstrated concordance of clonality testing between BM and PB samples supports the use of PB samples for a non-invasive detection and monitoring of clonality in the management of patients with lymphoproliferative disorders.

Clonality analysis in PB requires fresh whole blood and reliable transport to laboratories with specialized equipment. As such, an easy blood collection and conservation method could improve diagnosis in most cases. One option relies on the use of filter paper for blood collection; its analysis was implemented as early as the 1960s by Guthrie et al. using dried blood spot (DBS) for newborn phenylketonuria detection.[5] DBS on “Guthrie card” filter paper is widely used in many types of tests, including chemical, serological, and genetic applications.[6-8] These filter papers are made from high-purity cotton linters, manufactured to give accurate and reproducible absorption of blood specimens according to Clinical and Laboratory Standards Institute (CLSI) specifications.[9]

The simple puncturing of the skin for blood collection onto paper has become an important tool in screening individuals for clinical purposes and epidemiological studies. DBS ensures easy sample handling, transport, and storage, especially for samples collected at remote sites where the laboratory equipment, personnel, or infrastructures necessary for the correct handling of blood samples may not be available. Viral RNA and DNA, antibodies or other proteins and antiviral drugs or their metabolites remain stable in DBS cards for relatively long periods when stored with desiccant in closed bags at ambient temperature, as compared with wet plasma or serum stored under the same conditions.[8, 10]

Here we present a new application of DBS for the detection of clonal B-cell populations in PB for the diagnosis and monitoring of B-cell malignancies. DBS sampling still faces technical limits that need attention, especially for diagnostic analyses. The small quantity of spotted blood (50 µl) might have been a major limitation for the use of the DBS in the detection of clonal lymphocytic populations. We hypothesized that by using serial dilutions of a clonal B-cell line in fresh blood, it might be possible to define a sensitivity cut-off for the clonality assay, i.e. the lowest number of malignant cells reliably detectable as a cellular clone.

We also used DBS to detect Epstein-Barr Virus (EBV) viral load. EBV infects B lymphocytes, has a potent transforming ability, and has been implicated in the development of a wide range of B-cell malignancies, including Burkitt's lymphoma (BL), Hodgkin disease (HD), and lymphomas arising in immunocompromised individuals, i.e. post-transplant lymphoproliferative diseases (PTLD) and Human Immunodeficiency Virus (HIV)-associated lymphoproliferative disorders.[11, 12] In Africa, primary infection with EBV occurs during infancy and early childhood; EBV-associated lymphomas are an important cause of mortality and morbidity in children, and BL in African children account for up to 75% of all childhood malignancies.[13]

Our results demonstrated that the use of DBS sampling is a feasible tool for the molecular analysis of PB in leukaemia/lymphoma or virus-infected (i.e. EBV) patients. Its application could

therefore be extended to the diagnosis and monitoring of lymphoproliferative disorders, particularly in resource-limited settings.

Materials and Methods

Blood, cell line and DBS samples.

Blood was taken from five consenting healthy volunteer donors at the Instituto Oncologico Veneto, IOV-IRCCS, of Padova, Italy. Six B-cell chronic lymphocytic leukemia (B-CLL) samples were referred to the diagnostic laboratory at the IOV-IRCCS for routine clonality testing to complete the pathologist's diagnosis of B-CLL. All venous blood samples were collected in EDTA vacutainer tubes and parallel samples were prepared: 50 μl of blood were spotted on Protein Saver TM 903 Card (Whatman GmbH, Hahnestra, Germany) dried overnight at room temperature, and 500 μl of whole blood were transferred to Eppendorf tube for DNA extraction.

The Namalwa cell line, an EBV-positive BL cell line that is known to carry two integrated EBV type 1 genomes per cell,[14] was established at the initial concentration of 10^6 cells/ml and diluted into whole blood from healthy donors to obtain 200-150-100-50-40-20-2-0 Namalwa cells per μl of blood. Fifty μl of each dilution were spotted on filter paper card. All DBS samples were prepared in duplicate. A pellet with only Namalwa cells was used as a positive control of monoclonality.

DNA extraction

Genomic DNA was isolated using the automated MagNA Pure Compact instrument (Roche Applied Science, Indianapolis, IN). Briefly, 500 μl of fresh blood were processed according to the manufacturer's protocol included in the MagNA Pure Compact Nucleic Acid Isolation kit I-Large Volume (Roche Applied Science), with the elution volume set at 200 μl. For DNA extraction from DBS cards, one spot with 50 μl of dried blood was added to a 1.5-ml Eppendorf tube, and manually cut into pieces, avoiding cross-contamination. External lysis step was performed in 180 μl of

MagNA Pure DNA Tissue Lysis Buffer (Roche Applied Science) and 20 μl of proteinase K solutions, at 56°C overnight. After inactivation of proteinase K at 90°C for 10 minutes, and adjustment of final volume to 500 μl with additional phosphate buffered saline (PBS), the DNA was extracted using the automated MagNA Pure Compact Nucleic Acid Isolation kit (Roche Applied Science) protocol with the elution volume set at 50 μl, which is the minimum elution volume permitted by the protocol. The concentration of DNA extracted was determined using a spectrophotometer (NanoDrop ND-1000, Wilmington, Delaware, USA). We recovered approximately 11-24 ng/μl of DNA from DBS cards.

