

**B lymphocyte prevalence in the brain in health
and disease**

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For Sally

Declaration of authorship

This thesis was composed by the undersigned candidate. The work included is the candidates own with contributions from other researchers as described in the acknowledgments section.

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Abstract

In recent years evidence has accumulated which suggests that the brain may not be the immunologically privileged site it was once considered to be. It is now widely accepted that T lymphocytes perform surveillance functions in normal brain parenchyma. However as yet there are no reports of B lymphocytes entering brain parenchyma in the healthy state.

This study aimed first to determine the prevalence of B lymphocytes in normal human brain, and subsequently whether advancing human immunodeficiency virus (HIV) infection leads to changes in the brain B lymphocyte population which might contribute to the increased risk of lymphoma seen in acquired immunodeficiency syndrome (AIDS).

Our results show that B lymphocytes do enter all areas of normal brains in very low numbers and that the B lymphocytes within the brain parenchyma display an activated (CD23 positive) phenotype. In contrast intravascular B lymphocytes had a much lower expression of activation markers. B lymphocytes were found in increased numbers in both the brain parenchyma and perivascular spaces of pre-AIDS brains. However brains from the majority of AIDS subjects, including those with primary central nervous system lymphoma (PCNSL) (out with the area of neoplastic involvement) contained fewer B lymphocytes than normal or pre-symptomatic HIV infected brains. A subset of AIDS brains, previously shown to have pleomorphic lymphoid infiltrates in the perivascular spaces had significantly increased numbers of B lymphocytes in both the brain parenchyma and perivascular spaces, which led us to hypothesise that these may represent a pre-malignant PCNSL state. These pleomorphic lymphoid infiltrates were shown to be polyclonal, as defined by immunoglobulin gene re-arrangements, in contrast to PCNSL in which two of three tumours were found to be monoclonal.

Virtually all AIDS related PCNSL are known to be EBV positive, in contrast to non-HIV PCNSL and non-CNS AIDS related lymphomas. We examined the EBV status of brain parenchymal B lymphocytes to investigate whether EBV positive B lymphocytes are more frequent in HIV infected brains than normal thus explaining the propensity for CNS lymphomas in AIDS. In-situ hybridisation (ISH) studies showed EBV positive cells only in the tumours of AIDS related PCNSL cases. PCR based studies detected high EBV copy numbers in PCNSL tumour tissue and low copy

numbers from AIDS cases with pleomorphic lymphoid infiltrates. As none of the B lymphocytes in this latter group were EBV ISH positive, and this appears to be a prerequisite for PCNSL development, we find no evidence that pleomorphic infiltrates represent a pre-malignant PCNSL state. However both PCNSL and AIDS associated pleomorphic infiltrates were associated with expression of the B lymphocyte chemoattractant, stromal cell derived factor-1 (SDF-1) in the brain.

Humoral responses have been shown to play an important part in the immune response to many neurotropic viruses, therefore we assessed the role of B lymphocytes in HIV encephalitis (HIVE). Our results show that a slight increase in the brain B lymphocyte population occurs in HIVE compared to non-HIVE AIDS brains. However this response is far smaller than that seen either in pre-AIDS non-HIV encephalitis or in non-immunocompromised viral encephalitis.

Finally we have shown that the presence of B lymphocytes in the brain is not associated with damage or disruption to the blood-brain barrier.

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List of abbreviations

AIDS	Acquired Immune Deficiency Syndrome
BL	Burkitt's Lymphoma
BLPD	B cell Lymphoproliferative Disease
CSF	Cerebral Spinal Fluid
CNS	Central Nervous System
EBNA	Epstein Barr Nuclear Antigen
EBV	Epstein Barr Virus
HIV	Human Immunodeficiency Virus
Ig	Immunoglobulin
IgH	Immunoglobulin Heavy chain
ISH	In-Situ Hybridisation
K	Kappa (Immunoglobulin light chain)
L	Lamda (Immunoglobulin light chain)
LMP	Latent Membrane Protein
MHC	Major Histocompatibility Complex
PBMC	Peripheral Blood Mononuclear Cells
PCNSL	Primary Central Nervous System Lymphoma
PCR	Polymerase Chain Reaction
SDF-1	Stromal Cell Derived Factor – 1
VH	Variable Heavy (Immunoglobulin chain)

1 Introduction

1.1 Background

In December 2001 the World Health Organisation (WHO) estimated that approximately 40 million people worldwide were living with the human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS). The majority of these (28.1 million) reside in Sub-Saharan Africa, while North America and Western Europe account for only 1.5 million. In 2001 alone an estimated 5 million people were infected worldwide (3.4 million in Sub-Saharan Africa, 75,000 in North America and Western Europe), and 3 million people died from the disease (2.3 million in Sub-Saharan Africa and 27,000 in North America and Western Europe). In Sub-Saharan Africa HIV is now the leading cause of death and worldwide it is the 4th highest cause of death (WHO/UNAIDS 2001).

Infection with HIV leads eventually to the development of AIDS, with a progressive destruction of the immune system. Consequently there is an inability to resist infection with opportunistic pathogens and a predisposition to certain types cancer. There is as yet no cure or effective vaccine for HIV and although the introduction of highly active anti-retroviral therapy (HAART) has allowed disease progression to be slowed considerably this treatment is expensive (approximately \$15,000 per year) and is therefore only available in affluent countries and not in the overburdened countries of Africa. Prevention of viral transmission through education of the populace in methods of safer sexual practices is undoubtedly the best way of halting the epidemic at present. There is evidence from Zambia, one of the worst affected countries, that a decreased incidence of HIV in the urban population correlates with the population reporting less sexual activity, fewer sexual partners and more consistent use of condoms (Fylkesnes *et al* 2001). This suggests that education can make a difference. A lack of education and understanding of the disease coupled with the lack of preventative measures in some of the worst affected countries is reflected in the prevalence of HIV in the adult population. In Sub-Saharan Africa 8.4% of adults are infected, mostly through heterosexual sex. In comparison, the prevalence in North America is 0.6% with infection occurring through needle sharing of intravenous drug users (IDU), homosexual receptive sex and heterosexual sex. In

Western Europe the rate is even lower at 0.3% of adults (WHO/UNAIDS 2001). In contrast to many African countries, UK education programs have been in place for nearly 20 years. Although initially these proved effective in limiting the spread of HIV, there now appears to be a growing complacency over the risks of HIV infection. This is represented in a recent increase in the number of HIV infected individuals in the country. The figures for the year 2000 published by the Public Health Laboratory Service (PHLS 2000) represented a 14% increase over the previous year (3,500 in 2000). HIV is therefore still a major health problem in the UK and worldwide a catastrophic problem.

Individuals infected with HIV are estimated to be at 1000 fold increased risk of developing primary central nervous system lymphoma (PCNSL) in comparison to the general population (Larocca *et al* 1998). PCNSL is reported at autopsy in 5-10% of HIV infected patients (Auperin *et al* 1994, Flinn & Ambinder 1996) and develops late in HIV infection with an extremely poor prognosis. As yet the pathogenesis of PCNSL is not fully understood, however in HIV there is a 100% association with the gamma herpes Epstein Barr Virus (EBV) (MacMahon *et al* 1992). All AIDS PCNSL are of B lymphocyte origin and although it would seem that B lymphocyte entry to the brain is a pre-requisite for tumour formation there are as yet no reports of B lymphocytes entering normal brain.

The majority of cases used in the study are taken from the Edinburgh brain bank, most of whom were local residents in the Edinburgh area. In Edinburgh a large number of HIV infected individuals are intravenous drug users (IDU) and therefore the abuse of drugs has to be considered as a potential confounding factor within this study.

The epidemic of injecting drugs in Edinburgh started around 1980 peaking in 1983-4 (Robertson *et al* 1986). HIV first infected IDUs in 1983 and was spread rapidly by sharing of contaminated needles (Peutherer *et al* 1985). Between 1982 and 1985 51% of 164 heroin injectors attending a general practice clinic were HIV positive (Robertson *et al* 1986). To March 1997, 1176 HIV infections had been reported in Edinburgh and the surrounding area of which 52% were due to injecting drug use (Davies, Cormack & Richardson 1999). A survey undertaken in 1992-94 found 19.3% of IDU HIV positive (Davies *et al* 1995), twice the rate of the nearby larger city of Glasgow where a higher percentage of young adults inject drugs. The high prevalence of HIV in IDUs in Edinburgh appears to stem from a limited supply

injecting equipment during the 1980's. The shortage came about from restrictions by pharmacists on the sale of needles and syringes, as well as from the removal of equipment by police during searches (Burns *et al* 1995). Drug suppliers then began forcing users to inject on site, so that they left free of incriminating evidence. This resulted in all the clients of a supplier using the same equipment (Burns *et al* 1995), which in turn allowed rapid spread of HIV among the drug using community. The HIV infected population of Edinburgh IDUs have been shown to be at increased risk of mortality with up to 15% of HIV IDU in one study dying before the onset of AIDS (Seaman, Brettle, Gore 1997). The Edinburgh brain bank contains brains from this group which provides a unique opportunity to study both the effects of HIV within the brain before the onset of AIDS and the progression from pre-AIDS to AIDS.

1.2 The immune system

The organs and cells of the immune system provide the body with protection against invading pathogens, and are essential to the survival of complex organisms. There are two arms of the immune system; innate and adaptive. The innate immune response is a non-specific immediate response that is directly activated by infectious agents, inflammation and tumours. It cannot be modified during life and is therefore essentially inherited. Although innate responses are fast they lack any specificity, have no memory of previously encountered pathogens and often induce bystander damage to nearby tissue. In contrast an adaptive immune response develops during the lifetime of an individual in response to a specific antigen and in most cases confers lifetime immunity to a pathogen once it has been encountered.

1.2.1 Innate immunity

Innate immunity is provided by phagocytic macrophages, natural killer (NK) cells and neutrophil cells (see glossary for definition of cell types). These form the first line of defence against invading pathogens. They recognise and can bind extracellular pathogens (e.g. bacteria) via receptors on their cell surface. Receptor binding induces phagocytosis of the pathogens and release of cytokines (proteins made by cells which affect the behaviour of other cells by binding to cytokine

receptors on the cell's surface) such as interferon γ (INF- γ) which attracts further immune cells into the area.

The innate system is non-specific and cannot always eliminate pathogens. Furthermore the cells that provide innate immunity cannot recognise all pathogens and therefore many pathogens are invisible to the innate system (e.g. most viruses). The innate system is however critical for early defence of the body after entry of pathogens such as bacteria and protozoa, as the second highly targeted arm of the immune system, the adaptive system, takes 4-7 days to respond to foreign antigens.

1.2.2 Adaptive immunity

Adaptive immunity is provided by B and T lymphocytes. In contrast to the phagocytes of the innate system, which bear multiple receptors specific for many different target antigens, the lymphocytes have only one receptor type, specific for one peptide. In simplistic terms there are 3 main types of lymphocytes: B lymphocytes which secrete antibody, CD8 T lymphocytes which kill virus infected cells, and helper CD4 T lymphocytes required to activate many B and CD8 lymphocytes. CD4 cells play a crucial role in determining whether an immune reaction will be cell mediated (CD8 driven) or humoral (antibody mediated). This is controlled by the differentiation of CD4 cells from T helper (TH) 0 cells to either TH1 (cell mediated) or TH2 (humoral) helper cells. Which type of cell TH0 CD4 cells differentiate into is determined by the type, size and amount of antigen presented to them, and by cytokines present in the microenvironment. TH1 cells secrete INF γ which suppresses TH2 cells and interleukin-2 (IL-2) which activates T lymphocytes. TH2 cells secrete transforming growth factor β (TGF β) and IL-10, which suppress TH1 cells, and also IL-4 and IL-6 which stimulate B lymphocytes. Therefore an immune reaction is a balance between TH1 and TH2 responses, with the predominant response determined by the pathogen and local cytokine levels.

The specificity of lymphocyte receptors is determined genetically during lymphocyte development in the bone marrow (B lymphocytes) and thymus (T lymphocytes). Each lymphocyte has a different specificity allowing detection of an almost infinite number of potential antigens. The specificity of each lymphocyte is determined by the construction of the surface immunoglobulin (Ig) receptor expressed

on B lymphocytes (see Figure 1) and the T cell receptor (TCR) expressed on T lymphocytes.

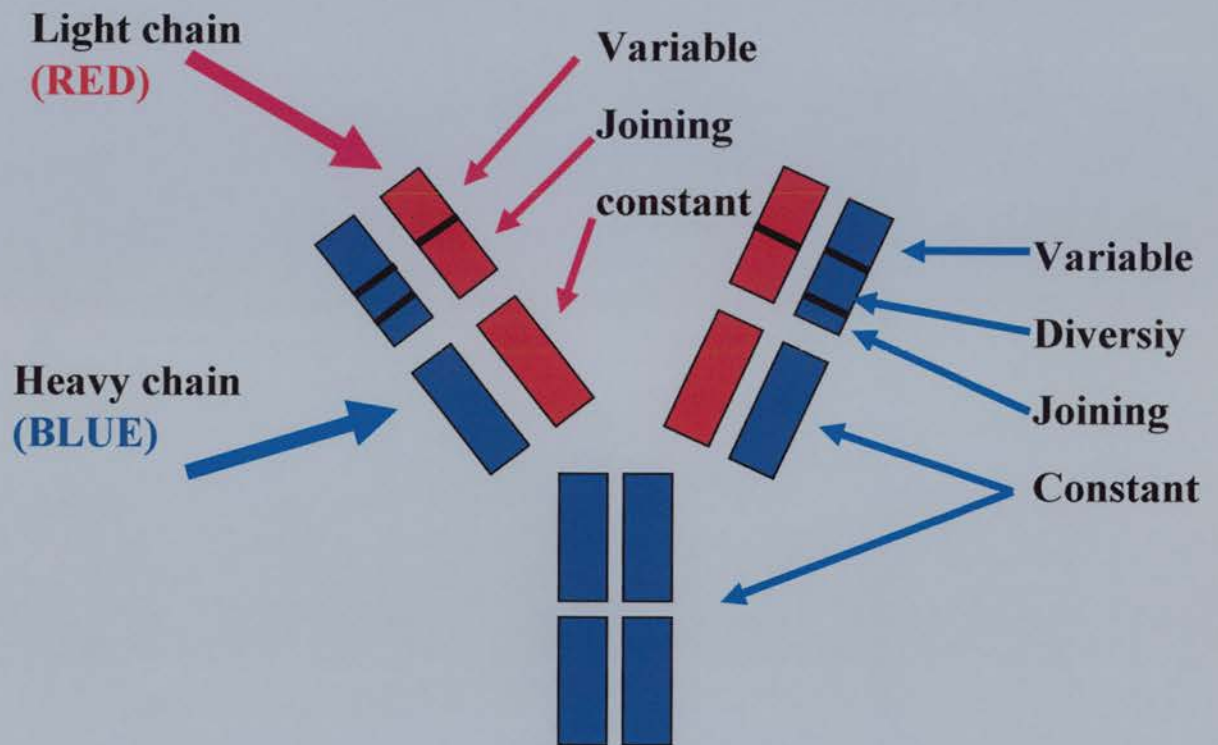
Lymphocytes are capable of binding antigen and recognising it when it is presented in the correct manner. CD4 T lymphocytes recognise antigen presented on major histocompatibility complex (MHC) class II molecules. Peptides displayed on MHC II are extracellular in origin and are degraded in cellular vesicles before presentation as peptide fragments. In contrast cytosolic antigens are presented on MHC class I to CD8 T lymphocytes. MHC class I is present on nearly all nucleated cells, with rare exception including neurones, although it can be induced on these cells (Fujimaki *et al* 1996, Neumann *et al* 1997). MHC class II is only expressed on specialised antigen presenting cells of the immune system including the professional antigen presenting dendritic cells and the non-professional antigen presenting macrophages and B lymphocytes.

1.2.2.1 Diversity of B lymphocyte Immunoglobulin and T lymphocyte T cell receptors

The B lymphocyte Ig receptor is composed of 4 protein chains, the 2 heavy and 2 light Ig chains (see Figure 1). The heavy chain is composed of 4 regions: a variable (V), diversity (D), joining (J) and constant (C) region. The light chain in contrast has only 3 regions: V, J and C. Each region of the chain is coded for by a family of genes. The heavy chain genes are located on chromosome 14 and in humans there are 86 V, 30 D, 9 J, and 11 C genes (Cook & Tomlinson 1995). There are 2 types of light chains, kappa (κ) and lamda (λ), either of which is paired with a heavy chain. There are 52 V λ genes, 7 J and 7 C genes located on chromosome 22. The κ genes are located on chromosome 2 where there are 76 V, 5 J and 1 C gene. Any of the individual V, J and C κ and λ genes can be combined to produce either a κ or λ light chain, giving a potential of 2548 λ and 380 κ combinations. In the same way, any of the heavy V,D,J and C genes can be combined to produce a heavy chain, giving a potential of approximately 255,000 Ig heavy chains. Any light chain can be paired with any heavy chain giving a possible ~734,000,000 combinations of Ig, although it should be noted that not all combinations produce functional Ig.

As discussed later, additional variability is introduced by the mechanism used to join the V,(D),J, and C genes together, further increasing the diversity of Ig

Figure 1: Structure of the immunoglobulin molecule



produced and increasing the number of potential foreign antigens which can be recognised. Although the genes used to create the TCR in T lymphocytes are different to the Ig genes, the principles of diversity generation and joining of the segments is the same. Construction of the TCR will not be discussed further as it is not central to this thesis.

1.2.2.2 Immunoglobulin genes

Figure 2 shows a schematic representation of the layout of the Ig heavy chain genes on chromosome 14. The genes coding for V, J and C in κ and λ are laid out in a similar way. The heavy chain variable genes provide a large percentage of the diversity of the heavy chains. The genes encoding V heavy segment of the Ig chain are divided into 6 families based on their DNA sequence homology, with members of each family showing at least 70% homology (Matsuda & Honjo 1996). The families are termed variable heavy (VH)-1 to VH-6 (see diagram 2). Within each family there are areas of highly conserved sequence, termed framework (FR) 1-4, and areas of high variability (HV). Figure 3 shows that the areas of high variability within each family corresponds to the areas of the protein involved in the binding to antigen.

There is a bias in the usage of variable heavy chain families, with the VH3 family providing the greatest contribution to the functional Ig repertoire (51 genes), followed by VH1 (17 genes) and VH4 (13 genes). VH2, VH5 and VH6 each contribute a relatively small number of functional members, with 4, 3 and 1 gene respectively (Cook & Tomlinson 1995).

1.2.2.3 Immunoglobulin gene re-arrangements

In order to create a viable Ig heavy or light chain, one gene from each section (V, [D], J and C) must be juxtaposed, to allow transcription of a single re-arranged gene and hence production of the Ig heavy or light chain protein. The mechanism by which the genes are brought together is called somatic recombination. This involves the splicing out of intermediate sections of DNA leading to the juxtaposition of genes from each of the sections of the chain. Figure 4 gives a representation of how this occurs. Cleavage of intervening DNA is carried out by a complex of proteins including the enzymes RAG1 and RAG2 (recombination activating genes), which are expressed only in lymphocytes.

Figure 2: Schematic representation of immunoglobulin heavy chain genes

Immunoglobulin heavy chain genes in humans can be grouped into families (VH1-VH6) by their nucleotide sequence, however their distribution in the genome is random as demonstrated below.

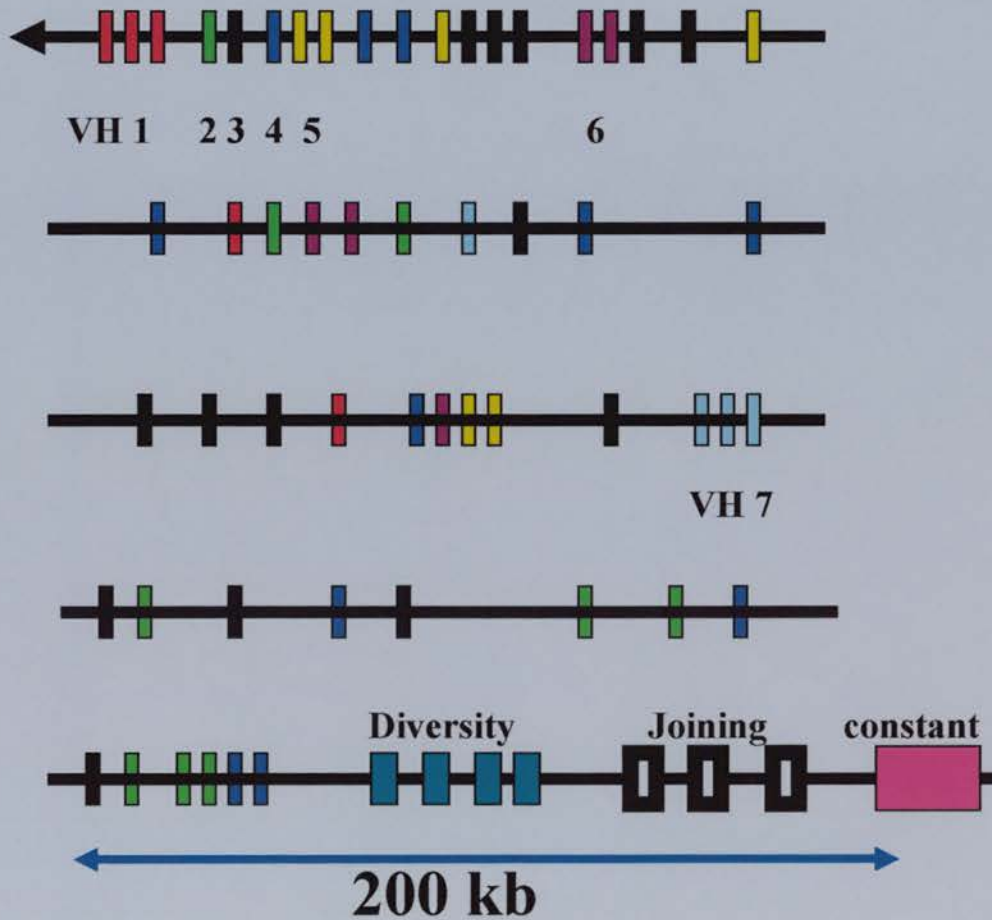
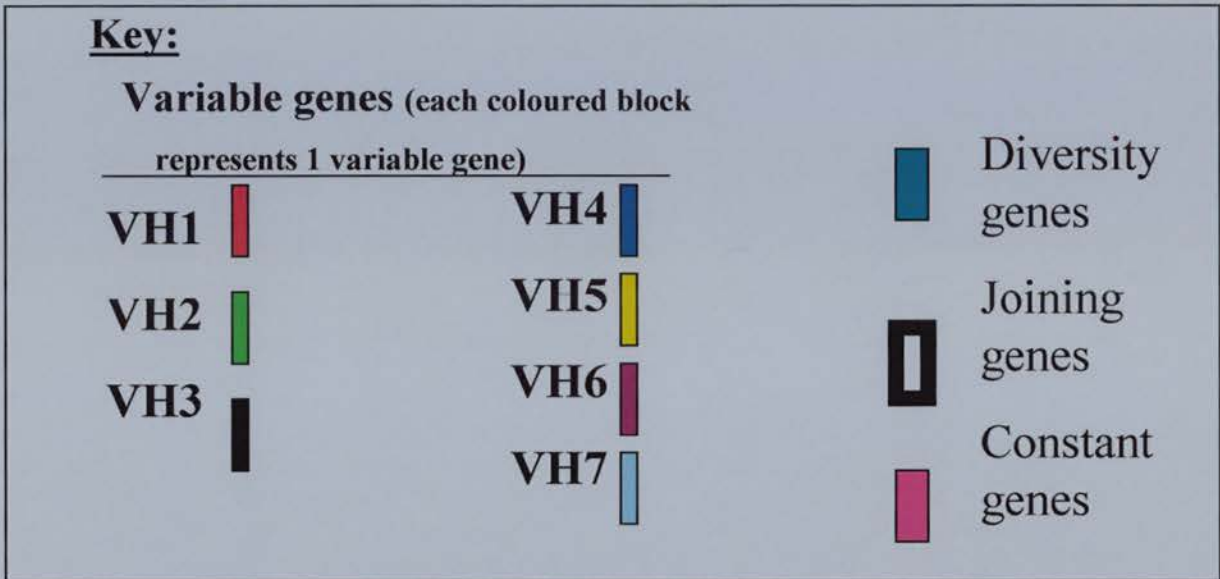
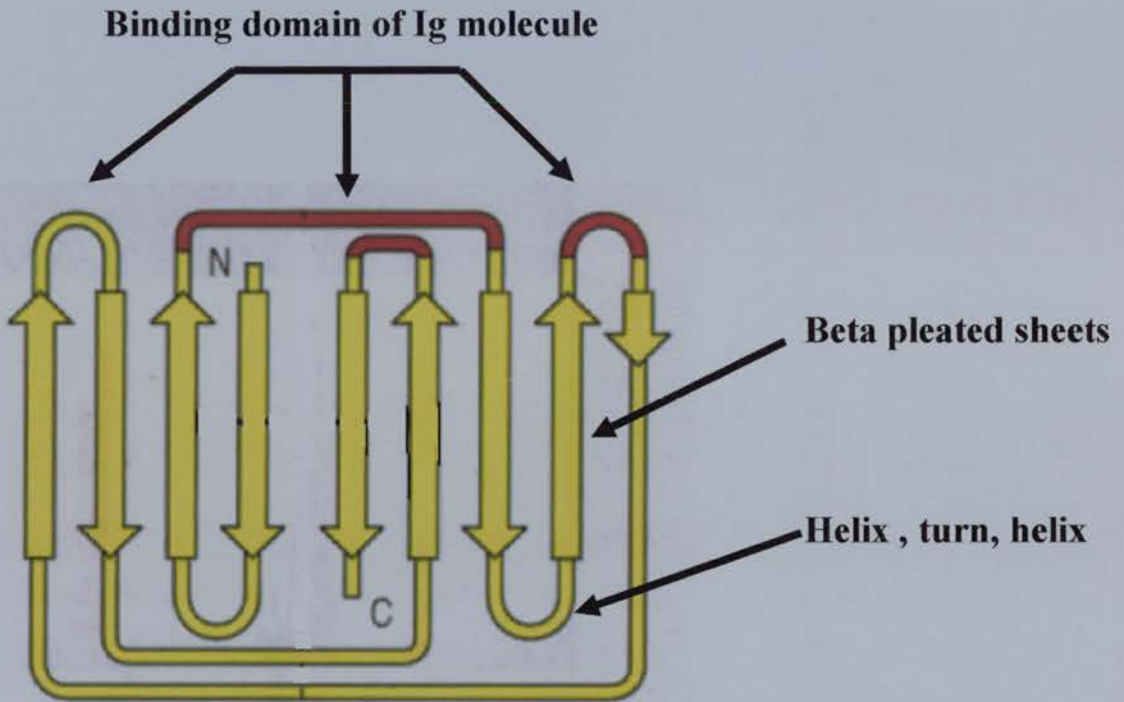


Figure 3: Variability of the immunoglobulin heavy chain genes

Folded protein structural model of Ig molecule



Unfolded Ig protein

Hypervariable regions in amino acid sequence coincide with binding domain of Ig molecule

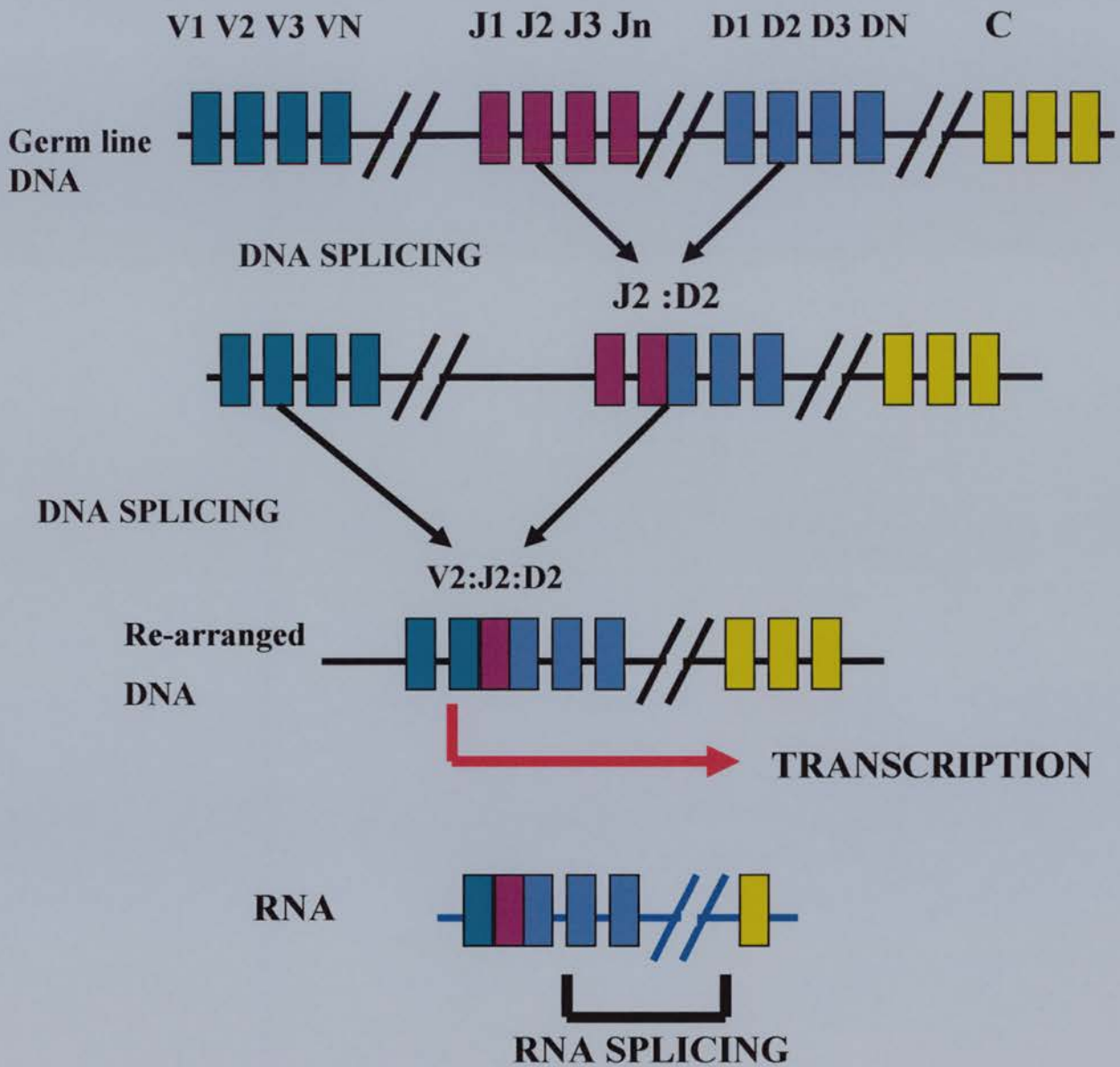
110 amino acid residues



FR **frame work region (conserved)**

HV **hyper variable region**

Figure 4: Representation of immunoglobulin heavy chain VDJ recombination



Key:

- V Variable gene
- J Joining gene
- D Diversity gene
- C Constant gene



The process of joining genes from each region is key to providing increased diversity and variation in the antibody repertoire. Figure 5 demonstrates how the cleaved ends of the two genes are joined together. The generation of P nucleotides (palindromic nucleotides are an inverse repeat of the sequence at the end of the adjacent gene segment) and N nucleotides (a variable number of nucleotides randomly inserted into the joint between variable genes by the enzyme terminal deoxynucleotidyl transferase) during the ligation of the two ends of DNA introduces further variability into the Ig heavy and light chains. This further increases the diversity of the Ig repertoire and hence increases the potential number of foreign antigens that can be recognised by the system.

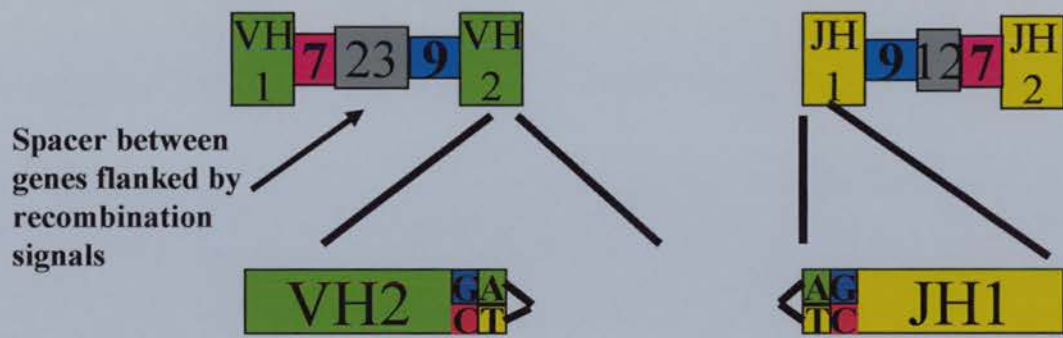
The whole process in B lymphocytes takes place in the bone marrow during B lymphocyte development, and the choice of Ig genes used is essentially random. There is however a bias in the use of κ light chains over λ light chains in humans. The heavy chain genes are re-arranged first, and production of a functional Ig heavy chain results in re-arrangement of the κ light genes. If there is a failure to produce a functional κ light chain then the cell will re-arrange its λ light chains (approximately 70% of Ig's are constructed using κ light chains in humans). Failure to produce a functional Ig results in apoptosis in B lymphocytes. (For a review of V(D)J recombination see Schatz, Oettinger & Schlissel 1992).

The huge potential repertoire of Ig antigen receptors and the random manner in which they are produced means that there is a high potential for creating lymphocytes able to bind to normal human proteins (self-antigens). Recognition of self-antigens leads to the induction of autoimmune disease, and therefore to avoid this occurring apoptosis is induced in B lymphocytes that recognise self-antigens before they leave the bone marrow.

1.2.2.4 Activation of lymphocytes

Lymphocytes continually re-circulate between the blood and secondary lymphatic organs (spleen, lymph node). In order for lymphocytes to become antigen specific effector cells able to act against their antigen, they must first be activated. This is triggered by antigen binding to lymphocyte receptors and is a complex multi-step process that ensures that immune reactions only occur when necessary, thus avoiding unnecessary bystander tissue damage which is associated with inflammatory immune reactions.

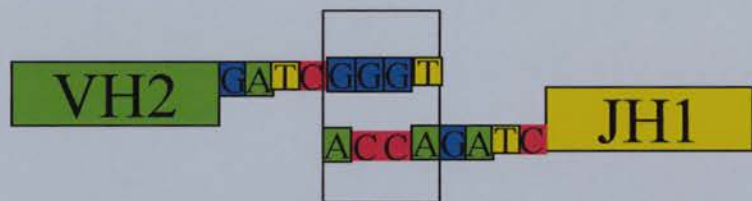
Figure 5: Joining of cleaved ends during VDJ recombination



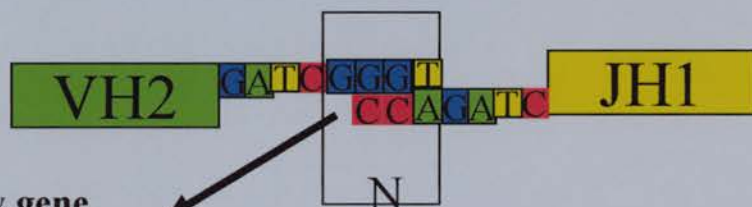
Hairpins form at the blunt ends



Endonuclease cleavage of hairpins generates P nucleotides



Variable number of N nucleotides are added by TdT



Key:

VH variable heavy gene

JH Joining heavy gene

G,C,A,T Nucleotides

A

Pairing of strands

Exonuclease cleaves un-matched nucleotides and DNA polymerase completes the new joint

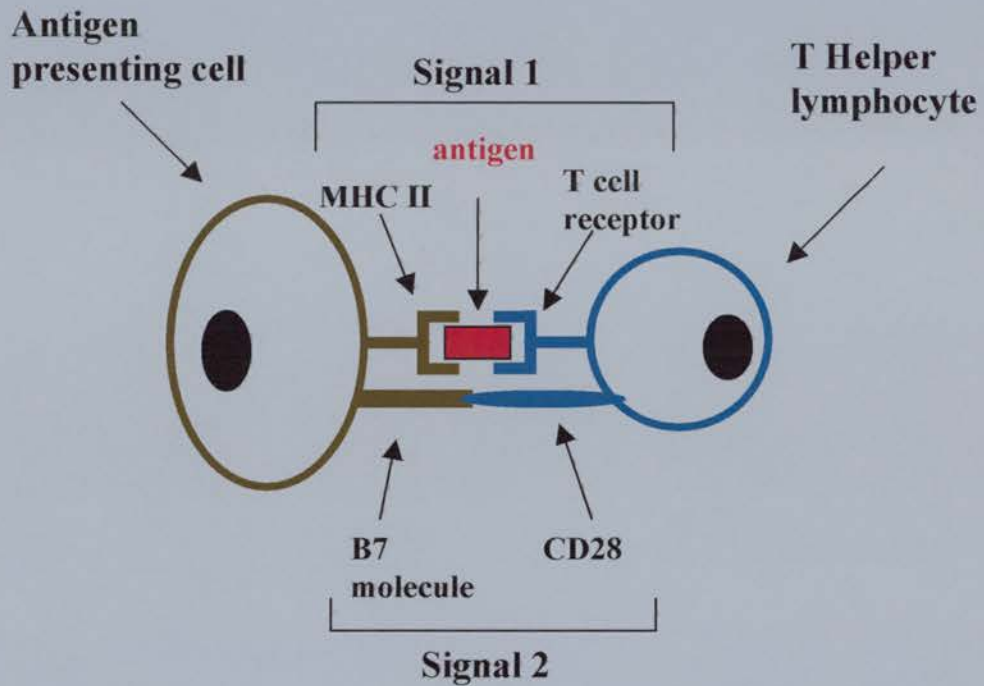
Phagocytes in the peripheral tissues take up antigen produced by infectious organisms. They then travel to the lymph nodes via the afferent lymphatic system where they present their antigen to B and T lymphocytes, causing lymphocyte activation.

CD4 T lymphocytes are activated by the presentation of their specific antigen by antigen presenting cells (dendritic cells, B lymphocytes and macrophages) on class II MHC which is only expressed on antigen presenting cells (Reviewed in Guermonprez *et al* 2002). The presented antigen is bound by the TCR of the CD4 lymphocyte, providing the first of 2 signals required for CD4 T lymphocyte activation. The second signal is also supplied by the antigen presenting cells, by expression of one or other of the B7 proteins (CD80 or CD86) on its surface which binds to CD28 on the surface of the CD4 T lymphocyte providing the second signal for activation (see figure 6). Activation leads to expression of IL-2 and IL-2 receptor in the CD4 cell, which operates in an autocrine system to stimulate proliferation and differentiation of the CD4 T lymphocyte.

CD4 helper T lymphocytes are required for most B lymphocyte responses to antigen. Activation of B lymphocytes also requires 2 signals, the first is supplied by the binding of antigen to the cell surface Ig molecule, and the second which is provided by CD4 cells by the ligation of CD40 on the B lymphocyte surface with CD40 ligand (CD154) on the CD4 cell surface. In addition, as mentioned above, CD4 cells also provide cytokines for proliferation and differentiation of B lymphocytes. Activated B lymphocytes display activation markers including CD23, a dual role cell surface protein involved in enhanced antigen presentation to CD4 cells and binding of both soluble cell free CD21 and cell surface bound CD21 present on other B lymphocytes; inducing B lymphocyte aggregation (Bonney *et al* 1995, Bjorck *et al* 1993). CD23 is displayed on mature B lymphocytes which have been activated, therefore it is a useful marker in determining the activation status of B lymphocytes.

B lymphocytes are capable of producing different classes of Ig each with differing properties. Figure 7 shows the maturation process for B lymphocytes in terms of Ig production and the specific functions of each class of Ig. The Ig class present on B lymphocyte surface is dependent on the class of Ig constant heavy chain

Figure 6: Two signals are required for Helper T cell activation

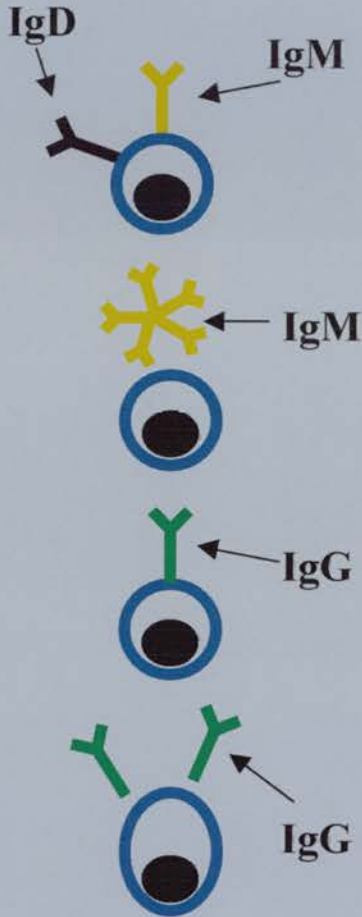


Signal 1 is provided through binding of the T cell receptor to antigen presented on MHC class II molecule.

Signal 2 is provided by ligation of CD28 molecule on the T cell surface with B7 (CD80 or CD86) molecule on the antigen presenting cell.

Both signals are required for activation of T helper cells

Figure 7: Maturation of naive human B lymphocytes







Mature naïve B lymphocyte with re-arranged VDJ heavy chain genes expressing μ and σ constant heavy chain genes by alternative splicing, which produces IgM and IgD on the cell surface.

B lymphoblast (which has been exposed to antigen) secretes IgM

Memory B lymphocyte has undergone isotype switching to express heavy constant γ, α or ϵ genes which produce IgG, IgA or IgE on the cell surface respectively.

Plasma cell secretes either IgG, IgA or IgE depending on constant chain used.

Properties of antibodies:

	IgM	weakly neutralising, strongly activates complement, found in plasma
	IgG (1-4)	Neutralising, opsonising (IgG1,3), sensitisation for killing by NK cells (IgG1,3), activate complement, diffuse into extra vascular sites
	IgA	Neutralising, diffuse into extracellular sites, transport across epithelium (particularly gut) into secretions
	IgE	Sensitisation of mast cells

being expressed. Figure 7 demonstrates the process of Ig constant heavy chain gene switching and the type of antibody produced by each gene. Upon activation B lymphocytes enter primary lymphoid follicles in lymph nodes, where they begin to proliferate and form germinal centres. Primary follicles contain follicular dendritic cells (FDC) (distinct from dendritic cells) which are thought to play an important role in controlling antibody responses to antigen. The function of FDC is to prevent apoptosis and support proliferation of germinal centre B lymphocytes, in contrast to the role of T lymphocytes in the germinal centre which induce differentiation of B lymphocytes. Rapid proliferation of B lymphocytes (centroblasts) in the germinal centre greatly increases the number of B lymphocyte centroblasts specific for the antigen that initiated the response. Most B lymphocytes in a germinal centre are therefore derived from 1 or a few founder cells. Re-arranged Ig genes of dividing centroblasts accumulate mutations at a rate of 1 base in every 10,000 per cell division. This high mutation rate (most other somatic cells have a rate of 1 base in 10^{10}) means that approximately every second cell produced will have 1 amino acid change in its Ig receptor. The consequence of these changes, called somatic hypermutation, is that occasionally a cell is produced which has a greater binding affinity for the antigen that invoked the immune reaction than the original founder cell. This cell is then selected over weaker binding cells for proliferation, whereupon the process begins again. Cells that accumulate mutations that weaken antigen binding undergo apoptosis. In contrast those with strong affinity for antigen have expression of anti-apoptotic genes induced (e.g. Bcl₂). The result of this process is a continual evolution of the Ig receptor and consequently greater antibody/antigen affinity.

CD4 T lymphocytes can only recognise antigen when it is presented on MHC class II molecules. The main class of antigen presenting cells are dendritic cells, which express MHC class II and reside in T cell areas of lymphoid tissue. Immature dendritic cells are found in non-lymphoid tissue, however these cells do not appear to stimulate T cell responses until they are activated and migrate to lymphoid tissues. An additional function of B lymphocytes is the ability to act as antigen presenting cells to CD4 T lymphocytes, when activated to express MHC class II. This allows CD4 cells to bind their antigen and be activated by the B lymphocytes. CD4 cells in turn further stimulate the pool of B lymphocytes enhancing and amplifying the humoral immune response. The process of antigen presentation by B lymphocytes is less efficient than that of dendritic cells.

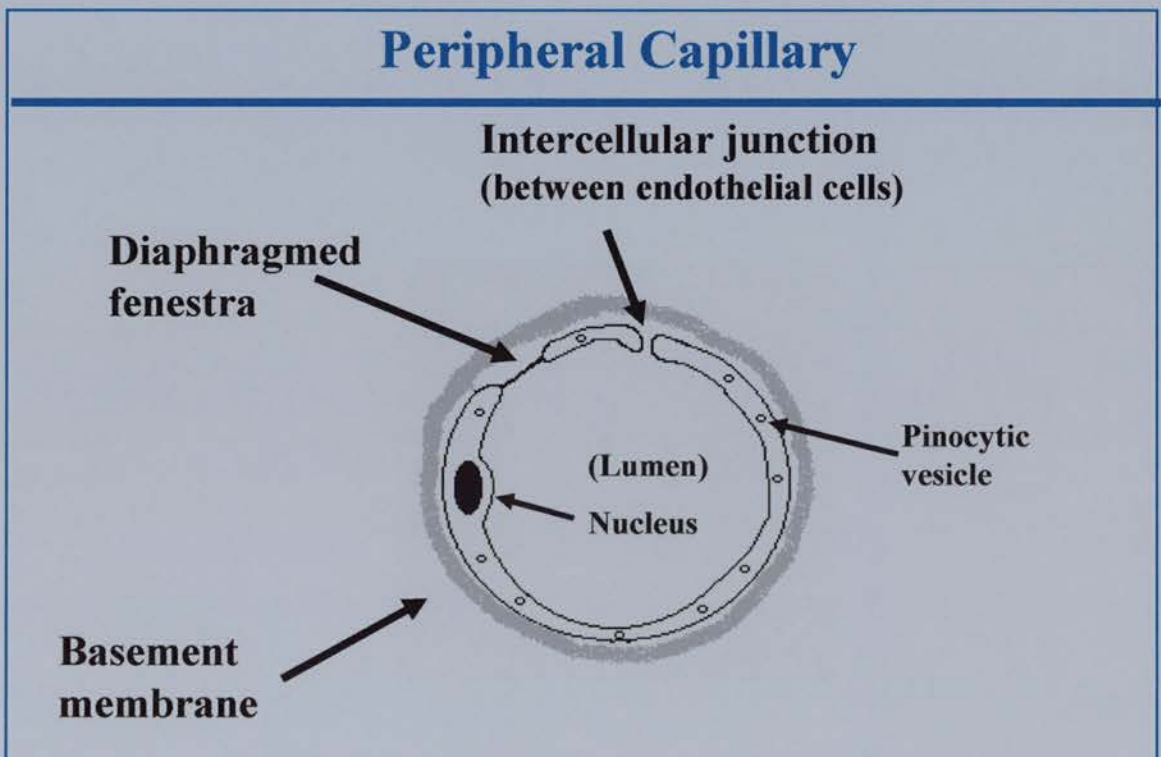
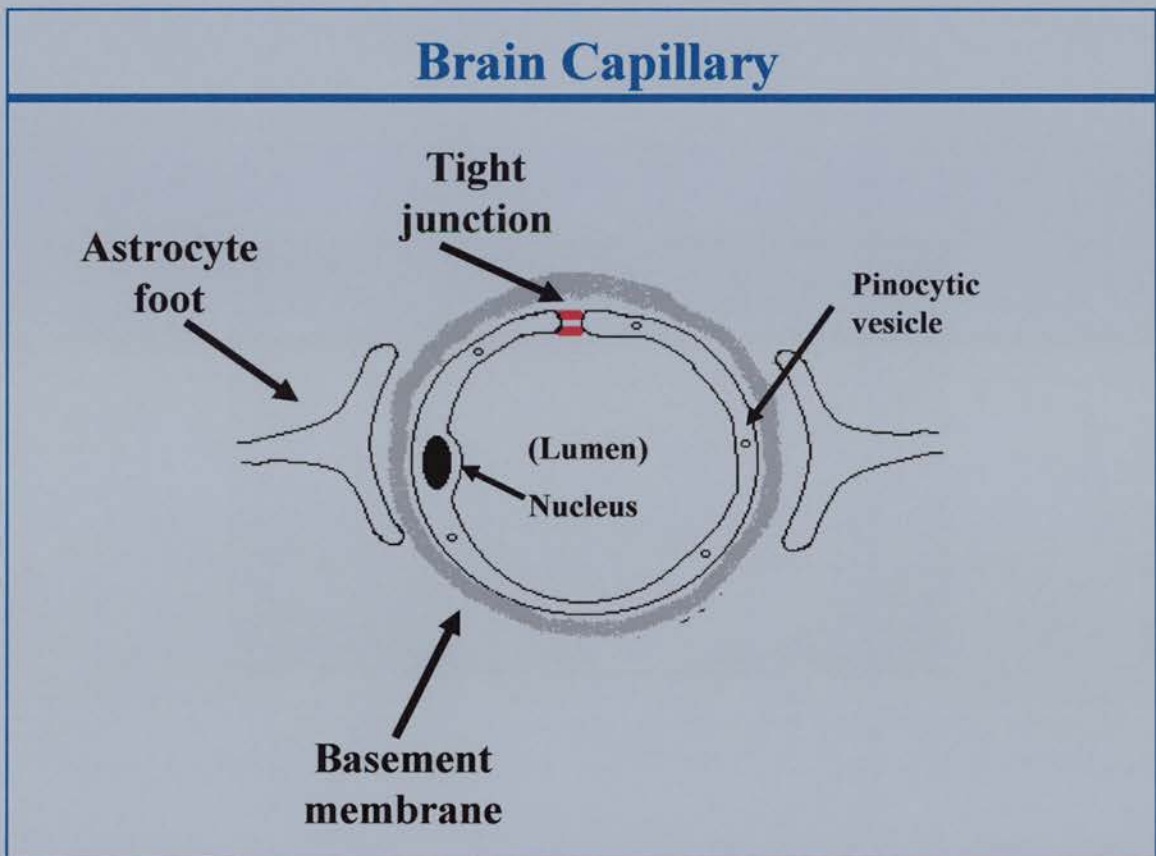
CD8 lymphocytes are activated through recognition of antigen presented on class I MHC in conjunction with expression of the B7 protein (CD80 or CD86) by the antigen presenting cell which is required for a second signal. Some CD8 cells require additional stimuli to become activated, this involves the binding of a CD4 cell to the same antigen presenting cell as the CD8. The CD4 cell can then release IL-2 to stimulate the CD8 cell or alternatively provide stimulation of the dendritic cell to induce B7 expression.

1.2.3 The Blood brain barrier (structure and function)

The BBB is essential for the maintenance and regulation of the brain microenvironment. It regulates and controls the entry of nutrients, electrolytes, vitamins, minerals, free fatty acids, peptides and regulatory proteins (Reviewed in Banks 1999). This regulation ensures that ionic composition of the interstitial fluid of the CNS remains stable despite any fluctuations in the blood circulating through the brain. In addition the BBB is involved in the regulation and control of entry of immune cells to the brain, via expression of adhesion molecules on the endothelial cell surface (Banks 1999).

The BBB is comprised of several different components, many of which are not found in the construction of peripheral blood capillaries. Figure 8 compares the capillaries found in the brain to those found at peripheral sites. Brain microvascular endothelial cells (BMVEC) form a continuous layer of cells and extracellular matrix. In addition BMVECs form tight junctions between the endothelial cells, these structures are absent in the intercellular junctions of peripheral capillaries (Reviewed in Kniessel & Wolburg 2000). Tight junctions are modified areas of cell surface membrane composed of numerous proteins including occludin and zonula occludin (ZO)-1 (Fanning *et al* 1998). These have been shown to link the extracellular component of the tight junction with the actin cytoskeleton of the cell (Fanning *et al* 1998). Antibodies against ZO-1 and occludin have been used to study the integrity of the BBB in healthy and diseased brains (Dallasta *et al* 1999). In healthy brains staining with either of these antibodies can be visualised as a continuous line of staining around blood vessels of the brain. In contrast diseased brains in which BBB breakdown has occurred (e.g. multiple

Figure 8: Diagram of a cross section of a brain capillary and peripheral capillary



sclerosis and HIV encephalitis) show fragmented staining or a complete absence of these tight junction proteins (Dallasta *et al* 1999, McQuaid 2002). The tight junctions between BBB endothelial cells leads to high endothelial electrical resistance, in the range of 1500-2000 Ω , as compared to 3-33 Ω in other tissues (Crone and Christensen 1981, Butt *et al*, 1990). The net result of this elevated resistance is low paracellular permeability.

Another important component of the BBB, which is not found in peripheral capillaries, is astrocyte foot processes. While these do not actually form a physical part of the barrier, their presence is essential for development of the BBB and for the decreased permeability of BMVECs compared to peripheral endothelial cells (Miller 1999). Without astrocytes, in-vitro cultured BMVECs show similar barrier properties to peripheral endothelial cells in terms of solute transport and cellular migration through a layer of endothelial cells. However, if the BMVECs are co-cultured with a layer of astrocytes below the layer of BMVEC cells many of the functions of the BBB seen in vivo are restored (Goldstein 1988, Hurwitz *et al* 1993)

Additional differences found between brain and peripheral capillaries include a decrease in the number of pinocytotic vesicles in BMVECs, an increase in the number of mitochondrial organelles found in BMVECs and a lack of fenestrations in the BMVECs (Broadwell 1989). There are also

The effectiveness of the BBB is demonstrated by the cerebrospinal fluid to serum albumin ratio of 1:200, which is one of the largest gradients found in the human body (Banks 1999).

1.3 Immunology of the brain

Until recent years the brain has been considered to be one of several immunologically privileged sites within the body; other sites include the anterior chamber of the eye, the testis, and the uterus (Janeway & Travers 1997). The immune privilege status results from the effects of both physical barriers to cell and antigen migration into the site, and from local soluble immunosuppressive mediators. The BBB effectively isolates the brain from changes in blood concentrations of ions and sugars maintaining a constant and regulated environment for the neuronal cells of the brain. Immune suppression within the brain results from production of cytokines such as TGF β which suppress expression of class II MHC on microglia and

suppresses microglial activation and proliferation (Suzumura *et al* 1991, Lodge & Sriram 1996, O'Keefe, Nguyen & Benveniste 1999). The combined effect of the physical BBB and immunosuppressive cytokines is to limit ionic changes in the extracellular fluid of the brain and to prevent immune reactions occurring, both of which may cause irreparable damage to non-regenerative neurones of the brain.

This concept of immune privilege of the brain was based on early studies that showed that allografts survived better in the central nervous system (CNS) than in peripheral sites (Woodruff 1960). The prevalent view at this time was that the CNS lacked both lymphatic drainage (Gell & Coombs 1968) and antigen presenting cells. In addition, the BBB was believed to prevent the entry of immune effector cells. However in recent years evidence has accumulated to suggest that the CNS is not excluded from regular surveillance by the immune system (see Perry *et al* 1997 for review). CD4 T lymphocytes enter the CNS in an apparently random manner (Hickey, Hsu & Kimura 1991). Only T lymphocytes that are activated are able to enter the normal CNS and those that fail to encounter antigen leave within one to two days of entry. T lymphocytes capable of reacting with antigen located in the brain parenchyma remain in the CNS, or cyclically re-enter, and thereby initiate inflammation (Hickey, Hsu & Kimura 1991).

Astrocytes, microglia, endothelial cells and pericytes are all capable of antigen presentation (Male, Pryce & Hughes 1987, Fabry, Raine & Hart 1994). Although in the normal brain there is little or no expression of MHC molecules, some cells of the CNS can be induced to express MHC. MHC class I can be induced on microglia, astrocytes and oligodendrocytes by the presence of $INF\gamma$. $INF\gamma$ and $TNF\alpha$ can also induce Class II MHC on endothelial cells, pericytes, microglia and astrocytes (Dories 2001, Male, Pryce & Hughes 1987). CD4 T lymphocytes are capable of supplying the inflammatory mediators necessary for upregulation of MHC in resident brain cells. The induction of MHC class II is of particular importance in microglia as these are the intrinsic immune cell of the CNS, they are the brain's resident macrophages and are the first line of defence should the BBB be breached. Microglia are capable of phagocytosis of both damaged tissue and foreign antigen, and of subsequent antigen presentation.

An important part of the normal immune response in a tissue is the transport of antigen from that tissue via the lymphatic system to local lymph nodes where lymphocytes can be activated and an immune reaction initiated. Although there is no

lymphatic drainage from the brain it is now recognised that antigen from the brain can drain to the cervical lymph nodes (Aloisi, Ria & Adorini 2000). The most likely path is via the cerebral spinal fluid (CSF) which constantly leaks at the junctions where the meninges meet the sensory cranial nerves; this is most prevalent at the cribriform plate where the olfactory nerves penetrate the base of the skull (Dorries 2001).

While some antigen drainage has been shown to occur from the CNS and certain cell types have been shown to be capable of antigen presentation, it should be noted that the brain is devoid of dendritic cells (the most potent type of antigen presenting cells). Dendritic cells can however be found in choroid plexus and in the meninges covering the brain (Aloisi, Ria & Adorini 2000).

In light of recent advances in our understanding of the functioning of the immune system within the brain, it seems that the concept of the brain existing as a completely immune privileged site must now be reconsidered. It is clear that the BBB tightly regulates entry of molecules, cells and pathogens to the brain in order to maintain a constant environment for neuronal cells. However regulation does not imply complete exclusion. In the case of the brain unnecessary inflammatory reactions could cause irreparable damage, therefore it appears logical that cells capable of mediating such damage are restricted in entry. Nevertheless, despite the presence of the BBB many pathogens are capable of entering the CNS and causing damage, therefore the immune system must be able to deal with these infections. In essence the brain would appear to have a restricted or lowered level of immune surveillance, rather than an absence.

With regard to the humoral arm of the immune response, there are as yet no reports of B lymphocytes entering the normal foreign antigen free CNS (Williams & Hickey 1995, De Angelis 1999, Hickey 2001). This contrasts with the previously mentioned evidence of random entry to the brain of activated T lymphocytes in an antigen independent manner. Several *in vitro* studies, using models of the BBB, have shown that while B lymphocytes may adhere to cerebral endothelium, it is CD4 T lymphocytes which migrate most effectively through the barrier (Pryce, Santos, Male 1994). These models are constituted from layers of endothelial cells cultured on membranes with astrocytes grown on the other side of the membrane. While these models are useful for studying individual aspects of cell transmigration into the CNS, e.g. adhesion molecule usage and interaction, the model can never fully replicate the *in vivo* functions of the BBB. It is known that B lymphocytes are capable of

responding to antigen within the CNS. For instance Knopf *et al* (1998) showed that infusion of antigen (OVA) through an indwelling brain parenchymal catheter, in rats naive to the antigen, was followed by recruitment of B lymphocytes and production of antibody despite the presence of an intact BBB. When the experiment was repeated in pre-immunised rats the B lymphocyte response was even stronger. This suggests that antigen can be detected behind the BBB and furthermore that antigen-specific B lymphocytes can respond to antigen within the brain.

1.3.1 Viral infections of the brain: role of B and T lymphocytes

Since the focus of this thesis is viral infections of the brain (in particular HIV and EBV) only immune reactions to viral entry and persistence in the CNS will be further discussed, however, there is evidence of immune reactions to other pathogens occurring within the brain for example toxoplasma (Deckert-Schluter *et al* 1999).

The main viruses capable of CNS infection are given in table 1.

Table 1: Neurotropic viruses

Viral family	Viruses	References
Enteroviruses	polio	Lohler 1981
Morbillivirus	measles	Lohler 1981
Paramyxovirus	mumps	Forsberg <i>et al</i> 1986
Herpes	Herpes simplex, cytomegalovirus, human herpes virus 6	Lohler 1981 Lohler 1981 Blumberg <i>et al</i> 2000
Flaviviruses	Yellow fever, West Nile virus, dengue virus	Eldadah <i>et al</i> 1981 Lanciotti <i>et al</i> 2000 Eldadah <i>et al</i> 1981
Lyssavirus	Rabies virus	Feiden <i>et al</i> 1985
Retrovirus	HIV-1, HIV-2 human T cell leukaemia virus	Budka <i>et al</i> 1987, Sankale <i>et al</i> 1996 Gartner <i>et al</i> 1986
Papovavirus	JC virus	Scweighardt & Atwood 2001

The detailed study of viral CNS interactions is not possible in humans except at autopsy, however there are a variety of good animal model systems that allow in depth study. In addition certain aspects of viral CNS infection can be studied in vitro.

Neurotropic viruses entering the body generally undergo several rounds of viral replication in the periphery before spreading to the CNS, and during this time the peripheral immune system begins to mount an immunological response to the virus. There are numerous potential routes of viral entry to the CNS from the periphery. Herpes simplex virus (HSV)-1 for example replicates in the epithelium of the oral cavity. Also present in the oral cavity are sensory receptors for taste, pressure and heat that are connected to neurones within the CNS. HSV nucleocapsids are capable of entering axons and are then transported to the neurones in the trigeminal ganglia, where the virus establishes a latent infection with few if any of its genes being expressed. Alternative routes of viral entry to the CNS include the use of adsorptive endocytosis by virus such as Japanese encephalitis virus (Liou & Hsu 1998), a mosquito born flavivirus, to cross the endothelial cells of the BBB in mice. The virus binds to endothelial cells of the BBB and is absorbed into endocytic vesicles, allowing transport into the brain endothelium from where it can spread into the CNS. In contrast HIV is thought to be carried into the CNS by infected blood mononuclear cells (CD4 T lymphocytes and macrophages) that normally traffic into the CNS (Nottet *et al* 1996, Persidsky *et al* 1997).