Clonality assay

The extracted DNA samples were tested for B-cell immunoglobulin heavy chain (IGH) clonality using the IGH Gene Clonality Assay (Invivoscribe Technologies, San Diego, CA), containing BIOMED-2 primers, in accordance with the manufacture's protocol. As with specimens with limited DNA the recommended multiplex PCRs for suspected B-cell proliferations are the three framework subregion (FR) of the IGH gene, preferably followed by the immunoglobulin kappa (IGK) targets,[15] we used the IGH FR1, FR2 and FR3 Master Mix, as well as the Specimen Control Size Ladder Master Mix for template amplification control. Each PCR reaction was prepared using the maximum volume of DNA provided in the protocol (5 μl) for DBS cards or 50 ng of DNA extracted directly from fresh blood. These post-PCR products were visualized by means of capillary electrophoresis on the ABI PRISM 3730XL Genetic Analyzer with subsequent software analysis using ABI GeneMapper 4.0 analysis software (Applied Biosystems). All samples were tested twice. A sample was considered clonal only if one or two reproducible peaks were detected within the valid range. Known nonspecific peaks were excluded to avoid false positives.[1]

EBV-DNA quantification

EBV-DNA levels were quantified by qPCR, exactly as previously described, using the standard curve and the probe for the quantification of EBV type 1.[16] The assay had a dynamic range from 5 to $2x10^5$ EBV-DNA copies.[16]

Results

Evaluation of polyclonal B cells on DBS.

One of the limitations that can occur using DBS for laboratory diagnosis is the small quantity of sample, especially in analysing different cellular populations present in the blood. Since a single cell DNA can be amplify generating a single (clonal) amplification product, if there are very few normal lymphocytes in the test sample and a PCR-protocol for rearranged immunogenic gene is used to detect the presence of polyclonal cell populations, a false positive result may occur. To rule out this problem, despite the fact that the BIOMED-2 protocol establishes the use of at least 100 ng of DNA in each PCR reaction, we first verified if 50 ng of DNA from normal whole blood (corresponding to about 7500 cells) were sufficient to detect a normal polyclonal pattern corresponding to B-lymphocytes carrying the different immunoglobulin gene rearrangements. DNA was extracted from 500 µl of whole blood taken from five donors and 50 ng of each sample were analysed for FR1-JH, FR2-JH, FR3-JH rearrangements of the IGH gene; a polyclonal pattern was observed in all cases. From these same donors, 50 µl of blood were spotted on DBS filter and 5 µl (about 50 ng) of the 50 µl DNA extracted were analysed; polyclonal results were concordant with those obtained with DNA from fresh blood (Figure 1). Thus, 5 µl of spotted blood contain sufficient lymphocytes to perform an IGH clonality assay without false positive results.

Lower Limit for Detecting Monoclonal IgH Gene Rearrangement on DBS.

We assessed the lower limit of clonal detection in the PCR-based analysis of DBS samples using Namalwa B cells, diluted in whole blood of a healthy donor; 50 µl of each dilution were spotted on

DBS filters in duplicate. DNA extracted from each spot was resuspended in 50 µl of water, and 5 µl (corresponding to 5 µl of blood) were employed in each reaction for FR1-JH, FR2-JH, FR3-JH rearrangements, applying the BIOMED-2 protocol. DNA from 100% Namalwa cells (N) revealed a monoclonal IGH gene rearrangement within all three framework regions. With DNA from Namalwa diluted in normal blood, the highest sensitive clonal signal was obtained with primers amplifying sequences between the FR2-JH and FR3-JH regions. As reported in Figure 2, a positive clonal signal could be detected in DBS samples containing 40 Namalwa cells per µl of blood. DBS samples with 20 or 2 Namalwa cells/µl revealed only a polyclonal pattern, similar to DBS spotted only with normal PB. Thus, clonal B-cell population on DBS can be detected by PCR-based methods when represented by at least 200 clonal cells in the sample.

Evaluation of EBV-DNA on DBS

Considering that Namalwa cells contain two integrated EBV genomes/cell,[14] the number of Namalwa B clonal cells contained in samples diluted from DBS and analysed for clonality, was also verified by testing them for EBV-DNA. The number of EBV copies was estimated by qPCR and the values were compared to those expected. A very good relationship was found between the detected and expected EBV-DNA copy numbers (Figure 3). Of note EBV-DNA was also found in the DNA sample with 10 Namalwa cells (2 Namalwa cells/µl of whole blood, corresponding to 20 EBV/5µl), indicating that viral DNA was quantified in DBS samples using qPCR methods with a higher sensitivity than analysis of clonality, likely because of the absence of background.

Clonality analysis on DBS from B-CLL.

Lastly, we proved the reliability of our approach for clonality detection on DBS from patients with B-CLL. Equal amounts of DNA either extracted from DBS or from fresh blood samples taken from 6 B-CLL patients were analysed using the same PCR-based approach. As reported in Figure 4, very

similar profiles, with similar migration distances and heights of clonal peaks, were present in both DBS and fresh blood samples from each patient.

Discussion

The use of DBS cards has been seen as a feasible tool for the diagnosis and monitoring of different diseases in the developing world. Initially they were used for metabolic disease screening in neonates,[5] but with the development of new techniques, the disease range and applications of DBS have expanded significantly. Today, DBS cards are used to measure and analyse nucleic acids, proteins and small molecules overcoming the limitations of small sample sizes.[6, 7] In this regard, we assessed if DBS sampling was also suitable to identify the presence of a B-cell clonal population circulating in the blood by immunoglobulin gene rearrangement analysis, with a view to offering a molecular valuation in the diagnosis and monitoring of lymphoproliferative diseases in resource-limited settings. Difficulties in making a final diagnosis of lymphoid malignancies arise in a proportion of cases (5-10%), even with extensive histomorphology or immunophenotyping, and additional diagnostics, such as molecular clonality, are needed to generate or confirm the final diagnosis.

The first issue to consider is whether or not the DBS contains a sufficient number of B lymphocytes to detect normal polyclonal B-cell populations by PCR-analysis. This is an important aspect to consider in order to avoid possible false positive results (pseudoclonality) due to the amplification of the few immunoglobulin gene rearrangements derived from a limited number of B-cells in the sample analysed. We applied the BIOMED-2 protocol for the analysis of B-cell clonality using PCR for three FR of the IGH. Thus, the DNA extracted from DBS had to suffice to perform at least the three PCR reactions specific for FR-IGH regions and the PCR-control for sample amplificability. Although the BIOMED-2 guidelines for the clonality assay suggest using at least 100 ng DNA, our experiments showed that about 50 ng of the DNA extracted corresponding to the DNA yielded from 5 µl of the spotted blood (corresponding to approximately 750-3600 B cells)

appeared to suffice to obtain the expected polyclonal pattern in each specific PCR reaction, as well as when normal fresh blood is analysed. Notably, while a previous study reported that 20 ng of DNA from normal tonsil, corresponding to 6000-9500 B cells, is required to detect a complete polyclonal pattern,[17] we have shown that by using the multiplex PCR method and fluorescent analysis, less than half this number of B-cells is sufficient.

The second issue to evaluate is the sensitivity of the DBS approach in detecting the presence of a clonal cell population circulating in the blood. Since limited sensitivity may hamper the detection of small clonal cell populations, we determined how many B clonal cells (Namalwa) must be present in the blood for detection using DNA corresponding to 5 μl of spotted blood. We demonstrated that it is possible to identify the monoclonal pattern even in the sample with 200 clonal Namalwa cells. Since a normal adult lymphocyte count is usually between 1000 and 4800 lymphocytes/μl of blood, of which approximately 15% are B cells (about 400 B polyclonal lymphocytes/μl of blood),[18] the detection of 40 clonal B-cells per μl of blood corresponds to a sensitivity of 10% using BIOMED-2 PCR protocol with DBS cards. Since the detection limit depends not only on the technique applied, but also on the relative size of the 'background' of normal (polyclonal) B-lymphocytes, it comes as no surprise that sensitivity is lower using DBS cards than fresh blood samples, in which the BIOMED-2 assay can reliably detect even 1-5 positive cell per 100 normal lymphocytes (1-5% of sensitivity).[1] Moreover, taking advantage of the presence of two EBV copies per Namalwa cell,[14] we investigated the size of the clonal Namalwa cells present in the DBS dilutions by means of qPCR for EBV-DNA. We found a good correlation between the expected and detected EBV copies and thus cell numbers. In addition, we detected EBV-DNA even in the sample containing 10 Namalwa cells (2 cells per μl of spotted blood, corresponding to 20 EBV copies in 5 μl). While DBS cards are already commonly used for the diagnosis and monitoring of different DNA/RNA viral loads,[8] no studies are available regarding EBV-DNA levels. Previously, EBV IgG antibodies were tested successfully in eluates of DBS cards to define EBV serostatus in neuroimmunology studies[19] and in nasopharyngeal carcinoma