Once within the CNS neurotropic viruses infect resident brain cells. Replication of viruses within the CNS causes activation of local microglial cells to produce INF α/β (Dorries 2001). The effects of this cytokine is to induce MHC class I expression on CNS cells (Njenga *et al* 1997), which allows infected cells to present viral peptides to CD8 lymphocytes (Morris *et al* 1987). Furthermore microglia phagocytose cellular debris that accumulates as a result of the cytopathic effects of viral infection of cells, inducing further cytokine release. The production of cytokines and chemokines within the brain leads to an increase in adhesion molecule expression on endothelial cells of the BBB allowing trafficking of activated T lymphocytes (Male *et al* 1990). Both CD4 and CD8 lymphocytes enter the brain, with CD4 lymphocytes secreting TNF α and INF γ which induces expression of class II MHC on microglial cells allowing local presentation of antigen to the CD4 lymphocytes (Hellendall & Ting 1997, Panek & Benveniste 1995).

Stohlman *et al* (1998) have demonstrated that CD4 lymphocytes are required for maintenance of CD8 T lymphocyte viability within the CNS. Wild type and CD4 T lymphocyte depleted mice were infected with neurotropic JHM strains of mouse

hepatitis virus. In the wild type mice CD4 T lymphocytes entered the brain but remained in the perivascular spaces. In contrast half of the CD8 T lymphocytes present migrated into the parenchyma. In the CD4 depleted mice the CD8 T lymphocyte migration into the brain was not inhibited but the CD8 cells underwent apoptosis, suggesting the CD4 cells were required for CD8 cell viability.

In addition to antigen presentation in the brain, there has been speculation that priming and activation of T lymphocytes may also occur in the brain during viral infection and multiple sclerosis. For this to occur expression of B-7 molecules (CD80 or CD86) is required in addition to MHC on microglial cells (Windhagen *et al* 1995, Soos *et al* 1999).

The role of T lymphocytes in eradication of virus from the CNS has been well documented (reviewed in Dorries 2001). However humoral immunity has also been suggested to play an important role in the control of viral infections in the CNS in several animal model systems.

Lewis rats infected with Borna disease virus develop a non-lytic and persistent CNS infection despite a vigorous immune response. The acute phase of the disease is characterised by infiltration of CD4 and CD8 T lymphocytes, macrophages and NK cells, with a few B lymphocytes also being found. Studies of cytokine expression during the acute phase show expression of IL-1, IL-2, IL-6, TNF α , INF γ . These TH-1 type cytokines are thought to be expressed primarily by CD4 T lymphocytes within the infiltrating mononuclear cells. As the infection progressed into the chronic phase the expression of TH-1 cytokines decreased and was replaced by expression of TH-2 cytokines; IL-4 and TGF β . The switch to TH-2 cytokine production is associated with a change in the composition of infiltrating mononuclear cells, with B lymphocytes becoming the dominant cell type and CD8 T lymphocytes almost disappearing (Hatalski *et al* 1998a, 1998b).

A similar phenomenon occurs during infection of mice with encephalitis inducing Sindbis virus. In the later stages of the inflammatory process there is a prominent B lymphocyte response, and recovery from the infection is dependent on the production of antiviral antibodies (Tyor, Moench & Griffin 1989). The first B lymphocytes taking part in the immune response to Sindbis virus encephalitis express surface IgM; this later switches to expression of IgG or IgA. In athymic mice (lacking mature T lymphocytes) infected with Sindbis virus, the number of B

lymphocytes found in the brain parenchyma in response to the CNS infection is decreased. Those that are present are mostly IgM positive and the switch to IgG or IgA is not seen in these mice (Tyor, Moench & Griffin 1989). This suggests that T lymphocytes, presumably CD4 positive cells, are required to aid the recruitment of B lymphocytes into the brain and also for Ig isotype switching during Sindbis virus encephalitis.

Other CNS viral infections in which antibodies have been shown to be essential components of the immune response include rabies and measles viruses. The transfer of specific antibodies into rats infected with measles virus results in a limited viral gene expression, decreasing the severity of infection. In severe combined immunodeficient (SCID) mice (which lack both B and T lymphocytes) infected with rabies virus, the transfer of rabies specific antibodies leads to clearance of the virus from CNS cells (Hatalski *et al* 1998a).

The preceding data demonstrate that both the cellular and humoral arms of the immune system play important roles in control or eradication of viral CNS infections.

1.3.2 Lymphocyte transmigration into the CNS

In recent years a significant amount of research has been undertaken to elucidate the mechanisms by which lymphocytes (particularly T lymphocytes) home to the CNS (and other tissues) and cross the BBB. As yet there is no evidence of CNS specific homing signals or adhesion molecules displayed on either lymphocyte cell surfaces or by BMVECs. There are however data demonstrating at least parts of the process of T lymphocyte transmigration from the blood into the brain parenchyma. This is summarised in figure 9 (see Pryce, Male & Sarkar 1991 and Hickey 1999 for review of leukocyte trafficking in the CNS).

1.3.3 Effects of drugs on the brain

The cohort of HIV brains used in this study were mainly from the Edinburgh brain bank. Edinburgh has a particularly high incidence of HIV amongst its population of IDU, and many of the cases included in this study were IDU. Therefore it is important to consider the effects of drug use on the brain as well as possible interactions between HIV and intravenous (IV) drugs.

Figure 9 legend:

Step1: Tethering

As cells pass through the terminal capillaries into venules their transit speed slows and the shear force of blood flow decreases allowing those cells expressing appropriate adhesion molecules to tether to the endothelium, providing it to is expressing the correct adhesion molecules.

Step 2: Rolling adhsion

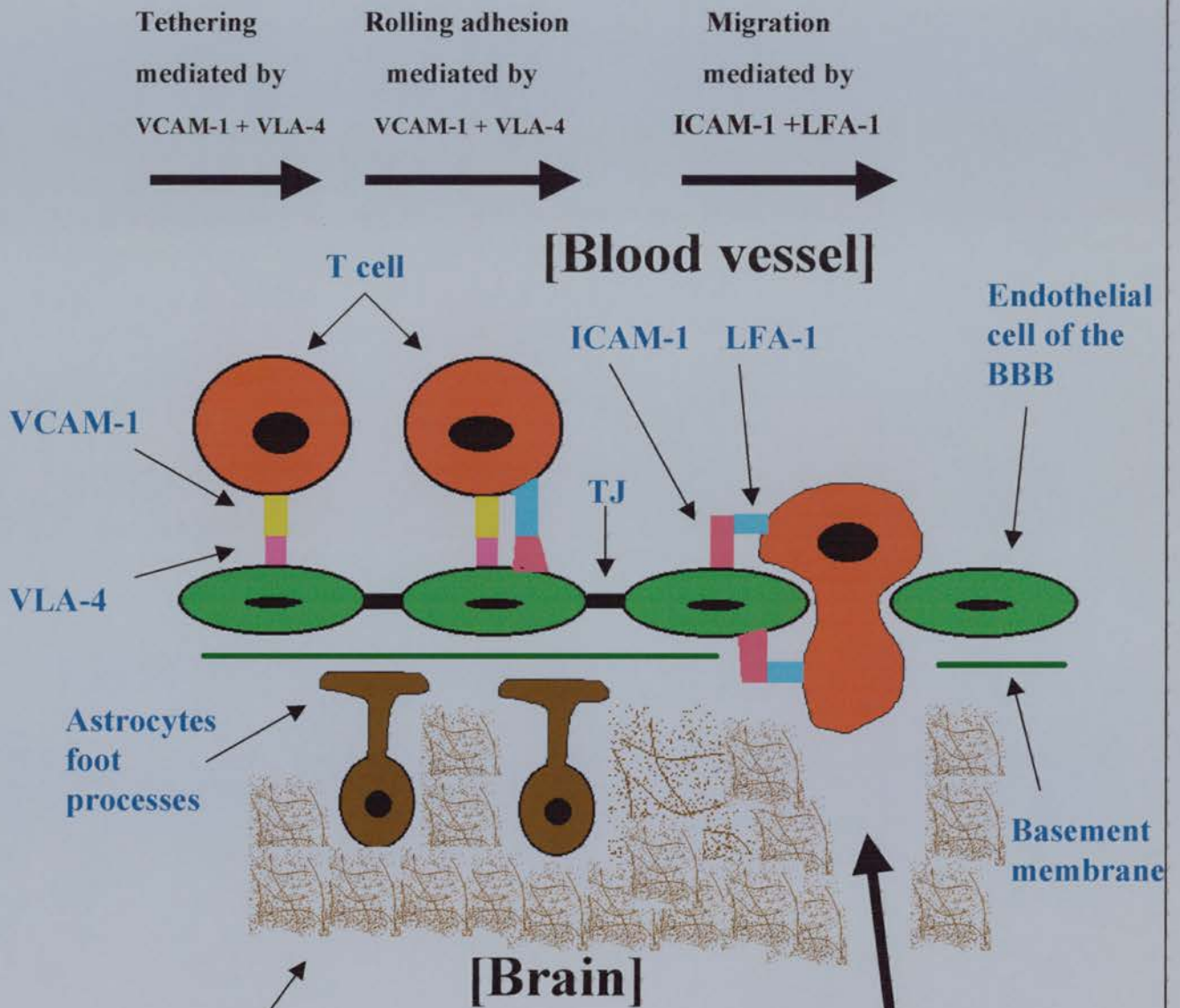
Tethering of the cell to the endothelium allows the cell to role along the endothelium in constant contact.

Step 3: Migration

At areas of endothelium expressing the correct migratory signals, or leaky areas of the BBB in disease, cells can migrate through the barrier into the tissue. This requires further expression of adhesion molecules and release of matrix metalloproteinases which degrade the matrix protein network behind the endothelium allowing passage of the immune cell into the tissue.

The process of transmigration of lymphocytes from the blood into brain is controlled by the expression of adhesion molecules on the endothelial cells, astrocytes and lymphocytes, in addition to the release of chemoattractants by cells within the brain.

Figure 9: T cell transendothelial migration through the BBB into the brain perivascular space



Degradation of matrix proteins by metalloproteinase-9 secreted by T cell

Key:

- VCAM-1 Vascular cell adhesion molecule -1 (CD106)
- ICAM-1 Intercellular adhesion molecule -1 (CD54)
- VLA-4 Very late antigen -4 (CD49D)
- LFA-1 Leucocyte function associated protein -1 (CD11a)
- TJ Tight junction

The literature concerning the effects of drugs and drug addiction on the BBB is surprisingly small. In vitro models of the BBB exposed to opiates have increased permeability to sugar and fluid, suggesting a partial disruption of the BBB. Opiates could act directly on the BBB or act indirectly by releasing histamine or cytokines (Banks 1999). Fiala *et al* (1998) showed that cocaine increases both monocyte migration across in vitro BBB models and upregulates expression of adhesion molecules on brain endothelial cells; most notably intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and platelet/endothelial cell adhesion molecule-1 (PECAM-1). Gan *et al* (1999) have also demonstrated an up-regulation of adhesion molecules on brain endothelial cells in conjunction with increased leukocyte migration in response to exposure to cocaine. Morphine has been shown to decrease the expression of IL-8 and macrophage inflammatory protein 1 β (MIP1 β) while increasing expression of their receptors (CCR3 and CCR5) in astrocytes (Mahajan *et al* 2002). The effects of this may be to enhance the susceptibility of the CNS to HIV infection. HIV utilises several co-receptors in order to enter cells including CCR5 and CCR3, expression of the normal ligand for these receptors (IL-8 and MIP1 β) provides competition for receptor binding sites preventing HIV binding and entering cells, hence the effects of morphine may increase HIV entry to CNS cells.

Within the brain tissue, Tomlinson *et al* (1999) have shown an increase in the number of activated, CD68 positive and MHC II positive, microglial cells in both HIV negative and HIV positive (pre-AIDS) IDU. However, they found no significant difference between IDU and non-IDU in the number of astrocytes or T lymphocytes present within the brain unless HIV was also present.

While the effects of drug abuse on the brain are of importance, of equal importance is the failure, or partial failure, of many therapeutic drugs used in the treatment of HIV and other conditions to be effective within the CNS. The BBB presents a major obstacle to the treatment of AIDS associated CNS conditions in terms of its function in isolating the brain from systemic therapeutic drug regimes. This may result in the CNS becoming a reservoir for HIV and allows the virus to evade the effective systemic treatments now being administered. While it has generally been assumed that many of the drugs used to treat HIV have not been able to penetrate the BBB (Aweeka *et al* 1999), there is some evidence to suggest that

penetration is less of a problem than the highly efficient efflux systems, which remove the drugs from the brain before they can induce any therapeutic effects (Banks 1999).

1.4 HIV

HIV is the casual agent of AIDS; it is a retrovirus with a 9.7kb RNA genome, which was discovered in 1981 when the disease began to be evident in the United States and Europe. The virus itself however has probably been circulating in the human population for some time before this and evidence exists to show it was present in humans in central Africa in the 1950's (Marx, Alcabes & Drucker 2001, Sharp *et al* 2001). Two forms of HIV are found in humans; HIV-1 and HIV-2. The two viruses have a similar genome structure however they differ by more than 45% at the nucleotide sequence level (Levine 1992). They also differ in their distribution and pathogenicity, with HIV-1 found throughout the world in contrast to the less pathogenic HIV-2 which is more localised in Western Africa. Although rare cases of HIV-2 are found in the Europe and North America, the overwhelming majority of infections are HIV-1. All of the HIV positive cases studied in this thesis were infected with HIV-1 and therefore HIV-2 will not be discussed further and the abbreviation HIV will be used for HIV-1. Figure 10a shows a schematic diagram of HIV and its constituent components, while the layout of HIV genes is given in figure 10b. The functions of each viral gene are given in table 2.

HIV has several modes of transmission, mostly via contact with blood. The main routes are:

- Homosexual sex (particularly receptive sex)
- Heterosexual sex
- Mother to child either via blood during birth, transplacental infection or via breast milk (30% of untreated mothers pass the virus to their child)
- Needle sharing amongst IDU
- Use of contaminated blood products, e.g. Factor VIII required by haemophiliacs, prior to 1985 when blood screening for HIV started to be introduced in western countries.

In the worldwide epidemic it is estimated that 75% of HIV transmission is due to heterosexual contact, with the chances of transmission and infection being increased

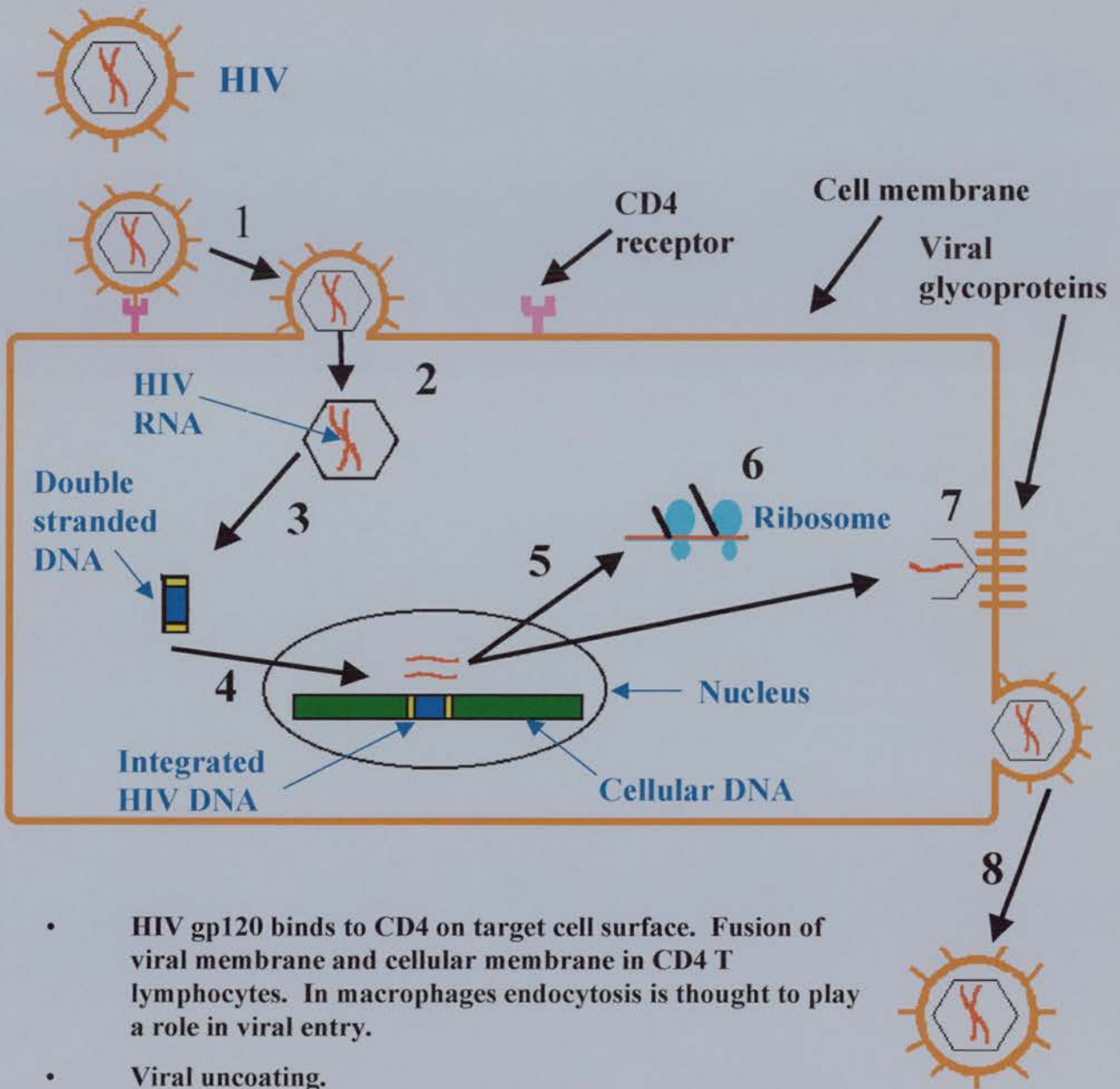
Table 2: HIV genes coding for proteins, their function and cellular/virion location

Gene	Protein	Function	Location	Required for replication
gag	Pr55 ^{gag}	Polyprotein precursor for viron core proteins MA (p17), CA(p24), NC(p9), p7	Viron nucleocapsid	Yes
pol	Pr160 ^{gag-pol}	Polyprotein precursor for viron enzymes: protease (p10), reverse transcriptase (p64), RNase H (p51/66), integrase (p32).	Viron nucleocapsid	Yes
vif	p23	Viral infectivity factor (function unresolved)	Cell cytoplasm	No
vpr	p15	Viron protein (function unresolved)	Virion	No
tat	p14	Transcriptional transactivator, binds tat response element and cell factors (initiation and elongation of viral transcripts)	Primarily in cell nucleus	No
rev	p19	Posttranscriptional transactivator, binds rev response element and cell factors (splicing, transport and translation of viral mRNA).	Primarily in cell nucleus	Yes
vpu	p16	Influences virus release, augments turnover of CD4 antigen.	Integral cell membrane protein	No
env	gp160	Precursor for envelope glycoprotein: gp120 (CD4 receptor binding), gp41 (membrane fusion).	Virion envelope plasma protein	Yes
nef	p27	Negative effector. Down regulates CD4 receptor, increases T-cell activation by increasing IL-2 production (Schibeci <i>et al</i> 2000) and protects cells against p53 mediated apoptosis (Greenway <i>et al</i> 2002), enhances viron infectivity.	Cell cytoplasm, plasma membrane	No
Data taken from Fields Virology 1996				

by the presence of other sexually transmitted diseases (STD) such as syphilis, gonorrhoea or genital herpes (Dukers *et al* 2002). Sores and ulcers caused by STD's increase the likelihood of blood contact and make access easier for HIV, while the ongoing local infection ensures the presence of large numbers of inflammatory cells in the area, including macrophages and CD4 T lymphocyte; the main target cell types for HIV.

Once within the body HIV infects its target cells via binding of the viral gp120 surface protein to CD4 molecules present on the surface of T helper lymphocytes and cells of the macrophage lineage. Entry requires binding of HIV via additional co-receptors (CXCR-4 on CD4 T lymphocytes and CCR5 for macrophage lineage cells, both of which are chemokine receptors present on the cell surface), before the virus fuses with the cell membrane and enters its host cell. When HIV enters a cell its RNA is reverse transcribed to DNA by a virally encoded enzyme reverse transcriptase (RT). The double stranded DNA is then integrated into the host genome and is therefore replicated with the cellular genome. Viral replication results in production of new virions and eventually lysis of the cell. In addition to productive infection HIV is capable of entering a latent state in which the viral DNA is preserved in the host genome but no virus progeny or viral proteins are produced. The virus can remain dormant in this state for long periods of time until some regulatory signal initiates viral gene expression and hence viron production. Figure 11 shows a schematic diagram of the entry of HIV to a susceptible cell. Langerhans cells (LC) are a specialized type of DC present in mucosal tissues which migrate from mucosal sites to lymphoid tissue after activation. Infection of LC at mucosal sites therefore leads to HIV being carried to the T cell areas of lymphoid tissues giving the virus easy access to its primary target cell type; CD4 T lymphocytes. Lytic infection is quickly established and the virus is able to enter CD4 T lymphocytes, replicate and lyse the cell within 24 hours. In an ongoing infection HIV is able to produce 10^{11} new virions per day and can destroy up to 10^9 CD4 T lymphocytes per day (Ho *et al* 1995). At the same time as the virus is spreading by lytic infection some virions establish latency in a pool of CD4 T lymphocytes, probably before immune responses to HIV are mounted by the host (Soudeyns & Pantaleo 1999). These latently infected cells are crucial for the long term survival of the virus, as the virus remains hidden within them from both the immune system and anti-retroviral therapies.

Figure 11: HIV cell entry and replication



- HIV gp120 binds to CD4 on target cell surface. Fusion of viral membrane and cellular membrane in CD4 T lymphocytes. In macrophages endocytosis is thought to play a role in viral entry.
- Viral uncoating.
- Reverse transcription of viral RNA to double stranded DNA
- Double stranded DNA is transported to the nucleus where it integrates into the host cell genome
- Transcription of viral DNA to mRNA
- Translation of viral mRNA by cellular machinery to produce viral proteins.
- Viral proteins accumulate at the cell surface and viral capsid is assembled
- Virus buds out of cell and is released.

1.4.1 Clinical features of HIV

The majority of HIV seroconversions are asymptomatic. In a small number of individuals, a non-specific mononucleosis-type illness occurs 4-8 weeks after exposure, lasting approximately 3 weeks. Symptoms include: fever, lethargy, lymphadenopathy, sore throat, myalgia, maculopapular rash. Neurological symptoms including headache, photophobia, and neuropathy are common, and occasional cases of encephalitis are also seen. Following recovery, the duration of the asymptomatic period varies between patients with a mean of 10 years.

In the absence of effective treatment the change from asymptomatic pre-AIDS to AIDS is marked by the development of opportunistic infections and the onset of one or more AIDS defining illness given below, and/or a decrease in the patient's CD4 T lymphocyte count below 200 cells/ml.

AIDS defining illnesses: (in the absence of other causes of immunodeficiency)

(Alphabetical order)

- Bacterial chest infection (recurrent in 12 month period)
- Candidiasis
- Cervical carcinoma
- Cytomegalovirus disease (not in liver, spleen, or lymph nodes)
- Encephalopathy (dementia) due to HIV
- Herpes simplex ulcers for 1 month or bronchitis, pneumonitis, or oesophagitis
- Kaposi's sarcoma
- Lymphoma
- Mycobacteriosis, pulmonary tuberculosis
- Pneumocystis carinii pneumonia
- Progressive multifocal leucoencephalopathy
- Salmonella septicemia (recurrent)
- Toxoplasmosis of the brain
- Wasting syndrome due to HIV (weight loss greater than 10% with no other identifiable cause)
- (CD4 T lymphocyte count below 200 cells/ml)

(Information taken from Kumar and Clark 1995)

1.4.2 Treatment of HIV

Three types of drugs have been used to combat the effects of HIV.

- 1- Nucleoside analogue reverse transcriptase (RT) inhibitors (e.g. Zidovudine). These nucleotides are incorporated into the expanding DNA chain and stop synthesis of DNA by terminating further expansion of the chain.
- 2- Non-nucleoside RT inhibitors (e.g. Nevirapine) interfere with the viral polymerase thereby inhibiting replication.
- 3- Protease inhibitors (e.g. Ritonavir) prevent the viral protease from cleaving precursor gag and gag-pol proteins into their active form.

Individually these types of drug are effective only for short periods of time before resistant viral strains appear. HAART involves the use of at least 3 anti-retroviral drugs in combination. It is now the standard treatment of choice in countries able to afford the therapy. It has been shown to decrease the morbidity and mortality in AIDS and prevent further decline of CD4 T lymphocyte counts as well as decreasing plasma loads of HIV (Palella *et al* 1998, Detels *et al* 1998).

1.4.3 Host response to HIV

HIV poses an unparalleled challenge to the immune system. Not only is the virus able to evade the immune system by entering a latent state, but it infects and kills cells (CD4 T lymphocytes) which are critical in the control and regulation of immune responses. In addition the virus has a huge replicative potential and a high mutation rate that allows it to evolve quickly, again allowing viral evasion of both the immune system and targeted vaccines and drugs.

1.4.3.1 Humoral immunity to HIV

HIV infected individuals produce a high concentration of antibodies against many viral peptides throughout life. However most of the antibody responses have weak anti-viral effects. Two primary reasons for the ineffectiveness of antibody response to HIV are:

- High mutation rate of HIV proteins allows virus to escape antibody responses through antigenic variation.

- Glycosylation of the viral envelope protein may mask important antigens from recognition by antibody making neutralising antibodies ineffective.

Not only are humoral anti-HIV responses largely ineffective but they may inadvertently aid viral entry to certain cell types via antibody dependent enhancement (ADE). This involves non-neutralising antibodies to HIV binding to the virus and labelling it for phagocytosis by macrophages or follicular dendritic cells. The antibody virus complex binds via the Fc portion of the antibody to Fc receptors on the phagocytic cell surface (Levy 1990) and the complex is then internalised, thus providing viral entry to the cell without the need for binding to CD4.

1.4.3.2 Cellular immunity to HIV

Anti-HIV CD8 cytotoxic T lymphocytes (CTL) provide the most potent immune response to HIV. CTLs specific for HIV appear within weeks of infection and are maintained until the onset of AIDS. CTLs control HIV by two means; firstly by recognising and killing infected cells displaying viral peptides on their cell surface in conjunction with MHC I molecules, and secondly by secretion of β -chemokines e.g. MIP 1 α , MIP 1 β , RANTES which competitively bind to HIV co-receptors thereby blocking viral entry to cells. The importance of CD8 CTLs is demonstrated by the control of initial viraemia in acutely infected individuals when HIV specific CTLs appear in the blood (Koup *et al* 1994).

The hallmark of HIV infection is a progressive depletion of CD4 T lymphocytes. In HIV infected individuals who do not progress to AIDS (AIDS non-progressors) there are generally strong CD8 CTL response, which correlate with strong CD4 T helper cell responses (Mollet *et al* 2000, Picker & Maino 2000). Immunological control of HIV is therefore dependent on both CD4 T helper cell and CD8 CTL responses.

1.4.4 Effects of HIV on the immune system

1.4.4.1 Effects of HIV infection on B lymphocytes

Abnormalities in the control of B lymphocytes in HIV infected individuals are demonstrated by polyclonal activation (Birn, Redfield & Tosato 1986), hypergammaglobulinaemia (Nath *et al* 1987), a decreased response to T cell

independent antigens and production of auto antibodies. B lymphocytes from HIV patients spontaneously secrete IgG and IgM at high levels, produce increased levels of cytokines, particularly IL-6 and IL-10, show increased expression of activation markers (Martinez-Maza *et al* 1987), and decreased expression of MHC class II (Ginaldi *et al* 1998). Increases in cytokines such as IL-6 and IL-10 induce B lymphocyte proliferation and differentiation which results in an increased number of circulating activated B lymphocytes and subsequent decreased number of resting B lymphocytes (Martinez-Maza *et al* 1987). These B lymphocyte perturbations precede significant depletion of CD4 T lymphocytes (Miedema *et al* 1988) and are not corrected in vitro by the addition of normal T lymphocytes (Terpstra *et al* 1989), suggesting that intrinsic B lymphocyte defects occur during HIV infection.

VH3 B lymphocytes (the largest Ig variable heavy chain gene family) have been shown to be decreased during late stages of HIV (Berberian *et al* 1991 and 1994, Scamarra *et al* 2000). VH3 antibodies are particularly important in the clearance of bacteria from the body and therefore loss of this group would further predispose HIV patients to opportunistic infections. HIV gp120 has been shown to bind to VH3 Ig at a site other than the normal Ig antigen binding site (Goodlick *et al* 1995). Binding of gp120 induces Ig production in VH3 cells and eventually their depletion (Berberian *et al* 1993). This has led to the suggestion that gp120 acts as a superantigen for B lymphocytes (Muller & Kohler 1997, Karray & Zouali 1997), although the method of VH3 clonal deletion is as yet unclear. In addition to the decreased usage of VH3 receptor family genes, there is an increase in the use of VH1 and VH4 genes (David *et al* 1995, David *et al* 1996, Wisnewski, Cavacini & Posner 1996). gp41 has also been shown to induce polyclonal B lymphocyte activation. (Chirmule *et al* 1990). Recently it has been suggested that HIV Tat may affect the normal B lymphocyte differentiation process and increase proliferation of germinal centre B lymphocytes which could predispose to AIDS associated B cell lymphoma (Lefevre *et al* 1999).

In addition to the superantigen effect of gp120 and increased B cell proliferation induced by other HIV proteins, a combination of decreased T cell control and/or increased cytokine production, particularly IL-4, IL-6 and IL-10 can further stimulate B lymphocyte proliferation. Uncontrolled B cell proliferation, particularly in the presence of oncogenic viruses such as EBV (see section 1.5), may lead to malignant transformation of cells.

1.4.4.2 Effects of HIV infection on other immune cell types

CD4 T helper cells are most affected by HIV. There is a gradual decrease in cell numbers throughout the course of HIV infection due to both indirect and direct cytopathic effects of HIV. Direct viral cytopathic effects include cell lysis during viral replication, altered cell membrane permeability in infected cells, induction of apoptosis by binding of HIV gp120 to the cell surface (Banda *et al* 1992). In addition, gp120 released from infected cells can bind to CD4 on uninfected cells which can lead to their recognition by CD8 CTLs or NK cells and subsequently their destruction. Finally, CD4 T helper cells can be damaged indirectly by cytokine toxicity caused by the induction of aberrant cytokine production by HIV. CD4 T helper cells from HIV infected individuals show decreased proliferative responses to recall antigens and decreased production of IL-2 (required for T cell activation) (Creemers *et al* 1988, Chou *et al* 1994).

The total number of circulating CD4 T lymphocytes is determined by the number of cells lost in the periphery and the production of new CD4 T lymphocytes in the bone marrow. In addition to the effects of HIV on circulating T lymphocytes, HIV also disrupts the production of new T lymphocytes in the bone marrow. As with circulating CD4 T lymphocytes, HIV has direct and indirect effects on production of new T lymphocytes by haematopoietic progenitor cells. Direct effects result from infection of progenitor cells and subsequent cell death. Indirect effects result from immunosuppression which allows opportunistic infections such as CMV within the bone marrow leading to further disruption of blood cell production. Production of other blood cells by the bone marrow are often disrupted in untreated HIV patients resulting in anaemia, neutropenia and thrombocytopenia in addition to lymphopenia. These cytopenias are usually reversed with effective anti-retroviral therapy.

CD8 CTLs from HIV infected individuals show decreased antigen specific proliferation and decreased antigen specific cytotoxic activity, particularly in late stage AIDS. Prior to the onset of AIDS there is loss of CTL responses to HIV antigens. The loss in CD8 CTL activity has until recently been attributed to aberrant cytokine production and loss of CD4 T helper cell stimuli. However in recent years there have been reports of HIV infection of CD8 lymphocytes (Saha *et al* 2001, Imlach *et al* 2001). Imlach *et al* (2001) have demonstrated that activated CD8

lymphocytes can co-express CD4, allowing HIV infection. They demonstrated that CD4, CD8 double positive lymphocytes accounted for between 3 and 72% of total HIV pro-viral load in peripheral blood mononuclear cells from 5 HIV positive individuals. The specific targeting of responding CD8 T cells may provide a functional explanation for the previously observed impairment of CTL function disproportionate to their numerical decline in AIDS.

Macrophages isolated from HIV infected individuals demonstrate a decrease in antigen presenting ability, decreased responses to chemoattractants and aberrant cytokine production, in comparison to normal macrophages (Nath *et al* 1999). Dendritic cells also demonstrate a decreased capacity to act as antigen presenting cells, as well as a decreased ability to stimulate primary T lymphocyte proliferation and to increase antibody production in B lymphocytes.

1.4.4.3 Effects of HIV infection on lymphoid tissue

A sub-group of asymptomatic HIV infected patients develop persistent generalised lymphadenopathy (PGL) before the onset of symptomatic HIV disease and AIDS defining illnesses. Lymph nodes are enlarged early in HIV infection reflecting the increase in number of polyclonal B lymphocytes being activated and proliferating. The normal architecture of the lymph node is slowly lost as CD4 T lymphocytes are depleted, with lymph nodes becoming fibrous. In addition to CD4 T lymphocyte loss there is destruction of FDC due either to direct HIV infection (Spiegel *et al* 1992) or to toxic aberrant cytokine production by cells activated in response to HIV (Emilie *et al* 1990). Loss of FDC leads to a decrease in antigen presentation and hence the inability of the immune system to respond to HIV, while HIV infection of DC has been shown to block antigen presentation (Knight & Macatonia 1991).

1.4.4.4 Effects of HIV infection on cytokine production

HIV infection affects production of many cytokines in both peripheral systems and the CNS. Of fundamental importance to the pathogenesis of B lymphocyte proliferation and subsequent development of B cell lymphoma is the change in balance of TH1 and TH2 cytokines seen in HIV infection. In addition to the increased production of TH2 cytokine IL-6 by monocytes, described earlier, the other

major TH2 cytokines are also increased (Klein *et al* 1997). Levels of IL-4 and IL-10 both begin to rise after seroconversion, peaking at the onset of AIDS. In contrast TH1 cytokines e.g. IL-2 begin to fall soon after infection and continue to decrease until death (Levy 1993). The effects of these changes in cytokine production are reflected in the immune response mounted by HIV infected individuals being skewed towards TH2 (humoral) responses (Barcellini *et al* 1994, Clerici & Shearer 1993), which as discussed earlier are less effective at combating HIV than cellular responses and may predispose to B cell lymphoma formation.

1.4.5 Effects of HIV on the brain

80% of untreated and 60% of treated HIV infected individuals have CNS pathology at autopsy, with 20-30% of terminally ill AIDS patients having AIDS dementia (Jellinger *et al* 2000). The CNS of HIV infected individuals is particularly vulnerable to opportunistic infections (including cytomegalovirus and toxoplasma), progressive multifocal leucoencephalopathy and high grade B cell lymphomas are also common (Bell 1998a). In addition to opportunistic infections, pathological evidence of HIV encephalitis (HIVE) is found in 40% of AIDS patients (Bell *et al* 1998b). A number of autopsy studies have demonstrated damage and induction of apoptosis in neurons of HIV infected individuals, in addition to axonal damage (Hill *et al* 1993, An *et al* 1997, Gray *et al* 2000).

Although in pre-AIDS no evidence of HIVE, opportunistic infections or lymphoma has been found at autopsy, there is often a low grade T lymphocyte infiltration, particularly in the meninges and around blood vessels. This T lymphocyte infiltration is thought to be a response to early invasion of the CNS by HIV, although the exact timing of viral entry to the CNS is as yet unknown (Bell *et al* 1998a).

1.4.5.1 HIV entry to the CNS

The most plausible method of HIV entry to the CNS is via infected macrophages/monocytes migrating from the peripheral blood across the BBB. HIV infected CD4 T lymphocytes migrating into the CNS may also potentially introduce HIV to the brain. However infected macrophages/monocytes are a more likely source

of persistent viral infection as strains of HIV capable of infecting monocytes (M-tropic strains) are also able to infect the resident brain microglial population, unlike the T-tropic strains found in CD4 T cells (Gorry *et al* 2001). Monocytes infected with HIV have been shown to increase expression of adhesion molecules, which increases monocyte aggregation and in addition increases binding of monocytes to endothelial cells. There is also increased expression of matrix metalloproteinase 9 (MMP9), an enzyme required for matrix degradation during transendothelial migration (Nottet *et al* 1996). The increased adhesive properties of HIV infected monocytes to endothelium can be replicated by treatment of monocytes with HIV tat protein, suggesting this is responsible for the increased potential to bind and transmigrate (Lafrenie *et al* 1996a, Lafrenie *et al* 1996b). HIV infection within the brain induces increasing transendothelial migration of monocytes into the brain and secretion of pro-inflammatory cytokines which are neurotoxic (Nottet *et al* 1999). Increased transendothelial migration of monocytes is mediated by an increase in expression of adhesion molecules in astrocytes and endothelial cells of the BBB in response to either HIV infection of these cells or cytokines released by HIV infected microglia (Woodman *et al* 1999). In addition HIV infected microglia release a monocyte chemoattractant monocyte chemoattractant protein-1 (MCP-1) (Mengozi *et al* 1999).

While it is entirely plausible that monocyte derived cells carry HIV into the brain and initiate infection of the CNS, there has to be an initial stimulus to attract monocytes into the brain in the first place. It is therefore unclear whether HIV gains access via another route to the brain and then the initiation of HIV infection draws monocytes/macrophages into the brain. Alternatively some non-HIV stimulus may attract HIV infected monocytes/macrophages into the brain, thus initiating HIV infection of the CNS.

An alternative method of cell free virus entry to the CNS has also been proposed (Banks *et al* 2001). Gp120, an HIV surface glycoprotein, can act as a lectin at the BBB inducing virus binding to the endothelium and adsorptive endocytosis into endothelial cells. HIV is not destroyed in the lysosomes of the endothelial cells and therefore could escape and enter the cytoplasm and hence eventually spread to other brain cell types. There have been several reports of HIV infection of brain endothelial cells (Moses 1997, Stins *et al* 2001). However there remains doubt over the reproducibility of these studies. Nottet *et al* (1996) failed to infect BMVECs in vitro with either cell free virus or infected monocytes. This method could provide entry for

cell free virus to the brain and also enhance entry of virally infected circulating immune cells expressing gp120 on their surface.

1.4.5.2 HIV encephalitis (HIVE)

The hallmark of HIVE is productive viral replication in brain macrophages and microglia, with the formation of multinucleated giant cells (Persidsky *et al* 2000), which are found predominantly in the central white matter and deep grey matter of the brain (Bell 1998a). These cells express both CD4 and the β -chemokine receptor CCR5, making them permissive to macrophage tropic strains of HIV, although microglia normally only show low level expression of CD4 (Choe *et al* 1996, He *et al* 1997).

Several studies have shown the presence of HIV in astrocytes in brain tissue sections using in situ hybridisation with probes specific for HIV nef mRNA (Ranki *et al* 1995). However there is a consistent lack of evidence of structural HIV protein expression indicative of productive HIV infection in these cells. A restricted form of HIV infection of astrocytes has also been described in vitro (Brack-Werner *et al* 1992) despite the absence of surface CD4 expression. In HIV replication, structural proteins are produced from incompletely spliced and unspliced mRNAs which retain a specific recognition element for the HIV Rev regulatory factor (Rev response element (RRE)). Rev binds to RRE mediating nuclear export of these mRNAs and promoting their translation. In the absence of Rev only a very low level production of structural viral proteins occurs. Ludwig *et al* (1999) have shown that in astrocytes infected with HIV there is a diminished capacity for Rev to transactivate synthesis of HIV structural proteins and that an accumulation of Rev in the cytoplasm of these cells occurs. The significance of this restricted form of viral infection is as yet unclear.

Other neuropathological features seen in addition to giant cells in HIVE include microglial nodules, macrophage infiltrations, white matter atrophy, reactive gliosis, myelin pallor and slight neuronal loss in the basal ganglia (Petito *et al* 1986). Interestingly HIVE is more common in drug users than homosexual AIDS patients, with 59% of AIDS drug users developing HIVE compared to 15% of AIDS homosexuals in one study (Bell *et al* 1996).

The presence of HIVE does not correlate with the clinical signs and symptoms of HIV dementia. Patients may have evidence of HIVE at autopsy without having shown clinical signs of HIV dementia during life, and giant cells associated with HIVE are present in only half of patients with clinical signs of dementia (Wiley & Achim 1994).

The introduction of effective therapy for HIV has changed incidences and the spectrum of diseases HIV positive individuals develop. With respect to the CNS, HAART has been shown to have beneficial therapeutic effects on cognitive processing in HIV infected patients and is superior to Zidovudine monotherapy or dual anti-retroviral therapy (Husstedt *et al* 2002, Chang *et al* 1999). The introduction of HAART has also decreased the incidence of AIDS dementia complex (ADC), however a recent study in Australia (Dore *et al* 1999) has shown that the decrease in incidence of ADC does not parallel the larger decrease seen in other AIDS defining illnesses since the introduction of HAART. As regards the incidence of non-Hodgkin's lymphoma (NHL) since the introduction of HAART, several studies have indicated decreases in the incidence of NHL (Kirk *et al* 2001, Chow *et al* 2001, Rabkin 2001, Gates & Kaplan 2002).

1.4.5.3 HIV induced Blood brain barrier (BBB) damage

BBB disruption has been demonstrated in both HIVE in humans (Dallasta *et al* 1999) and in animal model systems utilising simian immunodeficiency virus encephalitis (SIVE) in macaque monkeys (Luabeya *et al* 2000). Disruption of the BBB was demonstrated by aberrant immuno reactivity of both occludin and ZO-1 tight junction proteins. Additional observations in regions of tight junction disruption included the perivascular extravasation of the plasma protein fibrinogen, and a decrease in the metabolic BBB marker glucose transporter isoform-1 (GLUT-1).

In both HIVE and SIVE, BBB disruption was associated with accumulations of perivascular macrophages (Dallasta *et al* 1999, Luabeya *et al* 2000). It cannot however be ascertained whether these changes in the BBB allow the accumulation of macrophages to occur or if the BBB changes observed occur as a result of accumulating macrophages. HIV infection of microglial cells has been shown to modulate the migration of monocyte/macrophages into the brain. In addition HIV

infected monocytes/macrophages have been shown to down regulate the expression of tight junction proteins at the BBB and also disrupt transport systems in BMVEC (Persidsky *et al* 2000).

1.4.5.4 Neurotoxicity in HIV infection

Neurodegenerative changes in AIDS include dendritic abnormalities, neuronal loss and increased vascular permeability (Petito *et al* 1999). Neuronal loss from the cerebral cortex in AIDS is not restricted to cases of HIVE and it does not correlate well with HIV dementia. The best correlate of HIV dementia is the presence of activated macrophages in brain tissue (Tyor *et al* 1995). The observed neurodegenerative changes, particularly neuronal loss, are thought to be an indirect result of HIV infection of the brain as neuronal cells are not permissive for HIV infection. The infected cells of the brain, macrophages and microglia, are thought to cause indirect damage to neuronal cells via the release of neurotoxic cytokines and chemicals such as tumour necrosis factor (TNF) α , stromal cell derived factor (SDF)-1 and quinolinic acid (Giulian *et al* 1990, Esser *et al* 1991, Smith *et al* 2001). TNF α is produced by activated microglial cells and reactive astrocytes and has been proposed as a neurotoxin that may be responsible for the neuronal apoptosis seen in AIDS brains (Petito *et al* 1999). TNF α mediates signalling events such as NF- κ B activation, calcium mobilisation and release of oxidative oxygen species which have been associated with neurotoxicity. In addition TNF α upregulates expression of IL-6 (Aliosi *et al* 1992). The β chemokine SDF-1 has been shown to be produced by astrocytes in culture (Bajetto *et al* 1999). SDF-1 can bind to CXCR4 receptors present on neurones which in turn can lead to neuronal apoptosis. Activated macrophages produce quinolinic acid, a neurotoxin, in several inflammatory brain diseases including AIDS. HIV proteins Nef, Tat and gp41 have been shown in vitro to increase production of quinolinic acid by macrophages, suggesting that some of the neurotoxic effects of Nef, Tat and gp41 may be mediated by this route (Smith *et al* 2001).

In addition to toxic cytokine release induced by HIV infection many of the proteins produced by HIV are also neurotoxic. HIV gp120 can bind to CXCR4 receptors present on neurones again inducing neuronal apoptosis (Kaiser, Offerman & Lipton 1990, Petito *et al* 1999). HIV Vpr protein has also been shown in vitro to be

neurotoxic, via induction of apoptosis in neuronal cells in a manner similar to gp120 (Patel *et al* 2002). HIV Tat has been shown to bind to the cell membrane of rat glioma cells and murine neuroblastoma cells inducing a large depolarisation of the cell membrane, which modifies cell permeability (Sabatier *et al* 1991). Tat has also been shown to induce TNF α primarily in macrophages but also in astrocytic cells (Chen *et al* 1997), inducing effects similar to those described above.

1.5 Epstein Barr Virus (EBV)

EBV is an oncogenic DNA double stranded gamma herpes virus with a 172kb linear genome. It was discovered in 1964 in B lymphocytes cultured from an endemic African Burkitt's lymphoma (Epstein 1964). The virus latently infects circulating B lymphocytes, with viral DNA being maintained as an episome, replicated together with host cell DNA and stably inherited in daughter cells. Viral infection of B lymphocytes is mediated by the binding of the viral envelope glycoprotein gp340 to the cellular CD21 complement receptor (CR2) (Nemerow *et al* 1987).

EBV persistently infects over 90% of the world's adult population (reviewed in Crawford 2001). Infection is normally sub-clinical, however under certain circumstances EBV can cause disease; particularly, although not exclusively, in the immunosuppressed where there are strong aetiological links between EBV and several tumour types (Beral *et al* 1991, Neri *et al* 1991, Hamilton-Dutoit *et al* 1993). Spread of EBV between individuals occurs through close oral contact (Yao, Rickinson & Epstein 1985), and primary infection usually occurs as a sub-clinical seroconversion in childhood (See Crawford 2001 for review of EBV biology). Certain individuals, particularly in affluent areas of western countries do not seroconvert until late in adolescence. In these individuals approximately 30-50% suffer from a benign lymphoproliferative disease called infectious mononucleosis (IM) (Steven 1996). IM presents with fever, pharyngitis, generalised lymphadenopathy, fatigue, and in some cases splenomegaly, and hepatomegaly. The disease is characterised by lymphocytosis consisting of atypical lymphocytes which are largely antigen driven oligoclonal CD8 T cells specific for EBV latent and lytic antigens (Callan *et al* 1996). The most probable cause of the symptoms is lymphokines secreted by these T lymphocytes. During IM up to $2000/10^4$ circulating

B lymphocytes are infected with EBV (Laroche *et al* 1995) in comparison to between 5 and 500 per 10^7 B lymphocytes in a healthy carrier (Miyashita *et al* 1995).

Following silent seroconversion or IM a persistent life long infection is established which includes virus production in the throat and release of virus into saliva. Persistent infection is controlled primarily by CD8 cytotoxic T lymphocytes, which are specific for latent and lytic EBV proteins.

In vitro EBV is capable of immortalising B lymphocytes. In vivo EBV is thought to be important in tumourogenesis and shows a variable association with a wide range of tumours including Burkitt's lymphoma, Hodgkin's lymphoma, non-Hodgkin's lymphoma, nasopharyngeal carcinoma and gastric carcinoma. Three types of viral latency pattern have been described expressing combinations of the latent viral genes.

1.5.1 Latent EBV proteins

Table 3 gives the viral genes expressed in each type of latency and the main tumour types with which each latency pattern is associated.

Table 3: EBV latent gene expression in vitro

Latency pattern (tumour types)	Latent genes				
	EBER 1, 2	EBNA-1	EBNA 2, 3a, 3b, 3c, LP	LMP-1	LMP 2a, 2b
I (Burkitt's lymphoma)	+	+	-	-	-
II (Hodgkin's lymphoma, and nasal pharyngeal carcinoma)	+	+	-	+	+
III (B Lymphoproliferative disease)	+	+	+	+	+

Key: EBER (Epstein-Barr viral small RNA), EBNA (Epstein-Barr nuclear antigen), LMP (Latent membrane protein), LP (leader protein)
 + Expression of viral gene
 - No expression of viral gene

EBERs 1 and 2 are untranslated RNAs and are the most abundantly expressed EBV transcripts in latently infected cells. Their function is unknown although Swaminathan, Tomkinson and Keiff (1991) demonstrated that deletion of viral EBERs genes has no effect on growth or immortalisation of B lymphocytes. However studies by Komano *et al* (1999) have suggested that EBERs may contribute to apoptosis resistance in malignant Burkitt's lymphoma cells.

EBNA-1 is the only EBV encoded protein found in all EBV associated malignancies (Klein 1994). Despite the fact that EBNA-1 is antigenic and induces antibody responses (Dillner *et al* 1984), CTL responses are usually absent (Rickinson & Moss 1997). This is due to inhibition of the ubiquitin/proteasome dependant degradation pathway by the glycine-alanine repeat sequence of EBNA-1. Ubiquitin labels proteins within a cell for degradation by proteasomes (cylindrical shaped complex of proteases which degrades protein), and this process is essential for antigen presentation to occur as only small peptide fragments of the original protein can be displayed on MHC. Certain protein N-terminal repeats, including glycine-alanine of EBNA-1, prevent degradation of the protein by proteasomes, therefore preventing fragments of the protein being displayed on class I MHC (Lavitskaya *et al* 1995).

EBNA-1 is essential for maintenance of the viral episome through binding to the EBV origin of replication, and is also responsible for the portioning of progeny EBV genomes to daughter cells during mitosis, through association with the host cell chromosome (Davenport & Pagano 1999). EBNA-1 has been shown to increase activity of recombinase activating genes (RAG) involved in Ig gene V(D)J rearrangements (Srinivas & Sixby 1995). Aberrant V(D)J recombination in EBV infected cells could potentially facilitate translocation of the c-myc oncogene to Ig loci, inducing dysregulation of myc (Klein 1986).

EBNA-2 expression is essential for B lymphocyte immortalisation by EBV. EBNA-2 is expressed early after infection of a B lymphocyte with EBV and upregulates LMP-1 and 2, transactivates a number of cellular genes including CD21, CD23 (Cordier *et al* 1990, Wang *et al* 1991) and the proto-oncogene c-myc (Kaiser *et al* 1999), as well as inducing cytokines including TNF α (a growth promoting cytokine) (Spender *et al* 2001). EBNA-2 can interact with components of the basal transcription machinery but has no intrinsic DNA binding activity. EBNA-2 also suppresses IgM expression in B lymphocytes (Jochner *et al* 1996) and inhibits B lymphocyte differentiation. In coordination with EBNA leader protein (LP) EBNA-2

can induce cell cycle progression from G₀ to G₁ (Sinclair *et al* 1994) inducing cell proliferation. The combined expression of EBNA-2 and EBNA-LP within B lymphocytes induces expression of a G₁ cyclin, cyclin D2. EBNA-2 activates the cyclin D2 promoter resulting in indirect activation of cyclin D2 (Spender *et al* 2001). G₁ cyclins bind to cyclin dependant kinases activating them, which induces DNA synthesis and moves the cell into S-phase of the cell cycle. Therefore EBNA-2 is vital in the process of immortalisation of B lymphocytes by EBV.

EBNA-3a and c are essential for B cell immortalisation by EBV. EBNA-3c has similar properties to cellular pRB, driving the cell cycle through G₁ under conditions which would normally signal growth arrest (Parker *et al* 1996). EBNA-3b upregulates expression of CD40 on B lymphocytes, which is required for B cell activation.

LMP-1 is expressed on the cell surface of infected B lymphocytes, with both protein termini residing in the cytoplasm (Liebowits, Wang & Kieff 1986). LMP-1 is an EBV oncogene and is essential for immortalisation of B lymphocytes (Kaye, Izumi & Kieff 1993). Expression of LMP-1 leads to upregulation of B lymphocyte activation markers (including CD23), adhesion molecules, and induction of DNA synthesis (Peng & Lundgren 1992). It has recently been shown, *in vitro*, to be secreted by infected cells and have a direct immunosuppressive effect through suppression of T lymphocyte activation (Dukers *et al* 2000). LMP-1 aggregates on the cell surface and mimics constitutively active CD40 cellular receptor inducing cell activation. It has also been shown to upregulate NF- κ B responsive genes (Mosialos *et al* 1995) and anti apoptotic genes including Bcl-2 (Rowe *et al* 1994). LMP-1 has diffuse and profound effects on B lymphocyte growth, inducing activation and differentiation through several different pathways (Izumi *et al* 1999), and also upregulation of expression of MMP-9 which aids B lymphocyte entry to tissues (Takeshita *et al* 1999).

In addition to the range of genes expressed during latency, given in table 3 (page 45), there is a family of transcripts derived from the Bam HI-A region of the EBV genome that may also be related to latency. These include the transcripts from the open reading frame BARF0 (Bam HI-A rightward open reading frame 0), the function of which is unknown. Deletion of the region of EBV genome containing this open reading frame has no effect on EBV immortalisation of B lymphocytes *in vitro*. However the consistent expression of these transcripts in EBV associated epithelial

and B cell tumours implies that they may be making a contribution in vivo (Chen *et al* 1999).

1.5.2 EBV persistence

Like all other human herpes viruses, EBV establishes persistent infection in the immunocompetent host. Seropositive healthy individuals harbour low levels of EBV infected B lymphocytes in the circulation, between 5 and 500 per 10^7 B lymphocytes are infected (Miyashita *et al* 1995). The majority of evidence favours B lymphocytes as the site of persistent latent infection, and Thorley-Lawson & Babcock have suggested that EBV utilises normal B lymphocyte biology to enter the long lived B lymphocyte memory compartment, where it can reside long term in a latent state undetected by the immune system (Thorley-Lawson & Babcock 1999). They have suggested that the different latent states it is able to utilise may allow EBV infected B lymphocytes to emulate activities that occur in normal B lymphocytes to allow the virus to enter the memory B lymphocyte compartment (Thorley-Lawson & Babcock 1999).

B lymphocytes activated by antigen enter lymph nodes and proliferate to form germinal centres, each with a clone of antigen specific B lymphocyte (centroblasts). Centroblasts eventually mature into either short lived Ig secreting plasma cells or recirculating long lived memory cells, however this maturation process requires 2 signals. The first is given by antigen binding to the Ig receptor and the second by CD40 on the B lymphocyte surface ligating with its ligand (CD40L) on CD4 T helper cells. Thorley-Lawson & Babcock have suggested that when EBV activated B lymphocytes reach germinal centres a switch from full latent gene expression (latency III), to latency II occurs. In latency II EBNA-1 and LMP 1 and 2a are expressed but not EBNA-2 which inhibits B lymphocyte differentiation (Rowe *et al* 1992). The infected cell can then differentiate into a centroblast, with the virus able to provide the two signals required for further maturation in the form of LMP-1 and LMP-2a expression. These viral proteins can mimic the functions of CD40 and Ig receptor respectively allowing infected cells to mature into plasma cells or memory cells. The process allows infected cells to survive long term in the memory B lymphocyte population where they express a restricted form of latent gene expression. As yet the

exact viral phenotype of these cells is unclear, but LMP2a expression is likely to inhibit further activation and lytic cycle entry.

Occasionally these infected memory B lymphocytes will be activated by antigen and therefore return to the lymph node to form new germinal centres and hence produce more infected short lived plasma cells and infected long lived memory cells, replenishing the circulating population of infected memory cells. The production of antibody in Ig producing cells (plasma cells) has been linked to viral entry into the lytic cycle (Crawford & Ando 1986). Infected plasma cells which home to epithelial sites or tonsil could therefore produce virions that may infect other B lymphocytes at the site and may also allow viral transmission from human to human via the saliva.

1.5.3 Host response to EBV infection

Despite the transforming potential of EBV the virus persists and is maintained as an asymptomatic infection in immunocompetent individuals. The humoral and cellular immune responses to EBV arise during primary infection, and are maintained throughout life. CD8 cytotoxic T lymphocytes are of particular importance in the control of EBV infection. Following primary infection long term memory T lymphocytes against EBV latent and lytic antigens provide immune surveillance to control persistent infection (Murray *et al* 1992). Cytotoxic T lymphocyte responses in healthy seropositive individuals can be detected against all EBNA proteins except EBNA-1 (Murray *et al* 1988 & 1992, Bogedain *et al* 1995, Steven *et al* 1997). Cytotoxic T lymphocytes also play a role in protection against lytic infection, with cytotoxic T lymphocyte directed against lytic immediate early proteins (BZLF-1, BRLF-1) and early lytic proteins (BMLF-1, BMRF-1, BALF-2) commonly found (Steven *et al* 1997). CD4 cytotoxic T lymphocyte responses may also play a role in control of both latent and lytic infection, with CD4 cytotoxic T lymphocyte responses detected against gp340 and EBNA-1 and 2 (Khana *et al* 1997, Pothen, Rickert & Pearson 1991, Paludan 2002).

EBV also induces a humoral immune response. During the acute phase of IM the humoral response is directed primarily towards the viral antigens of the lytic cycle, while antibody responses to EBNA-1 and EBNA-2 are delayed until the recovery period. In healthy seropositive individuals serum antibodies against lytic

antigens can be detected in addition to anti-EBNA-1 antibodies, but anti-EBNA-2, 3a, 3b, 3c, LP antibodies are not normally detected (Khanna, Burrows & Moss 1995).

1.5.4 EBV associated malignancies

EBV is associated with various proliferative and malignant diseases which are listed in table 4.

Table 4: EBV associated tumours

Disease	Approximate association with EBV	EBV proteins expressed
Burkitt's lymphoma (BL) (endemic African)	96%	EBNA-1
Burkitt's lymphoma (Sporadic)	10-70%	EBNA-1
B cell lymphoproliferative disease (BLPD)	90%	EBNA-1,2,3a,3b,3c, LMP-1,2a,2b
Hodgkin's disease	40-80%	EBNA-1, LMP-1,2a,2b
T cell lymphoma (subset - Nasal)	10% (100%)	EBNA-1, LMP-1,2a,2b
Nasopharyngeal carcinoma	100%	EBNA-1, LMP2a,2b
Gastric carcinoma	10%	EBNA-1, LMP-1,2a,2b
AIDS associated lymphomas		
Burkitt's lymphoma (BL)	* 30-50%	EBNA-1
Large cell (B cell) lymphoma of the CNS (BLPD)	100%	EBNA-1, LMP-1,2a,2b
Large cell (B cell) peripheral lymphoma (BLPD)	60%	EBNA-1, LMP-1,2a,2b
Hodgkin's lymphoma (rare)	100%	EBNA-1, LMP-1,2a,2b
T cell lymphoma (rare)	40%	EBNA-1, LMP-1,2a,2b
Data taken from Crawford 2001, * Beral 1991 and Hamilton-Dutoit 1993		

Of the diseases described above this thesis will focus only on those commonly associated with AIDS (BL and BLPD).

1.5.4.1 Non-HIV associated Burkitt's lymphoma

Burkitt's lymphoma (BL) is often multifocal and almost always arises in extranodal sites, most commonly the jaw, ovary, mammary gland, liver, intestine and kidneys. Tumours occur in high incidence in equatorial Africa and low incidence throughout the rest of the world. Histologically BL cells resemble germinal centre B lymphocytes with a non-cleaved appearance. In endemic African BL 96% of tumours are EBV positive. In contrast only around 20% of sporadic non-African BL are EBV positive (Sixby 2000). The EBV genome is present in BL as multiple nuclear episomes, and has been shown to be clonal, suggesting infection occurred before clonal proliferation and tumourigenesis (Raab-Traub & Flinn 1986). Only EBNA-1 protein, EBERs and BARF0 RNA are expressed in BL (latency I), which has raised doubts over the role of EBV in tumour formation, as EBNA-1 is not recognised as a potent oncogenic protein, at least in vitro and neither EBERs or BARF0 viral transcripts are required for transformation of B lymphocytes (Swaminathan, Tomkinson & Keiff 1991, Robertson *et al* 1994). On the other hand, however, Wilson, Bell & Levine (1996) have shown that EBNA-1 transgenic mice develop lymphoid tumours, suggesting EBNA-1 may have oncogenic properties in vivo. Furthermore, EBERs have been shown in vitro to induce Bcl-2 (Komano *et al* 1999) an anti-apoptotic gene, and BARF0 is consistently expressed in several EBV associated epithelial and B lymphocyte tumours (Chen *et al* 1999). These findings suggest that EBV may play a role in tumourigenesis in BL.

All BLs, EBV positive or negative, have 1 of 3 chromosomal translocations (8:14, 8:2, 8:22) which brings the c-myc oncogene on chromosome 8 under the influence of the constitutively expressed Ig heavy chain gene promoters on chromosome 14 or alternatively one of the light Ig chain promoters on chromosome 2 or 22 (Zech *et al* 1976). The expression of c-myc causes the cell to enter the cell cycle and begin proliferation, therefore bringing c-myc under the control of Ig promoters induces cell proliferation, aiding tumour growth. In addition Carbone *et al* (1997) has shown that 11 of 11 BL tumours expressed BCL-6.

The pathogenesis of BL differs depending on the type of BL, but regardless of type it is likely to be a multistep process. (The pathogenesis of BL is reviewed in Crawford 2001) In EBV associated African BL the process is thought to include the following steps:

- Recurrent bouts of malaria, which induces immunosuppression (Whittle *et al* 1984) leading to increased lytic EBV replication and hence increase infection of naive B lymphocytes
- Malarial antigens induce polyclonal B lymphocyte activation, causing blast transformation of EBV infected memory B lymphocytes (Wahlgren *et al* 1986)
- Expression of EBNA-1 and EBERs in EBV infected cells (Rowe *et al* 1986, Minarovits *et al* 1992)
- Reciprocal chromosomal translocation between Ig genes and c-myc, leading to constitutive expression of c-myc and increased cell proliferation (Manolov *et al* 1986)
- Additional genetic abnormalities e.g. p53 mutation in tumour cells (Farrell *et al* 1991)

1.5.4.2 Non-HIV B lymphoproliferative disease

Patients receiving allograft transplants require immunosuppression to prevent graft rejection by the host's body immune system. This predisposes a small number of patients to EBV associated BLPD (1-10%, Penn 1991). During immunosuppressive therapy there is an increase in EBV viral load. This is either due to an increase in proliferation of virus carrying latently infected cells or increased lytic replication with infection of bystander B lymphocytes. In either case this results from iatrogenic T cell suppression which induces a decrease in EBV specific CTL activity (Haque *et al* 1997). Patients who are EBV seronegative at transplant and become infected thereafter are at increased risk of BLPD. They can acquire EBV from the donor organ (Haque *et al* 1996) or from normal transmission routes via saliva.

BLPD presents clinically in 2 ways. In around 50% of cases there is an IM like illness in the first year after transplant, particularly in younger patients and in particular those seronegative at transplant (Epstein & Crawford 1998). The second type of presentation generally occurs in older patients, with localised masses

developing often in the gut, CNS or transplanted organ. These are usually large cell monoclonal lymphomas with a heavy infiltrate of CD4 T cells present (Perera *et al* 1998). Although tumours are monoclonal, polyclonal B cell proliferation probably occurs early in the disease leading to outgrowth of a monoclonal tumour. There is usually expression of all EBV latency genes (latency III)(Young *et al* 1989) suggesting that EBV plays a key role in the transformation process.

Remission can be induced by decreasing immunosuppressive therapy, however this can lead to organ rejection and re-introducing therapy again allows relapses to occur which become progressively more resistant to treatment. The death rate from BLPD is therefore high with 40-60% of organ transplant patients with BLPD dying and 90% of bone marrow transplant patients (Benkerrou *et al* 1998).

1.5.4.3 EBV associated malignancy in HIV infection

It is estimated that 20% of all HIV infected patients will develop B cell lymphoma at some point in their life (Rodriguez-Alfagame 1998). Furthermore lymphoma is the second most common malignancy in HIV infection (after Kaposi's sarcoma) (Goedert *et al* 1998), with infected individuals 60 times more likely to develop NHL than the general population (Tulpule & Levine 1999). HIV lymphomas differ from those seen in non-HIV infected individuals in terms of both the aetiology and the pathogenesis. The most characteristic feature of AIDS lymphomas is that 70-90% are located at extra nodal sites (Tulpule & Levine 1999), with CNS being the most common (26%), followed by bone marrow (22%), gastro-intestinal tract (17-25%) and liver (12%). Histologically 60% of lymphomas are peripheral large cell, 20% peripheral Burkitt's (small non-cleaved), and 20% PCNSL (Beral *et al* 1991, review of 2500 cases). In addition to the Burkitt's and large cell peripheral lymphomas, there are several rarer lymphomas which can occur in the setting of HIV. These are listed in table 4 above and will not be discussed further.

1.5.4.4 Primary central nervous system lymphoma (PCNSL) in AIDS

Presenting symptoms of PCNSL can include: seizures, focal neurological dysfunction, headache, and cranial nerve palsies, all attributable to a mass lesion in the brain. However patients can present with merely subtle changes in mental status

as the only clinical manifestation of disease. Patients who develop PCNSL generally have severe underlying HIV infection and median CD4 counts at diagnosis is only $34/\text{mm}^3$ (Tulpine & Levine 1999).

Diagnosis is usually by radiological assessment, which shows 1 or 2 2-4 cm lesions in the parenchyma. Although similar results can be seen with toxoplasma in the CNS, toxoplasma tends to produce multiple smaller lesions (Scaravilli & Cook 1997). Diagnosis is confirmed by brain biopsy or failure of toxoplasma treatment. Recently less invasive methods of diagnosis have been introduced. Assessment of EBV viral load levels in lumbar puncture CSF samples by PCR (polymerase chain reaction) has been shown to be useful diagnostic tool. Cingolani *et al* (1998) showed an 80% success rate in predicting PCNSL in HIV patients with focal brain lesions, with presence of EBV in CSF being very specific for PCNSL (sample size 122, of which 42 were diagnosed with PCNSL).

The prognosis for PCNSL is extremely poor with median survival with no treatment only 1.5 months. Radiation therapy can induce complete remission in up to 50%, however median survival is still 6.3 months, with death due to opportunistic infection accompanied by multiple neurological disorders. The introduction of HAART has further increased median survival times to 16 months (Hoffman *et al* 2001).

Morphologically these tumours are homogenous diffuse large cell lymphomas of B lymphocyte origin occurring in the brain parenchyma in a perivascular cuffing pattern, and may occur as a single or small number of parenchymal lesions. In contrast metastatic peripheral lymphomas involving the brain are typically localised to the meninges. The classification of diffuse large cell lymphoma can be subdivided into 2 groups. Depending on the presence of immunoblastic plasmacytoid features PCNSL may be classified as large non-cleaved cell lymphoma or immunoblastic plasmacytoid lymphoma. In HIV both types of PCNSL are found, however in non-HIV PCNSL only large cell non-cleaved lymphoma is seen.

Molecular features of these tumours are homogenous, with almost all being monoclonal as defined by Ig gene re-arrangements, in common with the tumours found in non-HIV infected individuals (Meeker *et al* 1991, Bashir *et al* 1993). There is a universal association with EBV (MacMahon *et al* 1992, Auperin *et al* 1994) in HIV associated PCNSL, in contrast to only 15% association in non-HIV PCNSL in immunocompetent individuals (203 cases studied) (Jellinger & Palus 1995).

Differences are also found between HIV and non-HIV PCNSL in terms of EBV gene expression in those tumours with virus present. Auperin *et al* (1994) showed that 10 of 11 EBV positive HIV PCNSLs expressed both LMP-1 and EBNA-2, while just 1 of 11 expressed only LMP-1 and not EBNA-2. In contrast 4 of 5 EBV PCR positive non-HIV PCNSLs examined showed expression of LMP-1, with no expression of EBNA-2 in any samples. Neither HIV nor non-HIV PCNSLs demonstrated any expression of lytic antigens (Auperin *et al* 1994). However Bashir *et al* (1993) reported expression of lytic antigen VCA (viral capsid antigen) in 1-5% of cells in 5 out of 5 HIV PCNSLs. This suggests that although most tumour cells present are latently infected, demonstrating latency III phenotype, perhaps a very few cells within the tumour switch to lytic replication at some stage in lymphomagenesis.

With regard to expression of oncogenes in PCNSL, there is no evidence of c-myc or Bcl-6 translocations (Meeker *et al* 1991, Larocca *et al* 1998). However there is data which suggests that a large percentage of tumour cells have mutation in the 5' non-coding region of the BCL-6 gene (Gaidano *et al* 1997). Bcl-6 gene codes for a transcription factor which is involved in regulation of both B lymphocyte activation and differentiation. In a study by Larocca *et al* (1998) 9 of 9 HIV associated large cell non-cleaved PCNSLs showed evidence of mutation in the 5' non-coding region of the Bcl-6 gene, furthermore 13 of 13 non-HIV large cell non-cleaved PCNSLs also demonstrated mutations in this region. Molecular analysis of the mutations showed that most consisted of between 2 and 4 transition or transversion mutations. No mutations were detected in any of the 7 HIV associated immunoblastic plasmacytoid PCNSLs tested. Inhibition of BCL-6 is required for a plasmacytoid cell phenotype (Shaffer *et al* 2000), which may explain why Bcl-6 expression is only seen in non-cleaved large cell PCNSL and not immunoblastic plasmacytoid PCNSL. Furthermore Bcl-6 is only expressed in germinal centre or post germinal centre B lymphocytes, suggesting that, as all non-cleaved large cell PCNSL appear to express BCL-6, these tumours are derived from a post germinal centre cell.

In addition to Bcl-6, the study by Larocca also examined expression of EBV LMP-1 and cellular Bcl-2 in the same cases. Bcl-2 is believed to prolong cell survival by blocking apoptosis rather than increase proliferation (Camilleri-Broet 2000). Bcl-2 expression was found only in tumours demonstrating expression of LMP-1, and furthermore the dual expression of Bcl-2 and LMP-1 was only detected in HIV

immunoblastic plasmacytoid PCNSL. LMP-1 has been shown *in vitro* to transactivate Bcl-2 (Henderson *et al* 1991, Finke *et al* 1992).

This data demonstrates that differences exist between the two histological types of PCNSL in terms of gene expression and pathogenesis.

1.5.4.5 Peripheral lymphomas in AIDS

The 2 main types of systemic lymphoma seen in HIV (Burkitt's and NHL) differ both in their morphology and pathogenesis. The pathogenesis of AIDS BL is thought to be similar to that of endemic African BL (see 1.5.4) with HIV infection replacing malaria in the process. HIV infection provides the immunosuppression allowing increased lytic replication of EBV, and also induces polyclonal B lymphocyte activation which provides the proliferative stimuli for EBV infected cells. Up to 50% of AIDS BL are associated with EBV (Beral *et al* 1991). HIV associated BL, in comparison to HIV associated PCNSL, generally occurs much earlier in HIV infection when median CD4 counts are still greater than 100cells/cm³.

In contrast to BL HIV associated large cell lymphomas occur much later in HIV disease and are associated with a far poorer prognosis. Molecularly, large cell lymphomas are more heterogeneous than BL. As with BL there is variable association with EBV with typical studies suggesting between 31% and 66% are EBV positive (Meeker *et al* 1991, Shibata *et al* 1993, Beral *et al* 1991). Unlike BL c-myc translocations and BCL-6 translocations occur in only a subset of large cell lymphomas. In contrast to both HIV associated BL and PCNSL, approximately 40% of large cell lymphomas are polyclonal as defined by Ig gene re-arrangements (Beral *et al* 1991). In a study containing 25 HIV associated large cell lymphomas Beral *et al* showed expression of LMP-1 in 12 of 25 lymphomas of which 5 also expressed EBNA-2. This suggests a latency III phenotype for 5 of 25 tumours, intermediate latency II phenotype for 7 of 25 and latency I for 13 of 25 (Beral *et al* 1991).

Development of NHL during AIDS is thought to be attributed to a loss in EBV specific cellular immunity. Recently Van Baarle *et al* (2001) have shown that during infection with HIV there is no loss of EBV specific cytotoxic T lymphocytes in terms of total cell numbers, however there is a decrease in the functional ability of these cells. Cytotoxic T lymphocytes showed a progressive loss in their ability to produce

INF γ in response to EBV peptides. This loss of function correlated with lower CD4 T lymphocyte numbers and was accompanied by an increase in the EBV viral load.

1.6 Summary

Infection with HIV produces an ideal environment for B lymphocyte tumours to develop. Hyperstimulation of the humoral arm of the immune system by HIV, EBV and other antigens leads to increased B lymphocyte proliferation. B lymphocyte proliferation is aided by aberrant production of TH2 cytokines. In addition the loss of FDC and CD4 lymphocytes leads to a loss in negative regulatory factors for B lymphocyte proliferation. Furthermore decreased T lymphocyte mediated cytotoxic killing allows inappropriate B cell proliferation to continue unchecked. While the effects of HIV infection are prominent throughout the body the CNS may be at increased risk due to its already restricted immune surveillance.

Given the oncogenic capability of EBV it is unsurprising to find that it is associated with a large number of malignancies in HIV. Furthermore as EBV shows a 100% association with PCNSL in AIDS it is clearly plays an important role in the pathogenesis. Both histological types of PCNSL are EBV infected and demonstrate a latency III phenotype, which includes expression of the viral oncogenes EBNA-2 and LMP-1.

HIV subverts the immune system and provides an environment favourable to B lymphocyte proliferation. EBV takes opportunistic advantage of this new environment further stimulating proliferation of B lymphocytes in order to promote its own replication and survival. Inevitably in some cases this uncontrolled proliferation leads to tumour formation.

The question, which remains unanswered, is why these EBV related tumours preferentially develop in the CNS? Given that no evidence exists of B lymphocytes entering the normal brain (Williams & Hickey 1995, De Angelis 1999, Hickey 2001) it seems paradoxical that these EBV related tumours should develop preferentially in the CNS in immunosuppressed individuals.

It is widely accepted that activated T lymphocytes routinely enter the normal CNS where they perform antigen surveillance functions (Hickey *et al* 1991). As yet there is no evidence of B lymphocytes entering the normal human CNS (Williams & Hickey 1995, De Angelis 1999, Hickey 2001). However it is known that antigen specific B lymphocytes are capable of responding to antigens within the CNS (Knopf *et al* 1998).

HIV infected individuals are 60 times more likely to develop PCNSL than non-HIV infected individuals (Tulpule & Levine 1999). These malignancies are all of B lymphocyte origin and are universally associated with EBV (MacMahon 1992).

This study aimed to determine the prevalence of B lymphocytes in normal brain when, presumably, no exogenous immunological challenge originates from CNS tissue. Subsequently we aimed to determine whether advancing HIV infection leads to changes in the brain B lymphocyte population which might contribute to the increased risk of lymphoma seen in AIDS. We sought to characterise any B lymphocytes found within the brain, in terms of activation status of the lymphocytes, clonality of any large populations of B lymphocytes found, and EBV infection status. Finally, we wished to assess the effects of damage to the BBB on B lymphocyte accumulation in the brain, and to determine if HIV infection of the brain enhanced expression of B lymphocyte chemoattractants or expression of B lymphocyte proliferative chemokines within the brain.

2 Material and Methods

2.1 Reagents

Product	Company
100bp ladder	Gibco BRL
ABC alkaline phosphatase	Vector
ABC peroxidase	Dako
Biotinamidocaproic acid 3- sulpho-N-Hydroxysuccinimide Ester	Sigma
Boric acid	Sigma
Citric acid	BDH
DAB	Vector
DNA extraction kit	Roche
EDTA	Sigma
Ethidium bromide	Gibco BRL
Ficoll-Hypaque	Pharmacia Biotech
Foetal calf serum	Sera Lab
Hydrogen peroxide	Fisher
New Fucsin Red	Dako
Nova Red	Vector
Nucleon HT DNA extraction kit	Amersham
Nu-sieve 3:1 agarose	FMC Bio products

Oligonucleotides	Invitrogen
PBS	ICN Bio medicals Inc.
PHA	Sigma
Protease	Sigma
RNAzol B	Cinna/Tel test Inc.
RT-PCR kit	Invitrogen
Sodium chloride	Fisher
Tissue culture medium	Gibco BRL
Tris	BDH
Trypan blue	Flow
Tween 20	Sigma
Tyramine Hydrochloride	Sigma
Utrapure water	Sigma
Vector Black	Vector
VIP	Vector
X ray developer	Photosol
X ray fixer	Photosol

2.2 Solutions

Cell culture:

Culture medium	L-glutamine	2mM
	Penicillin	100IU/ml
	Streptomycin	100µg/ml
	Fetal calf serum	10% v/v
	Made up in RPMI 1640	
Freezing medium	Fetal calf serum	90% v/v
	DMSO	10% v/v
Phytohaemagglutinin (PHA)	Made up in PBS	500µg/ml

Immunohistochemistry:

Tris buffered saline (TBS) x1	Tris-HCL, PH7.6	0.05M
	NaCl	0.15M
	Made up in dH ₂ O	
TNT	Tris HCL pH7.5	0.1M
	NaCl	0.15M
	0.05% Tween 20	
TNB	Tris HCL pH7.5	0.1M
	NaCl	0.15M
	0.5% blocking reagent	
Biotinylated tyramide	4ml Boric acid	50mM
	10mg Biotinamidocaproic acid 3-sulpho-N-Hydroxysuccinimide ester	
	3mg tyramine Hydrochloride	
Hydrogen peroxide		10% v/v
	Made up in dH ₂ O	
Ethanol		99%
		75%
	Made up in dH ₂ O	

PCR:

Loading buffer	Glycerol	49.9% v/v
	10xTBE	49.9% v/v
	Bromophenol blue (BPB)	0.2% w/v
Ethidium bromide (Et Br)	Made up in dH ₂ O	1% w/v
Tris-Borate-EDTA (TBE) x10	Tris	10.8% w/v
	Boric acid	5.5% w/v
	0.5M EDTA, pH 8.0	4% v/v
	Made up in dH ₂ O	
Denaturing solution	NaCl	1.5M
	NaOH	0.5M
	Made up in dH ₂ O	
Neutralising solution	NaCl	1.5M
	Tris-HCL, pH7.2	0.5M
	EDTA, pH 8.0	0.001M
	Made up in dH ₂ O	
Standard saline citrate (SSC) x20	NaCl	1.5M
	Tris-HCL, pH 7.2	0.5M
	EDTA, pH8.0	0.001M
	Made up in dH ₂ O	
Sodium Dodecyl Suphate (SDS)	Made up in dH ₂ O	10% w/v
Gel electrophoresis	NuSieve 3:1 agarose Made up in 1x TBE buffer	2.5-5%w/v
100bp ladder (in 10mM Tris-HCL, pH7.5, 50mM NaCl, 0.1mM EDTA)		200µg/ml

2.3 Consumables

Product	Company
3MM chromatography paper	Whatman
Cell culture flask and plates	Marathon (Falcon)
Cover slips	Chance proper Ltd
DIG detection kit	Amersham international Plc
DIG end labelling kit	Amersham international Plc
DIG film	Amersham international Plc
Disposable gloves	Johnson & Johnson Medical
Hybond N+ Nylon membrane	Amersham international Plc
microtome blades (MB35 premier 35°/80mm)	Thermo Shandon
parafilm	American national can
Plastic bijoux	Sterlin
Plastic universal tubes	Sterlin
Polypropylene conical tubes	Marathon (Falcon)
Sequenza holders	Shandon
Superfrost slides	BDH

2.4 Pathological samples and control material

Postmortem brain tissue from 8 patient groups was used in the study. These cases were part of the Edinburgh HIV brain bank and use of this resource in research is approved by the Lothian Ethics of Research committee.

- Group 1 - **Normal brains** (n=7). All these cases died as a result of accidents and had no evidence of CNS disease at autopsy, or history of illness relating to the CNS. Five cases were male and 2 female. The age range was 16–31 years (mean age 24 years).
- Group 2 - **Non-HIV Intravenous Drug abusers** (n=5). These individuals were all HIV negative drug users who died of drug related accidents. Four were male and one female; age range 20-34 years (mean age 27 years).
- Group 3 - **Pre-symptomatic HIV infected patients** (n=6). These individuals were all HIV positive drug users infected by needle sharing who died of drug related accidents before developing an AIDS defining illness. Post mortem examination confirmed a lack of AIDS defining illness in all cases. All were male and the age range was 28–40 years (mean age 32 years).
- Group 4 - **AIDS patients with no significant CNS pathology** (n=5). These patients died of non-CNS related AIDS conditions, but displayed no evidence of PCNSL, CNS opportunistic infections nor of HIV encephalitis. Two cases in this group had evidence of systemic lymphoma without CNS involvement. Three patients in this group were drug users (one female), the remainder were homosexual men. The age range was 32–45 years (mean age 37 years).

- Group 5 - **AIDS patients with non-neoplastic lymphoid infiltrates in the CNS** (n=5). Despite relatively high T lymphocyte counts in two individuals in this group, all had symptoms of AIDS. At routine neuropathological examination these subjects displayed pleomorphic perivascular lymphoid infiltrates in all or many areas of the brain. These infiltrates contained occasional plasmacytoid cells and were not considered morphologically malignant. Careful examination of these brains had revealed no evidence of other significant CNS pathology apart from one case that had a single ependymal focus of cytomegalovirus infection. Three cases were drug users, all female. The other two comprised a homosexual man and an African child. The child died of systemic cytomegalovirus and pneumocystis infection and was part of a study in Abidjan (Bell *et al* 1997). The age range of the adult cases was 23–38 years (mean age 32 years) and the age of the African child was 6 months.
- Group 6 - **AIDS patients with HIVE** (n=6). These patients were all diagnosed at autopsy as having HIVE, characterised by the presence of giant cells and positive immunohistochemical staining for p24 HIV antigen. Four were male and two female, four were drug users, and the age range was 28-59 years (mean age 40 years).
- Group 7- **AIDS PCNSL** cases (n=6). Five individuals had focal deposits of neoplastic B lymphoid aggregates in the CNS perivascular compartment and also invading the brain tissue locally, while one individual had widespread disseminated CNS B cell lymphoma. Full autopsy in these patients failed to reveal evidence of lymphoma outwith the CNS. None had HIV encephalitis but three displayed immunohistochemical evidence of a focal, low level cytomegalovirus infection in addition to lymphoma. Five of the group were homosexual men, one was

an IDU, and the age range was 28–36 years (mean age 31 years).

Group 8 - **Non-HIV viral encephalitis** (n=3). These patients were all diagnosed at autopsy with encephalitis of various viral origins. (1 Case of herpes simplex encephalitis, and in 2 cases the causative virus was not identified). All were HIV negative. None had a history of immunosuppression. The age range was 24-68 years (mean age 50 years).

Additional Case - One pre-AIDS drug user which did not fit the criteria for group 3 because there was evidence of non-HIV viral encephalitis. Female, age 32 years.

In all the subjects described above, the brain was removed at autopsy and fixed intact in formalin for 2-3 weeks. Blocks were removed for histology from the frontal, parietal and occipital lobes, central white matter, temporal hippocampus, basal ganglia, thalamus, midbrain, pons, medulla and cerebellum. All samples were paraffin embedded and 5µm sections were cut from each block. Relevant clinical data is given for each individual case in section 2.6.1 Table 5.

2.4.1 Clinical information

Table 5 below gives details of each of the cases used in this study and any relevant clinical information which was available.

Table 5: Clinical details of individual cases

Case number/type	AIDS status	Drug use	CNS Pathology	Age / sex	Final CD4/CD8 count
Group 1 Normal					
1	Neg	-	Nil in CNS	31M	NA
2	Neg	-	Nil in CNS	16M	NA
3	Neg	-	Nil in CNS	26M	NA
4	Neg	-	Nil in CNS	22M	NA
5	Neg	-	Nil in CNS	24F	NA
6	Neg	-	Nil in CNS	28F	NA
7	Neg	-	Nil in CNS	23M	NA
Group 2 Non-HIV drug users					
8	Neg	+	Nil in CNS	31M	NA
9	Neg	+	Nil in CNS	34M	NA
10	Neg	+	Nil in CNS	20F	NA
11	Neg	+	Nil in CNS	20M	NA
12	Neg	+	Nil in CNS	32M	NA
Group 3 Pre-AIDS					
13	HIV +ve	+	Mild lymphocytic meningitis	28M	90/1010
14	HIV +ve	+	Mild lymphocytic meningitis	31M	211/1267
15 #	HIV +ve	+	Mild lymphocytic meningitis	40M	NA
16 #	HIV +ve	+	Mild lymphocytic meningitis	31M	425/1316
17 #	HIV +ve	+	Mild lymphocytic meningitis	30M	NA
18	HIV +ve	+	Mild lymphocytic meningitis	33M	312/701
Group 4 AIDS with no CNS pathology					
19	AIDS	+	Nil in CNS	34M	4/27
20 #	AIDS	-	Nil in CNS	45M	41/110
21 #	AIDS	-	Nil in CNS	41M	3/73
22	AIDS	-	Nil in CNS (Systemic lymph)	32M	40/260
23	AIDS	-	Nil in CNS (Systemic)	36F	23/74

Case number/type	AIDS status	Drug use	CNS Pathology	Age / sex	Final CD4/CD8 count
			lymph)		
Group 5 AIDS infiltrate					
24	AIDS	+	Pleomorphic infiltrate	29F	248/1154
25 #	AIDS	+	Pleomorphic infiltrate	38F	85/302
26 #	AIDS	+	Pleomorphic infiltrate	23F	246/510
27	AIDS	-	Pleomorphic infiltrate (minimal CMV)	37M	22/662
28	AIDS	-	Pleomorphic infiltrate	NA	NA
Group 6 AIDS HIVE					
29	AIDS	-	HIVE	59M	6/unknown
30	AIDS	-	HIVE	28M	60/300
31	AIDS	+	HIVE	35F	23/418
32	AIDS	+	HIVE	28M	90/520
33	AIDS	+	HIVE	41M	10/767
34	AIDS	+	HIVE	49F	87/1779
35	AIDS	+	HIVE + Systemic lymphoma	31M	22/85
Group 7 AIDS PCNSL					
36	AIDS	-	CNS lymphoma	51M	18/276
37	AIDS	-	CNS lymphoma +CMV	28M	4/384
38	AIDS	+	CNS lymphoma +CMV	30M	4/u
39	AIDS	-	CNS lymphoma	36M	1/33
40 #	AIDS	-	CNS lymphoma +CMV	37M	8/57
41 #	AIDS	-	CNS lymphoma + CMV + HIVE	32M	23/440
42 #	AIDS	-	Disseminated CNS lymphoma	34M	NA
Group 8 Non-HIV viral encephalitis					
43	Neg	-	Subacute viral encephalitis	57M	NA
44	Neg	-	Post infectious viral encephalitis	24M	NA
45	Neg	-	Herpes simplex in neurones + microglia	68F	NA
Cases not fitting defined criteria for any one group					
46 #	Pre-AIDS	+	Non-HIV viral encephalitis	32F	240/480
Key: # indicates frozen tissue was available for this case in addition to FFPE tissue.					

In addition to the formalin fixed paraffin embedded (FFPE) tissue described above, frozen samples were available from some of the HIV cases in the groups above and additionally from some normal cases for

which no FFPE sections were available. Table 6 lists the additional normal frozen tissue and CSF samples used. HIV cases with frozen tissue available are marked in table 5 above with a #.

Table 6: Frozen tissue available from additional non-HIV cases

Case number	Case group	Frozen tissue available
47	Normal	CSF
48	Normal	CSF
49	Normal	CSF
50	Normal	CSF
51	Normal	CSF
52	Normal	Frontal lobe
53	Normal	Frontal lobe
54	Normal	Frontal lobe
No clinical data was available for any of these cases other than their HIV status, which was negative for all.		

2.4.2 Formalin fixed paraffin embedded tissues

All tissue samples used in this study were from the Edinburgh brain bank, and had been previously processed to paraffin for long term storage. All sections used in this study were cut using a microtome, to a thickness of 5µm for immunohistochemistry and in situ hybridisation, and 20-25µm for PCR. Sections were then floated on a waterbath (at 37°C) to flatten out the section before being placed on superfrost slides (BDH). Sections were then left overnight at 37°C.

Sections used for in situ hybridisation and PCR were cut and handled using latex gloves to prevent degradation of RNA/DNA or contamination.

2.4.3 Frozen tissues

All frozen tissue samples used in this study were obtained from the Edinburgh HIV brain bank. The samples removed from the brain bank were used only for DNA extraction. Due to the infectious nature of the material extra safety precautions were taken when handling this material. Samples were only removed from their storage containers inside a Class 2 extraction hood, and samples were kept here until well digested with proteinase K. Double gloves were worn at all time when handling tissue containers, and all plastic wear used was soaked in Tigidor disinfectant before being autoclaved and disposed of. The hood was cleaned after use with Tigidor and the built in U.V. lamp left on overnight.

2.4.4 Blood samples and peripheral blood mononuclear cell (PBMC) separation

Upon informed consent, 20-50 ml of heparinized blood was obtained from several healthy members of the laboratory staff at the Royal Dick Vet School.

Heparin treated fresh whole blood (10^3 IU/ml) was layered onto an equal volume of Ficoll-Histopaque and centrifuged at 540g for 20 minutes. The interface layer, between the plasma layer and Ficoll, containing PBMCs was harvested and washed twice in PBS by centrifugation at 160g for 5 minutes.

2.4.5 PHA stimulation of PBMCs

PBMCs were diluted to a concentraion of 4×10^6 in 4 ml of culture medium, then 100 μ l of PHA (phytohaemagglutinin) was added. The mixture was pipetted into 4 wells of a 24 well plate and incubated over night at 37°C with 5% CO₂. Cells were then transferred to sterile eppendorf tubes and centrifuged at 1300g for 5 minutes. The supernatant was removed and cells resuspended in PBS.

2.4.6 Cell lines

The cell lines shown in table 7 were used as experimental controls as described in the appropriate sections:

Table 7: Control cell lines

Cell line	Description	Reference
Namalwa	Burkitt's lymphoma tumour derived cell line containing 1-2 copy of EBV genome per cell	Klein & Dombos 1973. Lawrence, Villnave & Singer 1988
BJAB	EBV negative Burkitt's lymphoma derived cell line	Steinitz & Klein 1975
Akata	Burkitt's lymphoma tumour	Takada <i>et al</i> 1991
Raji	Burkitt's lymphoma tumour	Epstein <i>et al</i> 1966
P3HR1	Burkitt's lymphoma tumour	Hinuma <i>et al</i> 1967
MOLT-4	T cell leukaemia tumour	Takada <i>et al</i> 1974
HUT-78	T cell leukaemia tumour	Seon <i>et al</i> 1984
U937	Monocyte cell line	Larrick <i>et al</i> 1980

Cell lines were cultured in a humidified incubator at 37°C with 5% CO₂. They were feed twice weekly with RPMI containing 10% foetal calf serum, L-glutamine and penicillin/streptomycin.

2.4.6.1 Cell viability assay and cell counts

Cell viability was assessed using the principle that live cell are able to prevent the uptake of the dye Trypan blue, whereas dead cells take up the dye. 10µl of 0.5% w/v Trypan blue in PBS solution was mixed with 10µl of cell suspension, and the mixture placed in a haemocytometer chamber. Viable cells were counted by light microscopy.

2.4.6.2 Thawing cells

Frozen cells were thawed in a water bath at 37°C then added to 10ml of culture medium in a universal tube. Cells were centrifuged at 160g for 5 minutes, resuspended in culture medium at a concentration of 1×10^6 cells/ml, and transferred to a 25ml tissue culture flask. Cells were propagated at 37°C with 5% CO₂.

2.4.6.3 Cell line Dilution series

Ten fold dilution series of cell lines were created to provide a means of quantifying PCR sensitivity. Cells were counted as described above and diluted in culture medium to a concentration of 10^6 cells per ml. Serial dilutions of Namalwa (EBV positive) cells, from 10^6 cells to 1 cell, were made by removing 1ml from 10ml of medium containing 10^7 cells and adding it to 9ml of fresh medium to give 10^6 cells in 10ml and leaving 9×10^6 cells in the original tube. This was repeated down to 10 cells in 10ml, then 1 ml was removed from each dilution and added to 1 ml of EBV negative BJAB cells (concentration 10^6 cells/ml), to give a dilution series from 10^6 Namalwa cells in a background of 10^6 BJAB cells down to 1 Namalwa cell in a background of 10^6 BJAB cells. Cells were then centrifuged at 160g for 5 minutes and resuspended in 1 ml of PBS. DNA was then extracted from the cells as described in 2.6.3.1.

Akata cell line dilution series was created in the same way as described above by substituting Akata for Namalwa and U937 for BJAB

2.5 Techniques

2.5.1 Immunohistochemistry

A list of antibodies used for immunohistochemistry is given in table 8.

2.5.1.1 Preparation for use of formalin fixed paraffin embedded (FFPE) sections (Re-hydration of sections)

Paraffin wax sections were de-waxed by immersion in xylene twice for 4 minutes each time. Sections were re-hydrated by two immersions in 99% ethanol, and two immersions in 70% ethanol. This was followed by a 12 minute immersion in picric acid, and finally rinsing in running tap water.

2.5.1.2 Antigen retrieval pre-treatments

Fixation of tissue in formalin can create crosslinking bonds within the tissue which can make it difficult or impossible for antibodies to penetrate and reach their target antigens. There are several antigen retrieval pre-treatment methods which can be utilised to un-mask antigens. Table 9 lists the antigen retrieval pre-treatments used in this study.

2.5.1.3 ABC immunohistochemistry procedure (peroxidase and alkaline phosphatase)

Sections were de-waxed and re-hydrated as described above. Sections requiring antigen retrieval pre-treatments to un-mask antigen affected by formalin bond formation were treated at this point, see table 9. After pre-treatments sections were rinsed in running water, then incubated for 10 minutes in 3% H₂O₂ to block endogenous peroxidase enzyme activity. Sections were then rinsed in running water, followed by PBS, then loaded into a Sequenza. 150µL of filtered blocking serum, diluted 1 in 5 in PBS, was then applied for 10 minutes (see table 8). This was followed by incubation with the primary antibody diluted in appropriate filtered and diluted blocking serum (see table 8 for list of antibodies used and concentrations) for 30 minutes at room temperature. Sections were then rinsed twice for 5 minutes with PBS, and incubated with biotinylated

secondary antibody, again diluted in appropriate blocking serum, for 30 minutes at room temperature. The sections were again washed twice in PBS, then incubated with streptavidin/peroxidase complex (DAKO ABC) or streptavidin/alkaline phosphatase complex (Vector ABC-AP), for 30 minutes at room temperature. Sections were then rinsed twice with PBS and removed from the Sequenza. A visualising agent was then applied (see table 8). Sections were then rinsed in running water before being counterstained in haematoxylin for 20 seconds, washed again, blued in lithium carbonate, and finally rinsed again. Sections were mounted as described in 2.7.1.7

2.5.1.4 Tyramide signal amplification (TSA)

Sections were de-waxed and re-hydrated as described above. Sections requiring antigen retrieval pre-treatments were treated at this point, (see table 9). Sections were rinsed in running water, then immersed in H_2O_2 for 15 minutes, rinsed again in running water, then rinsed in PBS before being placed in a sequenza slide holder. 150 μ L of filtered blocking serum, diluted 1 in 5 in PBS, was then applied for 15 minutes (see table 8), followed by 150 μ l of TNB for 20 minutes. After a brief rinse in PBS the primary antibody was added (see table 8 for: concentration, incubation period and incubation temperature). Sections were then rinsed twice for 5 minutes with PBS, and incubated with biotinylated secondary antibody, diluted in appropriate blocking serum, for 30 minutes at room temperature. The sections were again washed twice in PBS, then incubated with streptavidin horseradish peroxidase (strept HRP) diluted 1/500 in TNB for 30 minutes at room temperature. Sections were rinsed 3 times for 5 minutes with TNT. Biotinylated tyramide was diluted 1/350 in boric acid and H_2O_2 (3ml boric acid + 1 μ l 30% H_2O_2). Sections were incubated with diluted biotinylated tyramide for 10 minutes at room temperature, then rinsed three times in TNT. StreptHRP diluted as above for 30 minutes, then rinsed 4 times for 5 minutes with PBS. DAB was then applied to sections, sections rinsed in water, counter stained in haematoxylin and

blued in lithium carbonate, and finally rinsed again. Sections were permanently mounted as described in 2.7.1.7.

Table 8: Antibody concentrations and pre-treatments

Primary antibody	Pre-treatment	Secondary antibody	ABC or TSA	Visualisation agent
CD3 1/100 (DAKO)	Trypsin	SARBO 1/200 (DAKO)	ABC	DAB
CD8 1/50 (DAKO)	EDTA microwave	RAMBO 1/200 (DAKO)	ABC	DAB
CD20 1/300 (DAKO)	Citric acid microwave	RAMBO 1/200 (DAKO)	ABC	DAB
CD20 in double staining and antibody stripping experiments 1/100 (DAKO)	Citric acid microwave	RAMBO 1/200 (DAKO)	ABC (for double staining) ABC-AP (for antibody stripping)	VIP (for double staining) New fucsin red (for antibody stripping)
CD23 1/30 (DAKO)	Citric acid microwave	RAMBO 1/200 (DAKO)	ABC	DAB
CD45 1/300 (DAKO)	No pre- treatment	RAMBO 1/200 (DAKO)	ABC	DAB
CD79a 1/30 (DAKO)	EDTA microwave	RAMBO 1/200 (DAKO)	ABC	DAB
BCA-1 1/200	Citric acid microwave	RAMBO 1/200 (DAKO)	TSA	DAB
Fibrinogen 1/7000 (DAKO)	Citric acid microwave	RAMBO 1/200 (DAKO)	ABC-AP	Vector black
P24 1/3000	Citric acid microwave	RAMBO 1/200 (DAKO)	TSA	DAB
SDF-1 1/15	Citric acid microwave	RAMBO 1/200 (DAKO)	ABC	DAB
Von Willebrand 1/200 (DAKO)	Citric acid microwave	SARBO 1/200 (DAKO)	ABC	DAB
ZO-1 1/200	Protease	SARBO 1/200 (DAKO)	TSA	DAB

Key: SARBO swine anti-rabbit biotinylated antibody, RAMBO rabbit anti mouse biotinylated antibody, ABC avidin biotin complex (horse radish peroxidase), AP alkaline phosphatase, TSA tyramide signal amplification, DAB diaminobenzidine, EDTA ethylene diamine tetraacetic acid

Table 9: Antigen retrieval pre-treatments

Pre-treatment	Procedure
Citric acid 0.01M pH6.0	Microwave sections in 500ml for 15 minutes, allow to cool in solution for 10 minutes
Citric acid 0.01M pH6.0	Pressure cook sections in 1L for 20 minutes, allow to cool in solution for 10 minutes
EDTA 0.5mM pH8.0	Microwave sections in 500ml for 15 minutes, allow to cool in solution for 10 minutes
EDTA 0.5mM pH8.0	Pressure cook sections in 1L for 20 minutes, allow to cool in solution for 10 minutes
Trypsin 0.1%, CaCl ₂ 0.1%	Digest sections in 500ml for 20 minutes at 37°C
Protease 25mg in 50ml TBS pH7.8	Digest sections in 500ml for 15 minutes at 37°C

2.5.1.5 Double antigen labelling immunohistochemistry

This technique was used to label two antigens on one section. In order to avoid cross reactions, in all experiments using this technique, the first antigen was labelled using a rabbit anti human antibody and the second antigen labelled using a mouse anti human antibody.

The procedure described in 7.2.2 was followed using streptavidin/peroxidase complex (DAKO ABC) for the first antigen. Sections were then re-incubated in 3% H₂O₂ for 10 minutes, rinsed in running water, followed by PBS, then re-loaded into the Sequenza. 150µL of filtered blocking serum, diluted 1 in 5 in PBS, was then applied for 10 minutes (see table 8). Then an avidin/biotin blocking kit (Vector) was used to block biotin and avidin molecules added to visualise the first antigen. The kit was used in accordance with the manufacturers instructions – 15

minute incubation with avidin blocking agent, 15 minute incubation with biotin blocking agent, sections were then rinsed with PBS. The primary antibody was then added, and the protocol in 7.2.2 followed from that stage. Streptavidin/alkaline phosphatase complex (Vector ABC-AP) was used in the visualisation of the second antigen. Sections were permanently mounted as described in 2.7.1.7.

2.5.1.6 Antibody removal from sections after antigen labelling

This technique was used where it was necessary to determine if two antigens were expressed on the same cell. It was used in preference to double antigen labelling (7.2.6) in situations where only two mouse monoclonal antibodies were available for labelling the antigens of interest. By photographing previously stained sections, then removing the antibody and stains before re-staining a second antigen, the problems of cross reactivity associated with double antigen labelling using two primary antibodies raised in the same species are avoided.

Sections which had been previously stained using the technique described in 7.2.2 using streptavidin/alkaline phosphatase complex (Vector ABC-AP), and mounted in aqueous mounting medium (see 7.2.8), were photographed, using an Olympus BX40 light microscope with Roper scientific camera and image pro plus computer software, and the coordinates of cells of interest noted. Sections were then left in running water for 1-2 hours until the coverslips slid off. Sections were then placed in an antibody stripping solution (see 5.1) for 12 minutes on a shaker. Following this they were de-stained in 0.5% $\text{Na}_2\text{S}_2\text{O}_5$ for 1 minute, on a shaker. Sections were then rinsed in running water for 10 minutes, before being re-stained for a second antigen as described in 7.2.2. After labelling of the second antigen sections were re-photographed and the areas of interest compared.

2.5.1.7 Mounting sections

Sections to be aqueous mounted were dehydrated by brief sequential immersion in 70% ethanol, 99% ethanol, 100% ethanol (absolute alcohol). They were then briefly air dried before coverslips were added using Aqua mount.

Sections to be permanently mounted were dehydrated by brief sequential immersion in 70% ethanol, 99% ethanol, 100% ethanol (absolute alcohol), followed by immersion in xylene. Sections were then removed from xylene and mounted while still damp using pertex. Excess xylene was removed with paper tissue.

2.5.2 In-situ hybridisation

Dako PNA detection kit was used in conjunction with DAKO PNA probes (EBER, kappa and lambda). The kit was used in accordance with the manufacturers instructions. Sections were de-waxed and re-hydrated as follows: 2 rinses in xylene (5 minutes), 2 rinses in 99% ethanol (5 minutes), 2 rinses in 95% ethanol (5 minutes). Sections were then air dried for 5 minutes and tissue encircled with DAKO PAP pen. Sections were rinsed in RNase free water then 150µl of the supplied proteinase K was added for 25 minutes. Sections were rinsed again in RNase free water (5 minutes) then briefly immersed in 95% ethanol for 10 seconds, then air dried. 2 drops of DAKO PNA probe was added, sections coverslipped and placed in 37°C incubator in a humidity chamber for 1.5 hours. Sections were then washed in the supplied stringent wash buffer (diluted 1/60 in RNase free water) for 25 minutes at 55°C with stirring. Sections were briefly immersed in TBS, then 3 drops of supplied anti-FITC/AP (alkaline phosphatase labelled antibody) were added for 30 minutes at room temperature. Sections were rinsed twice in TBS for 3 minutes, then twice in distilled H₂O for 1 minute. Three drops of supplied substrate was added for 30 minutes at room temperature. Sections were then rinsed in running water for 5 minutes and lightly counterstained with haematoxylin (not supplied in kit), before being aqueous mounted as described in 2.7.1.7.

2.5.3 Quantitation of cells in immunohistochemistry sections

Cells were quantified manually using a cell counter and light microscope. Sections were scanned at magnification of x100 in the pattern shown below. Care was taken not to omit any areas of tissue present on the slide. All quantitations were carried out 'blind' with regard to HIV status and group.

2.5.4 Polymerase chain reaction (PCR)

PCR amplification was used to detect and quantify EBV gene sequences, and to detect re-arranged immunoglobulin heavy chain genes.

2.5.4.1 DNA extraction

Nucleic acids were extracted from paraffin embedded sections, frozen brain tissue, frozen CSF samples and fresh PBMCs. During each extraction care was taken to avoid contamination of samples with amplified PCR products which could give rise to false positive results. This was accomplished by using separate areas in the laboratory for storage of PCR products and for extraction of DNA. In addition PCR reactions were performed in a designated PCR only area and using sterile plasticware kept only for the procedure.

DNA was extracted from 20µm paraffin embedded sections using a Nucleon HT kit. DNA was extracted as follows, in accordance with the manufacturers instructions.

All DNA extractions from frozen tissue, HIV infected and uninfected, were carried out in class 2 hood following the precautions described earlier. A stock solution of extraction buffer was made (5ml TNE + 250µl 19mg/ml proteinase K + 33µl 2mg/ml PolyA). A small piece of frozen tissue was removed from the frozen sample and placed in a sterile eppendorff tube. 500µl of extraction buffer was added to each tube. Tubes were then incubated at 55°C for 2.5 hours. 450µl of

phenol:chloroform:iso-amyl alcohol was added to each tube and tubes shaken for 2 minutes. Tubes were then centrifuged for 5 minutes at 1300g. 400µl of aqueous phase was transferred to a fresh tube. 40µl sodium acetate pH 5.2 (3M) was added, the 800µl of ethanol (chilled to -20°C), and the tubes stored at -20°C for over night. Tubes were then centrifuged at 1300g for 15 minutes, the supernatant discarded and 600µl of 80% ethanol (chilled to -20°C) added. Tubes were centrifuged at 1300g for 5 minutes, then the supernatant removed. The remaining pellet was dried at 37°C for 10-15 minutes, before being re-dissolved with 50µl of nuclease free water. Tubes stored at 4°C overnight to allow pellets to dissolve.

DNA concentrations reading were taken. Samples were then stored at -20°C until required.

2.5.4.2 DNA extraction from frozen CSF samples, cell lines and fresh PBMCs

DNA extractions from frozen CSF samples and PBMCs were carried out in a class 2 hood. PBMCs were separated from whole blood as described above. The pellet obtained was re-dissolved in 200µl of PBS ready for extraction. Culture cells were diluted to the appropriate concentration in culture medium, then centrifuged and re-suspended in 200µl of PBS. CSF samples were thawed by leaving them in the hood at room temperature. 200µl was then removed from each sample and transferred to a sterile eppendorf.

A High pure viral nucleic acid kit was used to extract nucleic acid from all sample types. In accordance with the manufacturers instruction, 200µl of binding buffer (binding buffer + Poly A) was added to each tube. Then 50µl of supplied proteinase K was added and tubes incubated at 37°C for 10 minutes. 100µl of isopropanol was then added. The contents of the tube were then transferred to a spin column and centrifuged at 1300g for 1 minute. 500µl of inhibitor remover was then added and the column re-centrifuged as before. This was followed by two rinses with wash buffer

again centrifuged through the column. A fresh collection tube was added to the column and 50µl of elution buffer was added and the column centrifuged. The final elutant was removed and transferred to a sterile eppendorff. The DNA concentration readings were taken. Extracted DNA was stored at -20°C until required.

2.5.4.3 β-Globin PCR

β-globin PCR was used to confirm the presence of amplifiable DNA in samples after DNA extraction. This PCR is routinely used in our laboratory and has been previously standardised. Primer sequences and product sizes are given in Appendix E, reaction conditions are given below:

- 5µl 10x PCR buffer
- 3µl 25mM MgCl₂
- 0.5µl 20mM dNTP
- 0.5µl of each primer 100pM/µl
- 0.5µl Taq polymerase 5U
- 40.5µl nuclease free water
- 1µg DNA

2.5.4.4 Semi-quantitative EBV (BamW) PCR

Bam W PCR used to detect the presence of EBV in samples and to quantify the number of EBV genomes in samples. This PCR is also routinely used in our laboratory and has been previously standardised. Primer sequences and product sizes are given in appendix E, reaction conditions are given below:

- 5µl 10x PCR buffer
- 3µl 25mM MgCl₂
- 0.5µl 20mM dNTP
- 0.5µl of each primer 100pM/µl
- 0.5µl Taq polymerase 5U
- 40.5µl nuclease free water
- 1µg DNA

In order to evaluate the EBV copy number a semi-quantitative PCR method was employed. A 10 fold serial dilution of Namalwa cells (1-2 copies of EBV genome per cell) from 10^6 cells down to 1 cell in a background of 10^6 EBV negative BJAB cells was created (see 2.6.5.3). DNA was extracted from the cell mixtures and PCR performed to amplify the highly conserved Bam H1 W region of the EBV genome (Wagner *et al* 1992). Following electrophoresis and southern blotting of the PCR products, autoradiographs were analysed by scanning densitometry to determine the density and area of amplified bands. The densitometry readings from the dilution series were plotted on a graph, and then densitometry readings from samples were read off the graph to give a semi-quantitative assessment of the number of EBV genomes present in a sample.

2.5.4.5 FR3 PCR

FR3 PCR amplifies re-arranged Ig heavy chain genes at the junction between the variable heavy chain gene and the diversity heavy chain gene. The two primers bind to highly conserved regions of the two genes. The technique has been previously described by Trainor *et al* 1990, and was standardised as described in section 3.4.2. The optimised PCR reaction mixture contained:

- 5 μ l 10x PCR buffer
- 4.5 μ l 25mM MgCl₂
- 0.5 μ l 20mM dNTP
- 0.5 μ l of each primer 100pM/ μ l
- 0.75 μ l Taq polymerase 5U
- 38 μ l nuclease free water
- 1 μ g DNA

The optimised reaction conditions were as follows:

- 92°C for 2 minutes
- 34 cycles of
 - 92°C for 45 seconds
 - 55°C for 36 seconds
 - 72°C for 45 seconds
- 72°C for 10 minutes
- cool to 4°C

2.5.4.6 FR1 PCR

FR1 PCR also amplifies re-arranged Ig heavy chain genes. Six PCR are used one specific for each variable heavy chain family of genes (VH1, VH2, VH3, VH4, VH5 and VH6). The primer sequences are given in appendix E. Each 5' primer is specific for 1 variable heavy chain family of genes and binds to a region highly conserved within that family, the 3' primer is common to all six reactions and binds to a highly conserved region of the Ig heavy diversity gene. This technique has been previously published by Inghirami *et al* (1993), and the reaction conditions were optimised as described in 3.4.3. Optimal PCR reaction mixture for each of the 6 PCRs is given below:

- 5µl 10x PCR buffer
- 4.5µl 25mM MgCl₂
- 0.5µl 20mM dNTP
- 1µl of each primer 100pM/µl
- 0.5µl Taq polymerase 5U + 0.5µl Taq start
- 38µl nuclease free water
- 1µg DNA

PCR programs are given in table 10.

Table 10: FR1 PCR programe

Program	VH1, VH2, VH5, VH6	VH3, VH4
Initial denature °C (time: minutes)	95 °C (5)	95 °C (5)
Denature °C (time: seconds)	95°C (78)	95°C (78)
Annealment °C (time: seconds)	61°C (18)	65°C (18)
Elongation °C (time: seconds)	72°C (72)	72°C (72)
Number of cycles	34	34
Extension °C (time: minutes)	72°C (5)	72°C (5)

2.5.4.7 Gel electrophoresis

PCR products were resolved and visualised using gel electrophoresis. A 2.5% gel was made by melting NuSieve 3:1 agarose (3 parts agarose, 1 part Sea Kem agarose) by microwaving in 1X TBE buffer. The gel was then cooled under running water and 10µl ethidium bromide (EtBr) (10µg/ml)/ 100ml gel was added. The gel was poured into a casting tray with combs inserted and allowed to set. The gels were placed in an electrophoresis tank (Scot Lab), covered with 1x TBE buffer, and the combs removed.

20µl of PCR products were mixed with 4µl of 10x loading buffer and pipetted into appropriate wells in the gel. Two sets of DNA size markers were used; 100bp DNA ladder and DIG VIII ladder (both Gibco BRL). Electrophoresis was carried out at 80mA and EtBr staining was visualised under a UV transilluminator system (UVP) and a photograph taken to record the image.

2.5.5 Reverse transcriptase (RT) PCR

RT PCR was used to assess cytokine gene expression in frozen brain tissue. RNA extracted from PHA stimulated fresh PBMCs was used as control material for all RT-PCR reactions.

2.5.5.1 RNA extraction

Total cellular RNA was extracted by homogenisation of small pieces of frozen brain tissue or PBMC cell pellets in RNazol B solution. Samples were homogenised using sterile RNase free homogeniser rods. Following disruption of cells/tissue in RNazol B, 100µl of chloroform was added per 1ml of homogenate, the samples were capped and shaken for 30 seconds, then left on ice for 5 minutes. The mixture was then centrifuged at 1300g for 15 minutes. The upper aqueous phase was then removed to a fresh RNase free eppendorf. RNA was precipitated by addition of an equal volume of isopropanol and the mixture left on ice for

15 minutes. The RNA was pelleted by centrifugation at 1300g for 15 minutes, and the supernatant removed. The RNA was washed once in 75% ethanol and centrifuged at 1300g for 8 minutes. The supernatant was removed and the RNA pellet re-suspended in 50 μ l of nuclease free water. RNA concentration was determined by spectrophotometry. Samples were then stored at -70°C.

2.5.5.2 Complementary DNA (cDNA) synthesis

cDNA was synthesised by reverse transcription of 5 μ g of total RNA using oligo dT primers (Invitrogen RT kit). Volume of RNA was adjusted to 38 μ l with DEPC water. 3 μ l of oligo dT primers were added and the mixture incubated at 65°C for 5 minutes. The reaction was then cooled at room temperature for 10 minutes. The following reaction components were then added in order:

- 5 μ l 10x first strand buffer
- 1 μ l RNase block ribonuclease inhibitor (40u/ μ l)
- 2 μ l of MMLV (Moloney murine leukaemia virus) Reverse transcriptase (50u/ μ l)

The mixture was incubated at 37°C for 1 hour. Then heated to 90°C for 5 minutes and finally placed on ice. cDNA samples were stored at -20°C.

2.5.5.3 RT-PCR

In order to confirm that amplifiable DNA had been produced in 2.7.4.2 a GapDH PCR was performed on each sample. PCR reaction mixture contained the following in each reaction tube:

- 5 μ l 10x PCR buffer
- 3 μ l MgCl₂
- 0.5 μ l dNTP μ l
- 0.5 μ l of each primer
- 0.5 μ l Taq polymerase 5U/ μ l
- 40 μ l nuclease free water
- 5 μ l cDNA

Reaction conditions were as follows:

- 94°C for 2 minutes
- 30 cycles of
 - 94°C for 30 seconds
 - 58°C for 30 seconds
 - 72°C for 30 seconds
- 72°C for 10 minutes
- cool to 4°C

Products were visualised on EtBr gels as described above. Primer sequences and band sizes are given in appendix E.

2.5.5.4 IL-2, IL-4, IL-6, IL-10, and INF γ RT-PCR

IL-2,4,6,10 and INF γ PCR were performed in cDNA generated from RNA extracted from frozen brain tissue. PHA stimulated PBMCs were used as a source of control RNA. Reaction conditions for each PCR are given below and PCR programs given in table 11. Primer sequences and band sizes are given in appendix E. All reaction mixtures consisted of the following:

- 5 μ l 10x PCR buffer
- 3 μ l 25mM MgCl₂
- 0.5 μ l 20mM dNTP
- 0.5 μ l of each primer 100pM/ μ l
- 0.5 μ l Taq polymerase 5U/ μ l
- 40 μ l nuclease free water
- 5 μ l cDNA

Products were visualised on EtBr gels as described above.

2.5.6 Southern blotting and probing of blots

EtBr gels from BamW PCR and all RT-PCRs were Southern blotted and probed.

2.5.6.1 Southern blotting

Following electrophoresis of PCR products, the DNA was transferred onto a nylon membrane using the technique described by Southern (1975). The gel was rinsed in distilled H₂O and soaked in an alkaline denaturing

solution for 15 minutes, then transferred to a neutralising solution pH 7.2 for 15 minutes. The gel was then placed on 3MM blotting paper (soaked in x10 SSC) laid across a plastic brace which was supported over a tray containing x10 SSC solution. Both ends of the paper hung over the edges into the solution to act as a wick. The area surrounding the gel was covered with cling film. A piece of nylon membrane (Roche) was cut to

Table 11: PCR programs for IL-2,4,6,10 and INF γ

Primers/ Program	IL-2	IL-4	IL-6	IL-10	INFγ
Denaturation temperature (time: seconds)	94°C (60)	94°C (45)	94°C (60)	94°C (45)	94°C (60)
Annealment temperature (time: seconds)	-	60°C (45)	-	60°C (45)	-
Extension temperature (time: seconds)	-	72°C (120)	-	72°C (120)	-
Annealment/extension temperature (time: seconds)	55°C (120)	-	65°C (120)	-	55°C (120)
Final extension temperature (time: minutes)	-	72°C (7)	-	72°C (7)	-
Number of cycles	40	35	40	35	40

the exact size of the gel and placed over the gel. This was followed by 3 layers of 3MM paper also cut to size. A stack of dry paper towels was placed on top followed by a glass plate and a 500g weight. The gel was left over night, then removed and the DNA linked to the membrane by crosslinking in a Stratalinker with 120,000 μ J UV.

2.5.6.2 (Digoxigenin) DIG probing

DIG end labelling kit and DIG detection kit was used to label and detect oligo probes.

Probes were labelled as follows:

- 1µl (100pmol) probe added to 8µl water, on ice.
- 4µl of buffer added
- 4µl CoCl₂
- 1µl ddUTP
- 1µl Terminal transferase
- 1µl dATP

Mixture was incubated at 37°C for 15 minutes, then cooled on ice and 2µl of 0.2M EDTA pH 8.0 added to stop the reaction. Labelled probe were stored at -20°C. The sequences of probes used and the expected band sizes are given in appendix E.

DNA was attached to nylon membranes as described in 2.7.5.1. DIG detection kit was then used as follows:

- Membranes were pre-hybridised at hybridisation temperature (see table 12) for 30 minutes in DIG easy hyb (20ml/100cm²)
- Pre-hybridisation buffer was poured off and 1.4µl of probe diluted in 3.5ml of pre-heated DIG easy hyb was added. Probe was incubated for 2 hours at hybridisation temperature.
- Probe was poured off and membranes rinsed twice in x2 SSC + 0.1% SDS at the temperatures given in table 12 for 5 minutes.
- Membranes were then washed twice in x0.1 SSC + 0.1% SDS for 15 minutes at the temperatures given in table 12.
- 100 ml of x1 block solution (diluted 12ml block in 108ml maleic acid pH 7.5) was added to each membrane for 45 minutes at room temperature.
- Block solution was poured off and anti-DIG AP (alkaline phosphatase labelled antibody) was added (diluted 2µl in 20ml of diluted block solution) for 30 minutes at room temperature.
- Membranes were then rinsed twice with wash buffer (maleic acid 0.1M pH 7.5 + 0.3% v/v tween) for 15 minutes.

- CSPD was diluted 1/100 in 2ml of detection buffer (0.1M tris-HCl + 0.1M NaCl pH 9.5) and membranes incubated for 5 minutes at room temperature.
- Blots were sealed in plastic bags and incubated at 37°C for 15 minutes, then exposed to DIG luminescent film for 1-2 hours.

Table 12: DIG detection conditions

Hybridisation conditions \ Probe	BamW	IL-2 / IL-4	IL-6	IL-10	INFγ
Pre-hybridisation/hybridisation temperature °C	50	50	60	65	65
Wash temperature (2x SSC + 0.1% SDS) °C	20	20	20	50	65
Wash temperature (0.1x SSC + 0.1% SDS) °C	20	20	20	50	65

3 Results

3.1 Prevalence of lymphocytes in brain tissue

Eight patient groups were used in the study. The cases were carefully selected to fit within the definition of the group as in outlined 2.1 page 59, thus minimising potential confounding variables. Tissue sections were stained using immunohistochemical techniques described in 2.2.1 page 67. All tissue sections were stained and assessed without prior knowledge of case groupings.

3.1.1 Prevalence of lymphocytes in normal brain

The aim of this experiment was to identify both the position and types of lymphocytes found in normal human brain tissue, and to subsequently quantify the different cell types. Immunohistochemistry was used to identify both B and T lymphocytes in normal brain. An anti CD45 antibody was used to identify all lymphocyte cell types. B lymphocytes were then identified using antibodies against CD20 and CD79a, while T lymphocytes were identified using antibodies against CD3 and CD8. No anti-CD4 antibodies were available which would work on FFPE brain tissue sections that had been fixed for long periods in formalin (over 2 weeks fixation).

Seven cases were used with an average of 6 sections from different areas of the brain available for each case.

3.1.1.1 Optimisation of staining techniques for antibodies used to assess numbers of lymphocytes in the brain

In order to optimise the antibody dilutions, each antibody was titrated on control tonsil sections to determine the optimal primary antibody concentration and their staining intensity was assessed with a variety of antigen retrieval pre-treatments. Antibodies were assessed using the concentrations and pre-treatments given in table 13. Each antibody was assessed at the given concentrations for each pre-treatment.

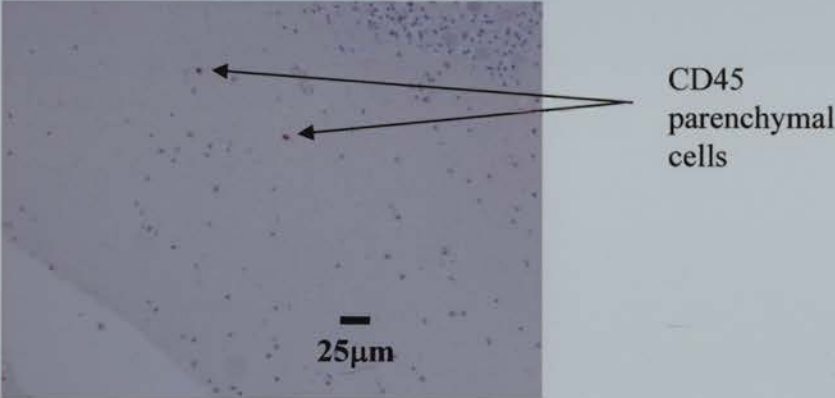
Table 13:**Techniques for optimisation of lymphocyte antibody staining reactions**

Antibody	Pre-treatments	Antibody concentrations assessed for each pre-treatment	Optimal staining method
CD3	No-pretreatment	1/25	Trypsin pre-treatment at 1/100 dilution
	Citric acid microwave	1/50	
	Citric acid pressure cook	1/100	
	EDTA microwave	1/200	
	EDTA pressure cook Trypsin enzymatic digestion		
CD8	No-pretreatment	1/25	EDTA microwave at 1/50 dilution
	Citric acid microwave	1/50	
	EDTA microwave	1/100	
		1/200	
CD20	No-pretreatment	1/50	Citric acid microwave at 1/300 dilution
	Citric acid microwave	1/100	
	EDTA microwave	1/300	
		1/500	
CD45	No-pretreatment	1/100	No pre-treatment required. 1/300 dilution
	Citric acid microwave	1/200	
	EDTA microwave	1/300	
		1/500	
CD79a	No-pretreatment	1/25	EDTA microwave 1/30 dilution
	Citric acid microwave	1/30	
	Citric acid pressure cook	1/50	
	EDTA microwave	1/100	
	EDTA pressure cook	1/200	

3.1.1.2 Quantitation of cells expressing CD45 in normal brain tissue

Sections from 7 normal brains (group 1) were stained using an anti-CD45 antibody. Figure 12 shows a representative photograph of the staining obtained using this antibody. The number of cells per section was deemed too great to accurately quantify by manual counting. Automated computer

Figure 12: CD45 positive cells in a normal brain



counting of cells was also not feasible as the computer is unable to distinguish between stained cells in the parenchyma and those present in blood vessels. In addition the antibody did not distinguish between B and T lymphocytes and therefore was felt to be of minimal use.