screening.[20] While serological tests confirm primary infection and document remote infection, quantitative EBV-DNA assessments are increasingly emerging as an important laboratory tool in supporting the diagnosis and management of EBV-associated disease.[21, 22] Measuring EBV-DNA levels is an important tool for identifying patients at risk of PTLD and is a parameter for monitoring immunosuppressive therapy and initiating pre-emptive or antitumor therapy.[23, 24] In some regions of Africa, such as Uganda, EBV seroprevalence is nearly universal, with acquisition in early childhood, and there is a striking association of EBV with BL.[25] EBV viral load assessment is generally used in PB compartments in clinical practice for the prevention, diagnosis and monitoring of EBV-associated lymphoproliferative diseases, even more in African children whereas EBV-associated lymphomas are an important cause of mortality and morbidity. DBS sampling allows us to extend EBV load assays not only to cases from the developing world, but also for giving the opportunity of collecting specimens from young children in whom venipuncture is difficult to perform.

Finally, we compared the use of DBS cards with fresh blood samples to perform clonality assay in B-CLL. In all samples analysed, we found the same clonal profile using DNA from both DBS cards and fresh blood. A clinical diagnosis of B-CLL is defined by absolute lymphocytosis of at least 5000/μl cells with an appropriate immunophenotype,[26] thus DBS sampling can be a suitable collection system for identifying the clonal population circulating in the blood. A diagnosis of leukaemia is not usually problematic: immnophenotyping and cytogenetic analysis and the use of histochemical stains can establish the diagnosis in virtually all leukaemia cases. However, in some patients diagnosis is more complicated, or molecular analyses complementary to the histomorphological evaluation are not always available, hence a clonality assay using DBS cards might prove to be a valuable tool. Our results show that it is possible to perform clonality testing and EBV load assessment on DBS sampling, thereby improving the diagnostic and monitoring capabilities of lymphoproliferative diseases in resource-limited settings.

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Figure legends

Figure 1. IGH gene polyclonality analysis. (A) IGH FR1-JH, (B) FR2-JH and (C) FR3-JH multiplex PCR GeneScan analysis using the polyclonal control (upper panels) and 50 ng of DNA extracted from DBS (middle panels) and fresh blood (lower panels) samples from one representative healthy donor, showing the typical polyclonal Gaussian patterns.

Figure 2. Minimal detection of B-cell clonality. Namalwa B-cells (N) were serially diluted in normal blood (panels with 200, 150, 100, 50, 40, 20, 2 N/μl of blood). DBS cards were prepared using 50 μl of each dilution, and 5 μl of the 50 μl DNA extracted were analysed by multiplex PCR for IGH gene rearrangements. From a representative case, GeneScanning revealed that a distinct monoclonal peak with the expected position of 100% N, could still be detected at a dilution of 40N/μl by PCR with master mix specific for (A) FR2-JH and (B) FR3-JH rearrangements.

Figure 3. EBV-DNA levels in DBS. Namalwa B-cells, containing two integrated EBV genomes/cell, were diluted in normal PB and spotted onto DBS. EBV-DNA was quantified by qPCR using DNA extracted from DBS. Relationship between detected and expected EBV-DNA copies is shown. Each plot represents the mean value of three replicates, and the bars show standard deviation.

Figure 4. IGH gene clonality analysis in B-CLL cases. Three representative B-CLL cases (B-CLL#1, B-CLL#2 and B-CLL#3) were analysed using fresh whole blood in parallel with DBS cards with 50 μl of blotted blood. In all cases, GeneScan analysis of multiplex PCR for FR1-JH rearrangements revealed a monoclonal peak using DNA from fresh blood as well as from DBS cards.

Figure 1

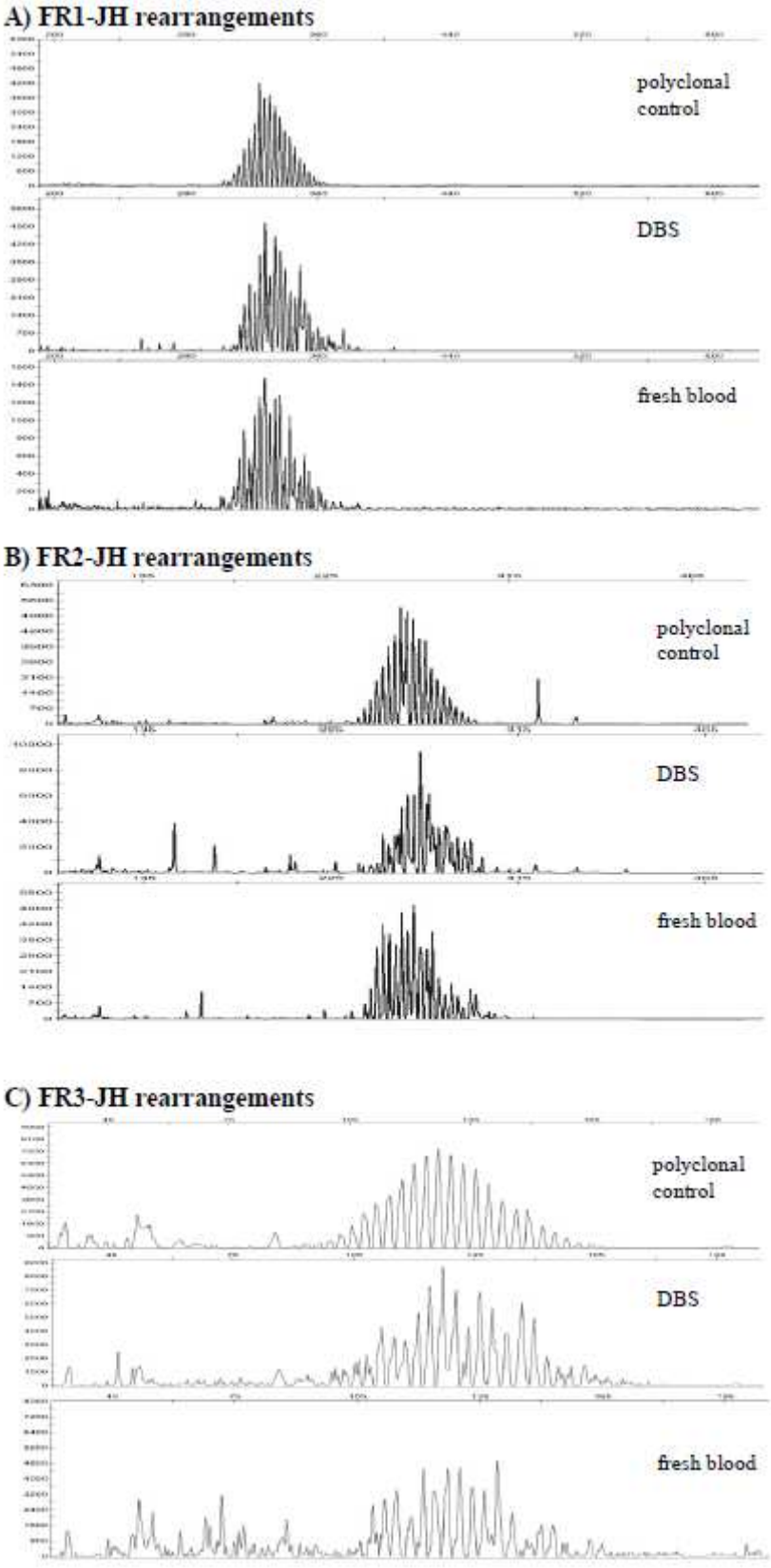

A) FR1-JH rearrangements
polyclonal control
DBS
fresh blood
B) FR2-JH rearrangements
polyclonal control
DBS
fresh blood
C) FR3-JH rearrangements
polyclonal control
DBS
fresh blood