3.1.1.3 Quantitation of cells expressing CD20 in normal brain tissue

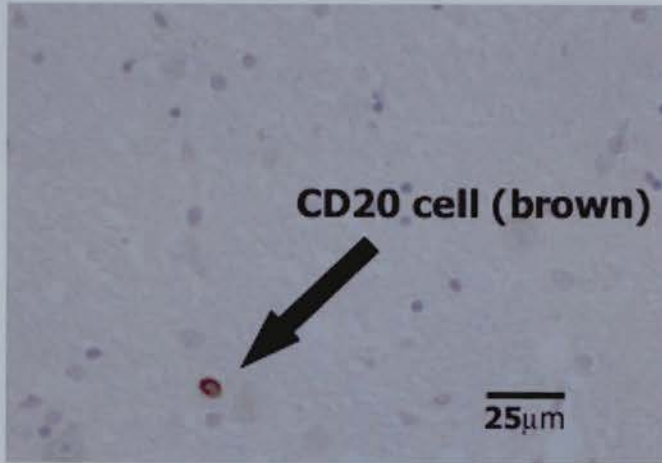
Sections from 7 normal brains (group 1) were stained using an anti-CD20 antibody. Where available sections from all 11 areas of the brain listed in section 2.4 (page 66) were used for each case studied. Positive cells were counted as using a light microscope and cell counter, and the average number of cells per square centimetre of the section calculated (Area was calculated by computer scanning sections then overlaying with a grid containing cm^2 and mm^2 boxes, complete boxes were then counted to give area). The results for each case are summarised in table 14 and the data from each individual section given in table A1 (appendix A).

Table 14: Quantitation of CD20 positive cells in normal brains

Case number	Number of parenchymal CD20 positive cells/ cm^2	Number of perivascular CD20 positive cells/ cm^2
1	0	0
2	0.111	0
3	0.093	0
4	0.1	0
5	0.134	0
6	0.111	0
7	0.238	0

The results show that occasional CD20 positive B lymphocytes were found in the parenchyma of 6 out of 7 normal brains (see figure 13). No CD20 positive B lymphocytes were found in the perivascular space in any of these brains. An

Figure 13: Single parenchymal CD20 positive B lymphocyte in a normal brain



average of 0.112 CD20 positive B lymphocytes were found per cm² of normal brain. Cells were found in all regions of the brains and there did not appear to be any obvious homing of these cells to particular region of the brain.

3.1.1.4 Quantitation of cells expressing CD79a in normal brain

A second B lymphocyte antibody (anti-CD79a) was used to assess the B lymphocyte population of the brain. As described earlier formalin fixation can prevent or hinder antibody attachment to antigen present within the tissue. The blocking effects of formalin fixation vary between different antibodies for reasons that are not clear. Therefore 2 antibodies were assessed in order to minimise the masking effects of formalin on B lymphocyte antigens.

Consecutive sections to those used in 3.1.1.2 were assessed for CD79a positive cells. The results for each case are summarised in table 15.

Table 15: Quantitation of CD79a positive cells in normal brains

Case number	Number of parenchymal CD79a positive cells/cm ²	Number of perivascular CD79a positive cells/cm ²
1	0.059	0
2	0.044	0
3	0.046	0
5	0.140	0
6	0	0
7	0.05	0

The results show that occasional CD79a positive cells were found in the parenchymal brain tissue of 5 out of 6 brains assessed. As with the CD20 antibody no perivascular CD79a cells were located. Overall the number of CD79a positive cells found was lower than the number of CD20 positive cells, an average of 0.057 CD79a cells/cm² compared to 0.112 CD20 cells/cm². However a paired T-test showed no significant difference between the two antibodies (p=0.163). The lower number of B lymphocytes found using the

CD79a antibody may perhaps be accounted for by the generally weaker staining obtained with this antibody and also the use of different sections.

3.1.1.5 Quantitation of cells expressing CD3 in normal brain

Consecutive sections to those used in 3.1.1.4 were assessed for CD3 positive cells (see figure 14 and 15). CD3 is a pan T lymphocyte marker present on both CD4 and CD8 lymphocytes. Results obtained using anti-CD3 antibodies were similar in terms of quantity to those obtained with the anti-CD45 antibody. Given the low number of CD20 and CD79a positive cells found in 3.2.1.3 and 3.2.1.4 this would suggest that the majority of cells detected with anti-CD45 antibody in normal brains were T lymphocytes. The number of CD3 positive cells was deemed too great to accurately count and therefore only sections from one case were quantified in order to allow a crude comparison of the number of B lymphocytes present compared to T lymphocytes. This showed approximately 50 fold greater number of T lymphocytes to B lymphocytes.

3.1.1.6 Quantitation of cells expressing CD8 in normal brain

Consecutive sections to those used in 3.1.1.5 were assessed for CD8 positive cells. The number of CD8 positive cells present in normal brain tissue was found to be similar to the number of CD3 cells present, suggesting that the majority of T lymphocytes present are of the CD8 phenotype. It was not possible to assess the number of CD4 positive cells present directly, as no antibody was available that worked reliably in material fixed for long periods in formalin, however based on these results the CD8 T lymphocytes appear to be dominant over CD4 T lymphocytes within the brain.

3.1.1.7 Double immunohistochemistry (CD20 and Von Willebrand's antibodies)

In order to be certain that B lymphocytes were located within the brain parenchyma, rather than within brain capillaries, double immunohistochemical staining was undertaken using an anti-CD20 antibody (B lymphocyte marker) and an anti-Von Willebrand antibody (endothelial cell

Figure 14:

CD3 positive cells in a normal brain

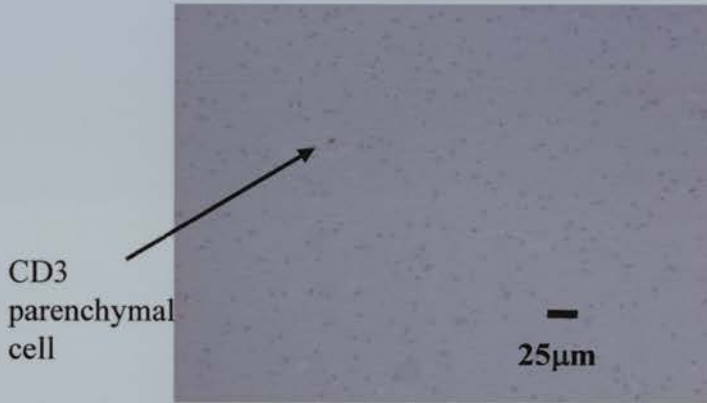
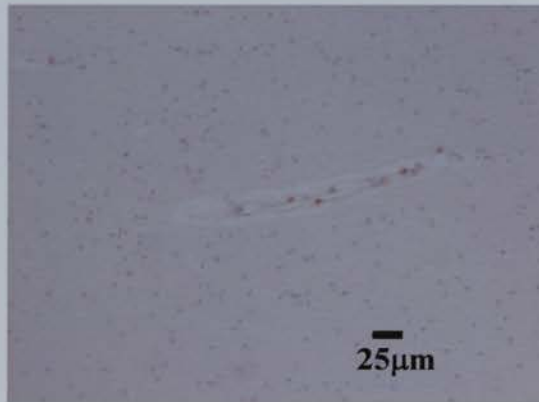


Figure 15:

CD3 positive cells in the perivascular spaces of a normal brain



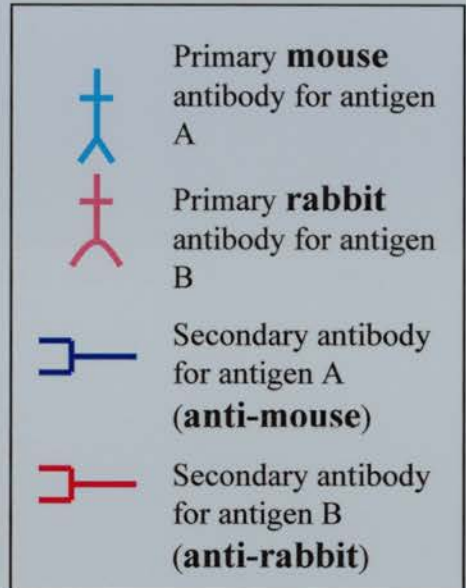
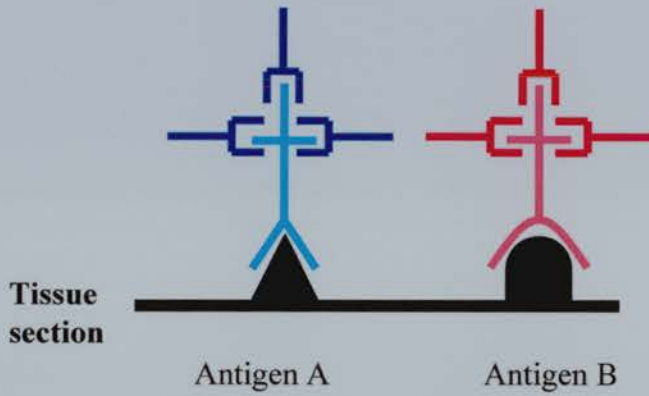
marker). Von Willebrand antibody binds to Factor VIII:Von Willebrand, a component of Factor VIII which is produced in endothelial cells and which binds to Factor VIII:C (produced in liver cells) to produce the complete Factor VIII molecule. Double immunohistochemical staining allowed the location of cells to be determined with reference to blood vessels, and to be sure that cells found within capillaries or small blood vessels were not being counted as parenchymal cells.

3.1.1.7.1 Optimisation of double antigen labelling immunohistochemistry

In order to use two antibodies effectively on a single section the primary antibodies must be raised in different species to avoid cross reactions between the secondary antibodies, which would make distinguishing between 2 labelled antigens on a single section impossible (see Figure 16 below). For this reason a mouse monoclonal CD20 antibody was chosen to label B lymphocytes and a rabbit polyclonal Von Willebrand antibody was chosen to label endothelial cells in the section. Secondary antibodies were biotinylated rabbit anti mouse for CD20 and biotinylated swine anti rabbit for the Von Willebrand's antibody. The antibodies were first titrated separately, CD20 on FFPE tonsil sections as described in 3.1.1.1 above, and Von Willebrand's on FFPE sections from normal brain. Von Willebrand's antibody was tested at each concentration given in table 16 for all of the antigen retrieval pre-treatment method given.

Figure 16: Cross reactions in double immunohistochemistry

No cross reaction using primary antibodies raised in different species



Potential cross reaction occurring using primary antibodies raised in the same species

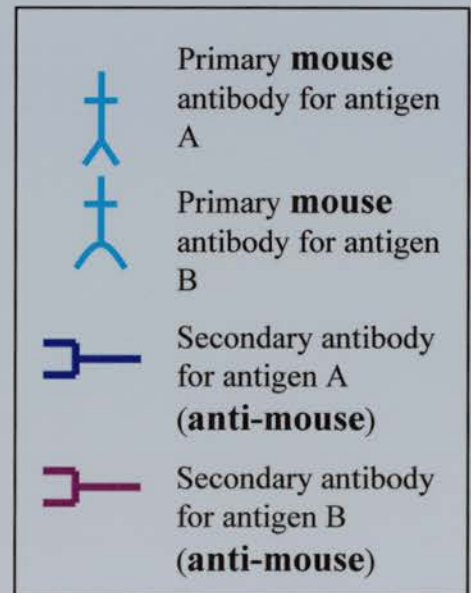
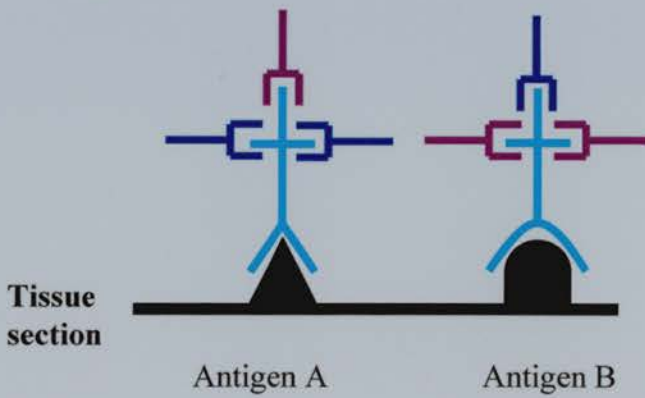


Table 16: Optimisation of Von Willebrand's antibody staining

Pre-treatments assessed	Antibody concentrations tested	Optimal method
No-pretreatment	1/100	Citric acid microwave at a concentration of 1/200
Citric acid (microwave)		
Citric acid (pressure cook)	1/200	
EDTA (microwave)		
EDTA (pressure cook)	1/400	

The two antibodies were then applied sequentially to the sections, Von Willebrand's first to avoid potentially binding of its secondary antibody (swine anti rabbit) to the secondary rabbit anti mouse used for CD20 if this was applied first. The full procedure is described in 2.2.1.5 page 71. Initial staining showed good staining of the endothelial vessels. However the B lymphocyte staining was weak and difficult to detect in the background of strongly stained endothelial cells. CD20 antibody concentration was therefore increased from 1/300 to 1/200 and finally to 1/100 to obtain a strong intensity of B lymphocyte staining.

3.1.1.7.2 Quantitation of CD20 positive cells in normal brain tissue using double immunohistochemistry

Figure 17a shows a photomicrograph of a single CD20 positive cell (purple) in the parenchyma of a normal brain, with nearby vessels stained brown. Figure 17b demonstrates a CD20 positive cell situated within a blood vessel. Figure 17c shows several perivascular CD20 positive cells in AIDS brain with pleomorphic infiltrates. Case 90/23 (normal brain) was re-assessed for numbers of CD20 positive cells using this technique. The same areas studied in 3.1.1.2 were used here although the sections were not consecutive to those used in 3.1.1.2. The results were remarkably similar with 1 extra parenchymal cell being found overall using the double

Figure 17a:

CD20 and Von Willebrand double immunohistochemistry on a normal brain (single parenchymal CD20 positive cell)

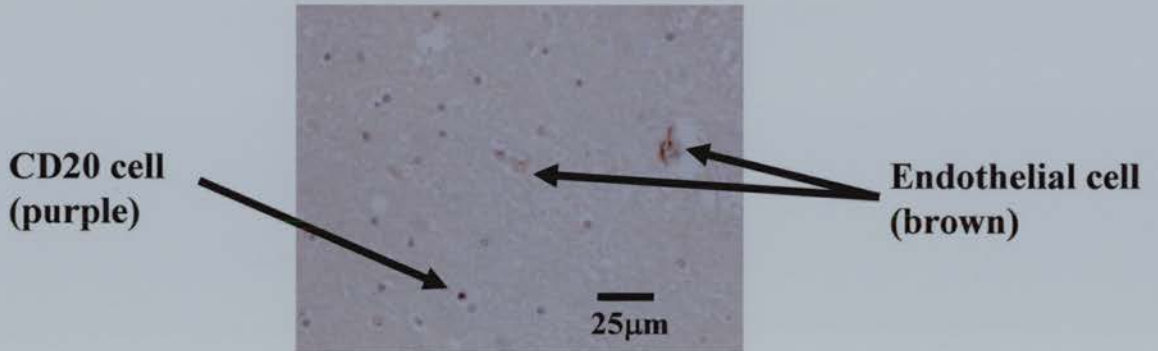


Figure 17b:

CD20 and Von Willebrand double immunohistochemistry on a normal brain (single CD20 positive intravascular cell)

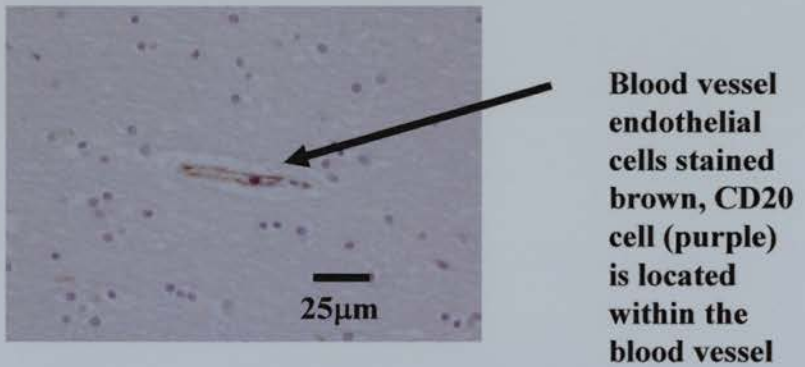
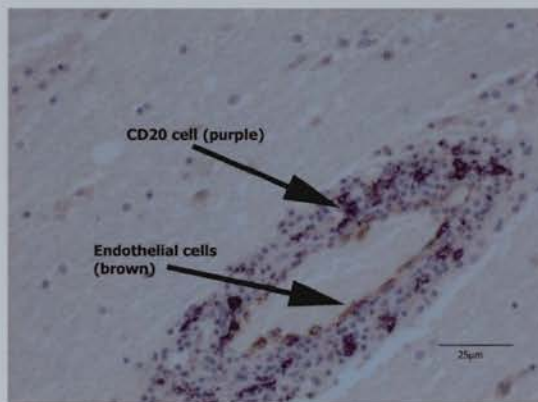


Figure 17c:

CD20 and Von Willebrand double immunohistochemistry on a AIDS brain with pleomorphic infiltrate



immunohistochemistry. This therefore suggested that cells counted using only the CD20 antibody in 3.1.1.2 had all been parenchymal cells, and that the subjective process of deciding on the location of CD20 positive cells had been carried out accurately.

3.1.2 Quantitation of CD20 positive cells in non-HIV drug users.

Since 16 out of 30 patients in the pre-AIDS and AIDS groups (groups 3-7) were IDUs it was essential to assess the effects of drug abuse alone on the CNS B lymphocyte population outwith the setting of HIV infection. Group 2 consisted of 5 cases of non-HIV infected IDUs who had died accidentally. Sections from all areas of the brain from each case were assessed for the number of CD20 positive B lymphocytes present. The results for each case are summarised in table 17 and the data from each individual section given in table A2 (appendix A).

Table 17: Quantitation of CD20 positive cells in non-HIV drug users

Case number	Number of parenchymal CD20 positive cells/cm²	Number of perivascular CD20 positive cells/cm²
8	0.959	0
9	0.293	0
10	0.052	0
11	0.102	0
12	0.174	0.116

CD20 positive parenchymal cells were found in sections from all 5 cases with a mean of 0.315 CD20 positive cells/cm² of brain tissue. Although this was higher than the number found in group 1 (normal brains) it was not significantly different from the results found in these cases (p=0.183). The majority of the increase was due to one outlier case (case 8) which showed far

greater numbers of cells than any of the other cases in the group (0.96 CD20 cells/cm² compared to an average of 0.154 CD20 cells/cm² for the other cases in the group). As with the normal cases CD20 positive cells were found in all areas of the brain and there appeared to be no preferential site for CD20 cells to accumulate. In one case (case 12) a single perivascular cell was found.

The fact that no significant difference was found between normal cases (group 1) and non-HIV drug users (group 2) allowed the valid inclusion of both drug users and non-drug users in groups 4,5,6 and 7 (AIDS no CNS pathology, AIDS infiltrate, AIDS PCNSL, and AIDS HIVE).

3.1.3 B lymphocytes in HIV brains

The aim of these experiments was to study the effects of advancing HIV disease on the B lymphocyte population of the brain. As before the CD20 and CD79a antibodies were used to quantify B lymphocytes in the brain. Six pre-AIDS cases (group 3), all drug users, and 24 AIDS cases were available for study. The AIDS cases were separated into 4 separate groups on the basis of their neuropathological findings at autopsy, as described in section 2.6.

3.1.3.1 Pre-AIDS brains

Sections from all areas of the brain from each case were assessed for the number of CD20 positive B lymphocytes present. Sections were then re-assessed using CD79a antibody.

The results for each case assessed for CD20 positive cells are summarised in table 18 and the data from each individual section is given in table A3 (appendix A).

Table 18: Quantitation of CD20 positive cells in pre-AIDS brains

Case number	Number of parenchymal CD20 positive cells/cm ²	Number of perivascular CD20 positive cells/cm ²
13	0.518	0.183
14	0.138	0.025
15	1.383	0.629
16	0.253	0.864
17	0.135	1.012
18	0.073	0

CD20 positive B lymphocytes were found in the parenchyma of all 6 cases and in the perivascular spaces of 5 out of 6 cases. The mean number of CD20 positive parenchymal cells for group 3 was 0.417 cells/cm², which did not differ significantly from the results found in group 1 (normal cases) (p=0.137) or group 2 (p=0.717). An average of 0.452 CD20 positive cells/cm² were found in the perivascular space, this was significantly raised when compared to the normal cases (group 1) and the non-HIV drug users (group 2), p=0.02 and p=0.05 respectively.

Sections were then re-assessed using the CD79a antibody. Table 19 shows a summary of the CD79a results, with the CD20 results from each case included for comparison.

Table 19: Quantitation of CD79a positive cells in pre-AIDS brains

Case number	Number of parenchymal CD79a positive cells/cm ²	Number of parenchymal CD20 positive cells/cm ²
13	0.105	0.518
15	0.105	1.383
16	1.345	0.253
17	0.123	0.135
18	0	0.073
Group average	0.336	0.473

N.B. Case 14 was omitted due to a shortage of tissue

CD79a positive cells were found in the parenchyma of 4 out of 5 pre-AIDS brains. As in group 1 (normal) the number of CD79a positive cells found in each brain was lower than the number of CD20 positive cells found, however the difference was less marked in this group with an average of 0.336 CD79a cells/cm² compared to 0.473 CD20 cells/cm² for the same cases. Paired T-test showed no significant difference in the number of cells detected with the two antibodies (p=0.738). The slightly lower number of CD79a cells may be due to the weaker staining achieved with this antibody in comparison to the CD20 antibody.

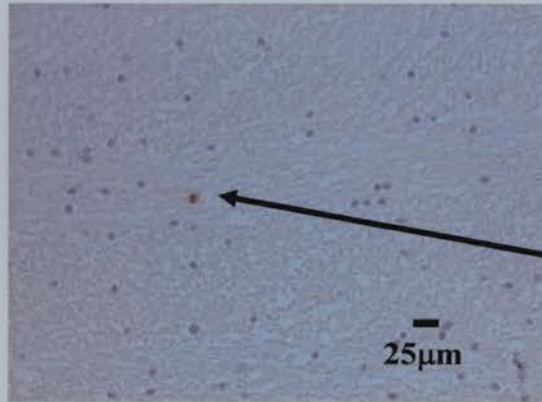
3.1.3.1.1 CD3 and CD8 Pre-AIDS results

As with the normal cases the number of CD3 and CD8 positive cells found per section was too great to quantify. Figure 18a and 18b demonstrates that as with the normal brains the majority of T lymphocytes present displayed a CD8 phenotype.

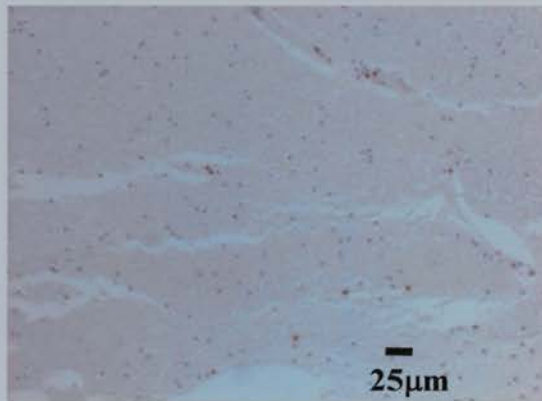
3.1.3.2 AIDS brains

Sections from all areas of the brain from each case in group 4 (AIDS no CNS pathology), group 5 (AIDS infiltrate), group 6 (AIDS PCNSL) were assessed for the number of CD20 positive B lymphocytes present. In addition, selected cases were assessed for the number of CD79a positive B lymphocytes present in the brain, and semi-quantitative assessment was made of the number of CD3 and CD8 positive T lymphocytes present in certain cases. In the AIDS PCNSL cases, the number of B lymphocytes was only quantified in areas of the brain that did not contain lymphoma. There were two reasons for this, firstly the number of cells in the neoplastic regions was too great to quantify accurately by subjective counting. Secondly we wished to assess the setting in which PCNSL had developed, in terms of the size of the B lymphocyte population forming a background in the affected brain.

Figure 18: CD3 sections of a pre-AIDS brain



CD3 positive
parenchymal
cell



Parenchymal and
perivascular CD3
positive cells (stained
brown) in a pre-
AIDS brain

The results for each case in each group are summarised in table 20 and the data from each individual section given in table A4, A5 and A6 (appendix A).

Table 20: Quantitation of CD20 positive cells in AIDS brains

Case number	Number of parenchymal CD20 positive cells/cm ²	Number of perivascular CD20 positive cells/cm ²
AIDS no CNS pathology		
19	0	0
20	0	0
21	0	0
22	0	0.023
23	0.039	0
AIDS pleomorphic lymphocytic infiltrate		
24	0.308	10.474
25	0	4.109
26	0.138	54.027
27	0.210	4.770
28	0.214	0.499
AIDS PCNSL (outwith the area of lymphoma)		
36	0	0.061
37	0	0
38	0	0
39	0.016	0
40	0	0
41	0.038	0
42	0	0.047

In AIDS cases with no CNS pathology (group 4), the brain was almost devoid of B lymphocytes, with only 1 case out of 5 containing any CD20 positive cells in the parenchyma or the perivascular spaces of the brain. In comparison to normal controls this represented a significant decrease in the number of B lymphocytes ($p=0.009$). The AIDS cases with pleomorphic lymphocytic infiltrations (group 5) showed higher levels of B lymphocytes in both the parenchyma and perivascular spaces of the brain. The mean number of parenchymal CD20 positive cells was 0.174 cells/cm² compared to 0.008 cells /cm² in the AIDS cases with no pathology.

All cases from group 5 contained blood vessels with infiltrations of lymphocytes in the perivascular space. Figure 19a shows a representative section stained using CD20 antibody. The majority of cells that were CD20

negative were shown to be CD3 positive T lymphocytes, with the vast majority of those being CD8 positive T lymphocytes (see figure 19b).

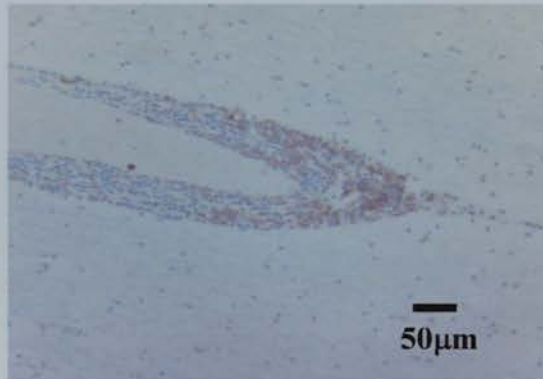
Focuses of perivascular infiltrations were found throughout the brains in this group, however there were areas in each brain devoid of B lymphocytes.

There did not appear to be any one anatomical area of the brain in which these infiltrations were more common. The average number of perivascular CD20 positive cells in this group was 14.776 cells/cm², a large increase over that seen in all of the other groups. In comparison to the normal cases and AIDS cases with no CNS pathology statistical significance was not reached. This was primarily due to the low numbers of cases, but also due to an outlier in the data. Case 26 had larger numbers of B lymphocytes than other cases in the group (54.027 compared to an average of 4.963 for the rest of the group). Removal of this outlier would give a significant difference between this group and both normal cases and AIDS cases with no CNS pathology, p=0.008 and p=0.029 respectively.

The AIDS cases with PCNSL, assessed outwith the area of lymphoma, were similar to the AIDS cases with no CNS pathology in terms of the presence of B lymphocytes. Only 2 out of 7 cases contained any B lymphocytes in the brain parenchyma or perivascular spaces. In both the parenchyma and perivascular spaces the overall average number of CD20 positive cells for the group was low, with the former having 0.003 cells/cm² and the latter 0.015 cells/cm². In terms of the number of parenchymal B lymphocytes this represented a significant decrease in comparison to normal controls, p=0.022.

In addition to the cases assessed for CD20 positive cells, 3 PCNSL cases and 1 AIDS case with no CNS pathology were assessed for brain B lymphocytes using CD79a antibody. The results are shown in table 21, which includes the CD20 results for each case to allow comparison.

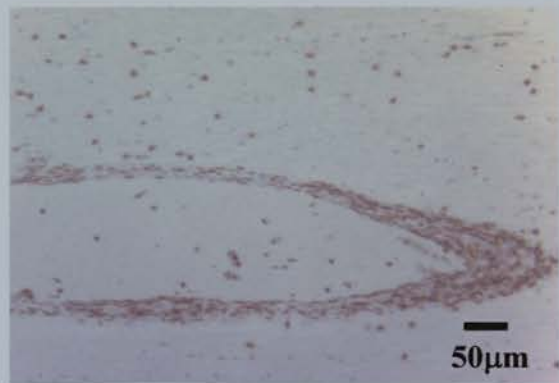
Figure 19: Section from AIDS case with pleomorphic infiltrate stained for CD20, CD3, CD8



CD20 stained section



CD8 stained section



CD3 stained section

The majority of cells found in pleomorphic infiltrates of AIDS brains are dual CD3 positive CD8 positive cells. Therefore the number of CD3 positive CD4 positive cells is likely to be low. CD20 B lymphocytes represent a smaller percentage of the inflammatory cells present than CD3 T lymphocytes.

In addition most of the parenchymal CD3 T lymphocytes in AIDS brains appeared to be dual CD3, CD8 positive cells.

Table 21: Comparison of CD79a and CD20 results from selected cases in AIDS groups

Case number	Number of parenchymal CD79a positive cells/cm ²	Number of parenchymal CD20 positive cells/cm ²	Number of perivascular CD79a positive cells/cm ²	Number of perivascular CD20 positive cells/cm ²
38 (PCNSL)	0	0	0	0
39 (PCNSL)	0.015	0.016	0	0
41 (PCNSL)	0.042	0.038	0	0
23 (AIDS no CNS pathology)	0	0.039	0	0
Group average	0.014	0.023	0	0

The data in table 21 shows that overall a slightly lower number of CD79a positive cells were found in AIDS brains compared to CD20 positive cells. As with the normal cases and the pre-AIDS cases the difference was not statistically significant ($p=0.437$).

3.1.4 Effects of encephalitis on brain B lymphocyte population

B lymphocytes have been shown to play a crucial role in the clearance of many viral infections from the CNS (Hatalski *et al* 1998a, 1998b, Tyor, Moench & Griffin 1989, Dorries 2001). Since there is a high prevalence of HIV encephalitis (HIVE) in AIDS patients, particularly in the drug using AIDS patients in the Edinburgh cohort, we sought to determine the effects of HIVE on the B lymphocyte population of the brain.

Brains which had pathological evidence of encephalitis (HIV related or otherwise) were available from 3 different categories: 6 AIDS cases with HIVE (group 6), 1 pre-AIDS case with non-HIV viral encephalitis, and 3 non-HIV cases with viral encephalitis (group 8). Sections from all areas of the brain were assessed for B lymphocytes using the anti-CD20 antibody. Table

22 shows a summary of the results in each group, data from individual sections in each case are given in table A7 and A8 (appendix A).

Table 22: Quantitation of CD20 positive cells in HIV and non-HIV brains with encephalitis

Case number	Number of parenchymal CD20 positive cells/cm ²	Number of perivascular CD20 positive cells/cm ²
AIDS patients with HIVE		
29	0.249	0.829
30	0.029	0.058
31	0	0.060
32	0.075	0.502
33	0.064	0.043
34	0.055	0.300
Pre-AIDS non-HIV viral encephalitis		
46	1.930	39.109
Non-HIV viral encephalitis		
43	3.181	12.511
44	1.378	24.664
45	14.852	155.779

An average of 0.075 CD20 positive cells/cm² was found in the parenchyma of AIDS HIVE brains. No significant difference was detected between the average number of B lymphocytes found in the brain parenchyma of AIDS patients with HIVE (group 6) and normal controls (group 1) (p=0.414) or AIDS patients with no CNS pathology (group 4) (p=0.130). Conversely, a significant increase was noted in the perivascular brain compartment of HIVE AIDS patients when compared to normal controls (p=0.028). Compared to AIDS patients with no CNS pathology (group 4) a large increase in the number of perivascular CD20 positive cells was noted in HIVE cases (an average of 0.005 CD20 cells/cm² compared to 0.306 CD20 cells/cm², p=0.07), however this did not reach statistical significance.

However in comparison to cases with non-HIV viral encephalitis in immunocompetent patients (group 8) HIVE cases had far fewer parenchymal

and perivascular CD20 positive cells (see figure 20a and figure 20b). No statistical tests were performed on group 8 due to the low number of cases available.

Case 46 was a pre-AIDS case with non-HIV viral encephalitis. In terms of both parenchymal and perivascular CD20 cells, this case contained more cells than the AIDS HIVE cases but less than non-immunocompromised viral encephalitis (see table 22 and figures 20a and 20b). The data shown in figures 20a and 20b suggest a rising trend in the number of both parenchymal and perivascular B lymphocytes in the brain from AIDS cases with no CNS pathology with the least B lymphocytes, to AIDS HIVE cases, then pre-AIDS encephalitis cases, and finally immunocompetent encephalitis cases with the greatest numbers of B lymphocytes.

3.1.5 Lymphocyte proliferation markers

The proliferation index of lymphocytes infiltrating the brain was assessed by staining sections with anti-Ki-67 and anti-PCNA (proliferating cell nuclear antigen) antibodies. Ki-67 is a nuclear protein expressed during all stages of the cell cycle (G_1 , S, M, G_2 phases) except G_0 , it is therefore a marker of cells in active cell division. PCNA is also a nuclear protein expressed during active cell division, with maximal production occurring during S phase.

Only sections from AIDS PCNSL cases and AIDS infiltrate cases were assessed, as the other groups had too few lymphocytes present. Sections were stained individually with both antibodies which revealed high numbers of proliferating lymphocytes in both infiltrate cases and in lymphomatous areas of PCNSL cases. However both cases contained a mixture of B and T lymphocytes and it was not possible to distinguish these morphologically, therefore it could not be determined which cell types were proliferating.

Figure 20a: Quantitation of Parenchymal CD20 cells in brains with encephalitis

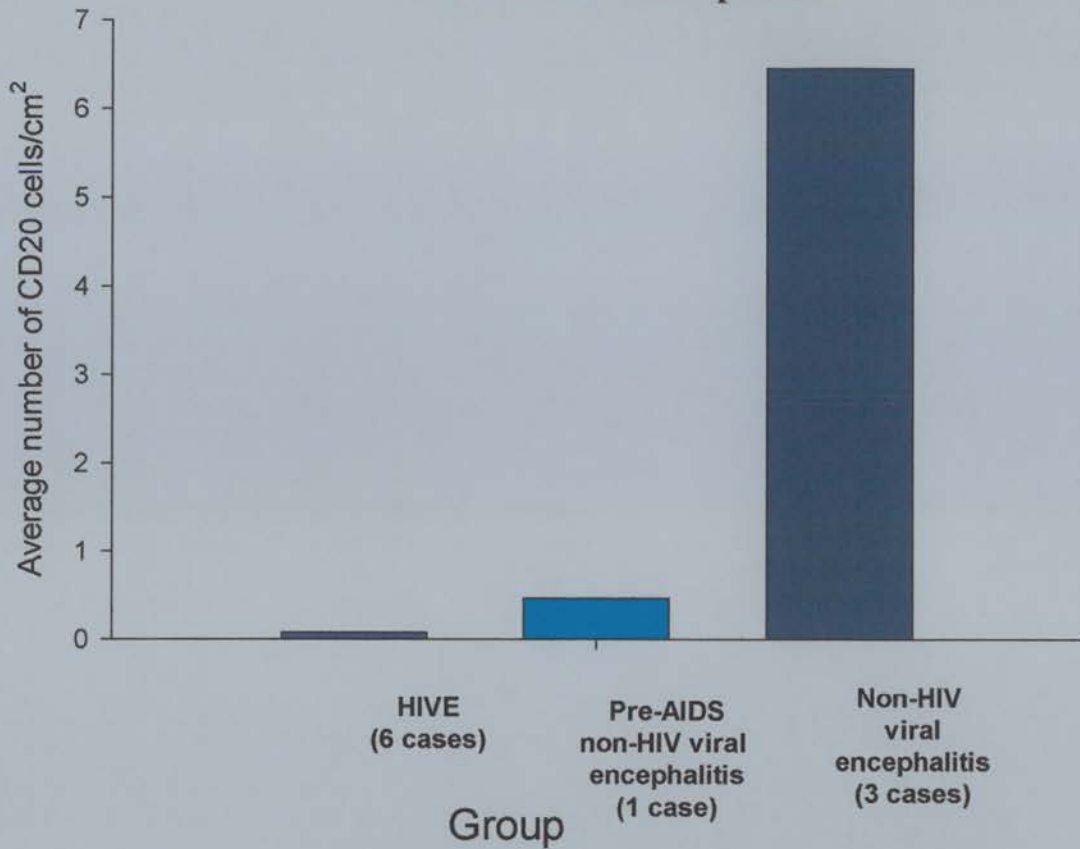
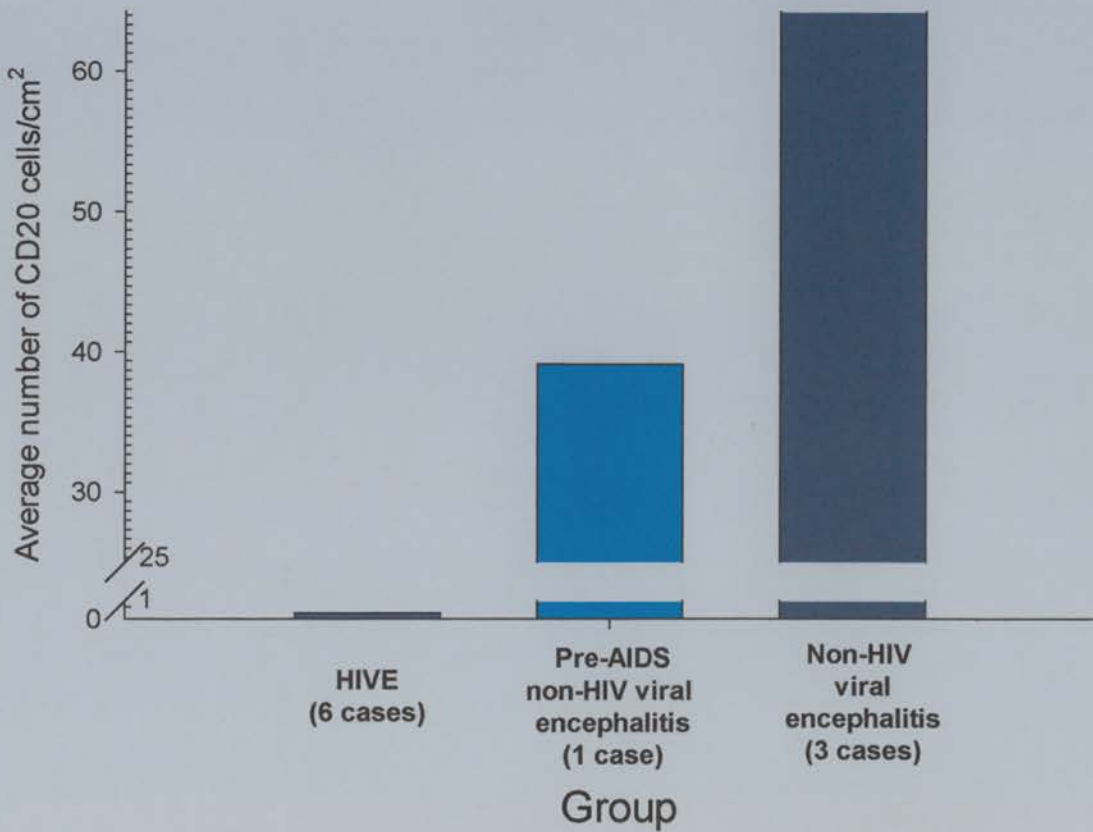


Figure 20b: Quantitation of perivascular CD20 cells in encephalitic brains



Double immunohistochemistry combining CD20 (monoclonal, B lymphocyte marker) and Ki67 (polyclonal, proliferation marker), and CD3 (polyclonal, T lymphocyte marker) and PCNA (monoclonal, proliferation marker), was then used to assess the proliferation of individual cell types. Results from this technique were poor as it was difficult to distinguish the two different stains on the same section. This was primarily due to the large numbers of stained cells and their close proximity to each other, and the fact that both antigens being labelled were potentially contained within the same small cell. An additional problem with this technique was the use of anti-PCNA antibody to mark proliferation in T lymphocytes (CD3) and anti-Ki-67 for B lymphocytes (CD20). Although both antibodies stain proliferating cells, PCNA shows weaker staining in cells that are not in S-phase of the cell cycle therefore making comparisons more difficult.

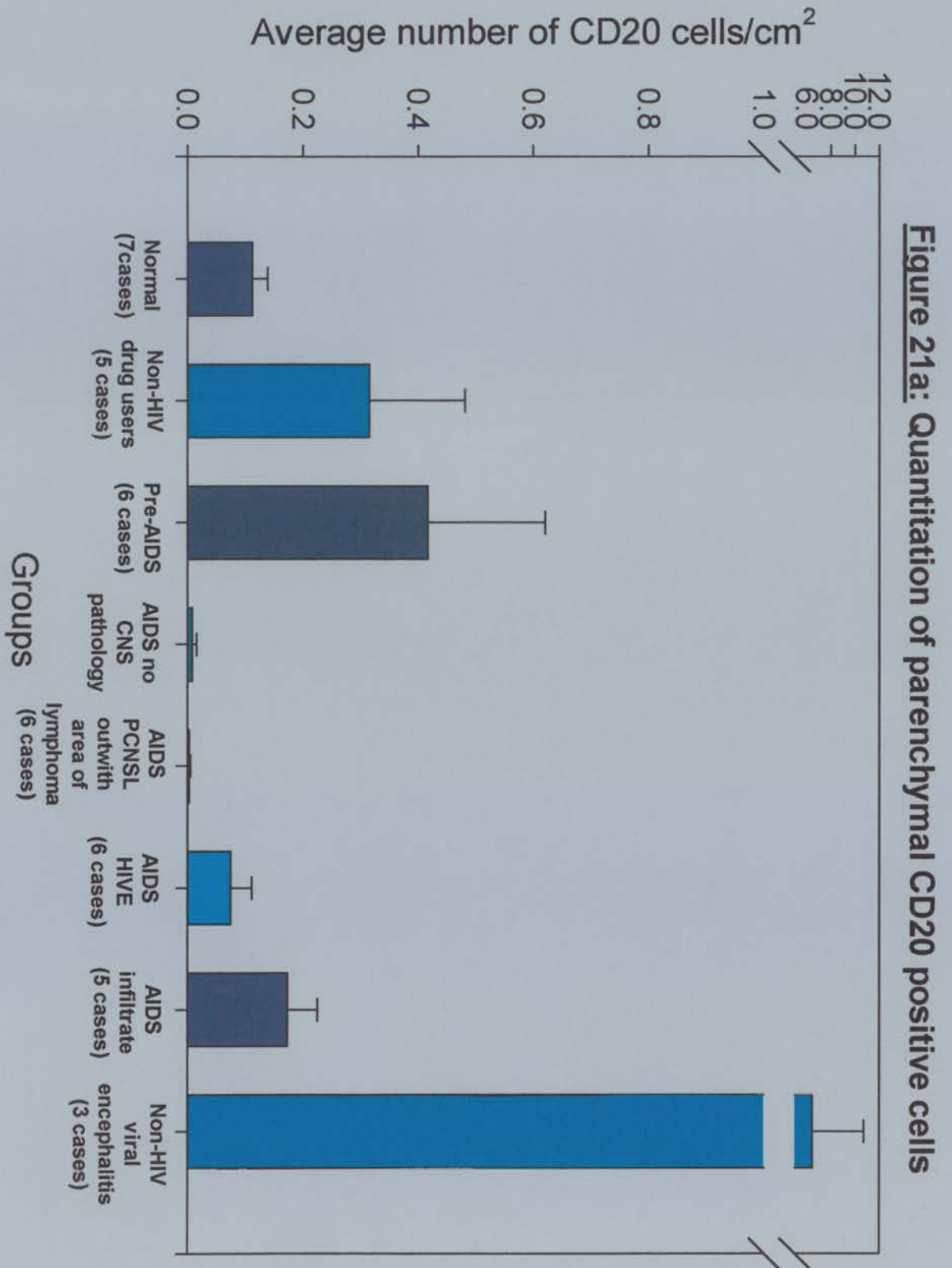
In general the results suggested that within lymphomatous lesions of PCNSL cases both B and T lymphocytes were proliferating, while in perivascular infiltrations of AIDS infiltrate cases only the T lymphocytes were proliferating.

In order to obtain more satisfactory results the double immunohistochemistry technique used above was repeated using immunofluorescence rather than traditional staining techniques. These results showed similar findings, however the intensity of staining was poor and although this could have been resolved with additional time it was felt that this would not alter the results.

3.1.6 Summary of study of B lymphocytes in the brain

Figure 21a and 21b show a summary of the parenchymal and perivascular B lymphocyte data for all 8 groups.

Examination of the brain in seven normal young subjects (Group 1) showed that B lymphocytes were present in the parenchyma in very small numbers but consistently so in all cases. Double staining with the Von Willebrand antibody confirmed that these occasional parenchymal B lymphocytes were not intravascular or perivascular in distribution.



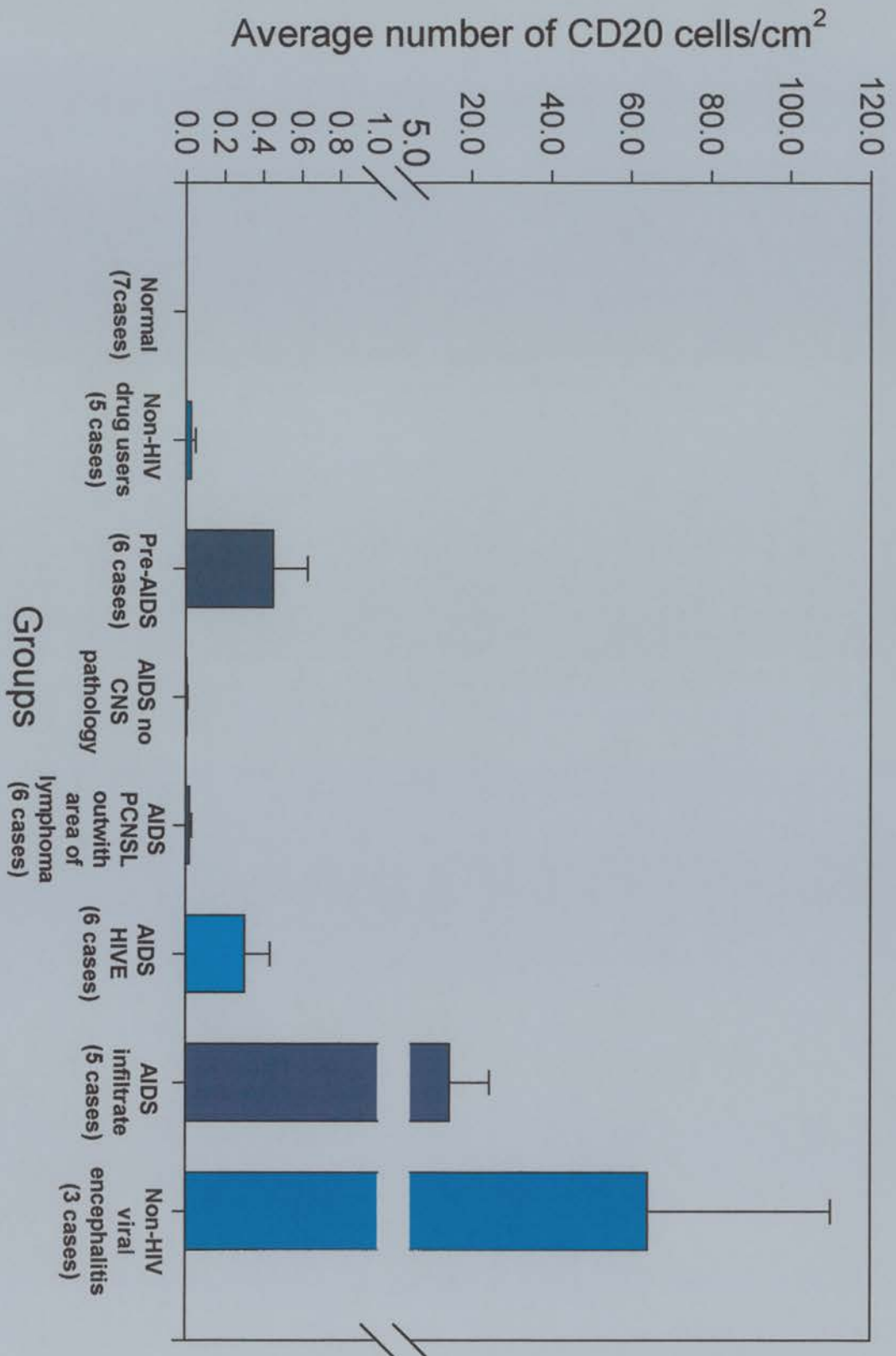


Figure 21b: Quantitation of perivascular CD20 positive cells

The number of parenchymal and perivascular B lymphocytes found in the brains of non-HIV drug misusers (group 2), does not differ significantly from that found in normal (non-drug misusing) controls (group 1).

On routine staining pre-symptomatic HIV infected patients (Group 3) displayed mild focal lymphocytic infiltrates in the leptomeninges and around some white matter vessels B lymphocytes appeared in the perivascular infiltrates and were also found in increased numbers in the parenchymal compartment. In contrast, once patients progressed to AIDS with a corresponding fall in the CD4 and CD8 blood lymphocyte counts, there was a decline in the brain parenchymal B lymphocytes to virtually zero, both in the parenchymal and perivascular compartments. Surprisingly, when the brains were examined from patients who had developed PCNSL (Group 7), the areas of the brain outwith the neoplastic foci showed a virtual absence of parenchymal and perivascular B lymphocytes. Group 5 included AIDS subjects in whom the brain displayed quite widespread pleomorphic lymphoid infiltrates which did not have the characteristics of lymphoma and which were accompanied by similar lymphoid infiltrates in non-CNS organs. These patients had somewhat higher CD4 and CD8 lymphocyte counts than the other AIDS groups in this study. Quantitation of B lymphocytes in multiple areas from these brains showed a widespread increase in parenchymal and perivascular B lymphocytes compared with other AIDS subjects and with pre-symptomatic HIV positive patients. In AIDS patients with HIVE the B lymphocyte response was greater than that seen in AIDS patients with no CNS pathology, but far smaller than that seen in encephalitis in the brains of non-immunocompromised patients.

Semi-quantitative assessment of the T lymphocyte presence in the brains studied showed low level T lymphocytes in normal brains, increased numbers in the pre-symptomatic HIV positive patients and a decline in most AIDS subjects. These results confirm previous reports by Bell *et al* (1993) and Tomlinson *et al* (1999) regarding T lymphocyte entry to the brain. Pleomorphic infiltrates included a high proportion of CD3 positive T cells. In subjects with PCNSL, the lymphomatous areas often had significant numbers of CD8 lymphocytes but these were not a feature of the non-lymphomatous areas. Proliferative activity, as confirmed by Ki-67 or PCNA positivity, was

seen in both the pleomorphic infiltrates and in the lymphomas, but was predominantly in T cells in the former group whereas in the latter, both T and B lymphocytes were Ki-67 positive although proliferating B cells greatly outnumbered T cells.

There is no evidence that B lymphocytes home to any specific region of the brain.

3.2 **Activation status of B lymphocytes in brain tissue**

In order to determine the activation status of B lymphocytes present in the brain a novel method of staining the same sections twice was utilised. Sections were stained using the first antibody, then both the antibody and stain were removed from the section before re-staining the same section with a second antibody. This method was used in preference to double immunohistochemical labelling of the two antigens on one section at the same time for two reasons. Firstly because the two antibodies available were both mouse monoclonal antibodies, and use of both antibodies on the same section would may have produced binding of the secondary antibodies (Rabbit anti mouse) to both primary mouse antibodies. Secondly, the antigens of interest co-localised to the same regions of the cell making double staining difficult to interpret. The antibodies used in this experiment were anti-CD20 and anti-CD23 (activated B lymphocyte marker). The anti-CD23 antibody was optimised as described in table 23.

Table 23: Optimisation of CD23 antibody staining

Pre-treatments assessed	Antibody concentrations tested	Optimal method
No-pretreatment Citric acid (microwave) EDTA (microwave)	1/15	Citric acid microwave at a concentration of 1/30
	1/30	
	1/60	
	1/100	

FFPE sections were stained using anti-CD20 antibody (B lymphocyte marker) with an alkaline phosphatase visualisation reagent. Sections were then examined under a light microscope and the position marked and co-ordinates taken of CD20 cells present in the parenchyma, perivascular space and intravascular cells. CD20 positive cells were then photographed, before the cover slips were removed and the sections placed in an antibody removal solution. Sections were then re-stained with an anti-CD23 antibody, which labels activated B lymphocytes. The previously noted co-ordinates were then used to locate the cells of interest and assess whether or not they were expressing CD23. Control sections for this experiment were from normal tonsil. Four sets of positive and negative tonsil control sections were utilized; firstly, positive and negative sections for CD20; secondly, positive and negative sections for CD23; and thirdly, positive and negative sections stained for CD20. These sections were then stripped of antibody and re-stained for CD23. The final set of control tonsils were stained for CD20, then stripped of antibody, before having the secondary antibody for CD23 applied followed by the ABC complex and the visualisation agent applied. Failure to remove all of the first primary antibody during the stripping process would have led to the CD20 antibody re-staining, in addition to the CD23 antibody, when the second round of immunohistochemistry was completed. Therefore the final control set was important to ensure that all of the first primary antibody had been removed from the section. Any staining seen in this final control set resulted in the entire run being discarded. The exact methods utilised in this technique are listed in section 2.2.1.6 page 72. The optimisation of the stripping technique required 4 test runs with alterations in each run to the length of time sections were immersed in the stripping solution and the strength of acid used. The technique described in 2.X provides full removal of all antibodies and stains present in the section, with minimal digestion/degradation of the tissue section.

Sections from 5 patient groups were used:

- Non-HIV - 6 normal cases with 39 sections and 4 drug users with 11 sections in total
- Pre-AIDS- 6 cases with 24 sections in total
- AIDS no CNS pathology- 3 cases with 11 sections in total
- AIDS infiltrate- 3 cases with 7 sections in total
- AIDS PCNSL - 6 cases with 18 sections in total, 9 with lymphoma tissue present in the section.

Each stained section was assessed for CD20 positive B lymphocytes in three locations: brain parenchyma, perivascular space, and intravascular space. A total of 110 sections were stained for CD20 cells, of these 43 contained CD20 positive cells whose location could be easily identified. Table 24 lists the sections from the above cases in which CD20 positive cells were found. Also shown in table 24 is the number of CD20 positive cells which then re-stained for CD23 after the CD20 antibody and staining had been removed from the section. Table B1 in appendix B lists the results of all the sections stained for CD20.

Five CD20 positive parenchymal cells were found in the non-HIV group, 4 of which proved to be CD23 positive (see figure 22a-d). All 5 belonged to two normal cases (1 and 2), no CD20 positive parenchymal cells were found in any of the drug user cases. Only one cell was found in the perivascular space of the non-HIV group and this cell failed to show a CD23 positive phenotype. Eighteen CD20 cells were located in blood vessels, 9 of which showed a CD23 positive phenotype. Thirteen of the CD20 positive intravascular cells were found in 5 normal cases and 5 in 2 drug user cases.

In the pre-AIDS group, 4 CD20 cells were found in the brain parenchyma and all 4 stained for CD23. Fifty one cells were found in the perivascular space and 50 of these were CD23 positive. The majority of perivascular cells in this group (44 of 51) were in sections from one case (case 46) which had evidence of a non-HIV encephalitis. Nine CD20 cells were found in the blood vessels of this group, 7 of which were CD23 positive.

Table 24: Results of sections stained for CD20, then stripped of antibody and re-stained for CD23

Case number (Case type)	Area of brain	CD20 parenchymal cells	CD23 parenchymal cells	CD20 perivascular cells	CD23 perivascular cells	CD20 intra vascular cells	CD23 intra vascular cells
Non-HIV Cases							
1	frontal	1	0	0	0	1	*
2	basal ganglia	2	2	0	0	1	0
2	temp hip	2	2	0	0	1	0
3	cerebellum	0	0	0	0	2	1
3	frontal	0	0	0	0	1	1
3	temporal hippocampus	0	0	0	0	2	1
5	bg	0	0	0	0	1	0
6	bg	0	0	0	0	5	5
8	frontal	0	0	0	0	1	0
8	frontal	0	0	0	0	1	0
9	thalamus	0	0	0	0	3	1
Pre-AIDS Cases							
13	parietal parasagittal	0	0	0	0	1	0
13	cerebellum	0	0	0	0	1	1
13	frontal	0	0	0	0	1	1
46	cerebellum	4	4	25	25	0	0
46	mid brain	0	0	19	19	0	0
17	cerebellum	0	0	7	6	3	3
17	parietal convex	0	0	0	0	1	0
18	temp hip	0	0	0	0	2	2
AIDS Cases with HIVE							
35	cerebellum	0	0	0	0	1	0
35	mid brain	0	0	0	0	1	0
35	pc	0	0	0	0	1	0
AIDS Cases with pleomorphic infiltrates							
24	cerebellum	2	2	0	0	1	0
24	occipital	0	0	14	14	0	0
25	basal ganglia	0	0	1	1	1	1
26	cerebellum	2	2	1	1	0	0
26	mid brain	0	0	56	56	1	1
26	temporal hippocampus	2	2	17	17	0	0
AIDS cases with PCNSL							
36	mid brain	Lymphoma	Lymphoma	0	0	0	0
38	medulla	Lymphoma	Lymphoma	0	0	0	0
38	frontal	Lymphoma	Lymphoma	0	0	0	0
38	cerebellum	Lymphoma	Lymphoma	0	0	0	0
39	mid brain	5 lymphoma cells	0	0	0	0	0
39	basal ganglia	2	2	0	0	1	0
40	basal ganglia	Lymphoma	Only few cells positive, most negative	0	0	0	0
40	frontal	Lymphoma	Lymphoma	0	0	0	0
41	cerebellum	Lymphoma	Lymphoma	0	0	0	0
41	medulla	Lymphoma	Lymphoma	0	0	1	1

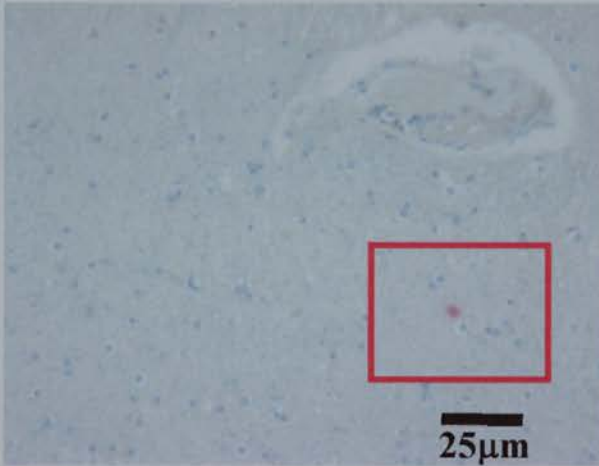
In sections from AIDS cases with no CNS pathology no CD20 cells were found in either the parenchyma or the perivascular spaces. Three cells were found in blood vessels, none of which showed positive staining for CD23.

Six parenchymal CD20 positive cells were examined from AIDS cases with pleomorphic infiltrates, with all 6 showing a CD23 phenotype. A total of 89 perivascular CD20 cells were assessed in this group, and again all cells showed positive staining for CD23 (see figure 22e and f). Only three intravascular cells were studied, none of which stained for CD23.

The final group of cases included sections from AIDS cases with PCNSL. Nine sections containing tumours were assessed from 5 different cases. Seven of these showed strong CD23 staining throughout the tumour, a representative picture of these seven sections is shown in figure 22g-j. Of the other two sections with lymphoma cells, the basal ganglia of case 40 showed only a minority of tumour cells expressing CD23, while the mid brain of case 39 had 5 parenchymal CD20 positive cells in the mid brain none of which stained for CD23. Given the rarity of CD20 cells in PCNSL brains outside the area of lymphoma, these 5 cells probably represent the very edge of a lymphomatous region. In addition case 39 also had 2 parenchymal cells in the basal ganglia, both of which showed a CD23 phenotype. No perivascular CD20 positive cells were found outwith the areas of lymphoma in any of the cases in this group. Two cells were located in blood vessels, one of which displayed a CD23 positive phenotype.

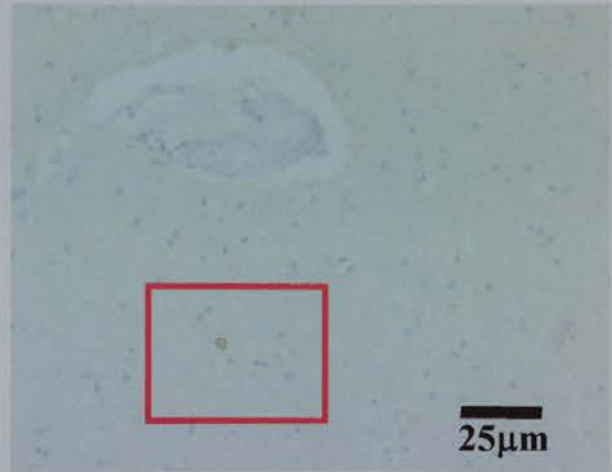
Figure 22:

Sections from normal brain stained for CD20 then stripped of antibody and re-stained for CD23



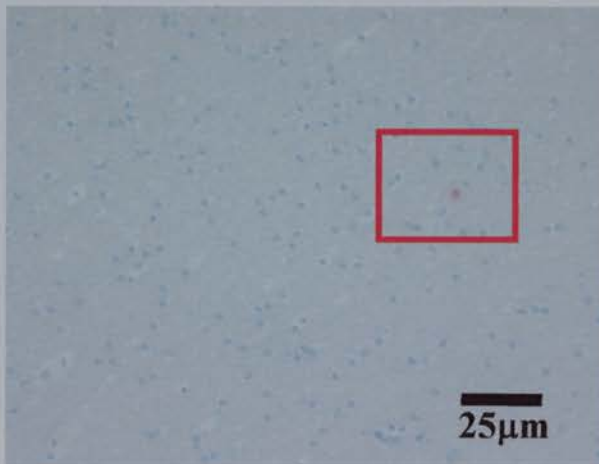
22a

Case 1 (Normal) basal ganglia
CD20 (purple staining)



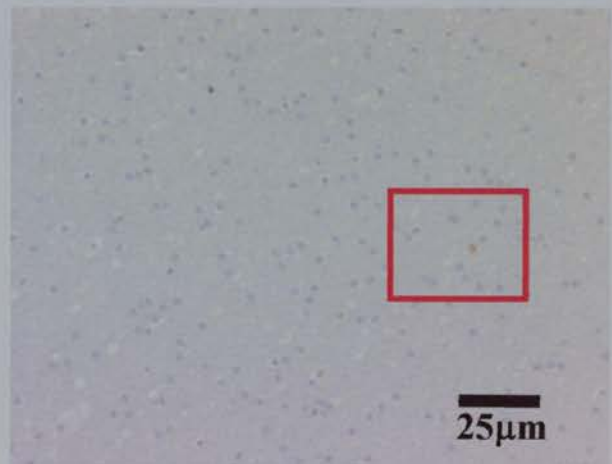
22b

Section from figure 22a stripped of
antibody and re-stained for
CD23 (brown staining). Red box
identifies the same cell in each
photograph.



22c

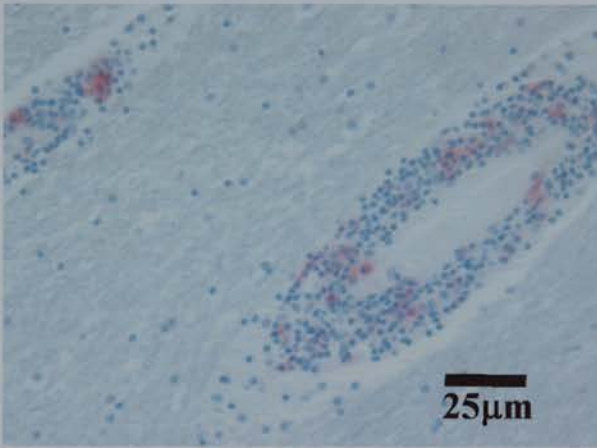
Case 1 (Normal) Temporal
Hippocampus. CD20 (purple staining)



22d

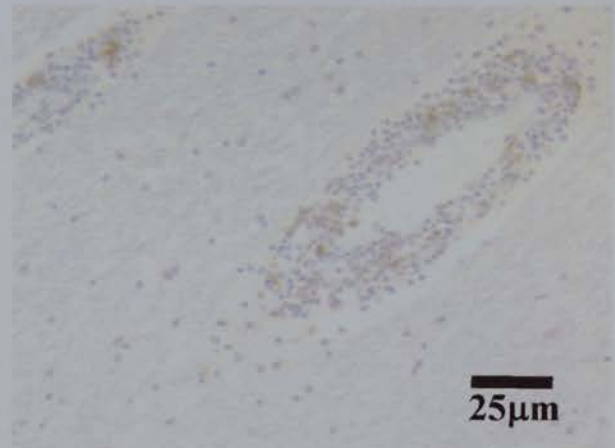
Section from figure 22c stripped of
antibody and re-stained for
CD23 (brown staining). Red box
identifies the same cell in each
photograph.

Figure 22 (continued): Sections stained for CD20 then stripped of antibody and re-stained for CD23



22e

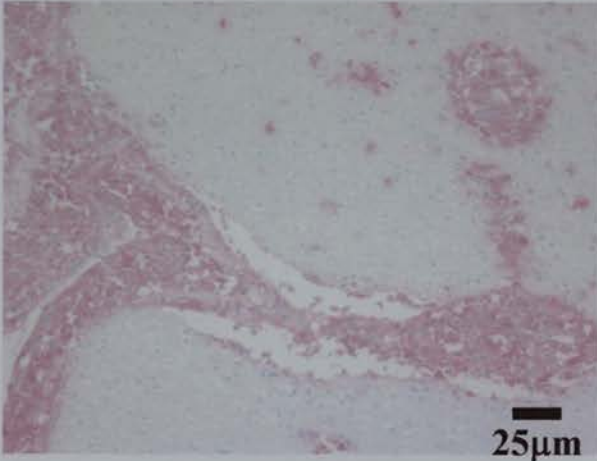
Case 25 (AIDS Infiltrate Case)
CD20 (purple staining)



22f

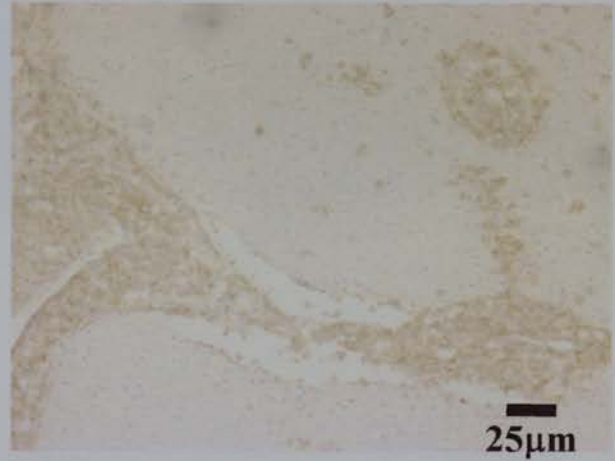
Section from figure 22e stripped of
antibody and re-stained for
CD23 (brown staining).

Figure 22 (continued): AIDS PCNSL sections stained for CD20 then stripped of antibody and re-stained for CD23



22g

Case 38 (AIDS PCNSL) Cerebellum
CD20 (purple staining)



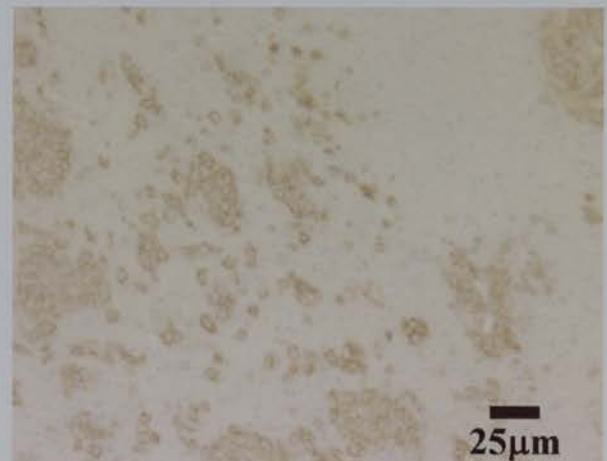
22h

Section from figure 22a striped of
antibody and re-stained for
CD23 (brown staining).



22i

Case 39 (AIDS PCNSL) Cerebellum
CD20 (purple staining)



22j

Section from figure 22a striped of
antibody and re-stained for
CD23 (brown staining).

A summary of the results from table 24 is shown in table 25. From the 17 parenchymal CD20 positive cells examined, from all cases, sixteen cells showed expression of the CD23 activation marker (94%). Of the 141 perivascular CD20 cells assessed, 139 showed a CD23 phenotype (99%). By contrast the intravascular cells assessed showed a much lower level of CD23 expression, with 17 out of 35 cells expressing both CD20 and CD23 (49%). It should be noted that although on average 49% of CD20 positive intravascular cells also expressed CD23, there was a wide variation between the different groups. The pre-AIDS cases showed quite a high level of CD23 expression, with 7 out of 9 cells (78%) displaying a double CD20/CD23 phenotype. In addition, 50% of the intravascular CD20 positive cells from the non-HIV group expressed detectable CD23, while only one of eight CD20 cells from all the AIDS cases together showed a CD23 positive phenotype (12.5%).

The this data suggests that in order for CD20 cells to enter the perivascular and parenchymal spaces of the brain they must display an activated CD23 phenotype, alternatively it is conceivable that all CD20 positive cells entering the brain are activated on entering the CNS rather than before entry.

Table 25: Summary of data from table 24 (Activation status of B lymphocytes in the brain)

Group	Parenchymal cells			Perivascular cells			Intravascular cells		
	Number of CD20 cells	Number of cells also CD23 +ve	% cells CD23 +ve	Number of CD20 cells	Number of cells also CD23 +ve	% cells CD23 +ve	Number of CD20 cells	Number of cells also CD23 +ve	% cells CD23 +ve
Non-HIV	5	4	80%	1	0	0%	18	9	50%
Pre-AIDS	4	4	100%	51	50	98%	9	7	78%
AIDS HIVE	0	0	NA	0	0	0	3	0	0%
AIDS infiltrate	6	6	100%	89	89	100%	3	0	0%
AIDS PCNSL (out with lymphoma areas)	2	2	100%	0	0	NA	2	1	50%
AIDS PCNSL (lymphoma areas) *	9 areas of lymphoma	8 areas of lymphoma	89%	0	0	NA	0	0	NA
Total	17 (excluding lymphomas)	16 (excluding lymphomas)	94%	141	139	99%	35	17	49%

* Of the nine areas of lymphoma, 8 had large areas of lymphoma (>100 cells), in 7 of which the CD20 cells showed ~100% CD23 expression, and one showed only a minority of cells CD23 +ve. The section which was negative had only 5 CD20 cells present.

NA Not applicable

3.3 Determination of EBV infection status of B lymphocytes in brain

The aim of these experiments was firstly to ascertain if B lymphocytes present in human brain tissue were infected with EBV, and secondly to assess the EBV viral load in brains from different patient groups. Samples consisted of 5µm and 20µm thick FFPE sections, frozen tissue samples and CSF samples taken at autopsy. FFPE sections were available from all of the cases

listed in section 2.1.1 table 5, frozen tissue and CSF samples was available for a limited number of HIV cases and from additional non-HIV cases not included in Section 1 (see section 2.1.1 table 6).

DNA extracted from FFPE sections was first screened using BamW PCR to detect any EBV DNA present in sections; in-situ hybridisation for EBERs was then used to localise EBV infected cells in sections positive for EBV by PCR. Semi-quantitative BamW PCR was then used on DNA extracted from frozen tissue samples and CSF to assess the viral load in brains.

3.3.1.1 BamW PCR on DNA extracted from FFPE sections

DNA was extracted from FFPE sections as described in 2.2.3.1 page 74. In order to verify that PCR amplifiable DNA had been extracted from samples, a β -globin PCR was performed. Samples positive for β -globin were then used in the Bam W PCR. Representative gel photographs of β -globin PCR are shown in Figure 23 (showing 7 out of 16 samples were positive). Most samples had to be tested at least twice in order to obtain a positive result (gel photographs not shown). Eventually positive signals were obtained from twenty samples. Control DNA extracted from B lymphocyte cell lines Namalwa and BJAB were used for the PCR. Samples that were PCR positive for β -globin were subsequently tested for EBV using BamW PCR, see Figure 24 for representative autoradiographs of BamW PCR. Controls for this PCR consisted of an EBV infected Burkits lymphoma cell line (Namalwa) and an EBV negative B lymphocyte cell line (BJAB). Table 26 summarises the results.

EBV DNA was not detected in any of the samples tested, including six sections taken from HIV positive PCNSL brains.

The control, EBV positive, Namalwa dilution series (see 2.1.6.3 page 67) showed that approximately $10^2 - 10^3$ EBV positive cells were required for a consistently detectable positive result. Therefore given the low number of B lymphocytes present in the sections tested, even in the lymphoma sections, it is unlikely that the PCR was sensitive enough to detect any possible EBV infected cells.

Figure 23 Legend:

Sample Number	Case Number (brain region)	Group
1	36 Mid brain	AIDS PCNSL
2	24 Cerebellum	AIDS HIVE
3	40 Frontal 2	AIDS PCNSL
4	38 Basal Ganglia	AIDS PCNSL
5	17 Parietal parasagittal	Pre-AIDS
6	44 thalamus	Non-HIV Viral Encephalitis
7	46 Cerebellum	Pre-AIDS
8	2 Basal Ganglia	Normal
9	25 Cerebellum	AIDS Infiltrate
10	41 Cerebellum	AIDS PCNSL
11	26 Parietal parasagittal	AIDS Infiltrate
12	13 Central White Matter	Pre-AIDS
13	41 Medulla	AIDS PCNSL
14	43 Temporal Hippocampus	Non-HIV Viral Encephalitis
15	40 Frontal 1	AIDS PCNSL
16	45 Cerebellum	Non-HIV Viral Encephalitis

Summary of all data from this set of experiments is given in table 26.

Key for figure 23:

- BJ - BJAB (EBV -ve lymphoma cell line)**
- H₂O - Water (PCR control)**
- N - Namalwa (EBV +ve Burkitt's lymphoma cell line)**

Figure 23:

**β -Globin PCR on DNA extracted from
Formalin Fixed Paraffin Embedded sections**

Samples

1 2 3 4 5 6 7 8 9 10 11 12

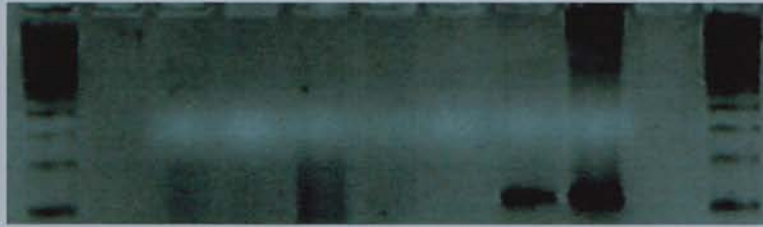
110bp →



Samples

13 14 15 16 H₂O BJ N

110bp →



**Negative
image**

Figure 24 Legend:

Sample Number	Case Number (brain region)	Group
1	36 Mid brain	AIDS PCNSL
2	24 Cerebellum	AIDS HIVE
3	40 Frontal 2	AIDS PCNSL
4	38 Basal Ganglia	AIDS PCNSL
5	17 Parietal parasagittal	Pre-AIDS
6	44 thalamus	Non-HIV Viral Encephalitis
7	46 Cerebellum	Pre-AIDS
8	2 Basal Ganglia	Normal
9	25 Cerebellum	AIDS Infiltrate
10	41 Cerebellum	AIDS PCNSL
11	26 Parietal parasagittal	AIDS Infiltrate
12	13 Central White Matter	Pre-AIDS
13	41 Medulla	AIDS PCNSL
14	43 Temporal Hippocampus	Non-HIV Viral Encephalitis
15	40 Frontal 1	AIDS PCNSL
16	45 Cerebellum	Non-HIV Viral Encephalitis

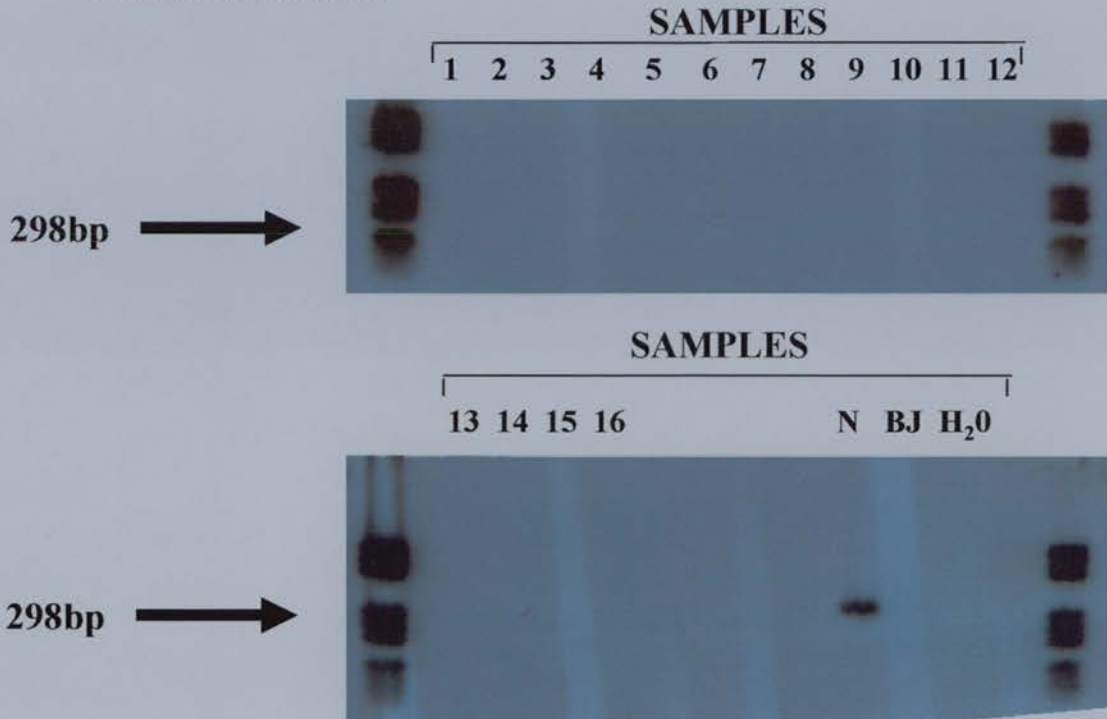
Summary of all data from this set of experiments is given in table 26.

Key for figure 24:

- BJ - BJAB (EBV -ve lymphoma cell line)**
- H₂O - Water (PCR control)**
- N - Namalwa (EBV +ve Burkitt's lymphoma cell line)**

Figure 24:

BamW PCR on DNA extracted from Formalin Fixed Paraffin Embedded sections



The problems in obtaining DNA from FFPE sections suitable for PCR are well documented (Bramwell & Burns 1988). Due to the long fixation periods required for whole brains this type of tissue is likely to suffer greatly from the effects of formalin DNA degradation which may partially explain the weak β -globin bands obtained.

Table 26: Bam W PCR on DNA from FFPE sections

Case Type	Case Number	Area	BamW PCR (Positive/ negative)
AIDS Lymphoma	36	Mid brain	-
	41	Brain stem	-
	38	Basal ganglia	-
	40	Frontal 1	-
		Frontal 2	-
41	Cerebellum	-	
AIDS infiltrate	24	Basal ganglia	-
	25	Cerebellum	-
	26	Parietal parasagittal	-
Pre-AIDS	46	Cerebellum	-
	13	Central white matter	-
	17	Parietal parasagittal	-
AIDS HIVE	34	frontal	-
Non-HIV encephalitis	43	Temporal hippocampus	-
	44	Thalamus	-
	45	Cerebellum	-
Normal	2	Basal ganglia	-

3.3.2 EBERs in-situ hybridisation

Due to the failure of the Bam W PCR to detect any EBV DNA in DNA extracted from FFPE sections it was decided to screen sections from all case types by in-situ hybridisation for EBV. Dako EBER PNA probe and detection kit (see section 2.2.2 page 73) was used to detect EBV infection of cells in FFPE sections. Sections shown to contain the highest numbers of B lymphocytes per section by CD20 immunohistochemical staining were selected for EBERs in-situ hybridisation staining, in order to maximise the number of B lymphocytes assessed for EBV infection. Controls for this

experiment included DAKO PNA positive and negative controls to show that the detection kit was working, and FFPE sections from a SCID mouse grown B lymphoblastoid cell line derived tumour previously shown to be EBV positive. The results are summarised in table 27 and a full list of sections stained and results are given in table C1 appendix C.

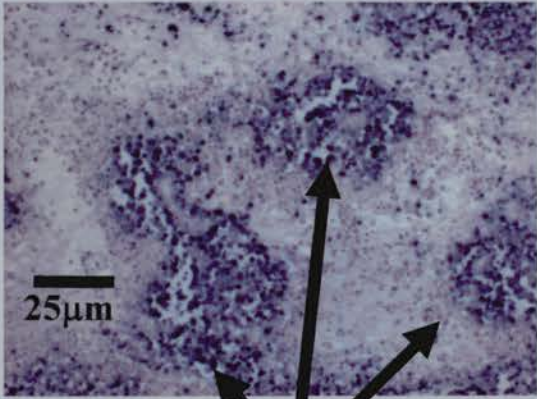
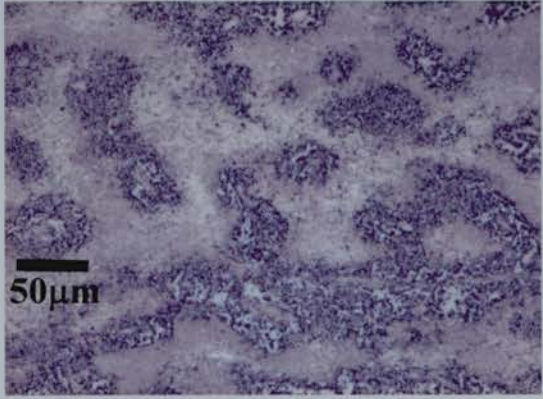
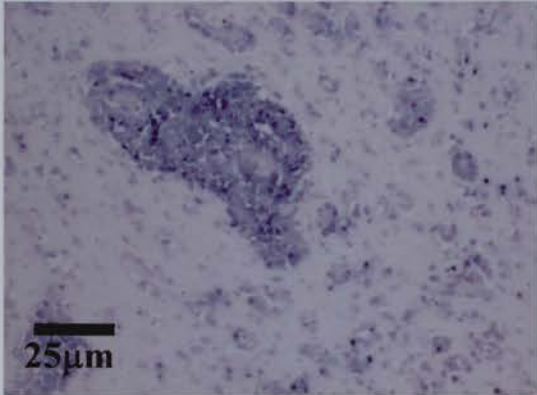
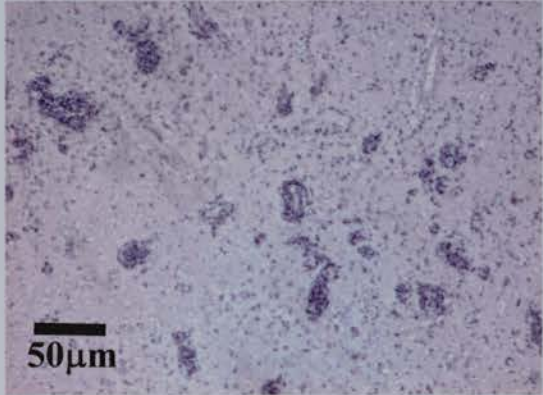
Table 27: Summary of EBERs in-situ results

Case Type	Number of sections stained	Number of sections with positive cells	Notes
AIDS Lymphoma	17	13	Negative sections had no lymphoma cells present
AIDS infiltrate	22	0	All sections tested had large infiltrates, all of which had been shown to have some B cells present
HIVE	4	0	
Pre-AIDS	11	0	Included several sections with large perivascular infiltrates
Non-HIV DU	9	0	
Non-HIV encephalitis	10	0	All sections tested had large numbers of B cells
Normal	14	0	

All 13 sections from lymphoma cases which contained neoplastic cells showed positive staining for EBERs (see figure 25). Approximately 90-100% of lymphoma cells in these sections stained strongly for EBERs. In addition to sections containing lymphoma tissue, 2 cases included sections with no lymphoma cells present, as defined by CD20 immunohistochemistry and assessment of haematoxylin and eosin sections. Case 38 included 2 sections with no lymphoma cells present. Only 2 sections were available from case 39 both without lymphoma cells. None of these 4 sections showed any positive staining.

Figure 25:

EBERs In-situ hybridisation staining

<p>Case 36 mid brain (AIDS PCNSL)</p>  <p>EBERs positive cells within lymphoma</p>	<p>Case 36 mid brain (AIDS PCNSL)</p>  <p>Almost all lymphoma cells are EBERs positive</p>
<p>Case 35 basal ganglia (AIDS PCNSL)</p>  <p>25µm</p>	<p>Case 35 basal ganglia (AIDS PCNSL)</p>  <p>50µm</p>

Results from these experiments are summarised in table 27.

Twenty two sections from 5 different AIDS cases with pleomorphic infiltrates were tested and all proved negative for EBERs, although CD20 immunohistochemical staining had shown consecutive sections to those used for EBERs staining to contain high numbers of B lymphocytes (see section 3.1).

Eleven sections from 3 pre-AIDS cases with mild lymphocytic meningitis were tested and all sections were EBERs negative.

Three groups of non-HIV cases were also tested: normal (3 cases, 14 sections), drug users (3 cases, 9 sections), and non-HIV viral encephalitis (3 cases, 10 sections). None of the sections stained in these three groups were positive for EBERs.

3.3.3 EBV Semi-quantitative Bam W PCR on DNA extracted from frozen tissue and CSF samples

A semi quantitative PCR (Bam H1 W) was used to determine EBV copy number in samples from frozen tissue and CSF samples. DNA was extracted from samples as described in 2.2.3.1. In order to verify that PCR amplifiable DNA had been extracted from samples, a β -globin PCR was performed. Samples positive for β -globin were then used in the Bam W PCR. An EBV positive Burkitt's lymphoma cell line (Namalwa) was used as a positive control and an EBV negative B lymphocyte cell line (BJAB) was used as a negative control. A series of Namalwa cells diluted in BJAB cells was used to allow a semi-quantitative assessment of viral load (see section 2.1.6.3).

Figure 26 shows representative gel photographs from β -globin PCR on both frozen samples and CSF samples, all 16 frozen tissue samples were β -globin positive and 15 out of 19 CSF samples were β -globin positive. All 16 frozen samples and CSF samples from 8 cases were subsequently tested for EBV. The CSF samples which were β -globin negative were also tested in order to make certain any cell free EBV present in these samples was not ignored. Figure 27 shows representative autoradiographs of BamW PCR.

Figure 26a Legend:

Sample Number	Case Number (brain region)	Group
1	42 Frontal	AIDS PCNSL
2	42 Cerebellum	AIDS PCNSL
3	42 Basal ganglia	AIDS PCNSL
4	42 CSF 1	AIDS PCNSL
5	42 CSF 2	AIDS PCNSL
6	42 CSF 3	AIDS PCNSL
7	42 CSF 4	AIDS PCNSL
8	39 CSF (pellet)	AIDS PCNSL
9	39 CSF (fluid)	AIDS PCNSL
10	46 CSF (fluid)	Pre-AIDS
11	22 CSF (pellet)	AIDS
12	22 CSF (fluid)	AIDS
13	25 CSF (pellet)	AIDS Infiltrate
14	25 CSF (fluid)	AIDS Infiltrate
15	20 CSF (pellet)	AIDS
16	20 CSF (fluid)	AIDS
17	40 CSF (pellet)	AIDS PCNSL
18	40 CSF (fluid)	AIDS PCNSL
19	23 CSF (pellet)	AIDS
20	23 CSF (fluid)	AIDS

Key:

H₂O – water (-ve control)

BJ – BJAB (+ve control)

Figure 26a:

β -Globin PCR on DNA extracted from frozen tissue samples and frozen CSF

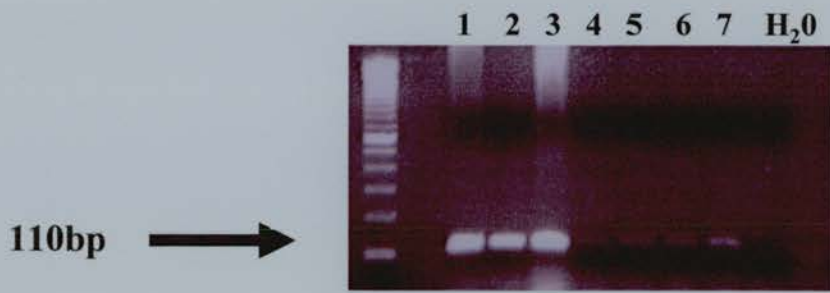


Figure 26b Legend:

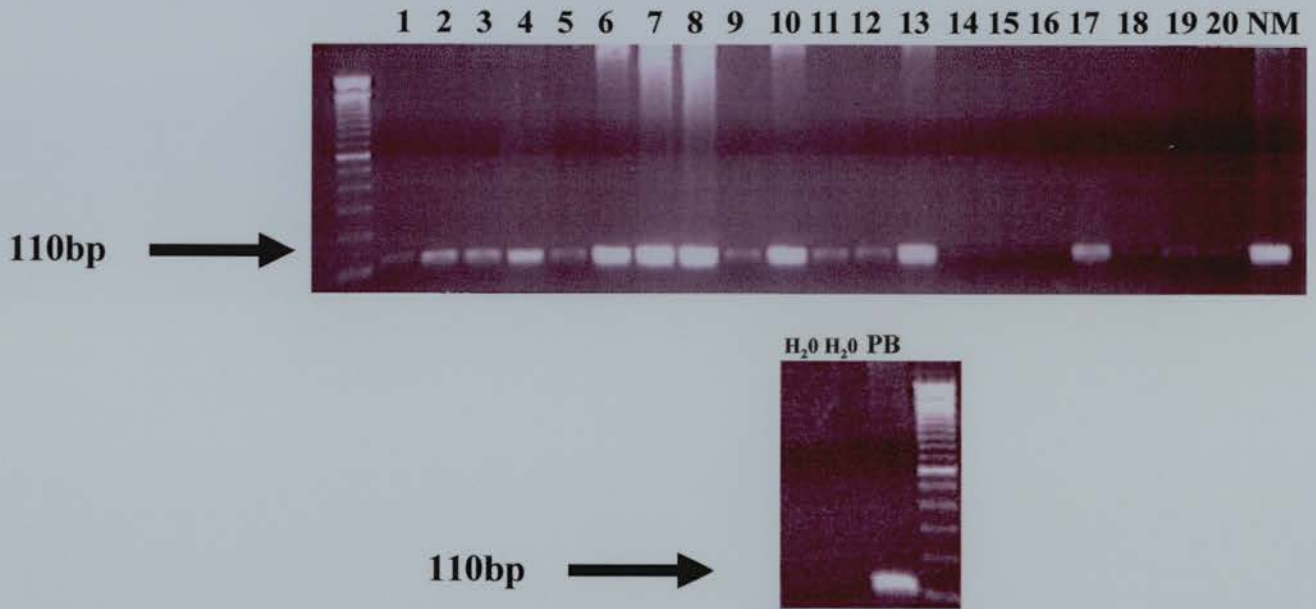
Sample Number	Case Number (brain region)	Group
1	39 Parietal	AIDS PCNSL
2	46 Cerebellum	Pre-AIDS
3	46 Basal Ganglia	Pre-AIDS
4	22 Parietal	AIDS
5	25 Occipital	AIDS Infiltrate
6	26 Cerebellum	AIDS Infiltrate
7	26 Brain stem	AIDS Infiltrate
8	20 Parietal	AIDS
9	40 Frontal	AIDS PCNSL
10	41 Cerebellum	AIDS PCNSL
11	41 Brain stem	AIDS PCNSL
12	21 Parietal	AIDS
13	52 Frontal	Normal
14	39 CSF	AIDS PCNSL
15	46 CSF	Pre-AIDS
16	22 CSF	AIDS
17	25 CSF	AIDS Infiltrate
18	40 CSF	AIDS PCNSL
19	20 CSF	AIDS
20	41 CSF	AIDS PCNSL

Key: H₂O – water (-ve control)
NM – Namalwa (+ve control)
PB – PBMC (+ve control)

See table 28 for a summary of data.

Figure 26b:

β -Globin PCR on DNA extracted from frozen tissue samples and frozen CSF



Key:

- H₂O – water (-ve control)**
- NM – Namalwa (+ve control)**
- PB – PBMC (+ve control)**

Legend for figure 27

Sample	Case number (Brain region)	Group
1	42 Frontal	AIDS PCNSL
2	42 Cerebellum	AIDS PCNSL
3	42 Basal Ganglia	AIDS PCNSL
4	42 CSF 1	AIDS PCNSL
5	42 CSF 2	AIDS PCNSL
6	42 CSF 3	AIDS PCNSL
7	42 CSF 4	AIDS PCNSL
8	39 CSF (pellet)	AIDS PCNSL
9	39CSF (fluid)	AIDS PCNSL
10	46 CSF (fluid)	AIDS PCNSL
11	22 CSF (pellet)	AIDS
12	22CSF (fluid)	AIDS
13	25 CSF (pellet)	AIDS Infiltrate
14	25 CSF (fluid)	AIDS Infiltrate
15	20 CSF (pellet)	AIDS
16	20CSF (fluid)	AIDS
17	40 CSF (pellet)	AIDS PCNSL
18	40 CSF (fluid)	AIDS PCNSL
19	23 CSF (pellet)	AIDS
20	23 CSF (fluid)	AIDS
21	39 Parietal	AIDS PCNSL
22	46 Cerebellum	Pre-AIDS
23	46 Basal Ganglia	Pre-AIDS
24	22 Parietal	AIDS
25	25 Occipital	AIDS Infiltrate
26	26 Cerebellum	AIDS Infiltrate
27	26 Brain stem	AIDS Infiltrate
28	20 Parietal	AIDS
29	40 Frontal	AIDS PCNSL
30	41 Cerebellum	AIDS PCNSL
31	41 Brain stem	AIDS PCNSL
32	21 Parietal	AIDS
33	52 Frontal	Normal
34	39 CSF	AIDS PCNSL
35	46 CSF	Pre-AIDS
36	22 CSF	AIDS
37	25 CSF	AIDS Infiltrate
38	40 CSF	AIDS PCNSL
39	20CSF	AIDS
40	41 CSF	AIDS PCNSL

Summary of data from figure 27 is given in table 28.

Figure 27: BamW PCR on DNA extracted from frozen tissue and CSF samples

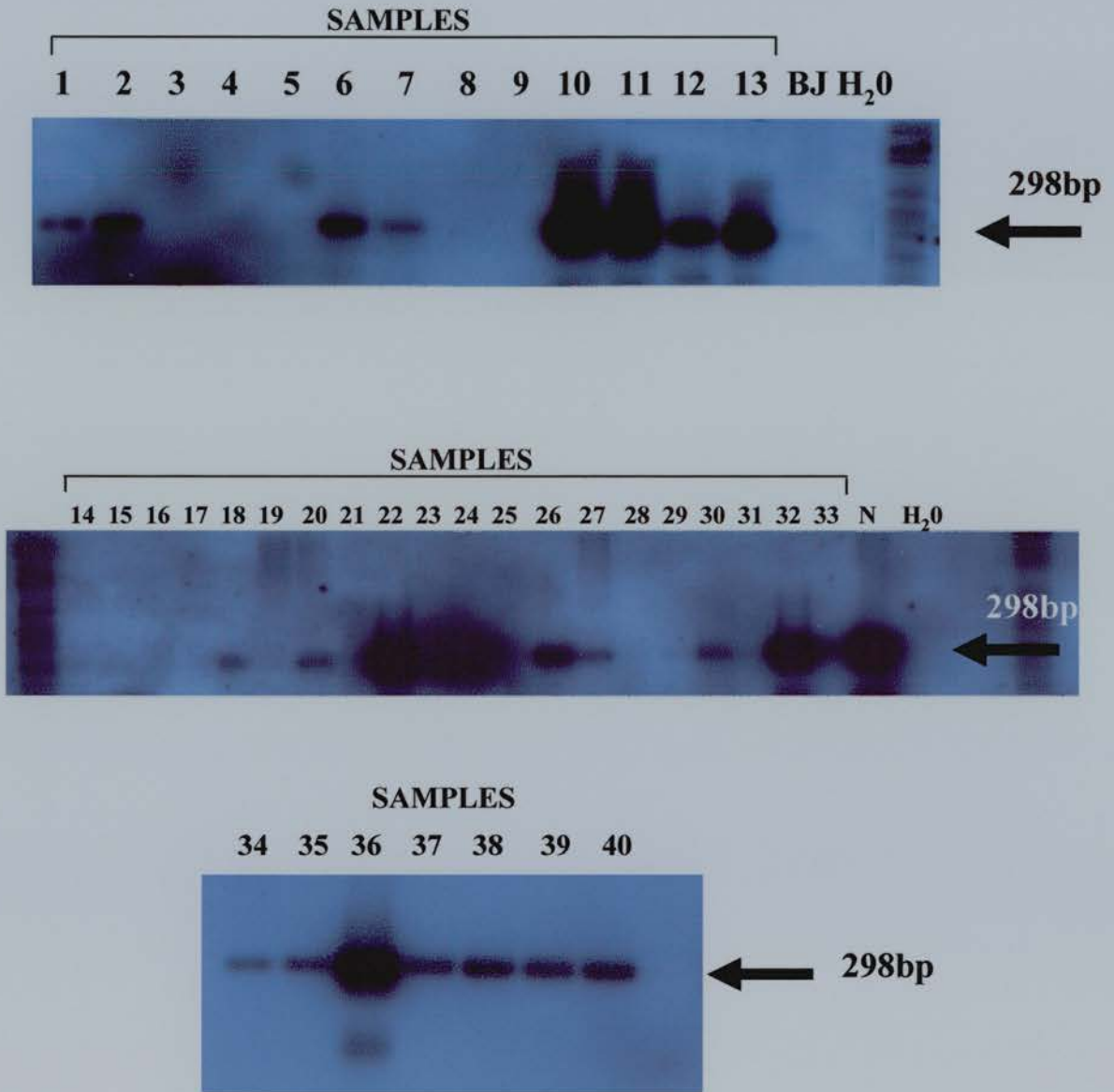


Figure legend over page

The Bam W PCR consistently detected between 10^3 and 10^2 EBV positive Namalwa cells in a background of 10^6 EBV negative BJAB cells. On two occasions, however, the BamW PCR detected 1 EBV positive cell in a background of 10^6 EBV negative cells (see Figure 28). Densitometry readings were taken from positive samples and from the Namalwa dilution series, and the dilution series data used to plot densitometry curves (see figure 29), the curves were then used to make semi quantitative assessment of the number of EBV genomes per sample.

Bam W PCR was repeated twice on all samples and an average of the two results calculated. In samples that were only positive in 1 reaction, only the results from the positive reaction are given. The results are summarised in Table 28.

No EBV DNA was detected in either of the normal frozen brains or in any of the 5 normal CSF samples. Likewise, no EBV was detected in any of the 3 pre-AIDS brains or in the 1 CSF sample in this group.

4 CSF samples and 7 frozen tissue samples were tested from the AIDS group with no CNS pathology (see table 28). 2 CSF samples were negative, 1 showed very low levels of EBV (2 copies/ μ g of DNA), and 1 demonstrated an intermediate level of positivity (12600 copies/ μ g of DNA). The case which showed higher levels of EBV, case 23, had evidence of a systemic lymphoma at autopsy but no evidence of CNS involvement. 6 of the 7 frozen samples were negative, and 1 sample very weakly positive (1 copy/ μ g of DNA).

3 frozen samples were tested from 1 case with HIV, all of which were negative.

The AIDS group with pleomorphic infiltrates had 2 CSF samples and 8 frozen tissue samples available. Both CSFs showed intermediate levels of EBV (6300 and 12600 copies/ μ g of DNA). 6 of the 8 frozen samples were negative and 2 weakly positive (110 and 160 copies/ μ g of DNA).

Figure 28: BamW PCR Namalwa dilution series

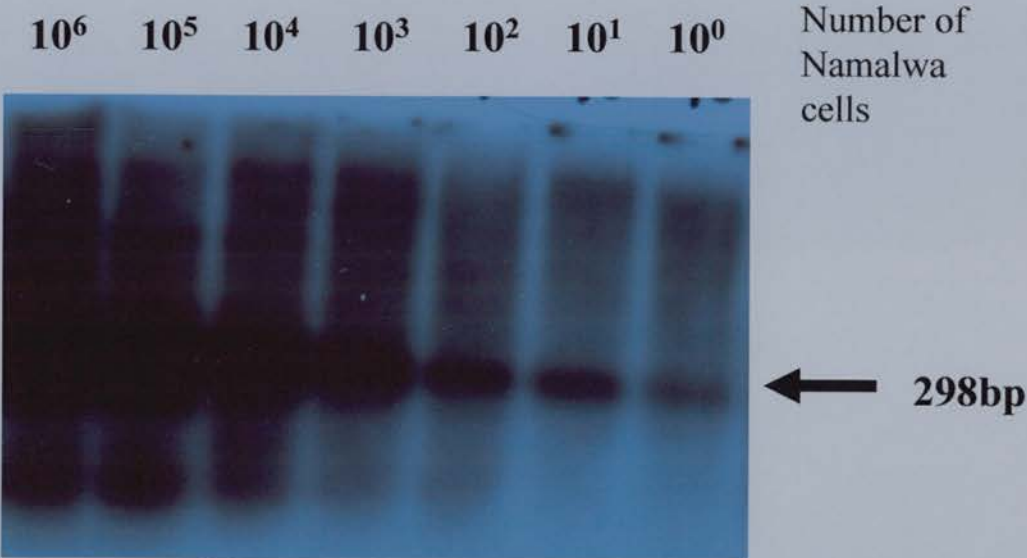
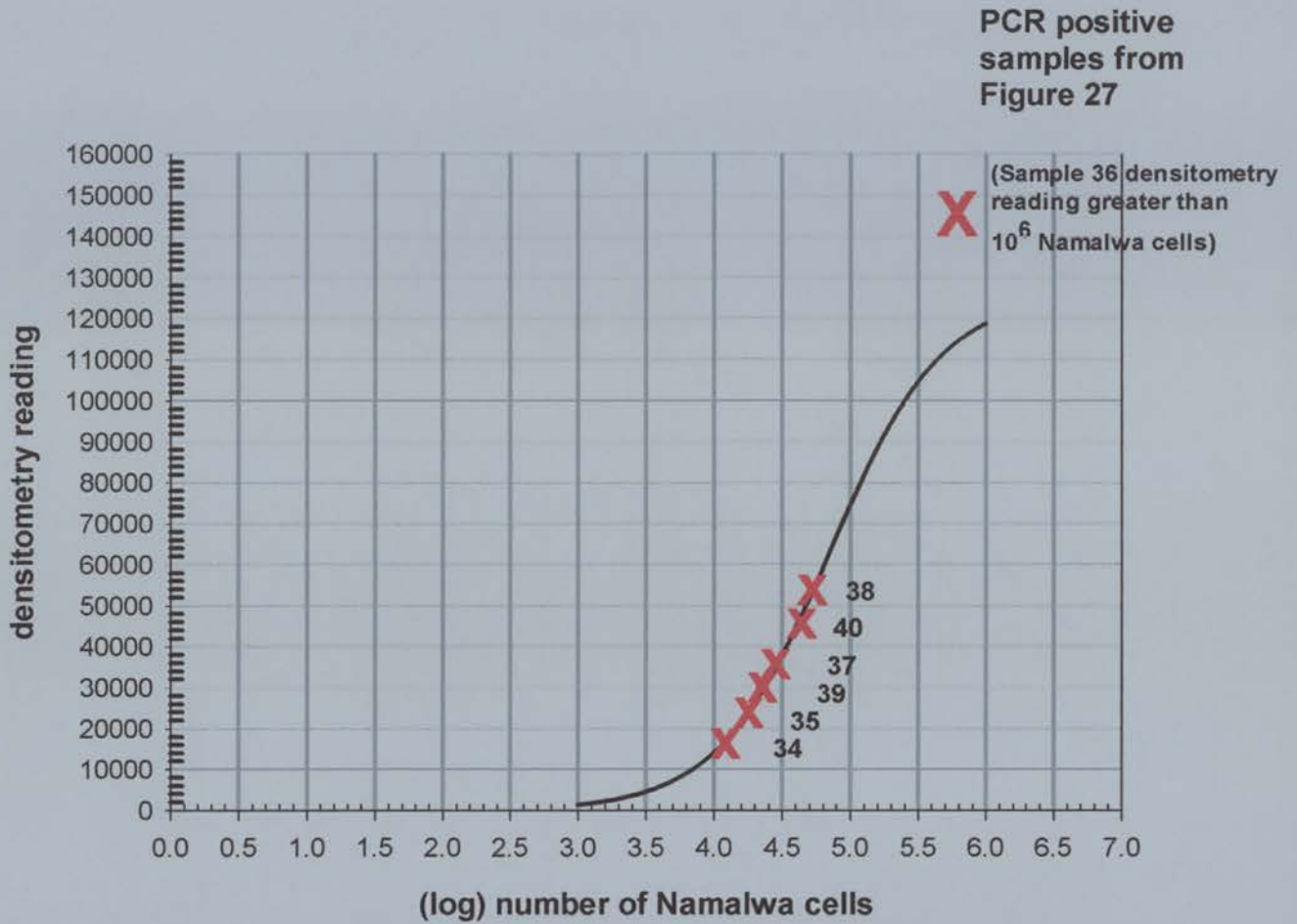


Figure 29: Densitometry readings from control Namalwa dilution series and BamW PCR positive samples



Graph plotted using densitometry readings taken from Namalwa dilution series

Key:

X Indicates densitometry reading of samples

Details of each sample are given in the legend for Figure 27

Table 28: Bam W PCR on DNA from frozen tissue and CSF

Case Number	Case Type	Area	BamW PCR 1 (Positive/negative)	BamW PCR 2 (Positive/negative)	No. of COPIES of EBV / μg DNA
47	NORMAL	CSF	-	-	0
48	NORMAL	CSF	-	-	0
49	NORMAL	CSF	-	-	0
50	NORMAL	CSF	-	-	0
51	NORMAL	CSF	-	-	0
52	NORMAL	BRAIN	-	-	0
		BRAIN	-	-	0
53	NORMAL	BRAIN	-	-	0
54	NORMAL	BRAIN	-	-	0
15	PRE-AIDS	BRAIN	-	-	0
17	PRE-AIDS	CEREBELLUM	-	-	0
		FRONTAL	-	-	0
		PARIETAL	-	-	0
46	PRE-AIDS INFILTRATE	CSF	-	-	0
		BASAL GANGLIA	-	-	0
		CEREBELLUM	-	-	0
19	AIDS NO CNS PATHOLOGY	CSF	-	+	2
		BRAIN	-	-	0
22 (systemic lymphoma)	AIDS NO CNS PATHOLOGY	CSF	-	-	0
23 (systemic lymphoma)	AIDS NO CNS PATHOLOGY	CSF	+	+	12600
		PARIETAL	-	-	0
20	AIDS NO CNS PATHOLOGY	CSF	-	-	0
		PARIETAL	-	-	0
21	AIDS NO CNS PATHOLOGY	FRONTAL	+	-	1
		CEREBELLUM	-	-	0
		TEMPORAL	-	-	0
		PARIETAL	-	-	0
32	HIVE DRUG USER	CEREBELLUM	-	-	0
		OCCIPITAL	-	-	0
		BRAIN STEM	-	-	0
25	AIDS INFILTRATE	CSF	+	+	6300
		CSF 2 (BLOOD)	-	+	12600
		CEREBELLUM	-	-	0
		OCCIPITAL	+	+	110
		PARIETAL	-	-	0
26	AIDS INFILTRATE	OCCIPITAL	-	-	0
		CEREBELLUM	-	-	0
		PARIETAL	-	-	0
		FRONTAL	-	-	0
		BRAIN STEM	+	+	160
37	PCNSL	CSF	+	+	283
40	PCNSL	CSF	+	+	$>10^6$
		FRONTAL	+	+	7.9×10^5
		BASAL GANGLIA	+	+	$>10^6$
41	PCNSL	CEREBELLUM	+	-	7.9×10^5
		BRAIN STEM	-	-	0
42	PCNSL	CSF	+	+	40000
		FRONTAL	+	+	140000
		BASAL GANGLIA	+	+	$>10^6$
		CEREBELLUM	+	+	20000
39	SYSTEMIC + CNS	CSF	+	+	1300
		PARIETAL 1+1	-	-	0

4 CSF samples and 8 frozen tissue samples were tested from the AIDS group with PCNSL. All 4 CSF samples were positive; 2 low level (283 and 1300 copies/ μ g of DNA) and two at higher levels (40000 and 10^6 copies/ μ g of DNA). 6 of the 8 frozen tissue samples showed high levels of positivity (between 20000 and 10^6 copies/ μ g of DNA), all 6 were from areas containing tumour. 2 samples were negative, both of which were from areas devoid of tumour.

In summary, BamW PCR failed to detect EBV in any of the normal, pre-AIDS, or HIVE cases assessed. All samples from AIDS cases with no CNS pathology were negative or very low level positive (1-2 copies/ μ g of DNA) with the exception of case 23 which showed higher levels. However this case had a potential confounding factor with the presence of a systemic lymphoma, and may not be representative of the rest of the group. The AIDS infiltrate group showed evidence of EBV in the CSF and in 2 of 8 brain tissue samples. However the areas of the brain showing positivity were not the areas containing the greatest numbers of B lymphocytes. Finally, in the AIDS group with PCNSL all CSF samples showed some PCR positivity, and all tissue samples from tumour areas contained high EBV copy numbers, while areas without tumour were negative. This suggests that within the brain EBV presence is restricted to infected B lymphocytes (lymphoma cells) and is not present as free virus throughout the brain.

3.3.4 EBV protein expression

Sections with high numbers of B lymphocytes or sections which were previously shown to be EBERs positive were assessed for the expression of two EBV latent proteins; EBNA-2 (Epstein Barr nuclear antigen 2) and LMP-1 (latent membrane protein 1). This allowed the latency state of virus in different cases to be assessed.

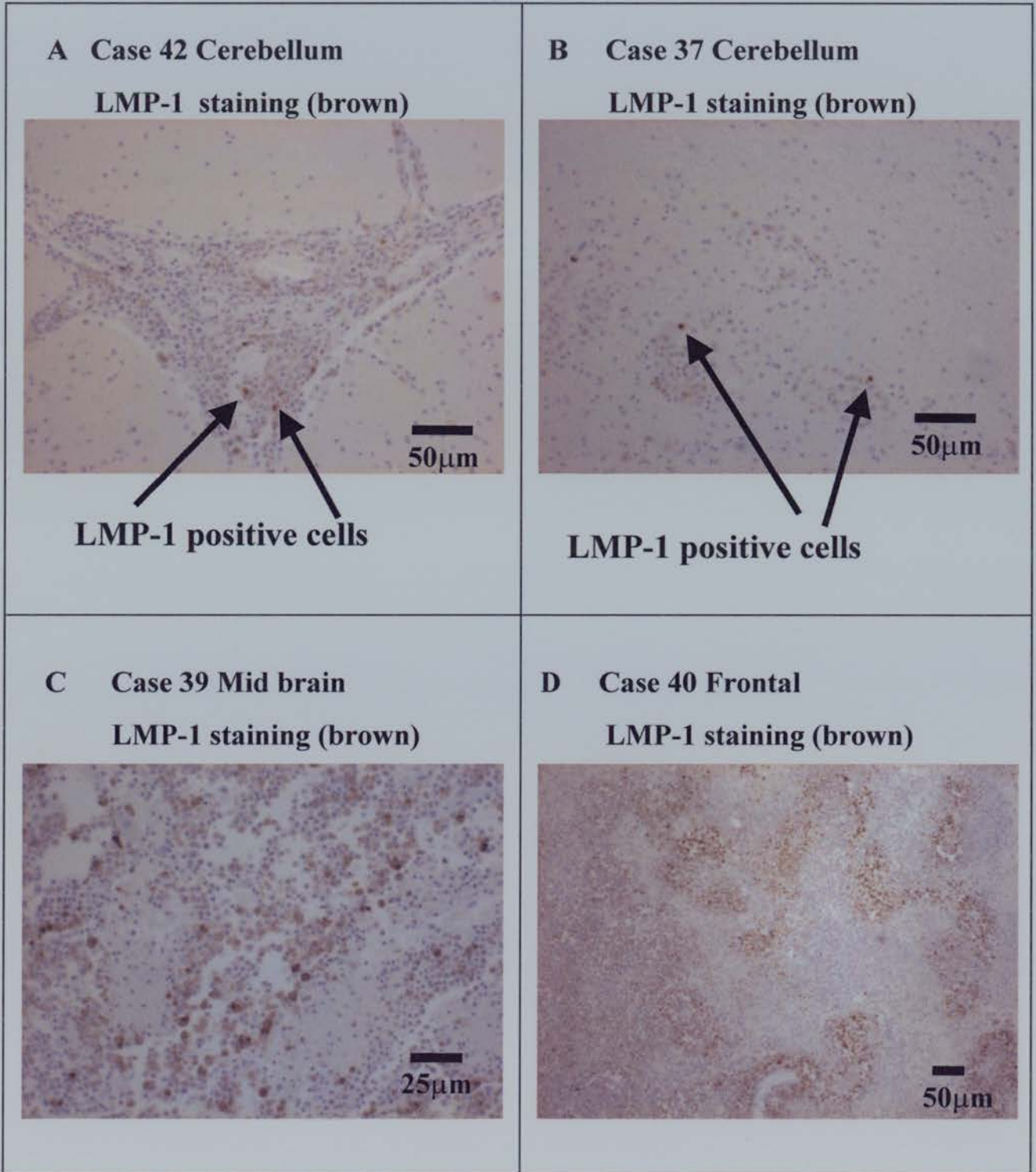
FFPE sections from five lymphoma cases and three AIDS infiltrate cases were assessed. Table 29 shows the results for both LMP-1 and EBNA-2 staining. In all sections assessed from lymphoma cases, which contained tumour cells, some positivity was found for both EBNA-2 and LMP-1. The number of cells staining positive for LMP-1 in each section varied greatly

between cases. Figure 30a and 30b shows sections from cases 42 (a) and 38 (b) in which only a few lymphoma cells stained for LMP-1. By contrast sections from cases 36 (figure 30c) and 40 (figure 30d) showed approximately half of the lymphoma cells stained for LMP-1. EBNA-2 staining was more consistent between cases, with all cases showing a high level of positivity and the majority of tumour cells staining positive (see figure 30e-h).

Sections from the AIDS infiltrate group showed no staining with either antibody, consistent with the negative in situ EBERs findings (3.3.2). The results from the lymphoma cases studied suggest a latency III phenotype for EBV in all of these lymphomas.

In summary, all PCNSL cases studied which had sections containing tumour cells, were EBERs positive. All showed strong EBNA-2 staining and variable LMP-1 staining, suggesting a latency III viral phenotype. Three of these cases had frozen tissue available which showed high levels of EBV DNA in tumour areas. Four cases had frozen CSF's available, all four of which showed evidence of EBV DNA. In addition one case with a systemic lymphoma but no CNS pathology had evidence of EBV DNA in the CSF. No EBERs staining was found in any other group, or in the AIDS lymphoma group outwith the areas of lymphoma. However, both of the AIDS infiltrate cases for which there was frozen tissue available showed evidence of low level EBV DNA in one area of the brain. In one case CSF was available, which also showed evidence of EBV DNA.

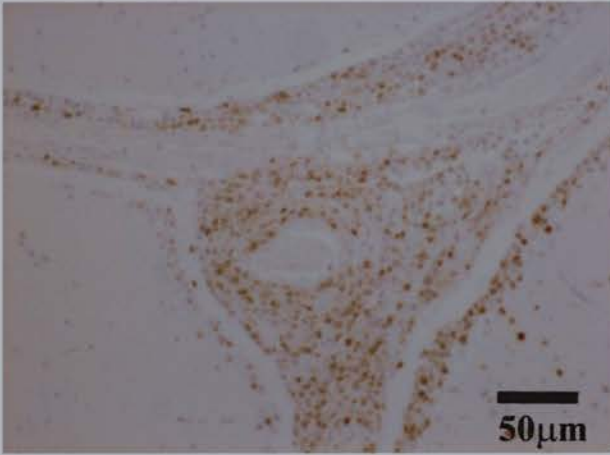
Figure 30: EBNA-2 and LMP-1 immunohistochemistry staining on AIDS PCNSL cases



Results of these experiments are summarised in table 29.

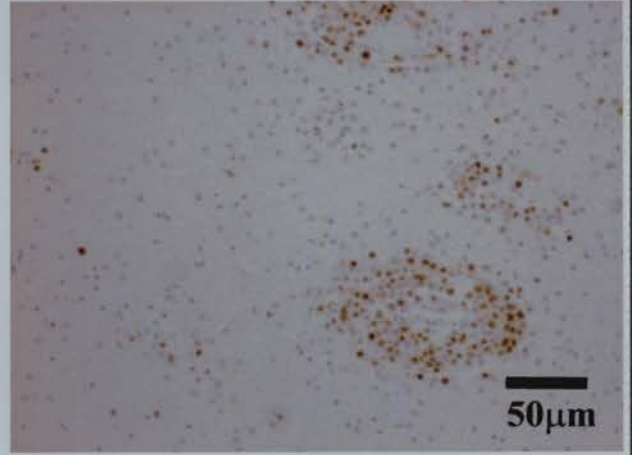
Figure 30 continued: EBNA-2 and LMP-1 immunohistochemistry staining on AIDS PCNSL cases

E Case 42 Cerebellum



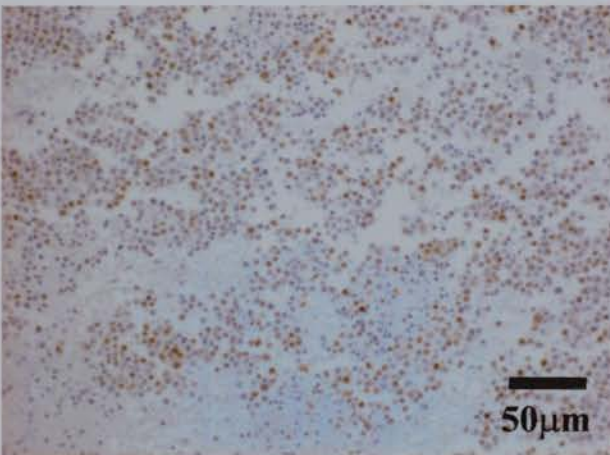
EBNA-2 positive cells stained brown

F Case 38 Cerebellum

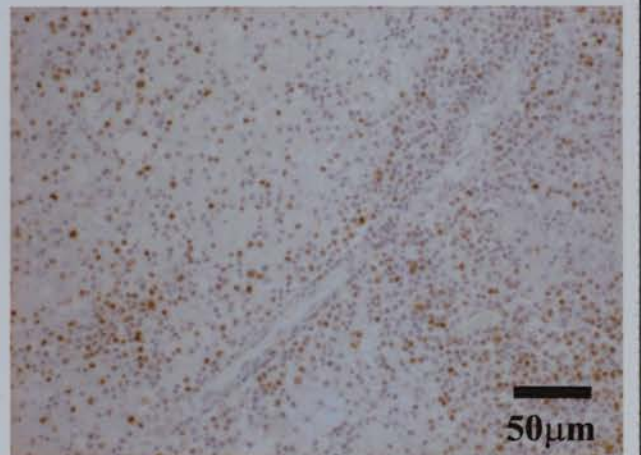


EBNA-2 positive cells stained brown

**G Case 39 Mid brain
EBNA-2 staining (brown)**



**H Case 40 Frontal
EBNA-2 staining (brown)**



Results from these experiments are summarised in table 29.

Table 29: EBNA-2 and LMP-1 Results

Case number / type	Area of brain	EBNA-2 positive / negative	LMP-1 positive / negative
36 Lymphoma	Mid brain	+++	++
38 Lymphoma	Cerebellum	+++	+
40 Lymphoma	Frontal	+++	+++
41 Lymphoma	Medulla	+++	+
	Basal ganglia	+++	+
42 Lymphoma	Basal ganglia	+++	+
	Cerebellum	+++	+
24 AIDS infiltrate	Occipital	-	-
25 AIDS infiltrate	Medulla	-	-
26 AIDS infiltrate	Temporal hippocampus	-	-

Key: - negative, + Only a few cells positive, ++ approximately half the cells positive, +++ majority of cells positive.

3.4 Clonality of B lymphocytes in tumours and pleomorphic infiltrates in AIDS brains

Large numbers of B lymphocytes have been shown to be present not only in PCNSL brains (within tumours) but also in AIDS brains with pleomorphic lymphocytic infiltrates. We had considered the possibility that these pleomorphic lymphocytic infiltrates might represent a pre-malignant lesion, and therefore to provide more data on potential mechanisms of pathogenesis of both groups, the clonality of the B lymphocytes present in the lesions was assessed.

Three methods were used to assess the clonality of large accumulations of B lymphocytes in the brain:

- 1- In-situ hybridisation using probes for kappa and lamda light Ig chains
- 2- PCR amplification of the frame work (FR) 3 region of Ig heavy chain genes (IgH)
- 3- PCR amplification of the FR1 region of IgH genes.