Figure 2

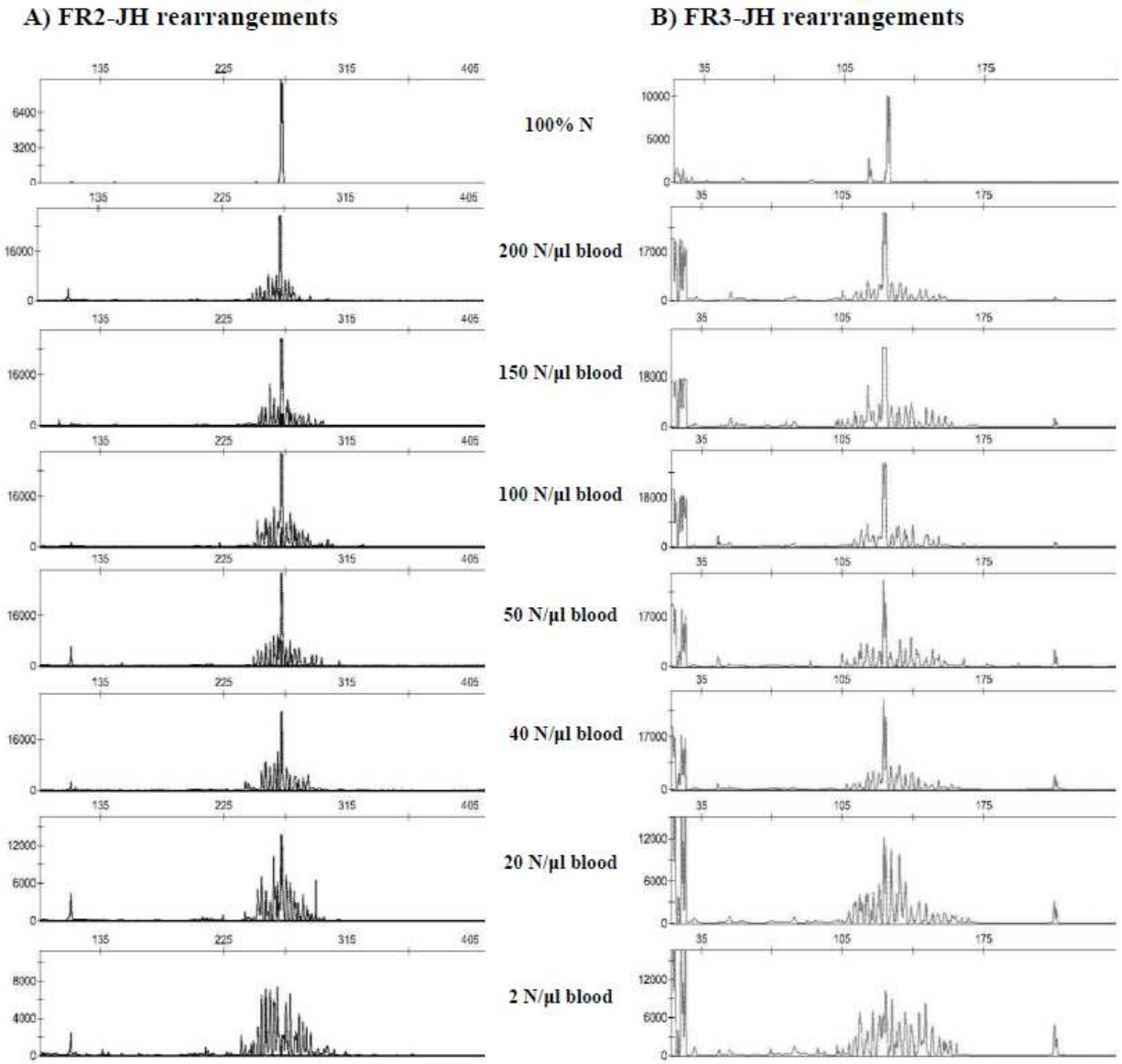

A) FR2-JH rearrangements
B) FR3-JH rearrangements
100% N
200 N/µl blood
150 N/µl blood
100 N/µl blood
50 N/µl blood
40 N/µl blood
20 N/µl blood
2 N/µl blood