3.4.1 kappa and lamda Ig light chain in-situ hybridisation

Dako PNA probe and detection kit were used in accordance with the manufacturer's instructions. Positive control material was FFPE tonsil sections which had been previously shown to contain large numbers of CD20 positive B lymphocytes. Despite numerous attempts, no staining was achieved on positive control sections using the manufacturers' reaction conditions. Conditions were then altered, increasing and decreasing the hybridisation temperature of the probe; again this failed to produce a positive result. The stringency of the probe washing was then altered, however this led to background staining but no specific staining. Finally the temperature at which sections were incubated in proteinase K was altered, but again no staining was produced. It was concluded that this technique was unsuitable for the material being tested and therefore the technique was abandoned and alternative methods of determining B lymphocyte clonality were pursued. It has since become apparent that a fault in the production of the proteinase K used in the DAKO PNA detection kit may have been responsible, and the kit has now been recalled by the manufacturer.

3.4.2 PCR amplification of the FR3 region of Ig heavy chain genes

This technique involved PCR amplification of the FR3 region of the Ig heavy chain genes, which is re-arranged during B lymphocyte development. PCR amplification yields a product of approximately 100bp from DNA derived from cells with re-arranged Ig genes. In cells which do not have re-arranged Ig genes the primer binding points are too far apart for PCR amplification and no product is produced. The experimental technique, PCR reaction conditions and primer sequences have been previously published (Trainor *et al* 1990, Trainor *et al* 1991, Steward *et al* 1994) but the technique

has not been used before in our laboratory. Therefore, using the published conditions as a starting point, the PCR was optimised for use. DNA was extracted from 3 B lymphoma cell lines, 2 T lymphocyte cell lines, a monocyte cell line and PBMCs from 4 healthy donors, for use as control DNA. The B lymphoma lines chosen represent monoclonal B lymphocyte populations, while PBMCs should have polyclonal populations present. T lymphocyte and monocyte lines acted as negative controls, as normally only B lymphocytes have re-arranged Ig genes.

3.4.2.1 Optimisation of FR3 PCR

The PCR reaction conditions were optimised using a Burkitt's lymphoma cell line (Akata). Altering one variable at a time, concentrations of the following components were optimised: MgCl₂, primers, dNTP, Taq polymerase, as well as annealing temperature of primers and quantity of DNA used per reaction.

3.4.2.1.1 MgCl₂ concentration

The enzyme Taq polymerase used for PCR is dependant on Mg²⁺ ions for its activity and therefore the concentration of MgCl₂ in the reaction mixture is critical. High concentrations of Mg²⁺ inhibit enzyme activity, while too low concentrations result in a poor yield of PCR product (Linz 1990). MgCl₂ was varied as follows: 1.5µl, 3µl, 4.5µl, 6µl of 25mM MgCl₂. The best results were achieved using 4.5µl of 25mM MgCl₂.

3.4.2.1.2 Primer concentration

An excess of PCR primer is required for efficient PCR amplification, however, a large excess can lead to amplification of non-target sequences, while too low concentrations will inhibit efficient PCR amplification. Primer concentrations were tested as follows: 25 pmol, 50 pmol, 75 pmol, 100 pmol per reaction. Optimum results were achieved using 50pmol/ reaction (50µl).

3.4.2.1.3 dNTP concentration

An excess of dNTPs is required for efficient PCR amplification, although a large excess can inhibit the reaction, and too low a concentration

leads to poor yields. dNTP concentrations were tested as follows: 0.25 μ l, 0.5 μ l, 0.75 μ l, 1 μ l of 20mM dNTP per reaction. Optimum results were achieved using 0.75 μ l of 20mM dNTP.

3.4.2.1.4 Taq polymerase concentration

The concentration of Taq polymerase is important for the PCR reaction as a low concentration leads to poor yield of product, while high concentrations can lead to amplification of non-target sequences. Taq concentrations were tested as follows: 1.25, 2.5, 3.75, 5 units per reaction. Optimum results were achieved using 2.5 units per reaction.

3.4.2.1.5 Annealing temperature of primers

The annealing temperature of the primers controls the efficiency of primer annealment to target sequences. Too high a temperature inhibits annealment, while a temperature which is too low can lead to primers binding to non-target sequences. Annealing temperatures were assessed using a robocycler (Stratagene) at temperatures of 59°C, 57°C, 55°C, 53°C, 51°C, with all other variables used at the optimum obtained above. 55°C produced the best results.

3.4.2.1.6 Quantity of DNA per reaction

The amount of template DNA used in a reaction is important to obtain a good yield of PCR product, however it is also important to optimise the quantity of DNA required so that precious resources of DNA from samples are not wasted. The following DNA amounts per reaction were assessed: 0.01 μ g, 0.1 μ g, 0.5 μ g, 1 μ g, 1.5 μ g, 2 μ g. The optimal DNA concentration was 1 μ g of DNA per reaction.

3.4.2.2 FR3 PCR on DNA extracted from control cells

Having optimised the FR3 PCR reaction conditions, the PCR was tested on 3 control B lymphoma cell lines (Akata, Raji, P3HR1), 2 control T

lymphocyte cell lines (Hut 78, MOLT 4), 1 control monocyte cell line (U937), and PBMC samples taken from 4 healthy donors. A representative gel photograph is shown in figure 31. All 3 B lymphoma cell lines are monoclonal, as expected. Both T lymphocyte cell lines also showed monoclonal bands, which was unexpected since normally only B lymphocytes re-arrange Ig genes. There are 2 possible explanations for finding T cell lines with re-arranged IgH genes. It is possible that the cell lines had been contaminated with B lymphocytes. Alternatively, there is evidence in the literature (Davey *et al* 1986, Szczepanski *et al* 1999) which suggests that some T cell leukaemias can re-arrange their Ig genes as well as their T cell receptor genes. We attempted to rule out contamination with B lymphocytes using immunocytochemistry with anti-CD3 and anti-CD19 antibody on cell cytopins from the T lymphocyte lines. Although both cell lines were CD3 positive (T lymphocyte marker), CD19 negative (B lymphocyte marker), the low number of cells tested means we cannot rule out low level contamination of the T cell line.

The monocyte cell line produced no band, indicating there were no cells present with re-arranged Ig genes. All 4 PBMC samples gave single bands indicating monoclonal populations of B lymphocytes were present in each. This result was unexpected as the samples were taken from healthy donors who are unlikely to have a single large monoclonal population of B lymphocytes in their blood. PBMCs would be expected to show either multiple bands or a smear of bands; indicating the presence of multiple clones.

The products produced by this PCR for different clones vary by as little as 1 base pair. This may explain the appearance of an apparently monoclonal band in the PBMC samples, and therefore we sought to ensure that the gel resolution being used was high enough to distinguish between such a small difference. An FR3 PCR was carried out on DNA from Akata and P3HR1 cell lines mixed in a 50:50 ratio. Again only one band appeared, suggesting that the gel resolution was too low to distinguish between bands of similar sizes (approximately 100bp). In order to improve the resolution the experiment was repeated and the PCR products run on 5% and 8% nu-sieve agarose gels and 6 % polyacrylamide gel instead of 3% agarose. Neither of these gels allowed resolution of the products.

Figure 31: FR3 PCR optimised conditions



Key:

- | | |
|----|------------------|
| A | Akata |
| R | Raji |
| P | P3HR1 |
| W | H ₂ O |
| PB | PBMC |

This technique was abandoned at this stage for 2 reasons. Firstly, the PCR was unable to differentiate two clonal cell lines. Secondly, all the bands produced seemed to be an identical size with no variation, suggesting that either the PCR could only detect re-arranged Ig genes and not differentiate between different clones, or that it was amplifying the wrong sequence. This technique has been previously used by Trainor *et al* 1990, and 1991, Steward *et al* 1994 who were able to demonstrate resolution of similarly sized bands.

3.4.3 PCR amplification of the FR1 region of Ig heavy chain genes

FR1 PCR requires six PCR reactions per sample, one to detect re-arrangement of the germ line DNA for each of the variable heavy chain Ig family genes (VH1,2,3,4,5 and 6). The primers are designed to bind to conserved regions within in each of the six families. As with the FR3 PCR, non re-arranged Ig genes are not amplified as the primer binding sites are too far apart for PCR amplification.

Control material used was DNA extracted from 7 different samples of PBMCs from healthy donors and the clonal B cell lines Akata, Raji, P3HR1, the T cell lines Molt 4 and HUT 78, and a monocyte line U937. All 7 PBMC samples gave positive reactions in at least 3 different FR1 PCRs, demonstrating their polyclonality. Akata was positive only in VH1 PCR, Raji only in VH3 PCR and P3HR1 only in VH4 PCR, showing the monoclonality of these cell lines. Figure 32 shows the sensitivity of the PCR (Akata dilution series). 10^2 - 10^3 cells were required to obtain a positive PCR reaction, therefore only relatively large clonal population will be detected by this method. Neither Molt 4, HUT 78, or U937 gave bands in any of the VH PCR reactions. None of the control samples showed positivity for VH5 or VH6 PCR, however given the low usage of these families by normal human B lymphocytes, and the fact that the same PCR reaction conditions and primers had been shown to work by Inghirami *et al* (1993), it was decided to proceed with testing samples using this technique.

Test DNA was extracted from frozen brain tissue and frozen CSF samples as described in 2.2.3.1. PCR was performed on those cases from

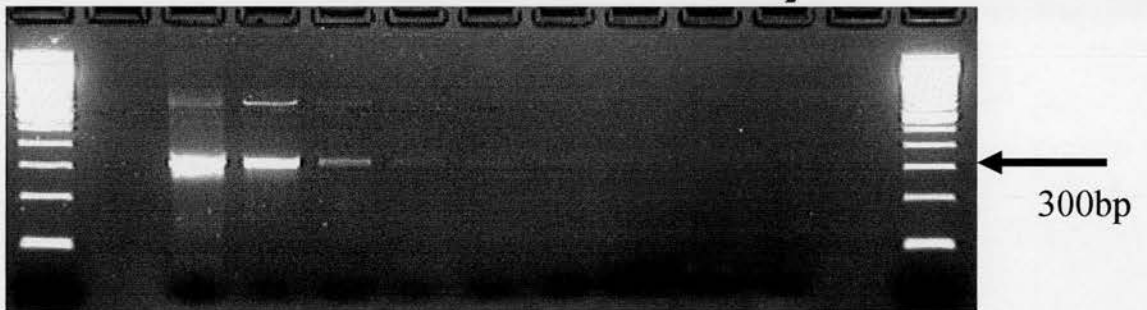
Figure 32:

FR1 PCR (VH1)

Akata dilution series

Number of Akata cells diluted in 10^6 U937 cells

10^6 10^5 10^4 10^3 10^2 10^1 10^0 U H₂O



Key:

U U937 (monocyte cell line)

H₂O Water (control)

which frozen tissue was available and which had previously been shown to contain large accumulations of B lymphocytes in the brain.

Figure 33 shows representative gel pictures from PCR for VH1,2,3,4,5 and 6. Where sufficient material was available, the 6 PCRs were repeated three times. The results are summarised in table 30. In two of the three PCNSL cases tested (41 and 42) only one variable immunoglobulin heavy (IgH) chain family gene re-arrangement was detected, both VH3. In case 41 both brain areas tested showed weak positivity for VH3 in one out of two PCRs, suggesting that this tumour may represent a clonal population but that the number of cells present was close to the limit of detection. In case 42 both the cerebellum and basal ganglia showed evidence of B lymphocytes with re-arranged VH3 family IgH genes, as did the CSF. In the frontal lobe of this case, no re-arranged IgH chain genes were detected. The third lymphoma case, case 40, showed positive reactions for germ line DNA re-arrangements of all six variable IgH family genes in the frontal lobe. In the basal ganglia of this case only re-arrangement of VH1 family gene was detected, however this was only detected on one of three tests. No clonal populations with re-arranged variable IgH family genes were detected in the CSF of this case. This data suggests that there are accumulations of polyclonal B lymphocytes in the frontal lobe in this case.

Two AIDS infiltrate cases were tested. Case 26 showed re-arrangements of all six variable IgH family genes in the cerebellum, re-arrangements of VH1,3 and 5 in the occipital lobe, re-arrangements of VH1,2 and 3 in one sample from the parietal lobe and re-arrangements of VH1 and 3 in a second parietal lobe sample. This suggests that the B lymphocyte accumulations seen throughout this brain were polyclonal. Case 25 showed re-arrangements in VH1,3,4 and 5 in the occipital lobe. In the cerebellum only one re-arrangement of VH5 family genes was detected. No re-arrangements were detected in the parietal lobe, while in the CSF VH1 and 3 re-arrangements were detected. This again suggests that the B lymphocyte accumulations seen throughout this brain were polyclonal.

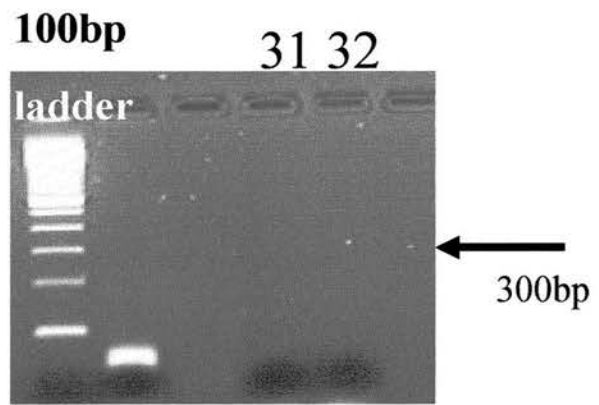
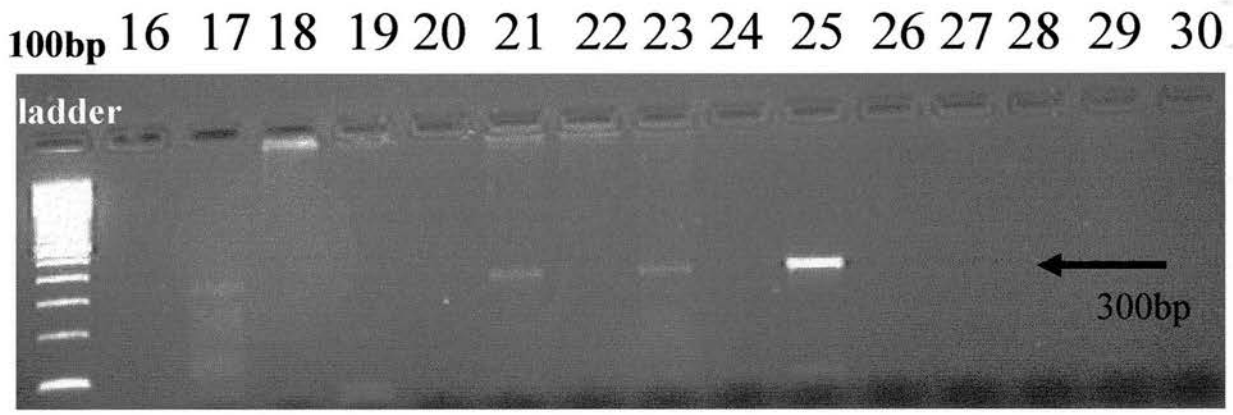
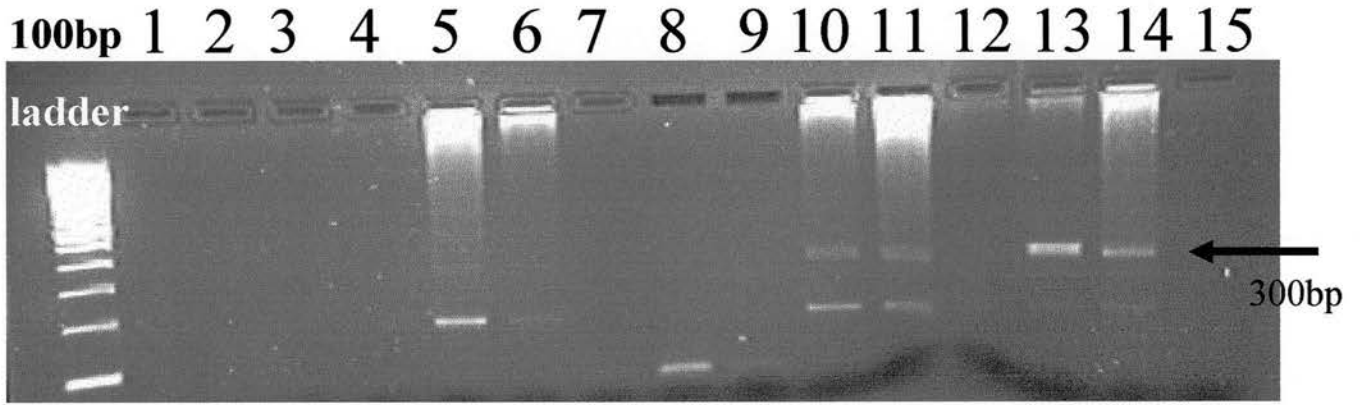
Figure 33 a legend:

Sample number	Case number	Area of brain / control cell line
1	42 (AIDS lymphoma)	Cerebellum
2		Basal ganglia
3		Frontal
4		CSF
5	40 (AIDS Lymphoma)	Frontal
6		Basal ganglia
7		CSF
8	41 (AIDS Lymphoma)	Cerebellum
9		Brain stem
10	26 (AIDS Infiltrate)	Cerebellum
11		Occipital
12		Parietal (1)
13		Parietal (2)
14	25 (AIDS Infiltrate)	Occipital
15		Cerebellum
16		Parietal
17		CSF
18	46 (Pre-AIDS viral encephalitis)	Cerebellum
19		Basal ganglia
20		CSF*
21	Control	PBMC
22	Control	PBMC
23	Control	PBMC
24	Control	PBMC
25	Control	Akata
26	Control	Raji
27	Control	P3HR1
28	Control	Hut 78
29	Control	Molt 4
30	Control	U937
31	Control	H ₂ O
32	Control	H ₂ O

Summary of results from these experiments is given in Table 30.

Figure 33a:

VH1 PCR (FR1 region of immunoglobulin heavy chain genes)



Target sequence size ~300bp

Figure 33b legend:

Sample number	Case number	Area of brain / control cell line
1	42 (AIDS lymphoma)	Cerebellum
2		Basal ganglia
3		Frontal
4		CSF
5	46 (Pre-AIDS viral encephalitis)	Cerebellum
6		Basal ganglia
7		CSF*
8	41 (AIDS Lymphoma)	Cerebellum
9		Brain stem
10	26 (AIDS Infiltrate)	Cerebellum
11		Occipital
12		Parietal (1)
13		Parietal (2)
14	25 (AIDS Infiltrate)	Occipital
15		Cerebellum
16		Parietal
17		CSF
18	40 (AIDS Lymphoma)	Basal ganglia
19		Frontal
20		CSF
21	Control	PBMC
22	Control	PBMC
23	Control	Akata
24	Control	Raji
25	Control	P3HR1
26	Control	Hut 78
27	Control	Molt 4
28	Control	U937

Key: Yellow box indicates location of positive result in this gel.

Summary of results from these experiments is given in Table 30.

Figure 33b:

VH2 PCR (FR1 region of immunoglobulin heavy chain genes)

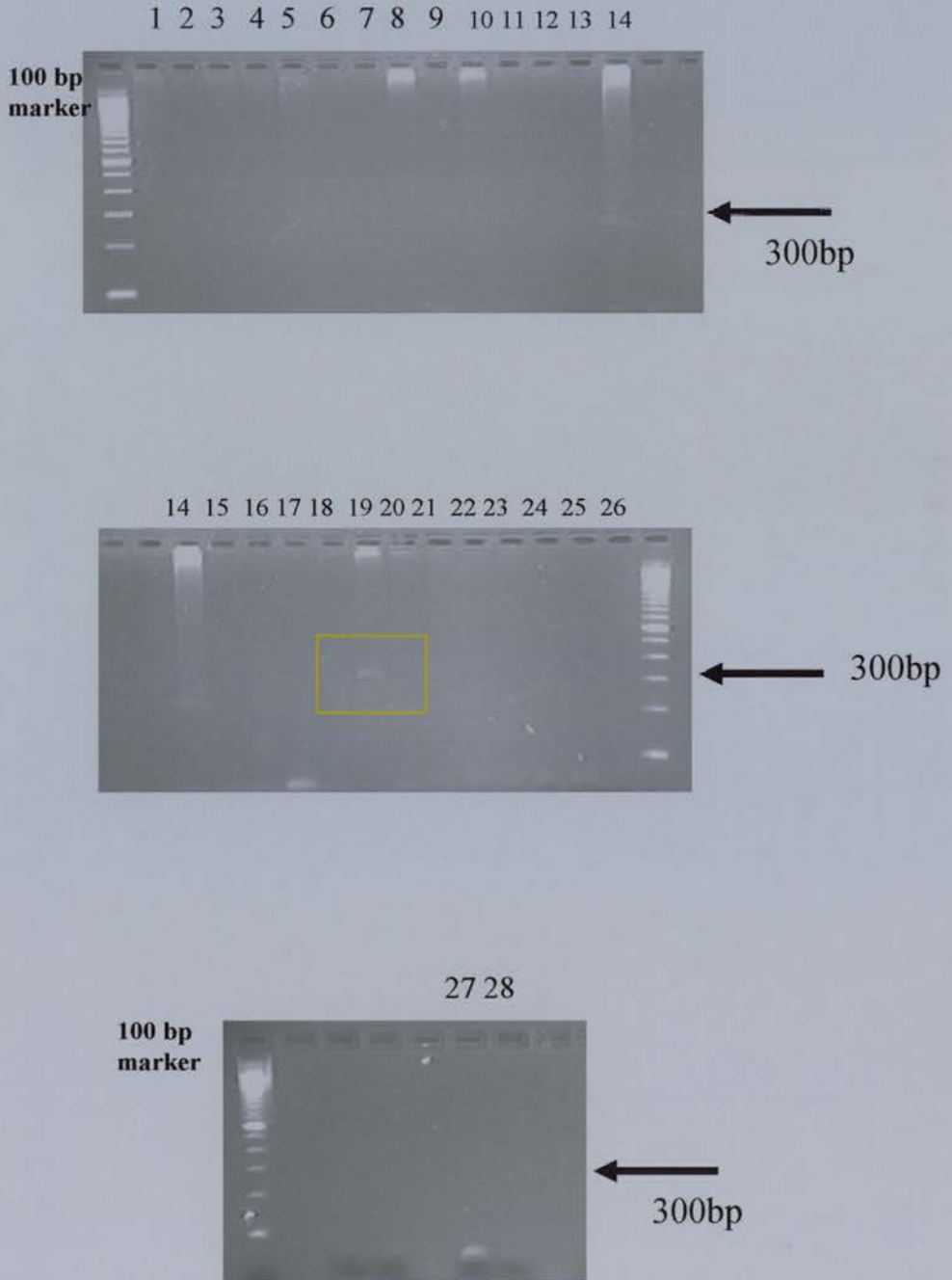


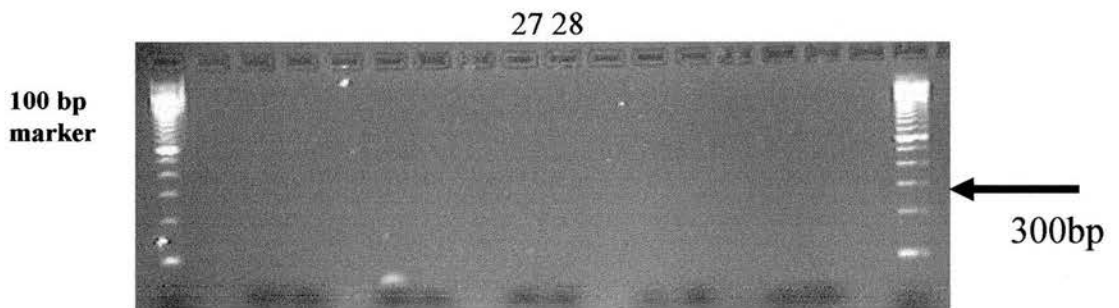
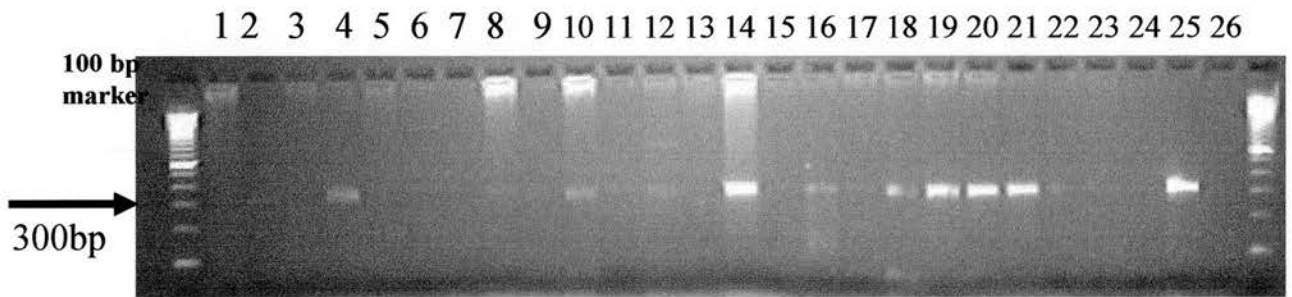
Figure 33c legend:

Sample number	Case number	Area of brain / control cell line
1	41 (AIDS Lymphoma)	Cerebellum
2		Brain stem
3	40 (AIDS Lymphoma)	Frontal
4		Basal ganglia
5		CSF
6	46 (Pre-AIDS viral encephalitis)	Cerebellum
7		Basal ganglia
8		CSF*
9	25 (AIDS Infiltrate)	Occipital
10		Cerebellum
11		Parietal
12		CSF
13	42 (AIDS lymphoma)	Cerebellum
14		Basal ganglia
15		Frontal
16		CSF
17	26 (AIDS Infiltrate)	Cerebellum
18		Occipital
19		Parietal (1)
20		Parietal (2)
21	Control	PBMC
22	Control	PBMC
23	Control	PBMC
24	Control	Akata
25	Control	Raji
26	Control	P3HR1
27	Control	Hut 78
28	Control	Molt 4

Summary of results from these experiments is given in Table 30.

Figure 33c:

VH3 PCR (FR1 region of immunoglobulin heavy chain genes)



Target sequence size ~300bp

Figure 33d legend:

Sample number	Case number	Area of brain / control cell line
1	42 (AIDS lymphoma)	Cerebellum
2		Basal ganglia
3		Frontal
4		CSF
5	40 (AIDS Lymphoma)	Frontal
6		Basal ganglia
7		CSF
8	41 (AIDS Lymphoma)	Cerebellum
9		Brain stem
10	26 (AIDS Infiltrate)	Cerebellum
11		Occipital
12		Parietal (1)
13		Parietal (2)
14	25 (AIDS Infiltrate)	Occipital
15		Cerebellum
16		Parietal
17		CSF
18	46 (Pre-AIDS viral encephalitis)	Cerebellum
19		Basal ganglia
20		CSF*
21	Control	P3HR1
22	Control	PBMC
23	Control	PBMC
24	Control	PBMC
25	Control	Akata
26	Control	Raji
27	Control	Hut 78
28	Control	Molt 4
29	Control	U937
30	Control	H ₂ O
31	Control	H ₂ O

Summary of results from these experiments is given in Table 30.

Figure 33d:

VH4 PCR (FR1 region of immunoglobulin heavy chain genes)

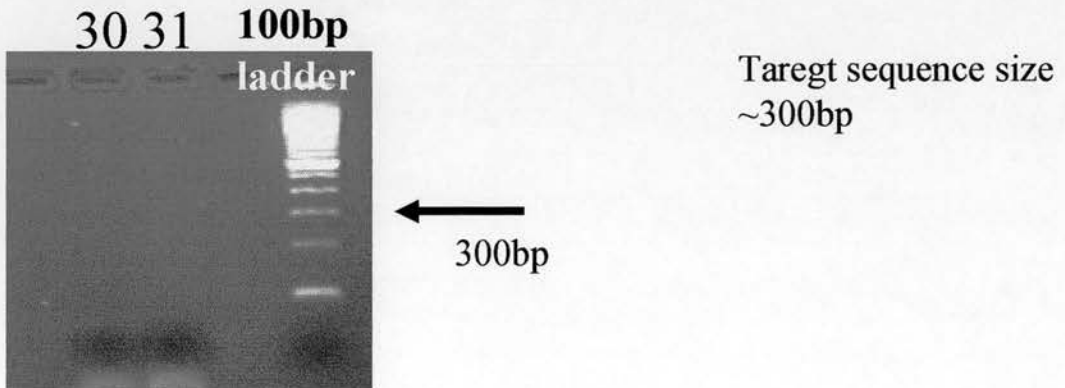
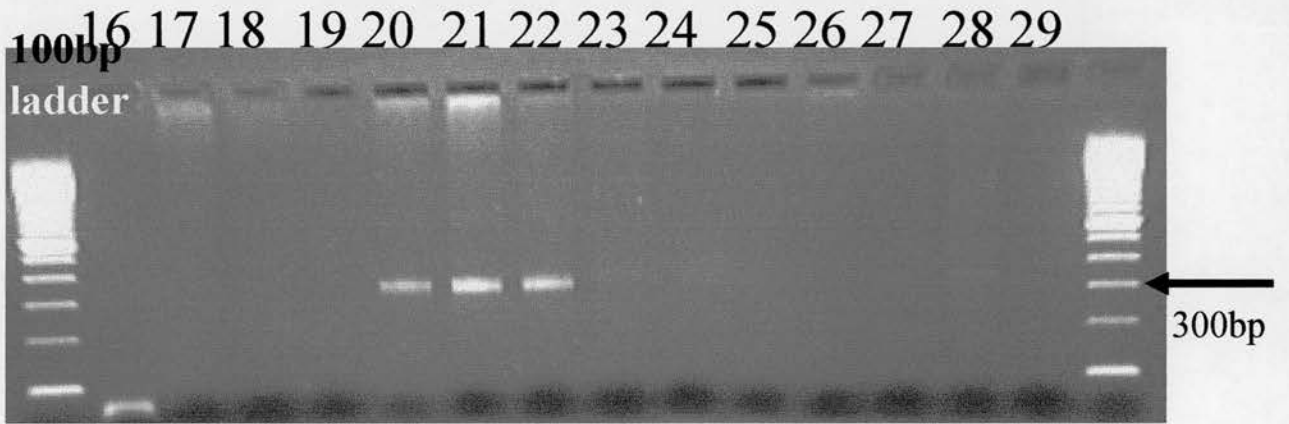
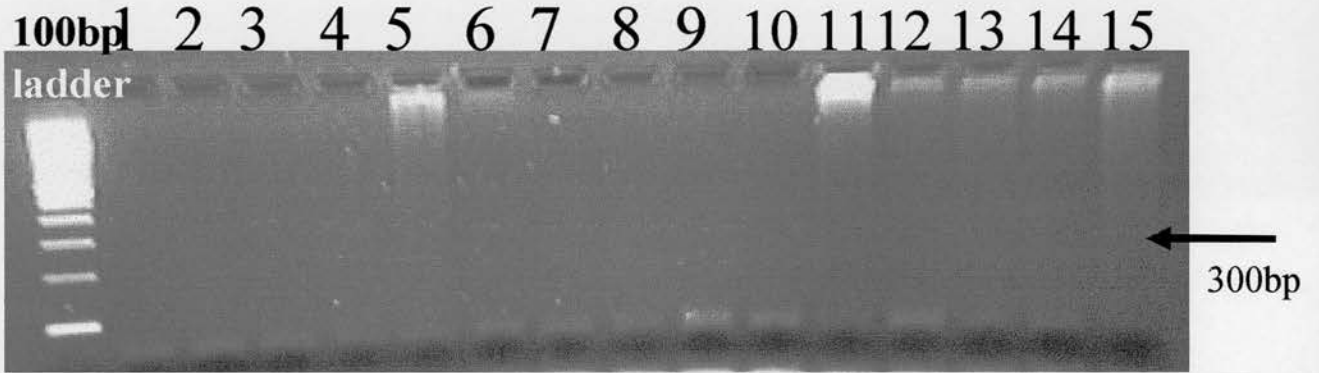


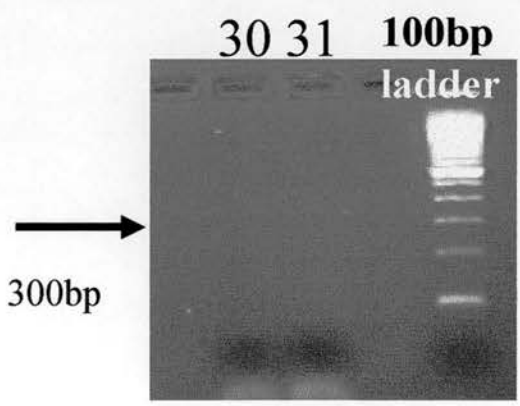
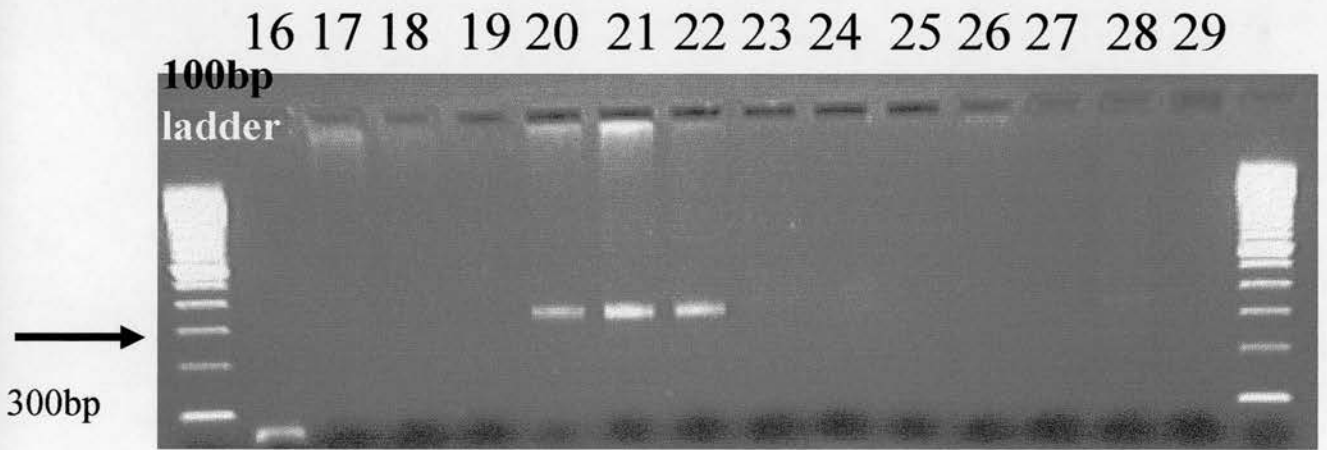
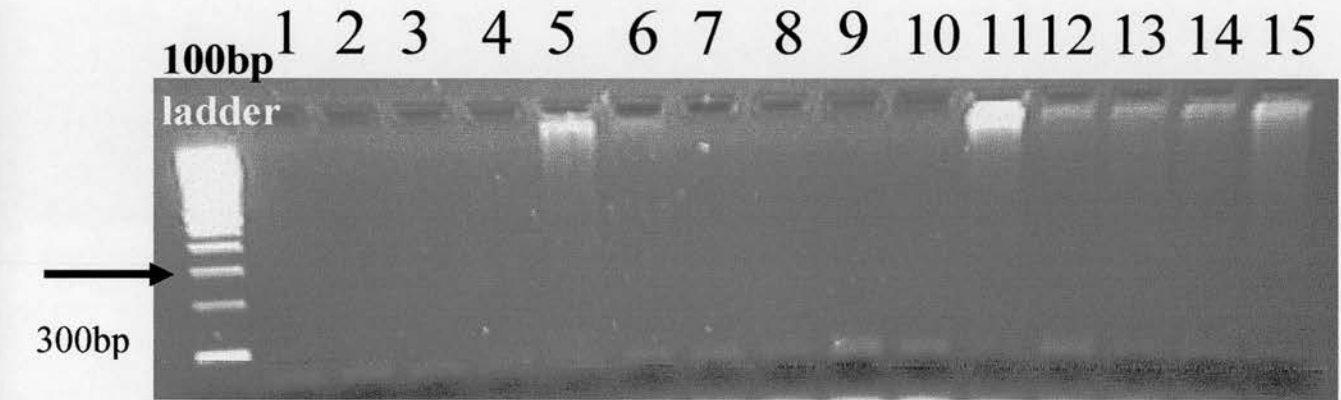
Figure 33e legend:

Sample number	Case number	Area of brain / control cell line
1	42 (AIDS lymphoma)	Cerebellum
2		Basal ganglia
3		Frontal
4		CSF
5	40 (AIDS Lymphoma)	Frontal
6		Basal ganglia
7		CSF
8	41 (AIDS Lymphoma)	Cerebellum
9		Brain stem
10	26 (AIDS Infiltrate)	Cerebellum
11		Occipital
12		Parietal (1)
13		Parietal (2)
14	25 (AIDS Infiltrate)	Occipital
15		Cerebellum
16		Parietal
17		CSF
18	46 (Pre-AIDS viral encephalitis)	Cerebellum
19		Basal ganglia
20		CSF*
21	Control	PBMC
22	Control	PBMC
23	Control	PBMC
24	Control	PBMC
25	Control	Akata
26	Control	Raji
27	Control	P3HR1
28	Control	Hut 78
29	Control	Molt 4
30	Control	U937
31	Control	H ₂ O

Summary of results from these experiments is given in Table 30.

Figure 33e:

VH5 PCR (FR1 region of immunoglobulin heavy chain genes)



Target sequence size
~300bp

Figure 33f legend:

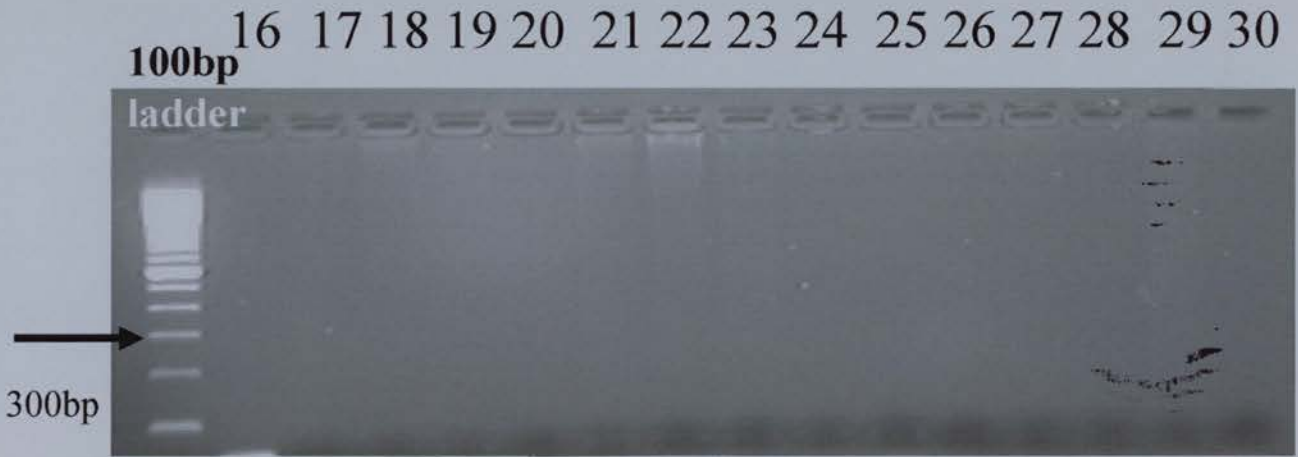
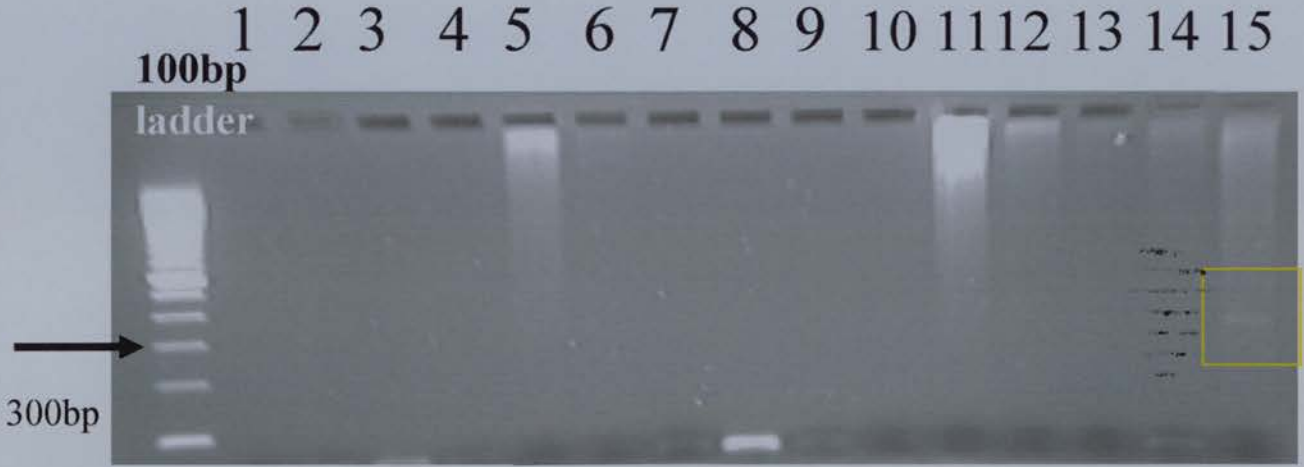
Sample number	Case number	Area of brain / control cell line
1	42 (AIDS lymphoma)	Cerebellum
2		Basal ganglia
3		Frontal
4		CSF
5	40 (AIDS Lymphoma)	Frontal
6		Basal ganglia
7		CSF
8	41 (AIDS Lymphoma)	Cerebellum
9		Brain stem
10	25 (AIDS Infiltrate)	Occipital
11		Cerebellum
12		Parietal
13		CSF
14	26 (AIDS Infiltrate)	Occipital
15		Cerebellum
16		Parietal (1)
17		Parietal (2)
18	46 (Pre-AIDS viral encephalitis)	Cerebellum
19		Basal ganglia
20		CSF*
21	Control	PBMC
22	Control	PBMC
23	Control	PBMC
24	Control	PBMC
25	Control	Akata
26	Control	Raji
27	Control	P3HR1
28	Control	Hut 78
29	Control	Molt 4
30	Control	U937
31	Control	H ₂ O
32	Control	H ₂ O

Key: Yellow box indicates location of positive sample.

Summary of results from these experiments is given in Table 30.

Figure 33f:

VH6 PCR (FR1 region of immunoglobulin heavy chain genes)



Target sequence size
~300bp

Table 30: FR1 clonality PCR Results

Case no. Case type	Area of brain	Immunoglobulin Variable Heavy Family genes (number of positive PCR reactions)					
		VH 1	VH 2	VH 3	VH 4	VH 5	VH 6
42 AIDS lymphoma	Cerebellum	-	-	2/3	-	-	-
	Basal ganglia	-	-	2/3	-	-	-
	Frontal	-	-	-	-	-	-
	CSF	-	-	3/3	-	-	-
40 AIDS lymphoma	Frontal	1/3	3/3	1/3	2/3	2/3	2/3
	Basal ganglia	1/3	-	-	-	-	-
	CSF	-	-	-	-	-	-
41 AIDS lymphoma	Cerebellum	-	-	1/2	-	-	-
	Brain stem	-	-	1/2	-	-	-
26 AIDS infiltrate	Cerebellum	2/3	1/3	3/3	1/3	1/3	2/3
	Occipital	1/3	-	3/3	-	1/2	-
	Parietal (1)	2/3	1/3	2/3	-	-	-
	Parietal (2)	1/3	-	3/3	-	-	-
25 AIDS infiltrate	Occipital	2/3	-	3/3	1/3	1/3	-
	Cerebellum	-	-	-	-	1/2	-
	Parietal	-	-	-	-	-	-
	CSF	3/3	-	2/3	-	-	-
46 Pre-AIDS viral encephalitis	Cerebellum	-	-	-	-	-	-
	Basal ganglia	-	-	2/3	-	-	-
	CSF*	-	-	-	3/3	-	-

* Sample only tested once due to limited supply of test material

One pre-AIDS case with viral encephalitis was tested. In this case no re-arrangements were detected in the cerebellum, while re-arrangement of the VH3 family genes was detected in the basal ganglia, and in the CSF re-arrangement of the VH4 family gene was detected. These data suggest that two monoclonal populations are present in different locations in this case, it is difficult to make conclusive assessments from the data as immunohistochemistry showed that this case has relatively few B lymphocytes

present and therefore smaller clones could potentially be on the limit of detection by this test.

3.5 Expression of B lymphocyte chemoattractants in the brain

Several brains investigated in this study contained high levels of infiltrating B lymphocytes. Although B lymphocytes were found scattered randomly throughout the brain parenchyma, the majority of cells were located in the perivascular regions surrounding blood vessels. A preliminary investigation was set up to detect possible chemoattractants that may be expressed in these particular brains and possibly play a role in attracting B lymphocytes into the brain.

3.5.1 SDF-1 expression in brain

Normal functions of SDF-1 include controlling movement of B lymphocytes in lymphoid tissues and also regulating early B lymphocyte movement in the bone marrow. A mutant version of SDF-1 has recently been linked to an increased incidence of NHL in AIDS patients (Rabkin *et al* 1999). In the brain the chemokine can be produced by both astrocytes and neurones (Bajetto *et al* 1999).

We therefore sought to determine if SDF-1 is over expressed in brains with PCNSL and if it therefore plays a role in the pathogenesis of AIDS PCNSL. In order to determine if SDF-1 is over expressed in PCNSL we also assessed the level of brain expression in other groups (group 1,3,4,5, and 8).

The antibody was optimised as described in 3.5.1.1 and then used to assess SDF-1 protein expression in the brain. The antibody used here binds to SDF-1 produced by both the wild type gene and mutant gene found in some AIDS patients.

3.5.1.1 Optimisation of stromal cell derived factor-1 (SDF-1) antibody

Table 31 shows the antibody concentrations, pre-treatments and immunohistochemical techniques used to find the optimal staining procedure using this antibody. In order to allow better access for antibodies to antigens affected by formalin cross linking the sections were subjected to 2 pre-

treatments: microwaving in either EDTA or citric acid. Full details of immunohistochemical methods are given in 2.2.1 page 67.

Table 31: Optimisation of SDF-1 antibody staining

Pre-treatments assessed	Antibody concentration tested for given pre-treatment	Results (intensity of staining)
No pre-treatment	1/10	++
	1/15	++
	1/20	+
	1/40	+
EDTA	1/10	++ high background
	1/15	++ high background
	1/20	++ high background
	1/40	+ high background
Citric acid	1/10	+++ high background
	1/15	+++ slight background
	1/20	++
	1/40	++
Key: +++ strong staining, ++ moderate staining, + weak staining		

An antibody concentration of 1/15 in combination with citric acid microwave pre-treatment gave the best overall result, and was therefore used for all sections stained in 3.5.2.2 below.

3.5.1.2 SDF-1 staining of brain sections

Cases with large numbers of B lymphocytes (PCNSL, AIDS infiltrate, non-HIV viral encephalitis) were chosen for this experiment together with AIDS brains with no CNS pathology and normal controls. In the cases with

large numbers of lymphocytes, areas of the brain with and without B lymphocytes were studied. AIDS cases with no CNS pathology were included to allow an assessment of the level of SDF-1 expression in AIDS compared to normal controls.

Table 32 shows the sections stained for SDF-1. Representative photomicrographs are shown in figure 34. In order to allow comparison of different groups the staining was quantified using an Olympus BX40 microscope linked via Roper Scientific digital camera to a computer, utilising image pro-plus software. Six individual, randomly selected, areas were assessed per slide and the area of staining summed for each slide. Figure 35 (SDF-1 graph) shows a summary of the results from the groups assessed. Sections from the normal group, non-HIV viral encephalitis group, and AIDS with no CNS pathology group had minimal or no staining, and the staining level shown in figure 35 for these groups is probably representative of the level of background staining seen with this antibody (see figure 34 photos of SDF-1). The AIDS infiltrate and AIDS lymphoma groups were divided into those sections which contained large numbers of B lymphocytes and those which contained none or very few lymphocytes. Both of these groups showed high levels of SDF-1 in the sections containing B lymphocytes (see figure 34), while the sections devoid of lymphocytes showed the base line level of staining seen in the normal group. Only two sections from one pre-AIDS case were assessed. This case had non-HIV encephalitis and contained occasional blood vessels with small numbers of B lymphocytes in the perivascular space. Figure 35 shows that this case had an increased level of SDF-1 expression over the base line level of the normal group, but a much lower level of expression than either the AIDS infiltrate group or AIDS lymphoma group (areas with B lymphocytes).

These data suggest an association between B cell accumulation in the brain of HIV patients and expression of SDF-1. Although the number of cases studied was low there does appear to be a link between the amount of SDF-1 expressed locally in HIV brains and the accumulation of B lymphocytes in these areas. Interestingly the non-HIV viral encephalitis group which contained large numbers of both parenchymal and perivascular B lymphocytes

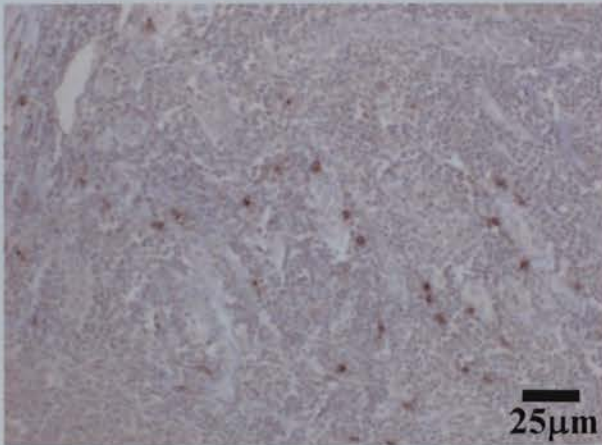
showed no or little expression of SDF-1, suggesting other mechanisms are also involved in the accumulation of B cells in the brain.

Table 32: Sections stained for SDF-1

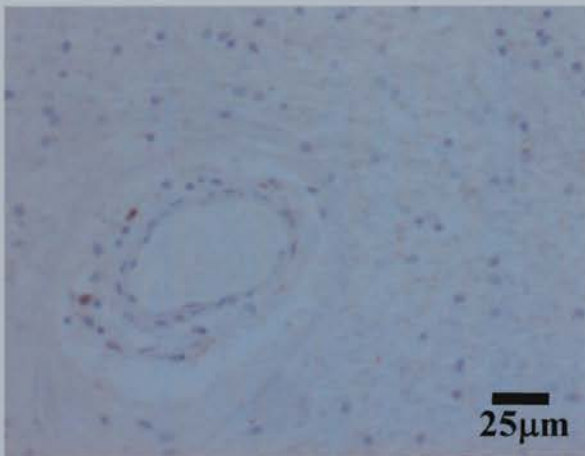
Case number	Area of brain	Area of section stained (total area for 6 fields/section) mm ²
Normal		
3	Cerebellum	2.6275
	Temporal hippocampus	0.175
	Mid brain	0.275
6	Basal ganglia	5.94
	Temporal hippocampus	0
AIDS no CNS pathology		
21	Cerebellum	7.455
	Temporal hippocampus	11.2475
	Mid brain	0
	Basal ganglia	0
19	Parietal convexity	10.3125
	Temporal hippocampus	0
	Medulla	0
AIDS infiltrate (sections with large numbers of B lymphocytes)		
25	Medulla	53.395
	Basal ganglia	0
26	Medulla	147.11
	Temporal Hippocampus	110.359
24	Medulla	132.228
	Temporal hippocampus	199.21
27	Pons	180.75
AIDS Infiltrate (sections with no or few B lymphocytes)		
24	Basal ganglia	1.465
25	Mid brain	0
27	Mid brain	0
	Parietal convexity	2.105
AIDS PCNSL (areas with lymphoma)		
42	Cerebellum	210.374
	Pons	178.21
36	Mid brain	78.236
38	Cerebellum	88.2
40	Frontal	85.81
41	Cerebellum	103.527
AIDS PCNSL (areas with no lymphoma)		
36	Temporal hippocampus	23.775
	Cerebellum	0
38	Temporal hippocampus	2.335
	Cerebellum	0
40	Pons	0.9425
	Temporal hippocampus	0
41	Frontal	2.0125
Pre-AIDS		
46	Cerebellum	15.6075
	Pons	5.885
Non-HIV viral encephalitis		
44	Pons	1.985
45	Basal ganglia	0.4675
	Frontal	0.045

Figure 34:

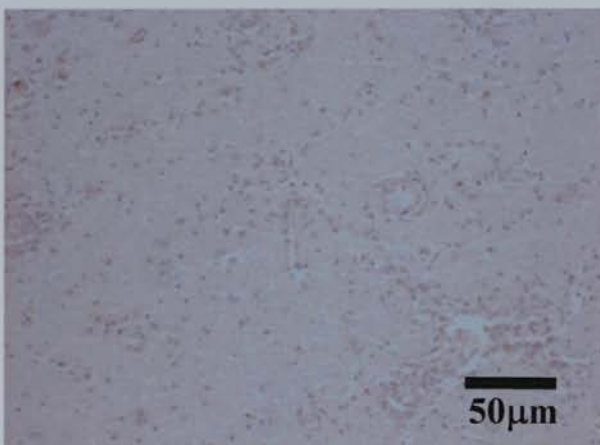
**Immunohistochemical staining
with SDF-1 antibody**



**34a: Control tonsil section
showing SDF-1 staining**



**34b: SDF-1 protein localised
to the perivascular
space of a blood vessel
that also contains
lymphocytes within the
perivascular space
(AIDS brain)**

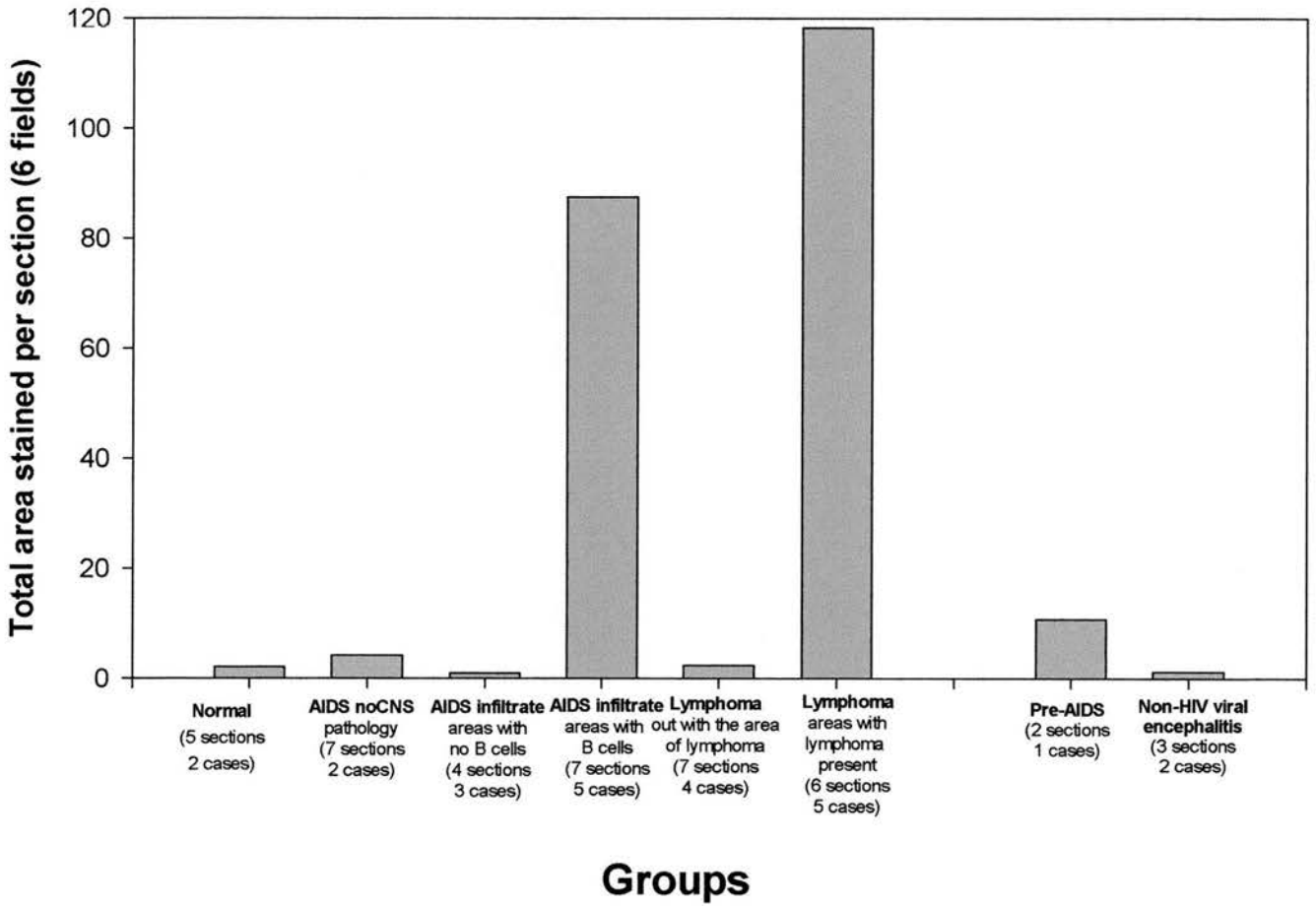


**34c: SDF-1 protein localised
to area of lymphoma in
an AIDS PCNSL brain**

Data from these experiments are summarised in table 32.

Figure 35:

Graph of SDF-1 expression in different patient groups



3.6 Expression of cytokines in the brain

This experiment was a preliminary study to assess cytokine gene expression in different brains. The expression of different cytokines has an influence on the type of immune response that develops. IL-4, IL-6 and IL-10 were chosen to be studied as these are all linked with a TH2 (humoral) immune response. IL-2 is required for activation and proliferation of T lymphocytes and therefore its presence suggests a TH1 (cellular) immune response. Finally, INF γ is another TH1 derived cytokine which inhibits TH2 responses and is known to upregulate expression of MHC molecules. 10 frozen brain samples from 8 different cases were assessed:

- 3 AIDS PCNSL cases (with tissue being taken from areas of the brain known to contain lymphoma)
- 2 samples from 1 AIDS infiltrate case (case had large numbers of perivascular B lymphocytes)
- 2 AIDS cases with no CNS pathology (which had few or no B lymphocytes)
- 2 normal brain which were assumed to have few B lymphocytes.

RNAzol B was used to extract RNA from frozen tissue samples (full procedural details for RNA extraction are given in 2.2.4.1 page 79). RNA was then converted to cDNA using an Invitrogen superscript kit (see 2.2.4.2 page 79). A GapDH PCR was used to confirm the presence of amplifiable DNA in samples. Samples were then analysed in separate experiments for the expression of: IL-2, IL-4, IL-6, IL-10, INF γ . Primer sequences and reaction conditions for PCRs are described in 2.x.x. IL-10 primers had been previously shown to bind only to human IL-10 and not EBV viral IL-10 transcripts (Johannessen 1997). Positive control material was RNA extracted from PHA stimulated PBMCs (see section 2.1.5), and a cDNA free reaction was included with each run as a negative control with sterile water replacing the target DNA. PCR products were run on agarose gels and then Southern blotted onto nitrocellulose paper, which was then probed using cytokine specific probes (see 2.2.4.4 for probe sequences, and hybridisation conditions). Probes were

labelled and detected using DIG non-radioactive labelling and detection kits (see 2.2.5.2).

Figure 36 shows representative gel images of GapDH PCR. All samples assessed were PCR positive for GapDH.

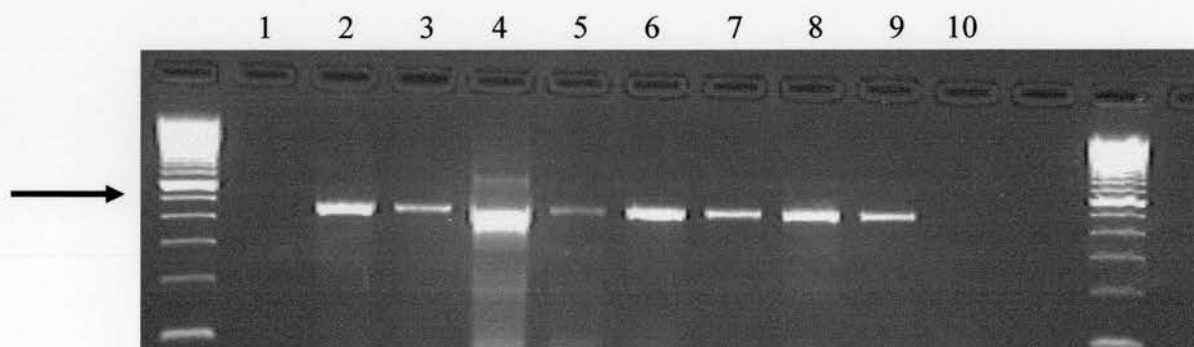
Figure 37a shows representative probed gel blots for samples assessed for IL-2 expression, figure 37b for IL-4, figure 37c for IL-6, figure 37d for IL-10, and figure 37e for INF γ . In each PCR reaction non-specific bands (bands of incorrect size) appeared on the gel photographs, but these disappeared when the gel blots were probed in all cases except INF γ . The results are summarised in table 33.

Table 33: Cytokine gene mRNA expression in brain tissue

Case type	Case Number / Area of brain	IL-2	IL-4	IL-6	IL-10	INF γ
AIDS PCNSL	40 Frontal	-	+	+	+	-
	41 Cerebellum	-	-	+	+	+
	42 Cerebellum	-	-	+	+	+
	42 Basal ganglia	-	-	+	+	+
AIDS Infiltrate	26 Frontal	-	+	+	-	-
	26 Cerebellum	-	-	-	-	+
AIDS no CNS pathology	20 Parietal	-	-	+	-	-
	21 Parietal	-	-	+	+	-
normal	52 Frontal	-	-	-	+	-
	53 Frontal	-	-	-	-	-

Figure 36:

Photomicrograph of GapDH PCR products



Number	Case number / area of brain	Case type
1	No sample	
2	50 Frontal	Normal
3	41 Cerebellum	AIDS PCNSL
4	51 Frontal	Normal
5	42 Basal ganglia	AIDS PCNSL
6	42 Cerebellum	AIDS PCNSL
7	26 Frontal	AIDS Infiltrate
8	26 Cerebellum	AIDS Infiltrate
9	40 Frontal	AIDS PCNSL
10	21 Parietal	AIDS no CNS pathology

Figure 37a legend:

Sample number	Group	Case Number	Area of brain
1	AIDS PCNSL	40	Frontal
2		41	Cerebellum
3		42	Cerebellum
4		42	Basal ganglia
5	AIDS Infiltrate	26	Frontal
6		26	Cerebellum
7	AIDS no CNS pathology	20	Parietal
8		21	Parietal
9	Normal	52	Frontal
10		53	Frontal
11	Control	PBMC	
12		PBMC	
13		PBMC	
14		H ₂ O	

Data from these experiments are summarised in table 33.

Figure 37b legend:

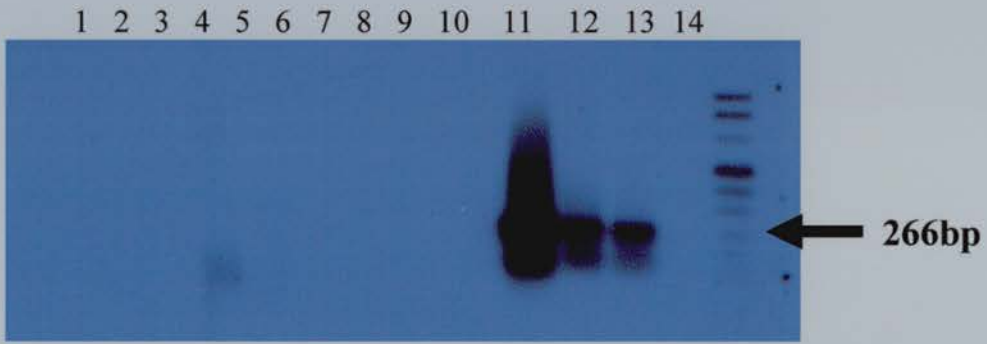
Sample number	Group	Case Number	Area of brain
1	AIDS Infiltrate	26	Cerebellum
2		26	Frontal
3	AIDS PCNSL	40	Frontal
4		41	Cerebellum
5		42	Cerebellum
6		42	Basal ganglia
7	AIDS no CNS pathology	20	Parietal
8		21	Parietal
9	Control	PBMC	
10		PBMC	
11		H ₂ O	

Data from these experiments are summarised in table 33.

Figure 37:

**mRNA cytokine gene expression
(Probed Southern blots)**

37a: IL-2



37b: IL-4

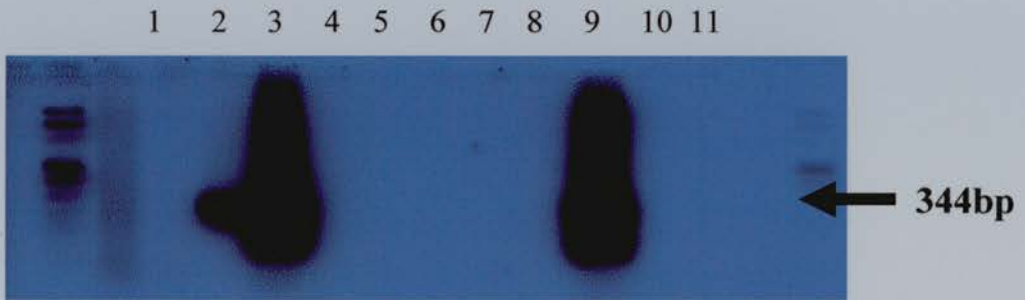


Figure 37c legend: (see table 33 for summary of data from these experiments.)

Sample number	Group	Case Number	Area of brain
1	AIDS PCNSL	40	Frontal
2		41	Cerebellum
3		42	Cerebellum
4		42	Basal ganglia
5	AIDS Infiltrate	26	Frontal
6		26	Cerebellum
7	AIDS no CNS pathology	20	Parietal
8		21	Parietal
9	Normal	52	Frontal
10		53	Frontal
11	Control	PBMC	
12		PBMC	
13		PBMC	
14		H ₂ O	

Figure 37d legend: (see table 33 for summary of data from these experiments.)

Sample number	Group	Case Number	Area of brain
1	Control	H ₂ O	
2	Normal	53	Frontal
3	AIDS PCNSL	40	Frontal
4	AIDS PCNSL	41	Cerebellum
5	AIDS PCNSL	42	Cerebellum
6	AIDS PCNSL	42	Basal ganglia
7	AIDS Infiltrate	26	Frontal
8	AIDS Infiltrate	26	Cerebellum
9	AIDS no CNS pathology	20	Parietal
10	Control	PBMC	
11	Control	PBMC	
12	Control	PBMC	
13	AIDS no CNS pathology	21	Parietal
14	Normal	52	Frontal

Figure 37e legend: (see table 33 for summary of data from these experiments.)

Sample number	Group	Case Number	Area of brain
1	AIDS no CNS pathology	20	Parietal
2		21	Parietal
3	AIDS Infiltrate	26	Cerebellum
4		26	Frontal
5	AIDS PCNSL	40	Frontal
6		41	Cerebellum
7		42	Cerebellum
8		42	Basal ganglia
9	Normal	53	Frontal
10		52	Frontal
11	Control	PBMC	
12		PBMC	
13		PBMC	
14		H ₂ O	

**Figure 37 continued: mRNA cytokine gene expression
(Probed Southern blots)**

37c: IL-6

1 2 3 4 5 6 7 8 9 10 11 12 13 14



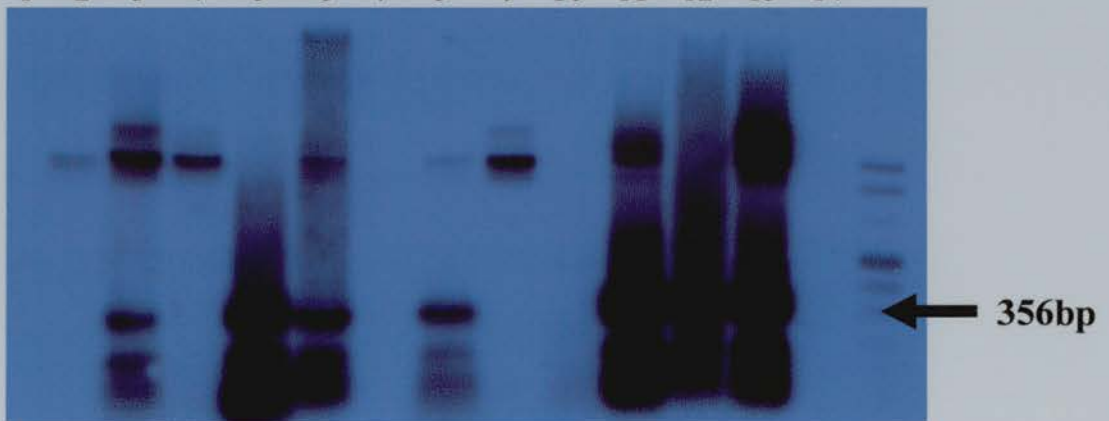
37d: IL-10

1 2 3 4 5 6 7 8 9 10 11 12 13 14



37e: INF-gamma

1 2 3 4 5 6 7 8 9 10 11 12 13 14



In the normal brain tissue one case showed expression of IL-10, while the other had no detectable expression of any of the cytokines assessed here. Both AIDS cases with no CNS pathology showed detectable expression of IL-6, while one also expressed IL-10. The AIDS infiltrate case showed expression of IL-4 and IL-6 in the frontal lobe, while only INF γ was expressed in the cerebellum. All of the AIDS PCNSL samples were from areas of the brain containing tumour tissue, and all samples showed detectable expression of both IL-6 and IL-10. In addition 3 of the 4 samples had detectable expression of INF γ , while IL-4 was expressed in only 1 of 4 samples. No IL-2 expression was detected in any PCNSL samples tested or in any of the other samples assessed from other groups.

The number of cases studied is too low to make any conclusive assessment of cytokine expression pattern in the groups studied. However it is interesting that 7 out of 8 samples from AIDS brains had detectable expression of IL-6 and that in the PCNSL cases all cases showed expression of both IL-6 and IL-10, with INF γ expressed in 3 of 4 samples. Although cytokine expression was detected in all but one brain sample the technique used was not quantitative and therefore no estimation could be made regarding the level of expression of each cytokine in individual brains or which cell types were producing the cytokines.

3.7 Study of the competence of the blood brain barrier in normal and AIDS brains

Damage to the BBB can allow entry to the brain of blood borne cells and compounds which are normally excluded or have restricted access. In order to assess the role of any BBB damage in the accumulation of B lymphocytes in the brain, FFPE sections were stained with an anti-fibrinogen antibody. Fibrinogen, a normal plasma component, is normally kept within the blood vessels of the brain by the BMVEC which constitute part of the BBB. Disruption of the BMVECs can allow fibrinogen to leak from the vasculature into the surrounding parenchymal tissue, which can be detected immunohistochemically indicating BBB damage.

A key difference between brain endothelial cells and non-brain endothelial cells is the expression of tight junction proteins, such as ZO-1, at the inter-endothelial junction between adjacent brain endothelial cells. Loss of expression of these proteins can also compromise the BBB aiding access for compounds and cells normally excluded from the brain tissue. In order to evaluate the level of tight junction protein expression an anti-ZO-1 antibody was used to stain FFPE sections.

3.7.1 Plasma fibrinogen leakage

Nine sections from 3 different AIDS PCNSL cases (7 with lymphoma present, 2 without), 7 sections from 5 pre-AIDS, and 7 sections from 4 normal cases were assessed for fibrinogen leakage into the brain tissue.

3.7.1.1 Optimisation of anti-fibrinogen antibody

The fibrinogen antibody was tested at the concentrations given in table 34 for each antigen retrieval pre-treatment method given. Sections from a brain with several recent cerebral infarcts, and therefore disruption of the BBB, were used for a positive control.

Table 34: Optimisation of fibrinogen antibody staining

Pre-treatment assessed	Antibody concentration tested for each pre-treatment	Result
No pre-treatment Citric acid microwave EDTA microwave	1/250	Strong background staining
	1/500	Strong background staining
	1/5000	Strong staining, strong background staining
	1/7500	Strong staining, slight background staining
	1/10000	Weak staining, no background staining
	1/15000	No staining

The results achieved using the above optimisation procedure were unsatisfactory. There was little or no difference between the different pre-treatment methods used. Antibody concentrations of 1/250 and 1/500 produced very strong background staining that masked any true staining present in the section. Concentrations of 1/5000, 1/7500 and 1/10000 gave strong staining of fibrinogen but still produced unacceptable levels of background staining. A concentration of 1/15000 had little background staining but fibrinogen staining was also very weak.

Using alkaline phosphatase labelled ABC (avidin biotin complex) rather than the standard peroxidase labelled ABC generally gives weaker staining and consequently less background staining. Therefore the optimisation procedure was repeated using alkaline phosphatase-ABC for all three pre-treatments at concentrations of 1/5000, 1/7500 and 1/10000, using Vector Black as the substrate. A concentration of 1/7500 using a citric acid pre-treatment produced the best fibrinogen staining with minimal background staining (see figure 38a). The full procedure used is given in section 2.2.1.

3.7.1.2 Results of fibrinogen staining

Sixty one sections from 7 groups of subjects were stained for fibrinogen and then assessed for evidence of fibrinogen leakage by scanning the entire section at x10 magnification with a light microscope. Table 35 gives a summary of the results of sections stained for fibrinogen, the individual data for each section stained is given in table D1 appendix D.

None of the 7 normal sections or 7 pre-AIDS sections tested showed any evidence of fibrinogen leakage into the parenchymal brain tissue. Two of the 8 sections from non-HIV drug users had small areas of weak staining in the parenchyma. Eleven sections from the AIDS HIVE group were tested, 7 of these had strong staining in the parenchyma, 2 had weak staining and two no staining. The areas of staining in this group were focal and were not linked to any obvious pathology. Fifteen sections from the AIDS infiltrate group were assessed. Five of these had strong parenchymal staining, 2 had weak

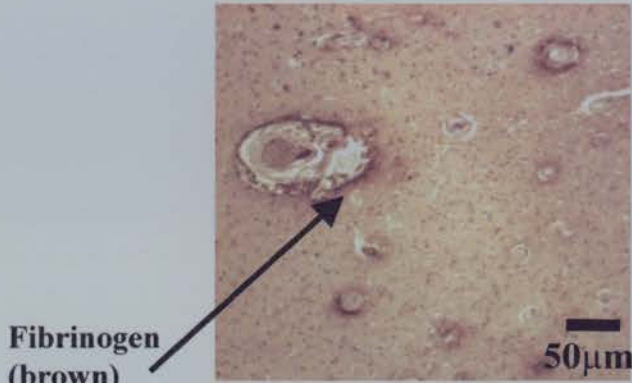
Table 35: Summary of fibrinogen staining

Group (Total number of sections stained)	Number of sections with strong parenchymal staining	Number of sections with weak parenchymal staining	Number of sections with no parenchymal staining
Normal (7)	0	0	7
Non-HIV drug users (8)	0	2	6
Pre-AIDS (7)	0	0	7
AIDS HIVE (11)	7	2	2
AIDS Infiltrate (15)	5	2	8
AIDS PCNSL (9)	7	0	2
Non-HIV viral encephalitis (4)	1	2	1

staining and 8 no staining. In some sections the staining was localised to the regions containing lymphocytic infiltrates, however other areas of lymphocytic infiltration showed no staining and staining was found in areas with no obvious pathology.

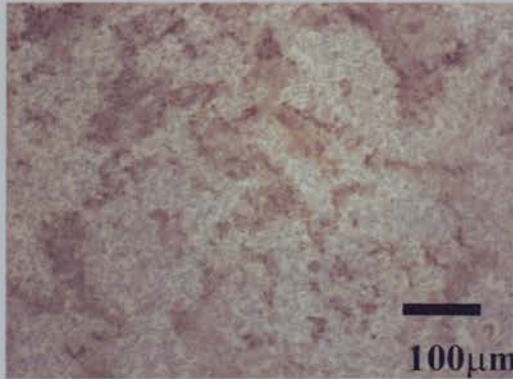
Of the 9 AIDS PCNSL sections, 2 had no lymphoma present and neither of these showed evidence of fibrinogen leakage. The 7 AIDS PCNSL sections all showed varying degrees of fibrinogen leakage into the tissue. Figure 38b shows a photograph of an AIDS PCNSL sections with fibrinogen leakage. In most of the fibrinogen positive AIDS PCNSL sections the fibrinogen leakage was associated with the presence of lymphoma. Staining of these sections for reticulin (Gordons and Sweet method) showed separation and disruption of the reticulin fibres of the blood vessels where lymphoma cells had infiltrated into the perivascular space. Thus the damage to the blood vessels and hence leakage of fibrinogen into the brain parenchyma may potentially be attributable to the expanding tumour (see figure 38b). Despite some fibrinogen leakage correlating with the presence of

Figure 38: Fibrinogen staining on control section and AIDS lymphoma sections

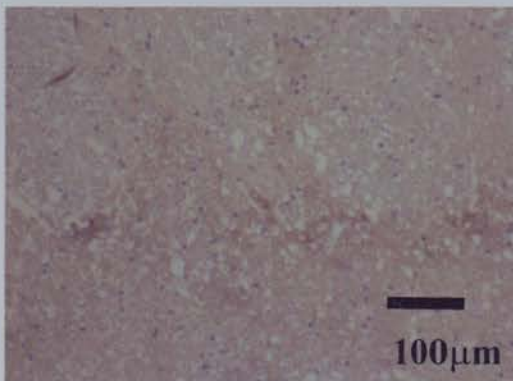


Fibrinogen (brown) leaking from blood vessel into brain parenchyma

38a: Fibrinogen
positive control brain
(x20 magnification)



38b: AIDS PCNSL
(area of brain with lymphoma)
(x10 magnification)



38c: AIDS PCNSL
(area of brain with no lymphoma)
(x20 magnification)

Data from these experiments is summarised in table 35.

lymphoma, many areas of lymphoma showed no evidence of leakage, even in areas where the lymphoma had infiltrated the perivascular space; while occasionally, in lymphoma cases, leakage could be found in areas with no lymphoma cells present (see figure 38c). Four sections from 1 non-HIV case with viral encephalitis were assessed and showed strong parenchymal staining in 1 section, weak staining in 2, and no staining in 1 section. Some of the areas of staining were localised to regions of the brain containing large numbers of lymphocytes, while other positive areas coincided with relatively few lymphocytes.

Overall there was no obvious pattern to the leakage of fibrinogen found in the brains assessed. In some cases the staining for fibrinogen localised to areas of brain pathology, while other areas of staining were found in regions of the brain with no obvious pathology. In addition there were areas of pathology, particularly lymphoma, which showed no evidence of fibrinogen leakage.

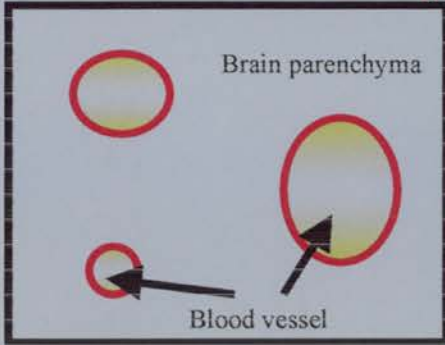
A potential complicating factor in assessing fibrinogen leakage by this method is that only one time point is assessed. Both the pathological processes leading to fibrinogen leakage from the blood vessels and the process of fibrinogen leakage and eventual fibrin clot production are dynamic, and therefore only assessing one time point in the process is a limitation of the technique which may bias the observed results.

3.7.2 Assessment of BBB tight junction protein expression in normal and AIDS brains

Sections were stained with anti-ZO-1 antibody to label tight junction proteins. Consecutive sections were then stained with anti-Von Willebrand antibody to detect endothelial cells. The total level of staining with ZO-1 and Von Willebrand sections was then assessed using a computer-linked microscope. A ratio of total ZO-1 staining (tight junction) to total Von Willebrand (endothelial cell) staining was calculated for each area of the brain. This allowed comparison of tight junction protein expression in several areas of different brains taking into account any potential disparity in the number of blood vessels and hence endothelial cells in each area of the brain and between different brains. Figure 39 gives a schematic example.

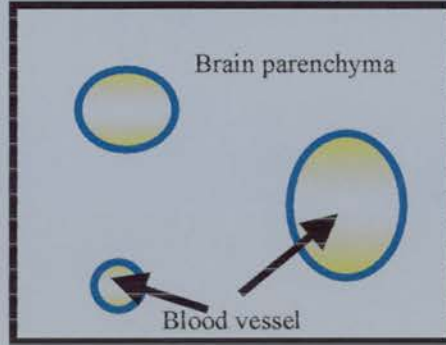
Figure 39: Schematic diagram of assessment of tight junction protein expression

Von Willebrand's staining



Total area stained = 90

ZO-1 staining



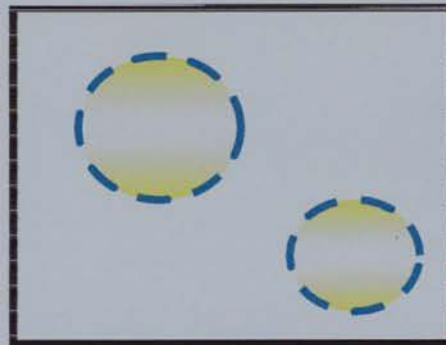
Total area stained = 90

Section 1

Therefore ratio tight junction to endothelial cell = 1



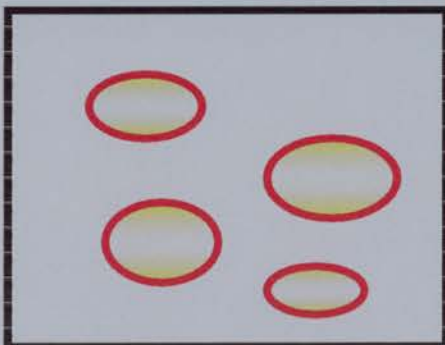
Total area stained = 80



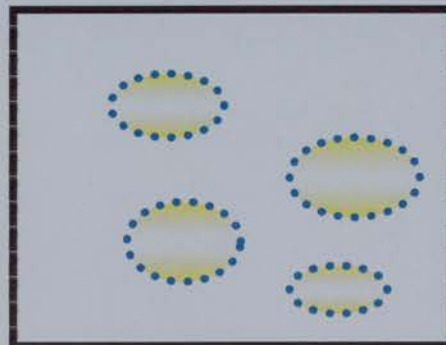
Total area stained = 40

Section 2

Therefore ratio tight junction to endothelial cell = 0.5



Total area stained = 100



Total area stained = 25

Section 3

Therefore ratio tight junction to endothelial cell = 0.25

- Endothelial cells (Von Williebrand)
- Tight junction protein (ZO-1)

3.7.2.1 Optimisation of ZO-1 antibody

The primary antibody concentrations given in table 36 were tested for each of the pre-treatments given using standard ABC immunohistochemistry methods. Sections from normal brain were used as positive controls with intact tight junction proteins, while sections from a multiple sclerosis (MS) brain with recently active plaques were used to demonstrate variable tight junction protein expression.

Table 36: Conditions used for optimisation of anti-ZO-1 antibody

Pre-treatments assessed	Antibody concentration tested for each pre-treatment
No pre-treatment	1/15
Citric acid microwave	1/20
Citric acid pressure cook	1/50
EDTA microwave	1/100
EDTA pressure cook	1/100
Protease digestion	1/200

No staining was seen on either normal or MS brains with any of the pre-treatments and concentrations assessed, with the exception of protease digested sections with an antibody concentration of 1/15 and 1/20, where some very weak staining was seen in patches on the normal brain sections. In order to improve the intensity of staining, the ABC technique was replaced by tyramide signal amplification (TSA) (see 2.2.1). TSA staining generally gives enhanced staining and requires approximately 100 fold less primary antibody. Sections were digested with protease and then stained using TSA with a primary antibody concentration of 1/20, 1/200, 1/2000. 1/20 and 1/200 dilutions gave a slight increase in staining intensity with no background staining. To further increase staining intensity, the incubation period of the primary antibody was increased from 30 minutes at room temperature to

overnight at 4°C. This produced good staining in the majority of blood vessels in normal sections (see figure 40), with only slight background staining. In MS control sections, the majority of vessels stained strongly throughout the section. In areas with active MS plaques, staining was absent or fragmented.

3.7.2.2 Results of staining with anti- ZO-1 antibody

Sections from the cases listed in table 37 below were assessed for ZO-1 protein expression.

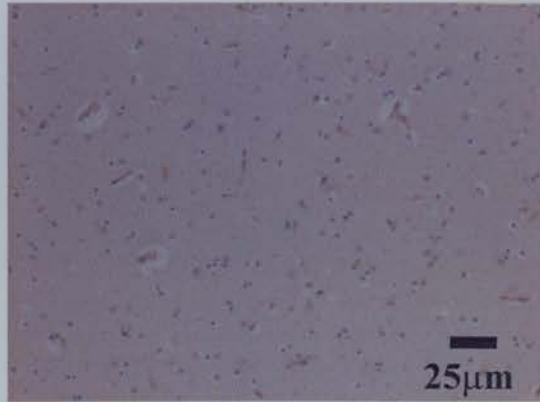
Table 37: Cases assessed for ZO-1 protein expression

Case number / case type	Number of sections stained
Normal cases	
2	6
3	5
5	7
6	5
Pre-AIDS cases	
15	6
16	8
46	4
AIDS PCNSL cases	
38	7
39	8
41	7

The sections were stained in 2 batches. On comparison of the positive controls from each batch it became apparent there was a considerable difference in staining intensity between the positive control sections in each batch (see figure 41a and 41b). The consequence of differing staining intensity between batches was that sections in different batches could not be compared with each other. In order to remedy this, all sections were re-

Figure 40:

ZO-1 immunohistochemical staining of a normal (control) brain

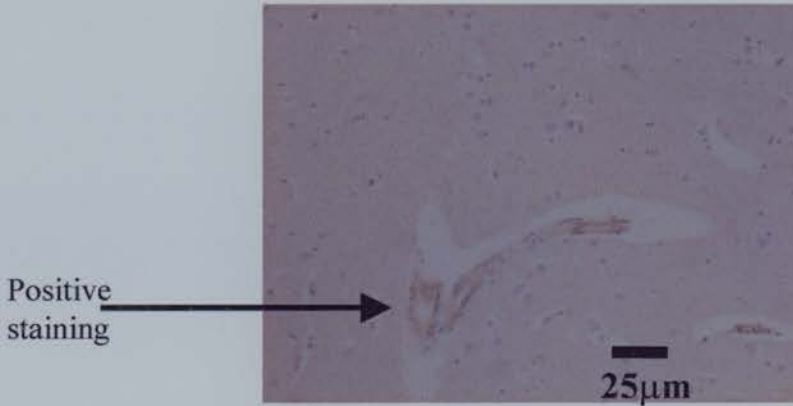


Endothelial cells of a normal brain stained using anti-ZO-1 antibody (brown)

(x20 magnification)

Figure 41:

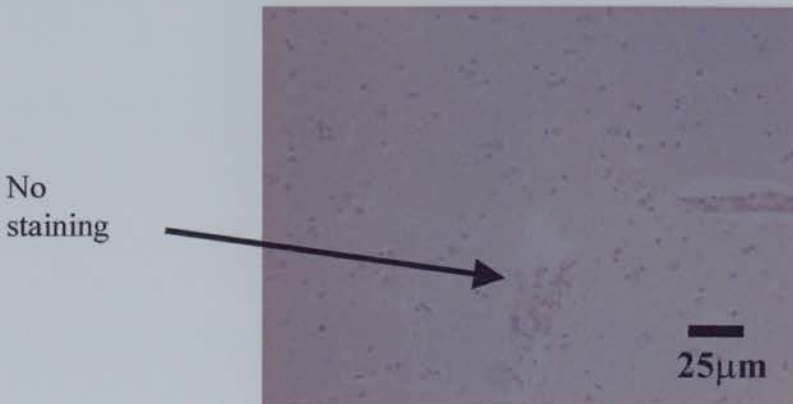
ZO-1 immunohistochemical staining of consecutive sections from the same AIDS brain



A:

ZO-1 staining on AIDS brain – Run number 1

(x20 magnification)



B:

ZO-1 staining on AIDS brain – Run number 2

(x20 magnification)

stained with particular care taken to ensure the staining procedure was identical for each batch. The third and fourth batches produced staining intensity which differed from both each other and from the original 2 batches. In order to be sure that the differences were not due to human error, both batches were re-stained for a third time. The first batch of this third run produced results similar to the first original run, however the second batch was not comparable with this. The results were assessed by two independent observers, both of whom reached the same conclusions.

The inconsistency in staining with this antibody meant that a meaningful comparisons of sections from different cases was not possible and therefore the technique was abandoned. While the reason for the staining variation could not be ascertained, there are two plausible explanations. Firstly, the antibody is extremely temperature sensitive and its function rapidly decreases if it is not stored below -20°C , therefore variations in room temperature and fridge temperature during the staining procedure may affect the antibody's functional activity. Secondly, the antibody only binds to sections which have been protease digested prior to staining. As protease activity is sensitive to both pH and temperature changes, minor changes in either of these may potentially affect the staining intensity.

In addition to tight junction staining of the BBB several sections, particularly in AIDS cases, also had strong nuclear staining in several large neurones. Gottardi *et al* (1996) have demonstrated ZO-1 staining in the nuclei of cells which are re-modelling their cytoskeleton in response to injury, so nuclear expression of this protein is possible. ZO-1 expression has also been noted in cells which never form tight junctions (Howarth, Hughes & Stevenson 1992) suggesting it may have secondary functions, however there are as yet no published descriptions of its expression in the nuclei of neurones.

3.8 Use of statistics analysis

The use of statistics in this thesis is limited to student T-tests comparing the means of different populations of lymphocytes. While the use of this test is valid it should be noted that in all cases the power of the test was extremely low and in most cases the confidence interval was also low.

Undoubtedly the reason for this is the relatively low number of cases in each of the study groups. In many instances there were obvious subjective differences between groups which because of the low case numbers did not reach statistical significance. In most pathological studies the number of samples and cases from one study are insufficient for statistical tests to be applied which will have enough power to provide meaningful results. However studies of this type are still of importance as several studies can be combined to produce statistically valid results, and in many cases these types of studies provide information on disease process within the body which can not be obtained from animal models or in vitro experiments.

4 Discussion

4.1 Prevalence of B lymphocytes in normal brain tissue

In recent years the concept of the brain existing as an entirely immunologically privileged organ has been challenged. The identification of T lymphocytes able to enter and patrol the CNS independent of antigen (Hickey *et al* 1991), the recognition that antigen presentation functions can be induced in several resident brain cell types including astrocytes, microglia and endothelial cells (Male, Pryce & Hughes 1987, Fabry, Raine & Hart 1994), and the realisation that CNS antigen can drain to cervical lymphatics (Cserr & Knopf 1992, Aloisi 2000) has led to this shift in attitude towards CNS immunology. As yet however the presence of B lymphocytes within the brain has only been studied in disease states and no studies have examined the prevalence in normal human brains. The deficit of studies in this area appears to stem from the failure of B lymphocytes to migrate across *in vitro* models of the BBB (Male *et al* 1992), which has led to a general assumption that they therefore do not cross the BBB *in vivo* in healthy brains (Williams & Hickey 1995, De Angelis 1999, Hickey 2001). However, while these models are undoubtedly of use in determining interactions between endothelial cells and lymphocytes, they can never entirely replicate the *in vivo* functioning of the BBB and therefore assumptions based on these models may be flawed.

Our results show for the first time that B lymphocytes do enter the parenchyma of normal brain albeit in extremely low numbers. We examined multiple areas of 7 normal brains and were able to find B lymphocytes in the parenchyma of 6 of the 7 brains, with an average of 0.112 cells/cm² (or 1 cell per 8.9cm² of brain). This finding indicates the potential for humoral as well as cellular immune responses within the CNS and further questions the concept of the brain as an immunologically privileged site (Anthony, Crawford & Bell, submitted). Although it is not possible from immunopathological studies alone to assess the trafficking pathways of lymphocytes to and from the CNS, B lymphocytes appear to move directly

into the parenchyma since, unlike T lymphocytes, they are not generally present in the perivascular space in the normal brain. Since no obvious stimulus for immune activation was detected in the normal brains studied, the presence of B lymphocytes in brain parenchyma presumably represents normal trafficking, although at a lower level than for T lymphocytes. A comparison of the prevalence of T and B lymphocytes within the brain was made, with T lymphocytes estimated to be approximately 50 times more common than B lymphocytes. However the functional role of B lymphocytes in the CNS under normal conditions remains unclear, and it is not known whether they leave the CNS via the blood circulation, drain via the CSF into the cervical lymph nodes or whether they are destined to die there. While the present data cannot provide an explanation for the presence of these rare B lymphocytes in the brain it is tempting to speculate on their function. In addition to the low numbers of randomly distributed B lymphocytes found in the brain, T lymphocytes can also be found in greater numbers in the normal brain as well as microglia which are distributed throughout the brain. Although all three cell types have been shown to be capable of participating in immune reactions within the brain, the relatively low numbers of cells and lack of focal accumulations suggests that these cells are not present in the brain responding to an ongoing acute or chronic inflammatory stimulus. A plausible explanation may be that perhaps many antigen/pathogens commonly encountered by humans are occasionally capable of breaching the BBB. If this were the case then the re-infection with many pathogens in the periphery may result in re-activation and homing of memory B lymphocytes to the CNS as well as other peripheral organs in antigen surveillance function.

We have also considered the possibility that the number of B lymphocytes within the brain may simply be a reflection of the number of circulating B lymphocytes in the blood. However, in the circulating blood of a healthy donor there are approximately 0.3×10^9 B lymphocytes and $1-2.5 \times 10^9$ T lymphocytes, giving an approximate 8 fold greater number of T than B lymphocytes. Our survey of the brain revealed a 50 fold higher incidence of T than B lymphocytes, suggesting that the number of lymphocytes within the brain is not simply a reflection of the number in the blood. Furthermore it suggests either that T lymphocytes are preferentially recruited into the normal

brain or that B lymphocytes are restricted in entry in comparison to T lymphocytes.

Our data further indicate that, as for T lymphocytes, only B lymphocytes that display an activated phenotype, defined by expression of CD23, are able to cross the BBB and enter the CNS. If activation is a prerequisite for B lymphocyte entry into the CNS, then facilitation by T lymphocytes would generally be required as an initial step in the process as CD4 T lymphocytes are required to activate most B lymphocytes.

The data produced in this study adds to a growing body of evidence which suggests that the status of the brain as an immunologically privileged site should perhaps be revised. While it is apparent that immune reactions are more restricted in the CNS than at most other peripheral sites, immune reactions can and do still occur within the CNS.

4.2 Prevalence of B lymphocytes in brains of HIV infected individuals

The dominant characteristic of HIV is a decrease in the number of CD4 T lymphocytes in the body (reviewed in McCune 2001). However immunodysregulation seen in HIV infection affects other cell lineages including B lymphocytes. B lymphocyte abnormalities includes a chronic, T lymphocyte independent, B lymphocyte activation (Lane *et al* 1983) and resultant hypergammaglobulinemia (Nath *et al* 1987). We therefore sought to establish whether HIV infection might alter the normal migration patterns of B lymphocytes within the CNS, leading to increased accumulation.

Our results show that in pre-symptomatic HIV patients there is an increase in the number of both parenchymal and perivascular B lymphocytes within the brain. An average of 0.417 CD20 cells/cm² (or 1 cell per 2.4cm² of brain tissue) was found in the pre-symptomatic HIV brains compared to 0.112 cells/cm² (or 1 cell per 8.9cm² of brain tissue) in normal brain parenchyma, although the difference was not statistically significant. In the perivascular space a significant increase was noted, with 0.452 cells/cm² in the pre-

symptomatic cases compared to no cells in the normal cases. This finding of increased number of B lymphocytes within the brain of pre-symptomatic patients is in line with the increased numbers of T lymphocytes found in pre-symptomatic brains of HIV infected individuals (Tomlinson *et al* 1999).

Brains from the majority of AIDS subjects, including those with PCNSL (outwith the site of malignancy) contained fewer B lymphocytes than normal brains. The exception was the group of AIDS brains with pleomorphic infiltrates which will be discussed separately. The AIDS brains with no CNS pathology were almost devoid of B lymphocytes, with CD20 parenchymal and perivascular cells each found in only 1 of 5 cases. There was an average of 0.008 cells/cm² in the parenchyma (or 1 cell per 125cm² of brain), which represented a significant decrease in comparison to controls 0.112 cells/cm² (p=0.009) and a non-significant decrease in comparison to pre-symptomatic HIV brains 0.417cells/cm². Common sense may predict that if the number of cells found in the pre-AIDS group is greater than that found in the normal group, and the normal group is statistically significantly different from the AIDS group then the pre-AIDS group should also be significantly different. However, the results from the normal group were very homogeneous in comparison to the more widely distributed results from the individual cases in the pre-AIDS group, hence combined with the low number of cases per group significance was not reached.

In the perivascular spaces there was no significant difference between the number of cells found in AIDS brains with no CNS pathology (average of 0.0046 cells/cm²) compared to normal controls, however there was a decrease in comparison to pre-symptomatic HIV brains (0.452 cells/cm²) (p=0.051). Similar results were found in the PCNSL brains, outwith the area of lymphoma. An average of 0.003 cells/cm² was found in the parenchyma and 0.015 cells/cm² in the perivascular space, neither of which differed significantly from the AIDS group which lacked CNS pathology. These results demonstrate that after an initial increase in both the parenchymal and perivascular brain B lymphocyte populations in pre-symptomatic HIV infected individuals compared to normal controls, there is a subsequent decrease in both populations at the onset of AIDS. Furthermore, as PCNSL is a late manifestation in AIDS, these results show that PCNSL lymphoma does not

develop in a background of increased B lymphocyte numbers in the brain. Therefore a generalised increase in B lymphocyte trafficking into the brain during HIV infection is not a predisposing factor for development of PCNSL.

The increase in number of lymphocytes found within the brain in pre-symptomatic HIV infected individuals may represent an increased surveillance of the brain and/or increased immune reactivity within the brain during the early stages of infection. Some of these lymphocytes are likely to be HIV specific as HIV is thought to enter the brain early in the infection process. Although this demonstrates the potential for immune reactivity within the CNS, the level of reactivity is likely to be lower than that seen in other organs due to the presence of the BBB and production of anti-inflammatory cytokines within the brain. The subsequent decrease in lymphocytes, below the level of the normal pathogen free brain, seen in most AIDS brains may therefore represent a decrease in the immune surveillance of the CNS, and hence decreased potential for immune reactivity within the CNS. Before the advent of effective treatment (HAART) this may have predisposed the CNS to opportunistic infections such as CMV infection. However with effective treatment CMV and other opportunistic pathogens are less common (Baril *et al* 2000). Conversely HAART has been shown to have no effect on the level of EBV DNA in HIV infected individuals (Stevens *et al* 2002), however its use does produce an increase in immune function which may be important in the control of EBV infection and prevention of development of EBV driven tumours. EBV driven lymphoproliferations are however still a major cause of mortality in HIV infected individuals.

4.3 Effects of encephalitis on the brain B lymphocyte population

Having shown that the brain B lymphocyte population increases in pre-AIDS and then decreases below normal levels as patients progress towards AIDS, we sought to determine the effects of productive HIV infection in the

CNS on the B lymphocyte population. We postulated that a B cell response would be present in the CNS of HIVE AIDS patients for three reasons:

- 1- Unlike T lymphocyte responses, B lymphocyte responses are preserved until the very late stages of AIDS. In addition B lymphocytes from HIV patients have been shown to produce high levels of anti-HIV antibodies (Amadori *et al* 1988)
- 2- B lymphocytes have been shown to enter the CNS in response to antigen, and can be found in large numbers in viral encephalitis in the non-immunocompromised patient and in animal models of encephalitis (Hatalski *et al* 1998a and 1998b, Tyor, Moench & Griffin 1989)
- 3- HIV antigens, which B lymphocytes are capable of recognising, can be found in the parenchyma of HIVE brains (Budha *et al* 1987, Chiodi *et al* 1988, Weber *et al* 1989).

In addition to studying cases with HIVE, a control group of 3 cases with viral encephalitis in non-immunocompromised patients was included. A single pre-AIDS case with a non-HIV viral encephalitis was also included. The HIVE cases had an average of 0.075 cells/cm² (or 1 cell per 13.3 cm² of brain tissue) in the parenchyma which although greater than that found in AIDS cases with no pathology (0.008 cells/cm²) was not significantly different (p=0.130). In the perivascular space a larger increase was noted, 0.306 cells/cm² (or 1 cell per 3.3cm²) in HIVE compared to 0.005 cells/cm² in AIDS brains with no CNS pathology (p=0.07). Although neither difference was statistically significant, there were clear differences between the brains particularly in the perivascular spaces and the lack of statistical significance is most likely a reflection of the low numbers of cases studied rather than there being no difference.

Brains from non-immunocompromised cases affected by viral encephalitis showed a large increase in the presence of B lymphocytes in both the parenchyma and perivascular space in comparison to HIVE cases: 6.47 cells/cm² in the parenchyma of the non-immunocompromised brains compared to just 0.075 cells/cm² in the HIVE brains, and 64.32 cells/cm² in the perivascular spaces of non-immunocompromised brains compared to 0.306 cells/cm² in HIVE. No statistical tests were performed on this data as only 3 cases of non-immunocompromised encephalitis were examined. The single

pre-AIDS brain with non-HIV encephalitis gave results which fell between those of HIVE and non-immunocompromised encephalitis, with 1.93 cells/cm² in the parenchyma and 39.109 cells/cm² in the perivascular spaces.

We have demonstrated that a moderate increase in brain B lymphocytes is seen in HIVE compared to other AIDS brains, however this increase is less than that seen in viral encephalitis in non-immunocompromised patients. A possible explanation for the decreased B lymphocyte response seen in HIVE is that CD4 T lymphocytes may be required to aid B lymphocyte entry to the CNS.

Tyor *et al* (1989) have suggested that, in mice, T lymphocytes are required for recruitment of B lymphocyte into the CNS in response to viral infection. Their assumption is based on data showing similar B lymphocyte responses to Sinbis virus encephalitis in BALB/c mice and athymic (nu/nu) mice upto day 7 of the infection. At this point B lymphocytes in the normal mouse represent approximately 10% of the inflammatory infiltrate and are composed mostly of IgM positive cells. The composition of inflammatory infiltrates in athymic mice at this point was comparable: however there was a tenfold decrease in the number of cells present. Beyond this point there is an isotype switch to IgG and IgA producing cells and a further increase in the number of B lymphocytes in the normal mice, all of which fails to occur in athymic mice. At no point in the infection was the percentage of B lymphocytes seen in the inflammatory response in the athymic mice greater than that seen in the normal mice. They suggest that if B lymphocyte entry to the inflammatory response was independent of T lymphocytes present, then B lymphocytes would be expected to be found in larger numbers in the inflammatory reactions in the athymic mice. Therefore T lymphocytes are required to facilitate B lymphocyte entry to the CNS.

However our data demonstrate that B lymphocytes are required to be activated in order to enter the CNS. The majority of B lymphocytes require signals from CD4 T lymphocytes for activation and therefore if this holds true in athymic mice the number of activated B lymphocytes would be lower. Fewer cells would consequently have the potential to enter the CNS, which is reflected in the decreased number of cells seen in the brains of athymic mice.

Therefore, CD4 T lymphocytes are indirectly required for B lymphocyte entry to the CNS through their role in B lymphocytes activation in the periphery.

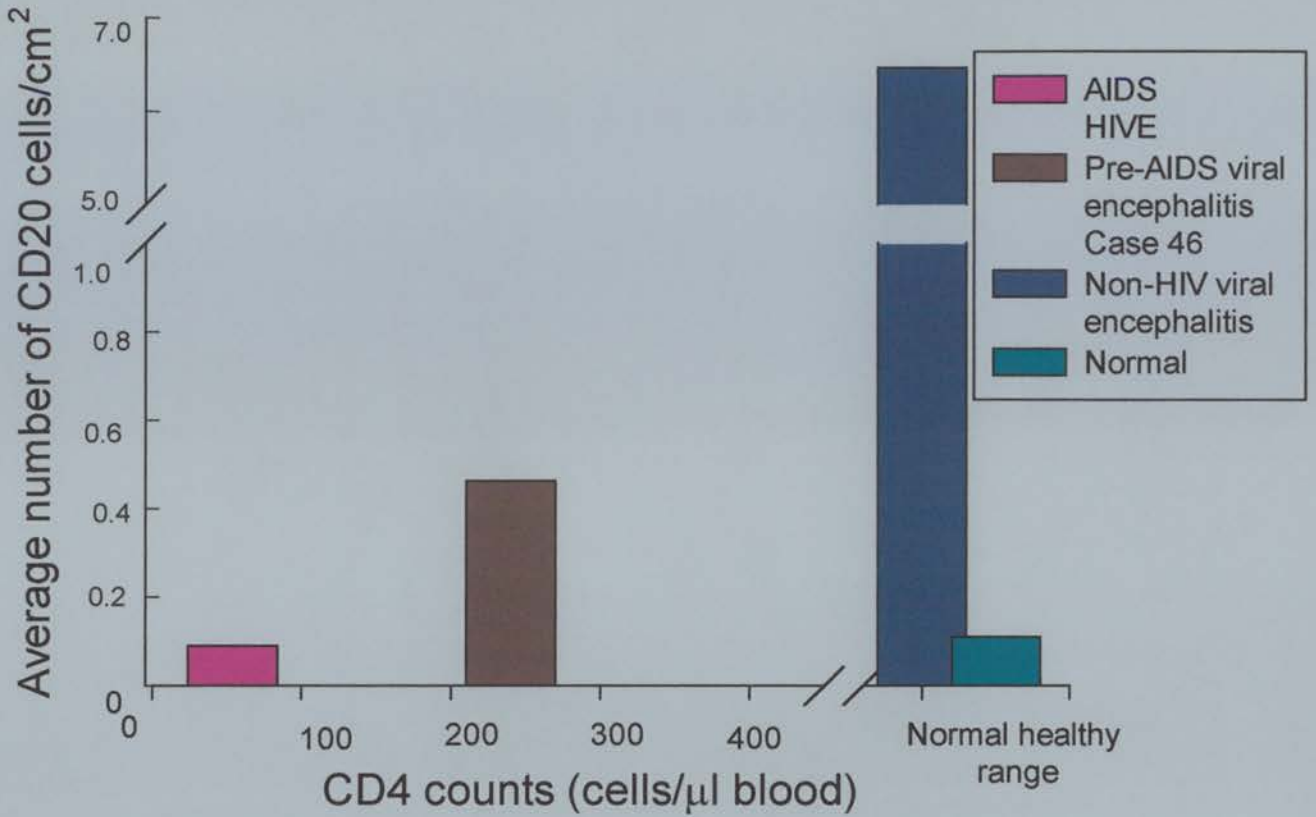
Figure 42 shows that in the HIVE cases studied here there is a general trend for the overall number of B lymphocytes in an HIVE brain (both parenchymal and perivascular B lymphocytes combined) to be higher in cases with higher final peripheral blood CD4 counts. In addition case 46, a pre-AIDS case with non-HIV viral encephalitis, showed greater numbers of B lymphocytes than the HIVE cases with overall numbers approaching the response seen in the non-immunocompromised encephalitis patients (figure 42). This case had a final CD4 count of 240 cells/ μ l, which was far greater than that seen in the AIDS HIVE cases, and adds strength to the argument that CD4 T lymphocytes have a role in B lymphocyte entry to the brain. These data cannot differentiate between the need for CD4 T lymphocytes for activation of B lymphocytes and a potential additional function of CD4 T lymphocytes within the brain recruiting B lymphocytes to the CNS. The situation is further confused in HIV infection as there is often hyper-activation of B lymphocytes in the periphery which is reported to be independent of T lymphocytes (Martinez-Maza *et al* 1987, Yarchoan *et al* 1986).

4.4 AIDS brains with pleomorphic lymphocytic Infiltrates

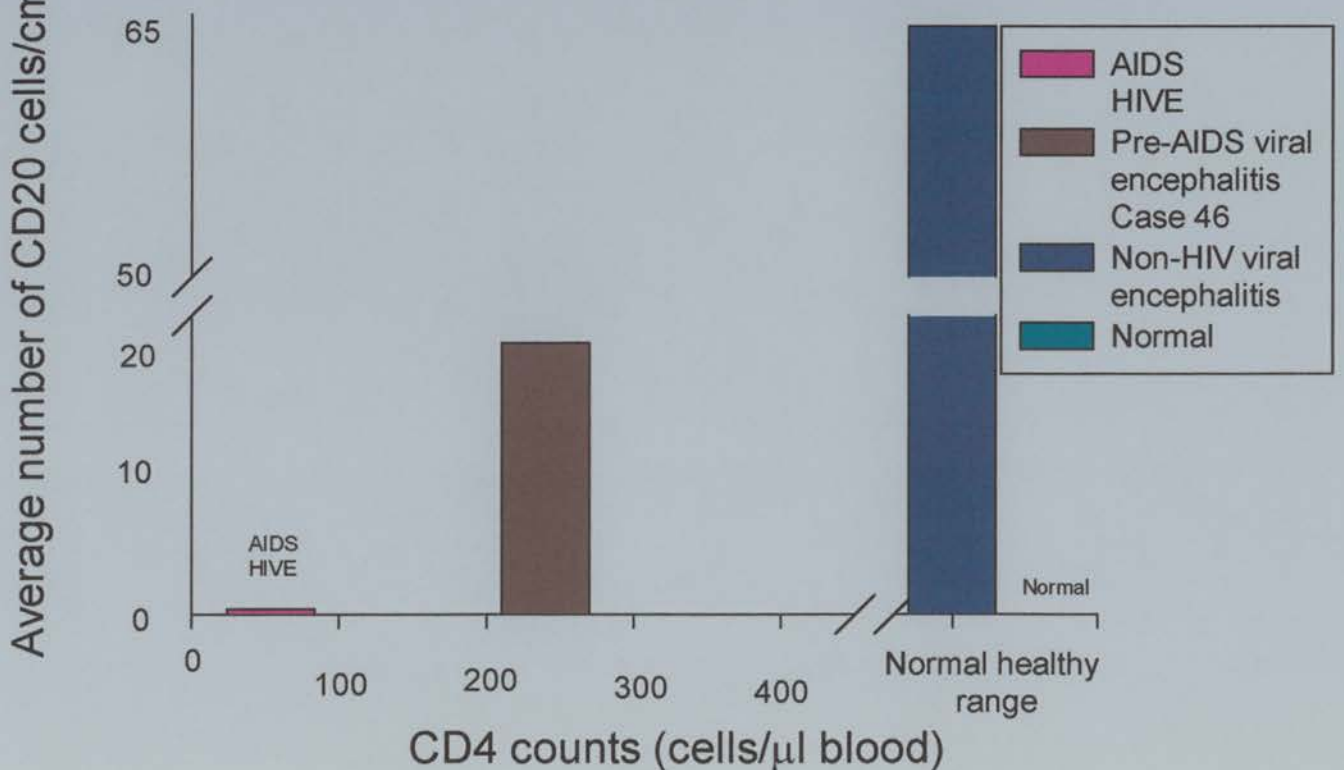
In a subset of AIDS brains, previously identified during routine neuropathological examination as having heavy pleomorphic lymphoid infiltrates in the perivascular spaces, there were greatly increased numbers of B lymphocytes in the brain tissue. The parenchyma of the AIDS infiltrate group contained an average of 0.174 cells/ cm^2 (or 1 cell per 5.7 cm^2 of brain tissue) in comparison to just 0.008 cells/ cm^2 (or 1 cell per 125 cm^2 of brain tissue) in the AIDS group with no CNS pathology. In the perivascular spaces the difference was even greater, with an average of 14.776 cells/ cm^2 in the infiltrate group compared to 0.009 cells/ cm^2 in the AIDS group with no CNS pathology. Neither of these results reached statistical significance for reasons

Figure 42:

CD4 count versus average number of brain parenchymal CD20 cells/group



CD4 count versus average number of brain perivascular CD20 cells/group



discussed in section 3.2.3.2. The infiltrating pleomorphic lymphoid cells did not localise to any specific area of the brain and were found in several regions of each brain. The composition of the infiltrates was mostly CD8 T lymphocytes with B lymphocytes accounting for between approximately 5 and 25% of the total cellular infiltrate. There was no obvious neuropathological stimuli for the accumulation of these cells within the brain.

A possible explanation for the existence of these infiltrates is that they merely represent an extension of the lymphocytic infiltration seen in the pre-AIDS brains. However in the infiltrate group, the number of perivascular lymphocytes is significantly higher than in the pre-AIDS group despite declining peripheral blood lymphocyte counts in the former. While the pre-symptomatic infiltrates consisted of small T lymphocytes (Tomlinson *et al* 1999) the pleomorphic infiltrates contained an admixture of larger lymphocytes, some of plasmacytoid appearance. Further differences were noted between the location of infiltrating cells in the pre-AIDS cases and infiltrate cases. Lymphocytes in the pre-AIDS cases were more frequently located in the meninges or blood vessels close to the surface of the brain, in comparison to the infiltrate cases where lymphocytes were located around deeper, more centrally located blood vessels. Analysis of these pleomorphic lymphoid infiltrates strengthens our impression that they do not represent merely an extension of the lymphoid infiltrate seen in pre-symptomatic patients but constitute a separate pathology.

The finding that the pleomorphic lymphocytic infiltrates contained substantial numbers of B lymphocytes raised the possibility that they represented a pre-malignant PCNSL state.

4.5 BBB studies

Abnormalities in the BBB may be important in mediating or permitting damage to the brain tissue during HIV infection, as well as facilitating viral entry to the CNS (Petito & Cash 1992). Several studies have addressed the issue of BBB disruption in HIV. Measurement of serum and CSF albumin ratios has demonstrated increased BBB permeability in 15% or pre-

symptomatic HIV infected individuals (Andersson *et al* 2001). In AIDS patients at post-mortem, fibrinogen extravasation from serum to brain tissue has been used as a marker of BBB damage/compromise. Fibrinogen extravasation has been shown in both HIVE AIDS brains and non-HIVE AIDS brains; including those with lymphoma and opportunistic infection (Petito & Cash 1992). Disruption of the tight junctions (TJ) of the BBB is a characteristic of several CNS pathologies including multiple sclerosis, stroke, Alzheimer's disease and HIVE (reviewed in Huber, Egleton & Davis 2001).

BBB damage has been shown to be at least partially attributable to the production of β -chemokines by HIV infected mononuclear phagocytic cells (for example microglia) and astrocytes. HIV infected mononuclear phagocytic cells have been shown to down regulate TJ proteins and transport systems on brain microvascular endothelial cells in both autopsy human brain tissue and in mouse models of HIVE (Persidsky *et al* 2000). Sporer *et al* (2000) have suggested that additional BBB damage may be caused by the induction of matrix metalloproteinase (MMP)-9 by HIV nef protein. Intracisternal injection of nef protein into rats led to increased MMP-9 activity in the CSF in addition to breaching of the BBB. The BBB damage could be inhibited by pre-treatment with the MMP inhibitor batimastat. The source of MMP-9 was not clear, with only slight induction *in vitro* in PBMCs and macrophages treated with nef, but no induction in endothelial cells or astrocytes (Sporer *et al* 2000).

Damage to the BBB during HIV infection has been shown to be associated with the accumulation of monocytes in the brain of patients with HIVE (Dallasta *et al* 1999). We therefore sought to determine if damage to the BBB was also associated with the accumulation of B lymphocytes in the brain. We hypothesised that B lymphocytes may be present in increased numbers at sites of BBB damage as these sites may be more readily penetrated than sites with intact BBB. There were 2 parts to the study:

- 1- Assessment of the tight junction protein ZO-1
- 2- Assessment of plasma fibrinogen leakage from blood vessels into the brain tissue.

The protein ZO-1 is an integral part of the tight junctions found within the BBB, and their presence or absence has been used in previous studies to

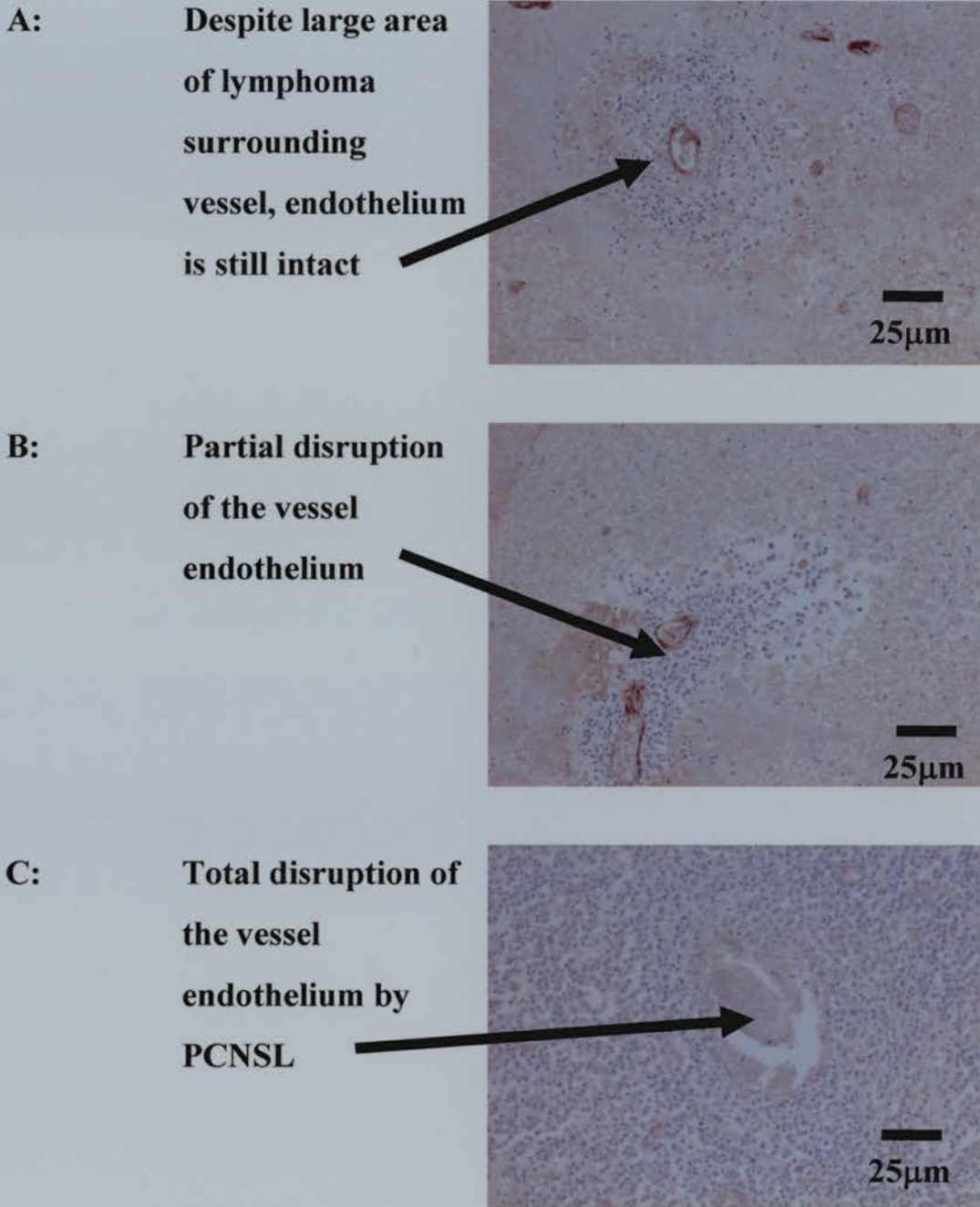
define BBB integrity (Dallasta *et al* 1999, Luabeya *et al* 2000). As discussed in the section 3.7.2, the results obtained using anti-ZO-1 antibodies to visualise the tight junctions of the BBB were inconsistent and therefore no valid data could be reliably extracted using the technique. Dallasta *et al* (1999) using the same technique found that ZO-1 immunostaining was disrupted or absent in HIVE brains and that monocytes were found in increased numbers in these sites. It is not clear however from these studies if monocytes accumulate in these areas and mediate the BBB damage or if they accumulate as a result of the BBB damage.

Plasma fibrinogen is normally contained within blood vessels and is not found within the brain tissue unless there is disruption of the BBB (Petito & Cash 1992). Our data from the study of plasma fibrinogen leakage showed no correlation between the accumulation of B lymphocytes in the brain and damage to the BBB as demonstrated by fibrinogen leakage. Fibrinogen staining of brain tissue was only noted in AIDS PCNSL cases, in which some of the fibrinogen positive areas coincided with the presence of tumour. The study of 6 individual cases of PCNSL revealed differences in the extent of the disruption of endothelial cells lining blood vessels by the tumours. In all tumours the malignant cells were found in concentric rings around the blood vessels, with most tumours showing no disruption of the endothelial cells of the blood vessels (see figure 43a). However in certain areas of several cases (particularly cases 36 and 41) the endothelial cells appeared to have been disrupted or destroyed by the tumour (see figure 43c). Intermediate stages were also observed where the endothelium was partially disrupted/destroyed (see figure 43b). While fibrinogen leakage was observed in all areas with endothelium disruption, it was variably associated with tumours which had not disrupted the endothelium. It is not clear whether the disruption of endothelium seen in some cases is due to tumours being observed at more advanced stage, or if these tumours are displaying more aggressive invasive properties. Interestingly the case with greatest spread of lymphoma throughout the brain (case 42) showed no evidence of disruption of blood vessel endothelium.

The absence of fibrinogen leakage in all brains assessed, with exception of PCNSL, suggests that B lymphocytes are able to penetrate the

Figure 43:

Disruption of endothelial cells by PCNSL



BBB without disruption of the endothelial cells, providing further evidence to suggest that B lymphocyte transmigration into the brain is a normal physiological process.

4.6 Activation status of B lymphocytes within the brain

In accordance with the results discussed earlier showing that B lymphocytes in normal brains all demonstrate an activated phenotype, the vast majority of B lymphocytes found in the parenchyma of HIV brains also showed an activated phenotype. This further strengthens the argument that activation of B lymphocytes is required for entry to the brain. The large numbers of perivascular cells found in AIDS infiltrate cases also demonstrated an activated phenotype. These data suggest that not only is activation required for entry into the parenchyma, but also for penetration of the BBB and subsequent entry to the perivascular spaces.

Eight of nine large areas of lymphoma from 4 cases of PCNSL showed the majority of tumour cells displaying an activated phenotype. There are two possible explanations for this finding. Firstly, if the original cell from which the tumour was derived migrated into the brain from the periphery then the preceding data would suggest that it must be activated to gain entry to the CNS. For this reason all PCNSL cells would be expected to display an activated phenotype. Alternatively, the tumour cells could be activated as part of the tumourogenesis process, particularly those infected with EBV which can activate cells via expression of viral proteins EBNA-2 and LMP-1.