Figure 3

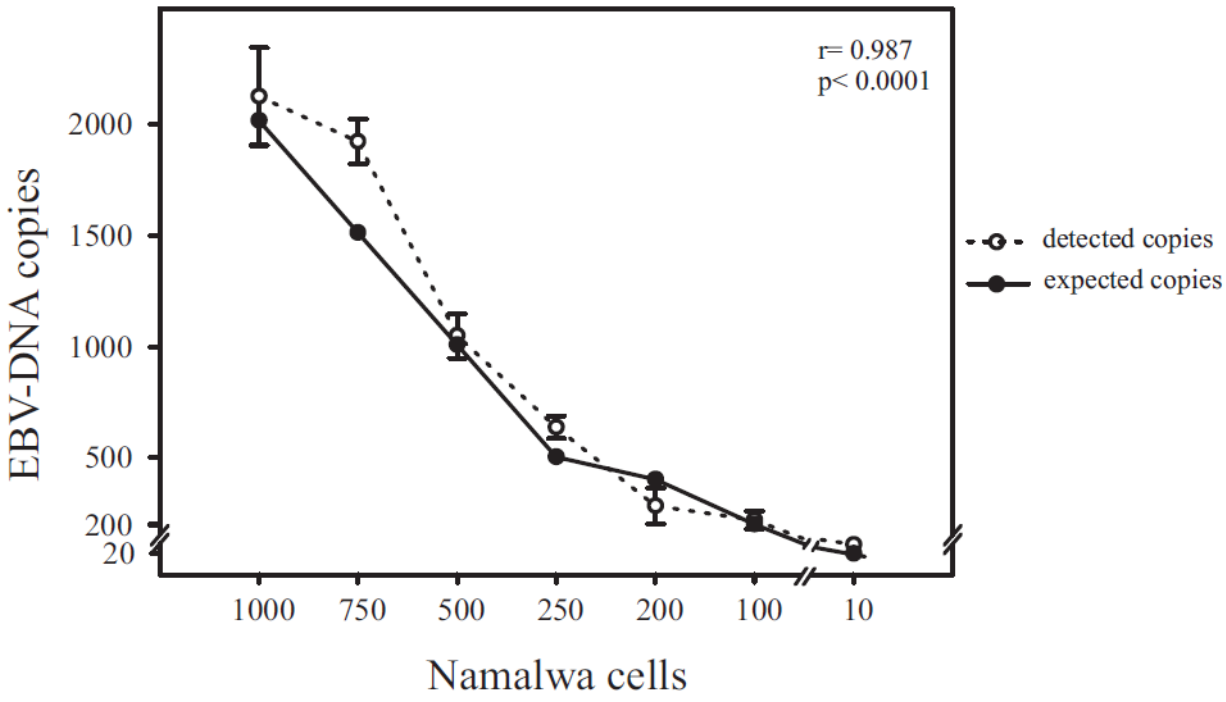
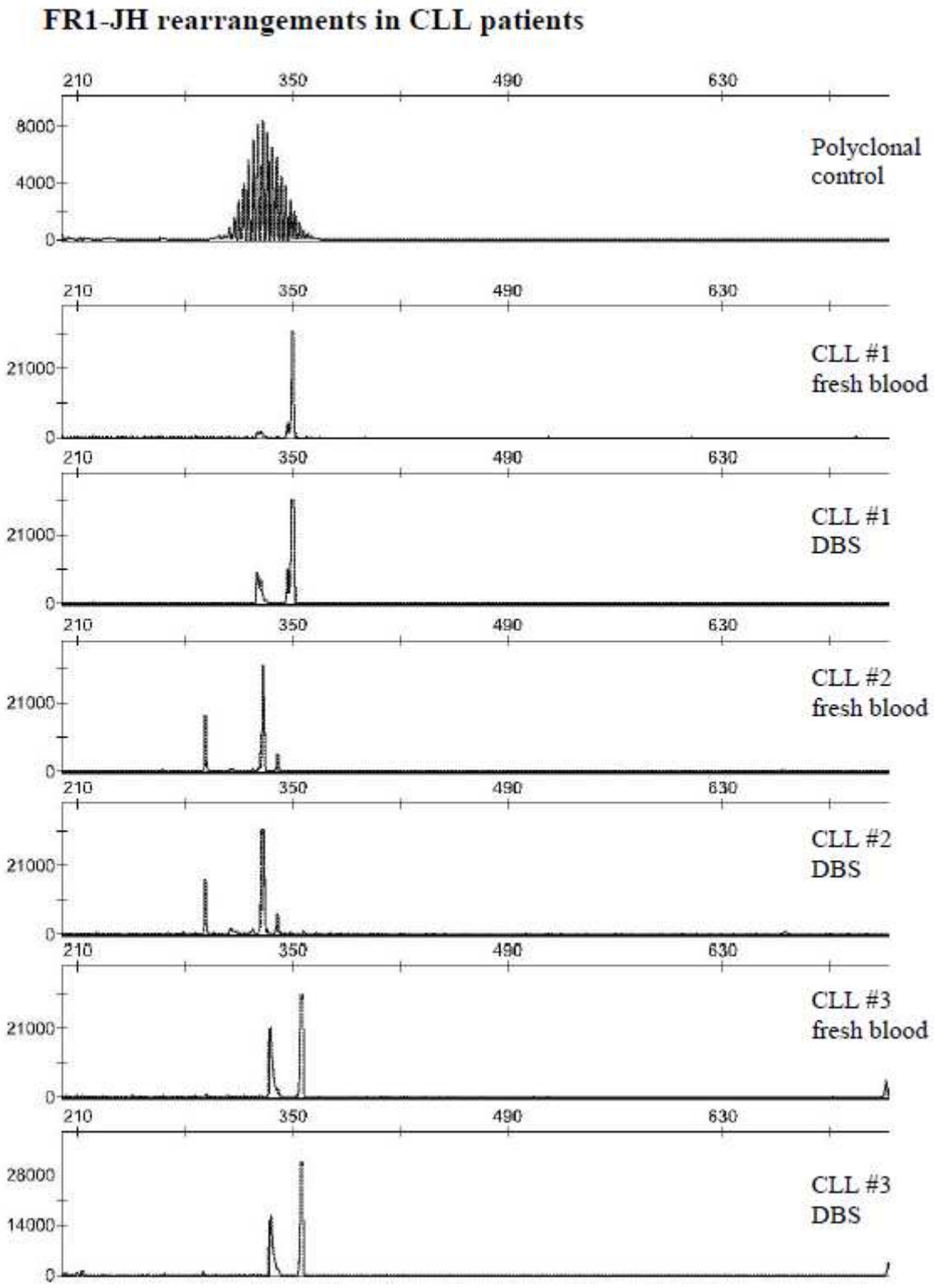

r= 0.987
p< 0.0001
EBV-DNA copies
2000
1500
1000
500
200
20
1000
750
500
250
200
100
10
Namalwa cells
detected copies
expected copies

Figure 4


FR1-JH rearrangements in CLL patients
210
350
490
630
8000
4000
0
Polyclonal control
21000
CLL #1 fresh blood
CLL #1 DBS
CLL #2 fresh blood
CLL #2 DBS
CLL #3 fresh blood
28000
14000
CLL #3 DBS

CHAPTER 6

General Conclusions

General Conclusions

Chronic immune activation, a hallmark of HIV-1 infection, may contribute to the expansion of EBV-infected B cells. In these studies, we described for the first time a strong association between HIV-1 infection, chronic immune activation and EBV load. In fact, we found a significant relationship between markers of microbial translocation, levels of pro-inflammatory cytokines and EBV-DNA levels in both HIV-1 infected adults and children, suggesting that cell activation driven by HIV-1 antigens may result in chronic B cell stimulation and expansion of EBV-infected B cells, a risk factor for the development of EBV-related malignancies. These findings were supported by a significant relationship between activated B-cells and EBV-DNA load observed in a subgroup of patients, suggesting that immune activation plays a pivotal role in the expansion of EBV-infected B-cells (Petrara MR et al, 2012). Persistence of plasma viremia during ART, despite immunoreconstitution, was frequently observed (Ometto L et al, 2002; De Rossi A et al, 2005; Lecossier D et al, 2001; Gilson RJ et al, 2010), and HIV-1 is known to stimulate the immune system, regardless of the status of peripheral CD4 depletion/repopulation (Anselmi et al, 2007; Brenchley et al, 2006). In agreement with previous studies (Righetti et al, 2002; Burighel et al, 2006), we found that EBV-DNA levels were higher in patients with a gain in CD4 lymphocytes but incomplete suppression of HIV-1 viremia than in patients with undetectable plasma viremia, regardless their CD4 cell number.

The relationship between immune activation and EBV levels was also supported by the findings in HIV-1 infected children in Uganda. One of the mechanisms by which HIV-1 activates B cells probably involves PAMPs. These are released into circulation after HIV-1-induced cellular/tissue damage, engage TLRs and induce a potent immune response, involving B cells. Furthermore, malaria may induce B cell activation, leading to the stimulation of EBV-infected B cells via TLR-9 engagement (Rochford R et al, 2005). In this study, we found that 16S rDNA levels, a marker of microbial traslocation (Jiang W et al, 2009) , significantly correlate with levels of EBV-DNA in ART-naive children, and EBV-DNA levels tend to be higher in children with clinical malaria.

Moreover, we found that EBV-DNA and 16S rDNA levels were significantly lower in children on ART than in those ART-naive, suggesting that treatment with ART, likely by limiting chronic immune activation, may prevent B cell stimulation and expansion of EBV-infected B cells. These observations may result of particular interest also for planning therapeutic strategies in HIV-1 infected patients.

We also found that NHLs are strongly associated with EBV load in peripheral blood, suggesting that high levels of EBV in blood might have diagnostic and prognostic relevance for the diagnosis and monitoring of EBV-related lymphomas. In this context we assessed that DBS sampling was also suitable to identify the presence of a B cell clonal population. DBS filter papers are already commonly used for the diagnosis and monitoring of different DNA/RNA viral loads (Snijdewind IJ et al, 2012). However, to date no studies investigated the quantification of EBV-DNA levels and B cell clonality from DBS. Previously, EBV antibodies (IgA and IgG) were tested successfully in eluates from DBS cards to define EBV serostatus in neuroimmunology studies (McDade et al, 2000) and in nasopharyngeal carcinoma screening (Fachiroh J et al, 2008). While serological tests confirm primary infection and document remote infection, quantitative EBV-DNA assessments are increasingly emerging as an important laboratory tool in supporting the diagnosis and management of EBV-associated disease (Abbate I et al, 2011). In some regions of Africa, such as Uganda, EBV seroprevalence is nearly universal, with acquisition in early childhood, and there is a striking association of EBV with BL (Orem J et al, 2007). Therefore, EBV viral load assessment in peripheral blood compartments may represent an important instrument in clinical practice for the prevention, diagnosis and monitoring of EBV-associated diseases, even more in African children where EBV-associated lymphomas represent an important cause of mortality and morbidity. DBS sampling allows us to extend B cell clonality assay in patients with a complicated diagnosis, or where molecular analyses complementary to the histomorphological evaluation are not always available. For the first time, we showed that clonality testing can be performed from DBS, thereby

improving the diagnostic and monitoring capabilities of B cell lymphomas in resource-limited settings.

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