We also compared the number of activated B lymphocytes found within the blood vessels of brains studied. In the normal brains far fewer B lymphocytes were activated within the blood vessels than within the brain tissue (50% intravascular, 80% parenchymal). In the pre-AIDS brains the percentage of activated cells found in the blood vessels increased to 78%. The number of cells studied in individual AIDS groups was too low to yield valid data. This data suggests that an increase occurs in terms of circulating activated B lymphocytes during the early stages of HIV infection, which is in accordance with previously published data (Martinez-Maza *et al* 1987, Lane *et*

al 1983) which demonstrate polyclonal B cell proliferation during early HIV infection. It should be noted however that the results presented here may be strongly influenced by post mortem effects such as drainage of blood from the tissues. Activated B lymphocytes display adhesion molecules (Bonney *et al* 1995) and are therefore more likely to adhere to endothelium and may be less affected by drainage of blood from tissues than non-activated cells. It would be interesting to perform similar studies, perhaps using FACS analysis, on blood samples from these cases to determine the extent of the post mortem effects. Post mortem blood may not be suitable for such a study in which case blood samples taken preceding death may be required.

4.7 Effects of drug use on the B lymphocyte population of the brain

A significant proportion of individuals infected with HIV in Edinburgh, are known drug abusers. Previous work by Tomlinson *et al* (1999) has demonstrated an increase in the number of activated microglial cells in the CNS of both HIV IDU and non-HIV IDU. Gan *et al* (1999) showed an upregulation of adhesion molecules at the BBB with exposure to cocaine. Activation of microglia may lead to expression of chemoattractants within the CNS and in combination with upregulation of adhesion molecules could potentially facilitate increased transmigration of lymphocytes into the CNS. Although Tomlinson *et al* (1999) showed no increase in the number of T lymphocytes present in the brain of drug users, it is possible that the number of B lymphocytes may be increased by these mechanisms and therefore it was important to establish if the abuse of drugs had an influence on the B lymphocyte population of the brain.

Our results demonstrated no significant increase in the number of parenchymal or perivascular B lymphocytes in non-HIV infected drug users in comparison to normal controls. We have demonstrated that the abuse of intravenous drugs has no significant effect on the B lymphocyte population of the CNS when compared to normal non-drug abusers. In addition there is no significant difference between the numbers of parenchymal B lymphocytes in the brains of pre-AIDS drug users as compared to non-HIV IDU. However

there is an increase in the number of B lymphocytes found in the perivascular space in pre-AIDS IDU which is not seen in non-HIV IDU. This increase in perivascular cells is therefore unlikely to be due to the effects of drug use alone, although a synergistic effect between drug use and HIV infection cannot be ruled out. Alternatively the B lymphocytes may be responding to the initial stages of HIV entry to the brain. This may explain the localisation of the B lymphocytes in the perivascular spaces rather than in the parenchyma, as the most likely entry point of HIV to the brain is via blood borne inflammatory cells which migrate across the BBB into these areas (Nottet *et al* 1996). Tomlinson *et al* (1999) have shown that an increase in activated microglia occurs in both non-HIV and HIV infected drug users, which they suggest may predispose drug users to HIV infection of the brain. In accordance with this Bell *et al* (1998b) demonstrated an increased incidence of HIV and generally higher CNS viral load in drug users than homosexuals with HIV.

4.8 EBV in the CNS

During HIV infection the number of circulating EBV latently infected B lymphocytes increases in comparison to healthy controls (Birx, Redfield, Tosato 1986, Stevens *et al* 2002). In addition lytic gene expression, which is not detectable in healthy carriers, has been detected in HIV infected individuals. Expression of the lytic gene BZLF-1 is detectable in 36% of HIV large-cell/immunoblastic lymphomas (Rea *et al* 1994). While in the serum anti-ZEBRA (lytic gene) antibodies were detected in both asymptomatic-HIV and AIDS patients (Joab *et al* 1991). Thus during HIV there is an increase in EBV latently infected cells and in some individuals lytic viral replication. Given the potential of EBV to drive B lymphocyte proliferations this increase in circulating EBV may have consequences for the subsequent development of EBV related tumours in HIV.

Almost all AIDS related PCNSL are EBV-driven lymphoproliferations and show expression of the EBV oncogenes EBNA-2 and LMP-1 (MacMahon

et al 1992, Auperin *et al* 1994). We were therefore interested to determine whether the increased number of B lymphocytes detected in the CNS in pre-AIDS and/or AIDS pleomorphic infiltrates contained EBV and therefore predisposed individuals to development of PCNSL. We hypothesised two mechanisms that could cause increased numbers of EBV infected B lymphocytes to enter the brains of HIV infected individuals:

- 1- The number of circulating latently infected B lymphocytes, which is between 5 and 500 per 10^7 B lymphocytes in a healthy carrier (Miyashita *et al* 1995), is significantly increased in HIV infection (Birx, Redfield, Tosato 1986). Therefore if B lymphocyte entry to the CNS is random process then there is an increased chance of EBV infected cells entering the brain. Even if the process of B lymphocyte entry is not random and only antigen specific B lymphocytes are able to enter the brain, then as EBV is present in the memory B lymphocyte population and is stably inherited in daughter cells (Babcock *et al* 1999, Thorley-Lawson & Babcock 1999), it is plausible that an EBV infected cell may be recruited to the CNS.
- 2- Like HIV, EBV is a potent T lymphocyte-independent polyclonal activator of B lymphocytes, and, although EBV gene expression is tightly controlled in healthy carriers (Miyashita *et al* 1997), expression of the B lymphocyte activating genes, EBNA-2 and LMP-1, has been detected in the immunocompromised host (Hopwood *et al* 2002). Our previous data indicate that activation is a pre-requisite for B lymphocyte entry into the brain and therefore activation by EBV is likely to facilitate the entry of EBV infected B lymphocytes to the brain.

We therefore sought evidence of EBV infection initially by screening for EBV DNA by PCR and then by *in situ* hybridisation to detect EBV RNA (EBERs) in tissue sections. The advantage of screening samples by PCR is that it allows rapid assessment of many samples for the presence of the virus, the disadvantage in using PCR is that it does not permit identification of which cells in a sample are infected. PCR may also detect cells present within the

blood vessels of the tissue which may give a false impression of the actual quantity of EBV within the tissue. In contrast *in situ* hybridisation allows determination of the cells infected with the virus, however the process is laborious and the screening of sections time consuming and subjective. In addition only small sample areas can be studied using *in situ* hybridisation. It is therefore important to use both techniques in order to obtain reliable data.

Despite an extensive search of both normal healthy brains and HIV infected brains by *in situ* hybridisation, EBV infected B lymphocytes were only found in the cells of PCNSL tumours. PCR detected high copy numbers of EBV DNA in all PCNSL samples and low copy numbers in occasional samples from infiltrate cases.

Entry of B lymphocytes in general to the brain is a comparatively rare event and our failure to locate any EBV infected cells within the brain B lymphocyte population (other than tumour cells) suggests that entry of EBV infected B lymphocytes is also a rare event. The failure to find any EBV infected cells (other than in tumours) is not unexpected given the relatively low number of circulating B lymphocytes infected with the virus, even in HIV. Given that in the peripheral blood EBV infects between 5 in 10^7 and 5 in 10^5 cells, and assuming random entry of B lymphocytes to the brain, it is predicted that at least 20,000 brain B lymphocytes within the brain would have to be studied to locate 1 EBV positive cell. Only if EBV infection of B lymphocytes induced a strong or absolute homing of cells to the brain would it be possible to locate EBV infected B lymphocytes in the brain by this technique. Despite the propensity for EBV related tumours to develop in the brain, there is as yet no evidence in the literature to suggest that EBV infected cells preferentially home to the brain, and our evidence does not contradict this. Whether EBV infected cells do specifically home to the brain or are occasionally randomly drawn into the brain, remains to be elucidated.

The fact that EBV driven tumours rarely develop in the brain of immunocompetent individuals (Jellinger & Paulus 1995) suggests however that the normal immune surveillance level in the brain is sufficient to prevent EBV driven proliferations occurring at this site. That EBV driven tumours develop in this site in immunosuppressed individuals, both in AIDS and post-transplant settings, suggests that either the CNS may suffer an earlier or larger

decline in immune surveillance than other organs in both these settings, or that the extracellular milieu within the CNS in these diseases is more favourable for EBV tumour development than at other sites. Most likely a combination of both occurs in HIV PCNSL.

All PCNSL assessed in this study were EBV positive, as determined by both *in situ* hybridisation for EBERs expression and PCR for EBV DNA, which is in accordance with most previous studies showing an almost complete association of EBV with PCNSL (MacMahon *et al* 1992, Auperin *et al* 1994). Furthermore, all tumours displayed strong EBNA-2 staining and some LMP-1 positivity, which is again in line with the majority of published data (Auperin *et al* 1994) and indicates that EBV infected PCNSL display an EBV latency III phenotype. In addition it suggests an aetiological association between EBV and PCNSL, since both EBNA-2 and LMP-1 are major viral oncogenes.

As well as studying the presence of EBV in brain tissue samples we also studied several CSF samples. Detection of EBV in the CSF of AIDS patients is considered diagnostic of PCNSL (Cingolani *et al* 1998) in the presence of other indicative clinical features. We therefore wished to assess the presence of EBV in CSF samples from AIDS PCNSL cases and AIDS infiltrate case in order to further determine similarities or differences between these two groups. The finding of low levels of EBV in the CSF and some tissue samples from the AIDS infiltrate group is interesting. All CSF samples from PCNSL cases were PCR positive for EBV (range 283 to $>10^6$ EBV genomes/ μ g DNA) as were all tissue samples which contained lymphoma (range 2×10^4 to $>10^6$ EBV genomes/ μ g DNA). In contrast only 2 of 8 tissue samples from the AIDS infiltrate group were EBV PCR positive (range 110 to 160 EBV genomes/ μ g DNA). However the CSF of case 25 showed higher levels of EBV (range 6,300 to 12,600 EBV genomes/ μ g DNA). It should however be considered that the CSF results may be misleading as these are post mortem samples and therefore may potentially be contaminated with blood (although visually only 1 sample appeared to be).

The discrepancy between results from the 2 techniques for tissue samples from AIDS cases with pleomorphic infiltrates may be due to detection

of EBV infected cells in the blood vessels of the brain tissue samples and may reflect an increased level of circulating EBV infected cells in these patients. Or, alternatively, given the low EBV copy number detected in these cases the differences may reflect the small area sampled by *in situ* hybridisation.

It is intriguing to speculate that these pleomorphic proliferations may actually represent pre-malignant lesions demonstrating a step in the process of PCNSL tumourigenesis. If in fact they do represent a pre-malignant PCNSL state, then there are two plausible routes of pathogenesis:

- 1- As part of the normal inflammatory responses to antigen with the CNS large numbers of B lymphocytes are attracted into the CNS. In the cases studied here the stimulus was not identified, however it is not likely to be productive HIV infection as all sections were negative for HIV p24 immunohistochemical staining. It is conceivable that as part of this B lymphocyte infiltration an occasional EBV infected cell may be recruited to the brain. Production of TH-2 type cytokines which occurs during HIV infection within the brain and in the periphery (Klein *et al* 1997) may stimulate proliferation B lymphocytes (including any EBV infected cells). The disruption of the immune system seen in HIV, may affect the CNS more than other organs as its immunity is already limited, and may lead to uncontrolled proliferation of these B lymphocytes. This could allow survival of any EBV infected cells utilising the latency III program which includes expression of the major EBV oncogenes EBNA-2 and LMP-1, which in turn would lead to uncontrolled proliferation of that clone. Unchecked, this proliferation might lead to outgrowth of a monoclonal tumour and hence to PCNSL.
- 2- Alternatively production of EBV within the CNS or entry of free virus from the peripheral blood, possibly via a disrupted BBB, may lead to infection of B lymphocytes already within the CNS which again may cause clonal outgrowth and PCNSL as outlined in point 1 above. A possible source of free EBV is from EBV infected B lymphocytes which have terminally differentiated to plasma cells, a process which induces lytic EBV replication (Crawford & Ando 1986), resulting in release of free virus.

Intriguing as it is to speculate on the pre-malignant nature of these infiltrates, there is no conclusive evidence to prove or refute this suggestion. Although the evidence accumulated in this study suggests that the pleomorphic infiltrates seen in some AIDS brains are not pre-malignant, the data are not conclusive and as discussed above there are possible mechanisms of tumourigenesis which could lead to malignancy arising from these infiltrates. In our study, despite obtaining positive EBV PCR signals, we found no evidence by *in situ* hybridisation of EBV infection of B lymphocytes within these infiltrates. However due to our samples size which is small compared to the overall size of a brain, the need for only 1 or few EBV positive cells to initiate a tumour, and the rarity of EBV infected B lymphocytes in the blood (1 per 10^5 B lymphocytes in HIV), it is highly improbable that our survey would have detected these very rare cells. To support the notion that occasional EBV infected B lymphocytes do enter the brain Bell *et al* (1997) have previously demonstrated occasional EBV positive cells in an infiltrate from 1 of the cases used in this study. Although the case in question (case 28) was an African child aged approximately 6 months and it should be considered that the child may have been experiencing a primary EBV infection and therefore have high numbers of EBV infected B lymphocytes circulating in the blood expressing all viral latent genes. (Given the prevalence of EBV in the adult population (over 90% world wide) it is unlikely that any of the adult cases of AIDS infiltrates were experiencing primary EBV infection and therefore they would have lower levels of EBV infected cells). Regardless of the source of the EBV, this case demonstrates that EBV infected cells can occasionally be found within the brain, which has implications for the pathogenesis of PCNSL.

We conclude that the PCR test used in this study (sensitivity of 1-10 EBV positive cell in 10^6 EBV negative cells) detected the occasional EBV infected B lymphocyte either in the brain tissue or blood vessels, but that EBV infection is not the primary cause of pleomorphic B lymphocyte infiltrates.

4.9 Comparison of AIDS pleomorphic infiltrate brains and AID PCNSL brains.

There are further differences between PCNSL and AIDS infiltrates which could have arisen because there is no link between PCNSL and the infiltrates, or, alternatively because PCNSL formation is a multi-step process of which we are only viewing two points. These differences include:

- 1- The ratio of B:T cells within the lesions
- 2- The cells proliferating within the lesion
- 3- The background level of B lymphocytes within the brain.
- 4- The location of B lymphocytes within the brain
- 5- EBV infection of B lymphocytes (discussed above, section 4.8).
- 6- Clonality of B cell lymphoproliferations (discussed below, section 4.10)

In terms of the ratio of B:T cells; the cells in PCNSL lesions are almost entirely B lymphocytes with a few infiltrating CD8 T lymphocytes, in contrast within the pleomorphic infiltrates B lymphocytes represent between approximately 10 and 40% of cells. The fact that infiltrates and PCNSL differ markedly in their B:T cell composition may suggest a difference in pathogenesis between the two. However, there is evidence from SCID mouse models of EBV driven B cell lymphoma in immunocompromised settings, that CD4 T lymphocytes are required for the initial stages of tumour development (Johannessen, Ashgar & Crawford 2000). While we were unable to look directly for CD4 T lymphocytes, the evidence obtained using CD3 (pan T lymphocyte marker) and CD8 markers would suggest that the majority of T lymphocytes present showed dual positivity for both CD3 and CD8 markers, therefore very few CD4 T lymphocytes are present in the pleomorphic infiltrates.

The two lesions also differ with respect to proliferation of cells. In PCNSL both B and T lymphocytes show expression of proliferation markers Ki67 and PCNA, in contrast only the T lymphocytes in infiltrate cases appear

to be proliferating. Again these differences could reflect genuine differences between the two lesions or alternatively the pleomorphic infiltrates could represent an initial early stage in the tumourogenesis process (before the B lymphocytes have begun to proliferate).

The background level of B lymphocytes within the brains also differs. PCNSL brains contained virtually no B lymphocytes other than the localised tumour cells, while brains containing pleomorphic infiltrates tended to show the presence of high numbers of B lymphocytes throughout the brain. Again this could reflect genuine differences in the pathogenesis of the lesions or it could be due to the fact that only one time point has been studied.

Finally, in PCNSL the neoplastic infiltrates were generally focal, being located in 1 or 2 regions of the brain. In contrast the pleomorphic infiltrates were diffusely spread and found in most regions of the brain. This could be accounted for if the infiltrates began as a polyclonal proliferation and develop into a single monoclonal tumour with eventual loss of the non-malignant polyclonal cells, or alternatively it may reflect true differences between the pathology of these lesions.

None of our data suggests that a link exists between the pleomorphic infiltrates seen in some AIDS brains and the development of AIDS PCNSL. However as stated throughout the discussion the limitations of this type of study, which allows only one time point in a pathological process to be viewed, means that a link cannot be entirely ruled out by our data.

4.10 Clonality of tumours

Several larger studies have previously addressed the issue of clonality in AIDS PCNSL (Meeker *et al* 1991, Bashir *et al* 1993, Julien *et al* 1999) with the consensus reached that the majority of these tumours are monoclonal as determined by Ig gene re-arrangements or κ/λ light chain usage. There is no reported preferential usage of any one VH gene family in AIDS PCNSL, however it is noteworthy that alterations in the usage of VH gene families are

observed in B lymphocytes present in the peripheral blood of HIV infected individuals.

Of the 3 PCNSL cases assessed for clonality in this study, 2 were monoclonal with VH3 family receptor gene usage while the third was a polyclonal tumour. The first 2 results (monoclonal tumours) fit well with results from previous larger studies which show the majority of PCNSL are monoclonal in terms of Ig gene re-arrangements (Meeker *et al* 1991, Bashir *et al* 1993, Julien *et al* 1999). As these tumours are predominantly monoclonal and always EBV positive they most likely develop from a single EBV infected B lymphocyte that undergoes the final stages of transformation within the CNS. However the polyclonal tumour found in our study does not fit this model. Formation of a polyclonal EBV driven tumour could occur in two ways. Either free EBV could infect several cells in a polyclonal B cell proliferation, with virus possibly being produced by terminally differentiated EBV infected plasma cells entering the lytic viral replication cycle. Or alternatively, more than one EBV infected cell could enter the CNS and undergo transformation. Neither hypothesis of polyclonal tumour formation can be proven from our data.

The use of VH3 family gene receptors by 2 of 3 PCNSLs was interesting given the evidence that HIV gp120 can act as a superantigen for VH3 expressing B lymphocytes (Muller & Kohler 1997, Karray & Zouali 1997) and that VH3 B lymphocytes undergo clonal deletion during the later stages of AIDS when PCNSL is most likely to develop (David *et al* 1995, David *et al* 1996, Wisnewski, Cavacini & Posner 1996). Although the data from our study suggest a high incidence of VH3 gene usage in PCNSL, too few cases were studied to enable any analysis of gene usage.

4.11 B lymphocyte Chemoattractants in the CNS

The mononuclear cell chemoattractant SDF-1 can be produced in the brain by neurones and astrocytes (Bajetto *et al* 1999). It acts on lymphocytes and monocytes, but not neutrophils, to promote cell migration *in vitro*. It is a

highly potent attractant *in vivo* and functions via binding to the chemokine receptor CXCR4 (Bleul *et al* 1996). With respect to B lymphocytes, it has been demonstrated in tonsil tissue that SDF-1 is a specific attractant for memory B lymphocytes (Casamayor-Palleja *et al* 2001). Expression of SDF-1 is not upregulated in inflammatory conditions in which B lymphocytes are present, which has led Bleul *et al* (1996) to propose that it may be involved in immune surveillance rather than inflammation.

Our preliminary study suggests an association between the accumulation of B lymphocytes in AIDS brains and SDF-1 expression, with both PCNSL brains in the regions of brain containing lymphoma and infiltrate brains with areas containing pleomorphic lymphocytes showing high levels of this chemokine. Interestingly increased expression of SDF-1 was only noted in areas that actually contained high numbers of B lymphocytes and not other areas of the same brain. Furthermore non-HIV encephalitic brains which also contained high numbers of B lymphocytes showed little or no expression of SDF-1, suggesting a different mechanism for lymphocyte recruitment may be involved here. As SDF-1 expression was only found at low levels in all brain groups other than AIDS PCNSL and AIDS infiltrate, there is a tendency to assume that these B lymphocytes are accumulating as a result of aberrant chemokine expression, possibly induced by HIV infection of resident brain cells. It is however conceivable that the chemokine expression is due to the presence of the B lymphocytes in these tissues rather than being the cause of the B lymphocyte accumulations, although if this were the case then the non-HIV encephalitis cases which contained high numbers of B lymphocytes would also be expected to demonstrate high levels of SDF-1 expression.

One of the limitations of immunohistochemistry is that it allows determination of the position of a given protein but cannot determine with certainty which cells are actually producing the protein if the protein is extracellular. The majority of staining found in this study was extracellular. An interesting follow up to this study would be to use *in situ* hybridisation techniques to locate the cells expressing SDF-1 mRNA. Given that astrocytes are a potential source of the chemokine and can be infected with HIV, it may be of further interest to determine if any link exists between astrocyte infection by HIV and expression of SDF-1.

Of interest are reports that a mutant version of the SDF-1 gene is associated with an increased incidence of NHL in HIV. Rabkin *et al* (1999) in a study of 746 HIV infected individuals reported an increased incidence of NHL in patients with a transition mutation in the 3' untranslated region of the SDF-1 gene. 19% of homozygotes for the mutation developed NHL, compared to 10% of heterozygous and 5 % with the wild type genotype. The physiological effects of this mutation are as yet undetermined.

4.12 Cytokines in the CNS

Our limited study of cytokine expression in the brain, using RT-PCR, showed detectable expression of several cytokines in AIDS brains, but only IL-4 in 1 of 2 normal brains. In particular the TH2 cytokines IL-6 (B lymphocyte growth factor (Tosato *et al* 1986, Emilie *et al* 1992)) and IL-10 (induces B lymphocyte proliferation) were detected in 4 of 4 PCNSL samples. The presence of these factors would be expected to enhance tumour growth. The present study did not allow us to determine which cells within the brain were expressing these cytokines. However monocytes have been shown to be a source of additional IL-6 during HIV infection (Clouse *et al* 1991, Nakajima *et al* 1989) and monocyte entry to the CNS has been demonstrated in HIV (Dallasta *et al* 1999). In addition to monocytes IL-6 and IL-10 can be produced by HIV infected astrocytes and microglia (Yeung *et al* 1995, Fiala *et al* 1996, Speth *et al* 2000). It should be noted that EBV positive tumours produce viral IL-10 (Benjamin, Knobloch & Dayton 1992) which acts in an autocrine manner to stimulate further tumour growth, however the PCR primers used in our assays had been designed to detect only human IL-10 and not the viral protein.

IL-6 expression was detected in 7 of 8 AIDS brain samples including 2 brains with no CNS pathology and 3 of 4 PCNSL, but was not detected in either of the normal brain samples. Although the number of samples studied was too low to draw firm conclusions, this data suggest that a consequence of HIV infection may be the expression of IL-6 within the brain. IL-6 has been shown in non-CNS B cell lymphomas to act as a growth factor for high grade

B cell lymphomas (Emille *et al* 1992), with production occurring mostly in non-malignant cells such as macrophages and endothelial cells. IL-6 production by these cell types can be induced by INF- γ (Leeuwenberg *et al* 1990, Sanceau *et al* 1991) and INF- γ production was detected in 3 of the 4 PCNSL samples assessed. As IL-6 promotes B lymphocyte proliferation and its production is increased in HIV particularly by monocyte derived cells (Klein *et al* 1997), this may be a predisposing factor to the development of PCNSL.

4.13 Pathogenesis of AIDS PCNSL

The exact pathogenesis of AIDS PCNSL is still unclear. However the 100% association of EBV with these tumours (MacMahon *et al* 1992) suggest EBV plays a significant part in the process of tumour formation. EBV infection of AIDS PCNSL shows a latency III phenotype, which includes expression of LMP-1, and EBNA-2. EBNA-2 transactivates cellular genes involved in proliferation and upregulates LMP-1. LMP-1 has profound effects on B lymphocyte growth, inducing activation and differentiation (Izumi *et al* 1999), it is also induces upregulation of the anti-apoptotic BCL-2 gene (Rowe *et al* 1994). The expression of oncogenic proteins by EBV within B lymphocytes is likely to provide at least some of the stimuli required in the multi-step process to tumour formation. In addition to the oncogenic properties of the EBV proteins expressed in tumour cells, there is evidence that during HIV infection anti-EBV CTLs become progressively less responsive to EBV antigens as AIDS develops and the CD4 count decreases (Van Baarle *et al* 2001). Thus EBV driven proliferation will be poorly controlled in late stages of AIDS, predisposing to B cell malignancies.

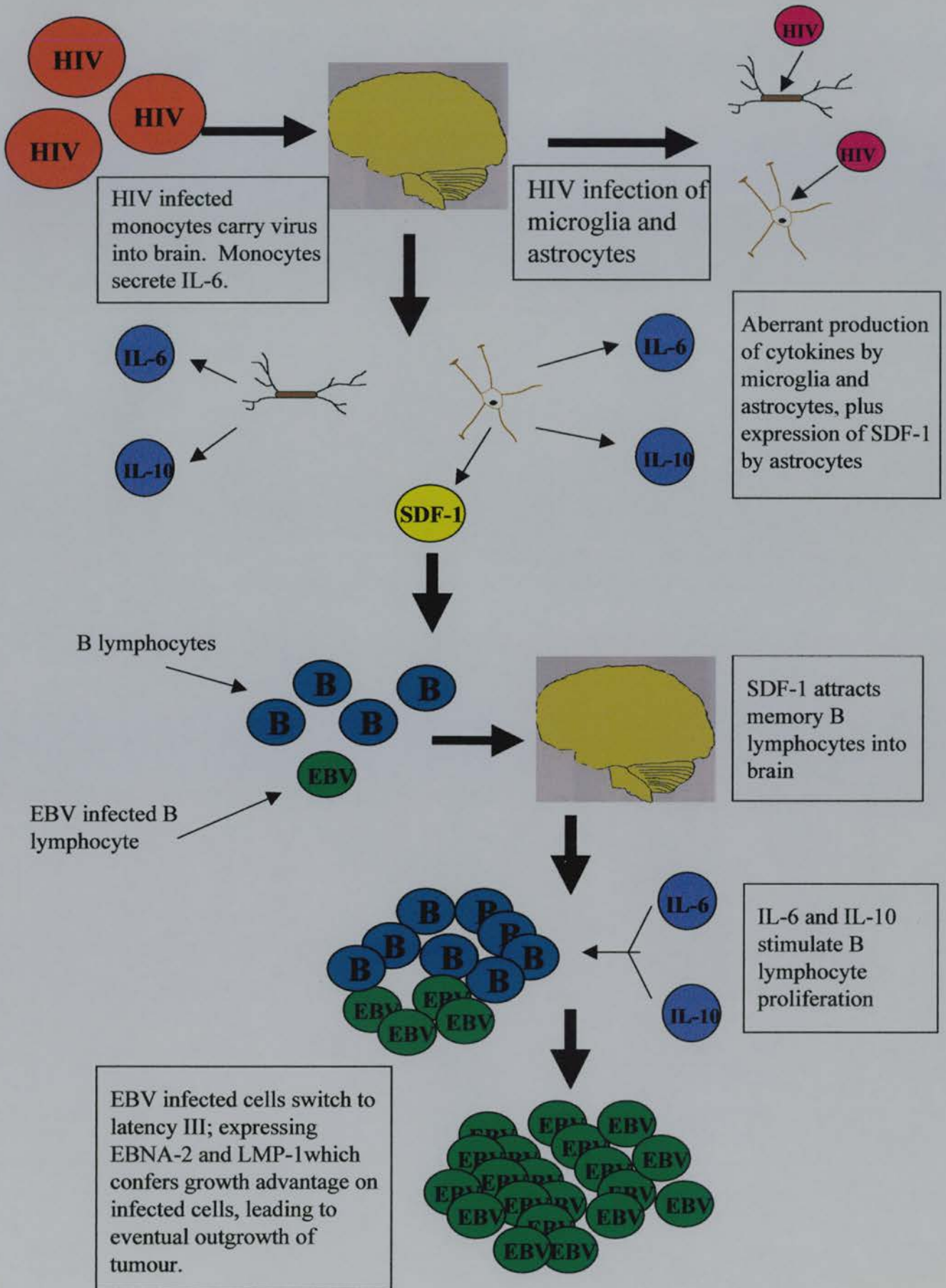
While it cannot be disputed that EBV plays an important role in the formation of these tumours, the fact that the majority are solitary monoclonal tumours suggests that EBV alone is not sufficient for transformation, even in an immunocompromised setting. If it were sufficient then given the increased number of EBV infected cells in HIV infected patients (Laroche *et al* 1995) it would be anticipated that more than one cell might be transformed.

A significant side effect of HIV infection is the aberrant expression of several cytokines. In particular, expression of the TH2 cytokines IL-4, IL-6 and IL-10 are increased during HIV (Martinez-Maza *et al* 1987, Klein *et al* 1997). The effects of this are to increase B lymphocyte proliferation, which combined with the loss of B cell apoptotic signals normally supplied by T helper 1 lymphocytes expressing Fas ligand and FDC, again predisposes to B cell tumour formation. Furthermore both HIV and EBV antigens are capable of acting as B lymphocyte superantigens, again increasing B lymphocyte proliferation (Muller & Kohler 1997, Karray & Zouali 1997).

It would seem therefore that HIV infection produces an environment favourable for uncontrolled EBV proliferation and subsequent tumour formation, in terms of both stimuli for proliferation and loss of viral control mechanisms. Figure 44 shows a schematic diagram of a model for the pathogenesis of PCNSL suggested by the results presented here.

The reason for the high incidence of B cell lymphoma formation in the CNS (20% of all NHL in AIDS) is still unclear. It is possible that EBV infection induces CNS homing signals in B lymphocytes, however there is as yet no evidence of this. Alternatively the extracellular milieu of the CNS in HIV may simply be more favourable for EBV driven B lymphoproliferations than other sites in the body. Furthermore HIV infection of the CNS may in some cases induce expression of chemoattractants, for example SDF-1, which recruit an increased number of B lymphocytes to the brain or preferentially recruit EBV infected B lymphocytes to the brain. Again however there are no strong data as yet to support this hypothesis.

Figure 44: Proposed model of pathogenesis of PCNSL



4.14 Summary

In summary we have demonstrated for the first time that B lymphocytes infiltrate the normal human brain, albeit in low numbers. The rarity of these cells within the normal brain, combined with the time consuming microscopy required to detect significant numbers has possibly resulted in these cells being overlooked or missed by previous studies. B lymphocytes in the normal brain are found only in the parenchyma and not in the perivascular spaces, which suggests that there is no impediment to movement of B lymphocytes from the perivascular to the parenchymal compartment of the brain. No evidence was found of BBB disruption in the normal brain, signifying that B lymphocytes are able to penetrate an intact BBB.

During pre-symptomatic HIV infection the B lymphocyte population of the brain increases in comparison to normal controls and a significant number of cells accumulate in the perivascular spaces in addition to the parenchyma. The progression to AIDS in most individuals leads to a decrease in the brain B lymphocyte population below that seen in the normal healthy brain for most patients.

Tumours involving B lymphocyte lineage cells are prevalent in HIV and are often associated with EBV. The development of these tumours is probably attributable to the disruption of the immune system caused by HIV and the shift in production of cytokines from TH1 to TH2, which promotes B lymphocyte proliferation. Disruption of the immune system permits survival of increased numbers of EBV infected B lymphocytes and in some cases lytic EBV infection. Increased numbers of EBV infected cells, present in an immunocompromised setting may predispose to EBV related malignancies. However, the lack of B lymphocytes found in most AIDS brains, in particular in PCNSL brains outwith the areas of malignancy, suggests that increased B lymphocyte infiltration of the CNS is not a predisposing factor for PCNSL development. Although in some pre-AIDS and AIDS subjects there is an unusual accumulation of B lymphocytes in the perivascular space, these cells

are not infected with EBV and probably do not represent a pre-malignant condition.

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Appendix A: Data from individual immuno stained slides

Table A1: Normal cases (Group 1)

Case no	Section type	number of cd20+ve cells in parenchyma	number of perivascular CD20+ve cells	Area of section (cm2)	CD20 cells in Parenchyma/cm2	CD20 perivascular cells/cm2
1	frontal	0	0	4.69	0	0
1	medulla	0	0	1.56	0	0
1	parietal convex	0	0	3.94	0	0
1	temporal hippocampus	0	0	4.19	0	0
1	mid brain	0	0	2.5	0	0
2	cerebellum	1	0	4.75	0.210526316	0
2	basal ganglia	1	0	5.44	0.183823529	0
2	frontal	1	0	5.5	0.181818182	0
2	parietal convex	0	0	3	0	0
2	temporal hippocampus	0	0	4.13	0	0
2	mid brain	0	0	4.13	0	0
3	mid brain	0	0	3.25	0	0
3	temporal hippocampus	1	0	4	0.25	0
3	parietal convexity	0	0	3.63	0	0
3	frontal	0	0	4.88	0	0
3	cerebellum	1	0	5.75	0.173913043	0
4	basal ganglia	1	0	5.81	0.17211704	0
4	cerebellum	1	0	6.44	0.155279503	0
4	frontal	0	0	4.19	0	0
4	medulla	0	0	1.94	0	0
4	mid brain	0	0	3.5	0	0
4	occ	0	0	5.19	0	0
4	parietal convexity	1	0	4.44	0.225225225	0
4	pons	0	0	4.1	0	0
4	th	1	0	4.56	0.219298246	0
5	temporal hippocampus	0	0	3.69	0	0
5	frontal	1	0	5.19	0.192678227	0
5	basal ganglia	0	0	5.19	0	0
5	cerebellum	1	0	4.44	0.225225225	0
5	parietal convex	1	0	3.44	0.290697674	0
5	occipital	0	0	5	0	0
5	mid brain	1	0	2.94	0.340136054	0

6	cerebellum	1	0	4.06	0.246305419	0
6	basal ganglia	1	0	5.88	0.170068027	0
6	temporal hippocampus	0	0	3.25	0	0
6	parietal convex	1	0	2.56	0.390625	0
6	pons	0	0	3.63	0	0
6	mid brain	0	0	3	0	0
6	frontal	0	0	4.75	0	0
7	parietal para	0	0	7.5	0	0
7	parietal convex	1	0	4.69	0.213219616	0
7	occipital	2	0	4.44	0.45045045	0
7	mid brain	0	0	4.07	0	0
7	thalamus	1	0	3.69	0.27100271	0
7	medulla	1	0	1.94	0.515463918	0
7	basal ganglia	0	0	6.13	0	0
7	pons	4	0	5.31	0.753295669	0

Table A2: Drug users (non-HIV) (Group 2)

Case no	Section type	number of cd20+ve cells in parenchyma	number of perivascular CD20+ve cells	Area of section (cm2)	CD20 cells in Parenchyma/cm2	CD20 perivascular cells/cm2
8	central white	1	0	5.63	0.177619893	0
8	pons	1	0	4.56	0.219298246	0
8	cerebellum	1	0	5.81	0.17211704	0
8	medulla	29	0	2.13	13.61502347	0
8	basal ganglia	1	0	4.81	0.207900208	0
8	mid brain	0	0	3.88	0	0
8	temporal hippocampus	1	0	3.88	0.257731959	0
8	parietal convex	1	0	4.44	0.225225225	0
8	frontal	5	0	4.5	1.111111111	0
8	occipital	1	0	3.13	0.319488818	0
9	medulla	1	0	3.44	0.290697674	0
9	occipital	0	0	4.63	0	0
9	basal ganglia	5	0	6.56	0.762195122	0
9	mid brain	4	0	4.12	0.970873786	0
9	central white	0	0	5.19	0	0
9	thalamus	3	0	4.5	0.666666667	0
9	temporal hippocampus	0	0	4.25	0	0
9	parietal convex	2	0	6.19	0.323101777	0
9	frontal	0	0	5.06	0	0
9	cerebellum	0	0	7.18	0	0
10	basal ganglia	0	0	5.44	0	0
10	frontal	0	0	4.25	0	0
10	medulla	0	0	2.62	0	0
10	cerebellum	0	0	6.06	0	0
10	central white	0	0	6.06	0	0
10	thalamus	1	0	5	0.2	0
10	parietal convex	0	0	3.94	0	0
10	temporal hippocampus	1	0	5	0.2	0
11	basal ganglia	0	0	5	0	0
11	parietal convex	0	0	3.62	0	0

11	occipital	0	0	4.56	0	0
11	medulla	0	0	1.81	0	0
11	cerebellum	0	0	5.19	0	0
11	temporal hipocampus	2	0	3.81	0.524934383	0
11	thalamus	2	0	4.31	0.464037123	0
11	frontal	0	0	5.81	0	0
11	central white	0	0	5.06	0	0
12	medulla	0	0	1.94	0	0
12	occipital	2	0	4.62	0.432900433	0
12	cerebellum	0	0	6.5	0	0
12	pons	1	1	4.56	0.219298246	0.219298246
12	parietal convex	0	0	4.88	0	0
12	mid brain	0	2	3.56	0	0.561797753
12	frontal	0	0	3.44	0	0
12	basal ganglia	3	1	4.94	0.607287449	0.20242915

Table A3: Group 3 pre-symptomatic HIV positive individuals

Case no	Section type	number of cd20+ve cells in parenchyma	number of perivascular CD20+ve cells	Area of section (cm2)	CD20 cells in Parenchyma/cm2	CD20 perivascular cells/cm2
13	pons	5	4	4.44	1.126126126	0.900900901
13	central white	4	2	7.56	0.529100529	0.264550265
13	medulla	0	0	1.75	0	0
13	temporal hipocampus	2	0	5.31	0.376647834	0
13	parietal para	2	0	6.19	0.323101777	0
13	cerebellum	4	0	7.56	0.529100529	0
14	temporal hipocampus	0	0	4.88	0	0
14	occipital	0	20	4.44	0	4.504504505
14	medulla	0	0	2.13	0	0
14	parietal para	1	1	6.44	0.155279503	0.155279503
14	frontal	0	6	4.94	0	1.214574899
14	parietal convex	0	0	4.35	0	0
14	pons	0	0	5.19	0	0
14	mid brain	65	1	3.18	20.44025157	0.314465409
14	cerebellum	0	2	7.34	0	0.272479564
14	basal ganglia	0	0	4.81	0	0
15	cerebellum	1	13	5.5	0.181818182	2.363636364
15	parietal convex	1	4	5	0.2	0.8
15	medulla	0	0	2	0	0
15	mid brain	4	0	3.44	1.162790698	0
15	occipital	0	2	3.75	0	0.533333333
15	temporal hipocampus	0	9	4.13	0	2.179176755
15	central white	2	7	6.63	0.301659125	1.055806938
15	pons	3	0	3.81	0.787401575	0
15	parietal para	0	0	4.69	0	0
15	Frontal	1	1	3.88	0.257731959	0.257731959
15	basal ganglia	0	5	4.63	0	1.079913607
16	basal ganglia	0	5	5.75	0	0.869565217
16	mid brain	0	4	3.38	0	1.183431953

16	parietal convex	1	2	4.19	0.238663484	0.477326969
16	parietal para	2	7	4.63	0.431965443	1.51187905
16	temporal hipocampus	0	4	3.75	0	1.066666667
16	frontal	0	6	3.19	0	1.880877743
16	cerebellum	1	0	6.94	0.144092219	0
16	occipital	1	4	4.31	0.232018561	0.928074246
16	central white	1	6	5.94	0.168350168	1.01010101
16	medulla	0	7	2.38	0	2.941176471
17	occipital	1	0	3.81	0.262467192	0
17	mid brain	0	0	4.63	0	0
17	parietal para	1	0	4.31	0.232018561	0
17	pons	0	0	4.75	0	0
17	basal ganglia	0	0	4.88	0	0
17	frontal	0	0	6.31	0	0
17	cerebellum	0	0	7.25	0	0
17	temporal hipocampus	1	0	5.25	0.19047619	0
18	basal ganglia	1	0	10.13	0.098716683	0
18	central white matter	1	0	9.31	0.107411386	0
18	cerebellum	1	0	8.94	0.111856823	0
18	frontal	0	0	6.13	0	0
18	medulla	1	0	1.94	0.515463918	0
18	mid brain	1	0	3.5	0.285714286	0
18	occ	1	1	7.38	0.135501355	0.135501355
18	parietal convexity	1	0	6.81	0.146842878	0
18	pp	1	0	6.75	0.148148148	0
18	pons	2	0	4.94	0.4048583	0
18	th	1	1	7.06	0.141643059	0.141643059
18	thal	0	0	6.63	0	0

Table A4: Group 4: AIDS patients with no CNS pathology

Case no	Section type	number of cd20+ve cells in parenchyma	number of perivascular CD20+ve cells	Area of section (cm ²)	CD20 cells in Parenchyma/cm ²	CD20 perivascular cells/cm ²
19	thalamus	0	0	3.31	0	0
19	temporal hippocampus	0	0	4.31	0	0
19	parietal convex	0	0	4.56	0	0
19	mid brain	0	0	3.5	0	0
19	basal ganglia	0	0	6.44	0	0
19	medulla	0	0	2.06	0	0
20	cerebellum	0	0	6.44	0	0
20	pons	0	0	5	0	0
20	thalamus	0	0	4.69	0	0
20	basal ganglia	0	0	5.75	0	0
20	frontal	0	0	5.5	0	0
20	parietal convex	0	0	3.69	0	0
20	temporal hippocampus	0	0	2.75	0	0
20	mid brain	0	0	3.5	0	0
21	medulla	0	0	2.13	0	0
21	central white	0	0	5.25	0	0
21	occipital	0	0	3.31	0	0
21	temporal hippocampus	0	0	3.06	0	0
21	basal ganglia	0	0	6.19	0	0
21	pons	0	0	4.13	0	0
21	mid brain	0	0	4.56	0	0
21	cerebellum	0	0	6.13	0	0
21	parietal para	0	0	6.06	0	0
22	thalamus	0	0	5.5	0	0
22	parietal convex	0	0	5.06	0	0
22	frontal	0	0	6	0	0
22	temporal hippocampus	0	0	5.63	0	0
22	pons	0	0	4.5	0	0
22	basal	0	0	6.34	0	0

	ganglia					
22	mid brain	0		3.69	0	0
22	cerebellum	0				
			0	5.5	0	0
22	medulla	0	1	1.82	0	0.024390244
23	temporal hipocampus	0				
			0	3.38	0	0
23	cerebellum	0				
			0	6.44	0	0
23	mid brain	0	0	4.13	0	0
23	pons	0	0	3.69	0	0
23	medulla	0	0	1.75	0	0
23	basal ganglia	1				
			0	4.94	0.20242915	0
23	parietal para	0				
			0	4.5	0	0
23	occipital	0	0	4	0	0
23	frontal	0	0	3.69	0	0
23	central white	0				
			0	6.44	0	0
23	parietal convex	1				
			0	5.19	0.192678227	0
23	thalamus	0	0	3.5	0	0

Table A5: AIDS patients with pleomorphic infiltrates in the brain (Group 5)

Case no	Section type	number of cd20+ve cells in parenchyma	number of perivascular CD20+ve cells	Area of section (cm2)	CD20 cells in Parenchyma/cm2	CD20 perivascular cells/cm2
24	temporal hippocampus	0	52	4.69	0	11.08742004
24	mid brain	0	3	5.13	0	0.584795322
24	cerebellum	0	15	7.69	0	1.950585176
24	basal ganglia	0	39	5.81	0	6.712564544
24	parietal para	0	1	6.31	0	0.158478605
24	occipital	0	46	5.13	0	8.966861598
24	central white	0	3	7.5	0	0.4
24	medulla	0	29	3.5	0	8.285714286
25	cerebellum	1	131	4.5	0.222222222	29.11111111
25	mid brain	1	266	2.62	0.381679389	101.5267176
25	temporal hippocampus	0	325	3.66	0	88.79781421
25	medulla	0	181	1.69	0	107.1005917
25	parietal para	1	26	4.57	0.218818381	5.689277899
25	basal ganglia	0	245	4.69	0	52.23880597
26	parietal convexity	0	20	4.75	0	4.210526316
26	cerebellum	4	5	5.94	0.673400673	0.841750842
26	medulla	2	91	2.19	0.913242009	41.55251142
26	occipital	2	58	4.63	0.431965443	12.52699784
26	temporal hippocampus	1	147	4.45	0.224719101	33.03370787
26	basal ganglia	1	16	6.19	0.161550889	2.584814216
26	pons	0	3	4.31	0	0.696055684
27	temporal hippocampus	1	0	3.81	0.262467192	0
27	thalamus	0	4	4.88	0	0.819672131
27	basal ganglia	5	143	5.19	0.963391137	27.55298651
27	cerebellum	0	9	5.5	0	1.636363636
27	mid brain	0	1	3.13	0	0.319488818
27	parietal convex	0	1	4.94	0	0.20242915
27	medulla	0	0	1.75	0	0
27	central white	1	1	4.13	0.242130751	0.242130751
28	basal ganglia	1	2	4	0.25	0.5
28	cerebellum	0	1	4.13	0	0.242130751

28	frontal	2	2	3.19	0.626959248	0.626959248
28	mid brain	0	0	2.94	0	0
28	parietal convexity	1	2	3.63	0.275482094	0.550964187
28	pons	1	2	3.25	0.307692308	0.615384615
28	Temporal hipocampus	1	4	3.96	0.252525253	1.01010101
28	thalamus	0	1	2.94	0	0.340136054

Table A6: AIDS cases with PCNSL (Group 7)

Case no	Section type	number of cd20+ve cells in parenchyma	number of perivascular CD20+ve cells	Area of section (cm2)	CD20 cells in Parenchyma/cm2	CD20 perivascular cells/cm2
36	frontal	0	0	3.69	0	0
36	temporal hippocampus	0	0	5.75	0	0
36	mid brain	0	0	3.94	0	0
36	pons	0	0	4.5	0	0
36	basal ganglia	0	0	5.94	0	0
36	parietal convex	0	2	5	0	0.4
36	cerebellum	0	0	5.56	0	0
36	medulla	0	0	2	0	0
36	central white	0	0	5.94	0	0
37	pons	0	0	6.13	0	0
37	parietal convex	0	0	4	0	0
37	cerebellum	0	2	5.63	0	0.355239787
37	thalamus	0	0	3.75	0	0
37	central white	0	0	5.75	0	0
37	medulla	0	0	2.56	0	0
37	temporal hippocampus	0	0	4.75	0	0
38	mid brain	0	0	3.69	0	0
38	temporal hippocampus	0	0	4.63	0	0
38	basal ganglia	0	0	5.63	0	0
38	medulla	0	0	1.69	0	0
38	occipital	0	0	6.81	0	0
38	frontal	0	0	3.75	0	0
38	parietal convexity	0	0	3.81	0	0
38	cerebellum	0	0	5.81	0	0
38	thalamus	0	0	5.13	0	0
39	mid brain	1	0	5.78	0.173010381	0
39	temporal hippocampus	0	0	5.63	0	0
39	parietal convex	0	0	5.94	0	0
39	medulla	0	0	2.81	0	0

39	basal ganglia	0	0	8.13	0	0
39	frontal	0	0	4.75	0	0
39	cerebellum	0	0	7.13	0	0
39	central white	0	0	6	0	0
39	parietal para	0	0	5.94	0	0
39	pons	0	0	6.13	0	0
39	occipital	0	0	6.31	0	0
40	basal ganglia	0	0	4	0	0
40	occipital	0	0	5	0	0
40	central white	0	0	5.94	0	0
40	pons	0	0	4.94	0	0
40	temporal hippocampus	0	0	4.56	0	0
40	cerebellum	0	0	6.31	0	0
40	frontal	0	0	3.19	0	0
40	medulla	0	0	2.44	0	0
42	basal ganglia	0	0	5.25	0	0
42	cerebellum	0	0	11.81	0	0
42	central white matter	0	0	6.06	0	0
42	frontal	0	0	4.31	0	0
42	mid brain/pons	0	0	4.31	0	0
42	occipital	0	0	6.31	0	0
42	parietal convex	0	0	5.63	0	0
42	pons	0	0	6	0	0
42	temporal hippocampus	0	0	4.69	0	0
42	thalamus	0	0	5.56	0	0

Table A7: AIDS cases with HIVE (Group 6)

Case no	Section type	number of cd20+ve cells in parenchyma	number of perivascular CD20+ve cells	Area of section (cm2)	CD20 cells in Parenchyma/cm2	CD20 perivascular cells/cm2
29	pons	7	10	6.81	1.027900147	1.468428781
29	parietal para	1	1	5.88	0.170068027	0.170068027
29	temporal hippocampus	1	1	5.31	0.188323917	0.188323917
29	mid brain	2	2	4.19	0.477326969	0.477326969
29	medulla	1	19	2.13	0.469483568	8.920187793
29	parietal convex	0	0	4.69	0	0
29	cerebellum	0	0	6.88	0	0
29	central white matter	0	2	6.5	0	0.307692308
29	thalamus	0	5	5.88	0	0.850340136
30	basal ganglia	0	2	6.25	0	0.32
30	cerebellum	0	0	6.87	0	0
30	mid brain	1	0	3.69	0.27100271	0
30	medulla	0	0	1.94	0	0
30	parietal convex	0	0	5.69	0	0
30	pons	0	0	4.43	0	0
30	parietal para	0	0	5.81	0	0
31	occipital	0	0	3.93	0	0
31	basal ganglia	0	0	4.62	0	0
31	parietal convex	0	0	3.88	0	0
31	medulla	0	2	2.5	0	0.8
31	cerebellum	0	0	4.88	0	0
31	thalamus	0	0	4	0	0
31	central white	0	0	5.81	0	0
31	mid brain	0	0	3.75	0	0
32	medulla	1	7	1.93	0.518134715	3.626943005
32	parietal para	0	2	5.81	0	0.344234079
32	temporal hippocampus	1	1	4.5	0.222222222	0.222222222
32	Mid brain	0	0	3.93	0	0
32	BASAL GANGLIA	1	0	6.22	0.160771704	0
32	pons	0	4	4.12	0	0.970873786
32	cerebellum	0	0	6.13	0	0
32	parietal convex	0	0	4	0	0

32	occipital	0	6	3.19	0	1.880877743
33	cerebellum	1	1	7.06	0.141643059	0.141643059
33	basal ganglia	0	0	5.5	0	0
33	thalamus	0	0	3.88	0	0
33	frontal	0	0	5.5	0	0
33	mid brain	1	0	4.38	0.228310502	0
33	temporal hipocampus	0	0	4.31	0	0
33	pons	1	0	4.13	0.242130751	0
33	occipital	0	0	4.5	0	0
33	parietal convex	0	1	5.25	0	0.19047619
33	medulla	0	0	2.37	0	0

34	medulla	0	0	2	0	0
34	mid brain	1	5	3.5	0.285714286	1.428571429
34	temporal hipocampus	0	0	5	0	0
34	cerebellum	0	1	6.31	0	0.158478605
34	parietal convex	0	0	4.44	0	0
34	pons	1	5	4.06	0.246305419	1.231527094
34	occipital	0	0	5.25	0	0
34	basal ganglia	0	0	6.13	0	0

Table A8: non-immunocompromised viral encephalitis (Group 8)

Case no	Section type	number of cd20+ve cells in parenchyma	number of perivascular CD20+ve cells	Area of section (cm2)	CD20 cells in Parenchyma/cm2	CD20 perivascular cells/cm2
43	pons	34	13	4.75	7.157894737	2.736842105
43	mid brain	12	9	3.94	3.045685279	2.284263959
43	parietal convex	2	4	5.19	0.385356455	0.770712909
43	temporal hippocampus	12	72	3.88	3.092783505	18.55670103
43	basal ganglia	14	193	5.5	2.545454545	35.09090909
44	parietal convex	3	92	5.94	0.505050505	15.48821549
44	medulla	10	114	2.25	4.444444444	50.66666667
44	central white	0	26	5.81	0	4.475043029
44	thalamus	7	228	3.81	1.837270341	59.84251969
44	mid brain	5	105	4.63	1.079913607	22.67818575
44	pons	13	115	5.13	2.53411306	22.417154
45	cerebellum	12	246	4.69	2.558635394	52.45202559
45	medulla	44	691	2.69	16.35687732	256.8773234
45	central white	91	1019	5.31	17.13747646	191.9020716
45	basal ganglia	155	1626	5.44	28.49264706	298.8970588
45	temporal hippocampus	8	64	5.69	1.405975395	11.24780316
45	pons	27	177	5.81	4.647160069	30.46471601
45	frontal	180	1600	5.18	34.74903475	308.8803089

Appendix B: Sections stained for CD20, then stripped of antibody and re-stained for CD23

Case number (Case type)	Area of brain	CD20 parenchymal cells	CD23 parenchymal cells	CD20 perivascular cells	CD23 perivascular cells	CD20 intra vascular cells	CD23 intra vascular cells
Non-HIV Cases							
90/101	frontal	1	0	0	0	1	*
90/101	mid brain	0	0	0	0	0	0
90/101	parietal convex	0	0	0	0	0	0
90/101	temporal hippocampus	0	0	0	0	0	0
90/23	basal ganglia	2	2	0	0	1	0
90/23	cerebellum	0	0	0	0	0	0
90/23	mid brain	0	0	0	0	0	0
90/23	parietal convexity	0	0	0	0	0	0
90/23	basal ganglia	0	0	0	0	0	0
90/23	mid brain	0	0	0	0	0	0
90/23	temporal hippocampus	2	2	0	0	1	0
90/23	parietal convexity	0	0	0	0	0	0
90/23	frontal	0	0	0	0	0	0
90/23	cerebellum	0	0	0	0	0	0
90/23	frontal	0	0	0	0	0	0
90/23	temporal hippocampus	0	0	0	0	0	0
90/24	cerebellum	0	0	0	0	2	1
90/24	frontal	0	0	0	0	0	0
90/24	mid brain	0	0	0	0	0	0
90/24	occipital	0	0	0	0	0	0
90/24	cerebellum	0	0	0	0	0	0
90/24	frontal	0	0	0	0	1	1
90/24	frontal	0	0	0	0	0	0
90/24	temporal hippocampus	0	0	0	0	2	1
90/24	temporal hippocampus	0	0	0	0	0	0
90/286	frontal	0	0	1	0	0	0
90/286	cerebellum	0	0	0	0	0	0
90/286	parietal convex	0	0	0	0	0	0
90/286	basal ganglia	0	0	0	0	1	0
90/99	frontal	0	0	0	0	0	0
90/99	parietal convex	0	0	0	0	0	0
90/99	basal ganglia	0	0	0	0	5	5
92/290	thalamus	0	0	0	0	0	0
92/290	occipital	0	0	0	0	0	0
92/290	pons	0	0	0	0	0	0
92/290	parietal convexity	0	0	0	0	0	0
92/290	parietal convexity	0	0	0	0	0	0
94/300	cerebellum	0	0	0	0	0	0
94/300	frontal	0	0	0	0	1	0
94/300	medulla	0	0	0	0	0	0
94/300	cerebellum	0	0	0	0	0	0
94/300	frontal	0	0	0	0	1	0
98/423	thalamus	0	0	0	0	3	1
98/423	medulla	0	0	0	0	0	0
98/423	basal ganglia	0	0	0	0	0	0
98/424	basal ganglia	0	0	0	0	0	0
98/430	frontal	0	0	0	0	0	0
98/430	parietal convexity	0	0	0	0	0	0
Pre-AIDS Cases							
91/283	parietal para	0	0	0	0	0	0
91/283	parietal parasagittal	0	0	0	0	1	0
91/283	cerebellum	0	0	0	0	1	1
91/283	frontal	0	0	0	0	1	1
91/283	thalamus	0	0	0	0	0	0
91/283	mid brain	0	0	0	0	0	0

Case number (Case type)	Area of brain	CD20 parenchymal cells	CD23 parenchymal cells	CD20 perivascular cells	CD23 perivascular cells	CD20 intra vascular cells	CD23 intra vascular cells
91/59	cerebellum	0	0	0	0	0	0
91/59	parietal para	0	0	0	0	0	0
91/59	occ	0	0	0	0	0	0
92/248	cerebellum	1	1	25	25	0	0
92/248	parietal convex	0	0	0	0	0	0
92/248	thalamus	0	0	0	0	0	0
92/248	mid brain	0	0	19	19	0	0
93/94	occipital	0	0	0	0	0	0
93/94	mid brain	1	*	0	0	0	0
93/94	cerebellum	0	0	0	0	0	0
93/94	frontal	0	0	0	0	0	0
95/10	frontal	0	0	0	0	0	0
95/10	occipital	0	0	0	0	0	0
95/10	cerebellum	0	0	7	6	3	3
95/10	parietal convex	0	0	0	0	1	0
97/101	cerebellum	0	0	0	0	0	0
97/101	temporal hippocampus	0	0	0	0	2	2
97/101	parietal para	0	0	0	0	0	0
AIDS Cases with no CNS pathology							
95/15	thalamus	0	0	0	0	0	0
95/15	cerebellum	0	0	0	0	1	0
95/15	mid brain	0	0	0	0	1	0
95/15	parietal convexity	0	0	0	0	1	0
96/160	basal ganglia	0	0	0	0	0	0
96/160	parietal convexity	0	0	0	0	0	0
96/160	cerebellum	0	0	0	0	0	0
96/160	temporal hippocampus	0	0	0	0	0	0
96/267	frontal	0	0	0	0	0	0
96/267	cerebellum	0	0	0	0	0	0
96/267	basal ganglia	0	0	0	0	0	0
AIDS Cases with pleomorphic infiltrates							
93/52	basal ganglia	0	0	1	1	1	1
93/52	cerebellum	0	0	0	0	0	0
95/66	cerebellum	2	2	0	0	1	0
95/66	occipital	0	0	14	14	0	0
95/270	cerebellum	2	2	1	1	0	0
95/270	mid brain	0	0	56	56	1	1
95/270	temporal hippocampus	2	2	17	17	0	0
AIDS cases with PARIETAL CONVEXITYNSL							
89/298	mid brain	Lymphoma	Lymphoma	0	0	0	0
92/218	medulla	Lymphoma	Lymphoma	0	0	0	0
92/218	frontal	Lymphoma	Lymphoma	0	0	0	0
92/218	cerebellum	Lymphoma	Lymphoma	0	0	0	0
92/218	occipital	0	0	0	0	0	0
92/344	mid brain	5 lymphoma cells	0	0	0	0	0
92/344	parietal convex	0	0	0	0	0	0
92/344	frontal	0	0	0	0	0	0
92/446	temporal hippocampus	0	0	0	0	0	0
92/446	frontal	0	0	0	0	0	0
92/446	medulla	0	0	0	0	0	0
92/446	basal ganglia	2	2	0	0	1	0
96/206	basal ganglia	Lymphoma	Only few cells positive, most negative	0	0	0	0
96/206	frontal	Lymphoma	Lymphoma	0	0	0	0
98/369	thalamus	0	0	0	0	0	0
98/369	parietal convexity	0	0	0	0	0	0
98/369	cerebellum	Lymphoma	Lymphoma	0	0	0	0
98/369	medulla	Lymphoma	Lymphoma	0	0	1	1

Appendix C

Table C1: EBERs In-situ hybridisation results

Case type/ number	Area of brain	Positive / Negative
Lymphoma		
00/261	Medulla	+
	Central white matter	+
	Basal ganglia	+
	Frontal	+
89/298	Mid brain	+
92/218	Central white matter *	-
	Thalamus	+
	Basal ganglia	+
	Cerebellum	+
	Frontal *	-
92/344	Parietal parasagittal	+
92/446	Medulla *	-
	Basal ganglia *	-
96/206	Frontal	+
	Basal ganglia	+
98/369	Medulla	+
	Basal ganglia	+
AIDS Infiltrate		
93/52	Temporal hippocampus	-
	Basal ganglia	-
	Occipital	-
	Medulla	-
93/87	Basal ganglia	-
95/270	Mid brain	-
	Medulla	-
	Parietal parasagittal 1	-
	Basal ganglia	-
	Temporal hippocampus 2	-
	Parietal parasagittal 2	-
	Temporal hippocampus 2	-
95/66	Medulla	-
	Cerebellum	-
	Temporal hippocampus	-
	Occipital	-
97/73	Central white matter	-
	Thalamus	-
	Mid brain	-
	Parietal convexity	-
	Pons	-
	Occipital	-
Normal (HIV negative)		
90/24	Temporal hippocampus	-
	Frontal	-
	Parietal convexity	-
	Cerebellum	-

* Sections from lymphoma cases which did not contain lymphoma cells

Case type/ number	Area of brain	Positive / Negative
90/286	Temporal hippocampus	-
	Occipital	-
	Frontal	-
	Basal ganglia	-
	Parietal convexity	-
	Cerebellum	-
	Mid brain	-
90/99	Temporal hippocampus	-
	Frontal	-
	Parietal convexity	-
HIV-ve Drug users		
94/300	Temporal hippocampus	-
	Parietal convexity	-
	Mid brain	-
	Medulla	-
98/423	Thalamus	-
	Basal ganglia	-
	Mid brain	-
	Parietal convexity	-
98/430	Occipital	-
Non-HIV viral encephalitis		
91/128	Basal ganglia	-
	Pons	-
	Temporal hippocampus	-
93/13	Pons	-
	Medulla	-
94/190	Central white matter	-
	Medulla	-
	Pons	-
	Basal ganglia	-
	Frontal	-
Pre-AIDS (HIV +ve)		
91/59	Occipital	-
	Pons	-
	Parietal parasagittal	-
92/428	Medulla	-
	Basal ganglia	-
	Mid brain	-
95/10	Central white matter	-
	Frontal	-
	Parietal parasagittal	-
	Temporal hippocampus	-
	Cerebellum	-
HIVE		
97/73	central white matter	-
	thal	-
	mid brain	-
	parietal convexity	-
	occ	-
	pons	-

Appendix D

Table D1: Sections stained using anti-fibrinogen antibody

Case number	Area of brain	Parenchymal fibrinogen staining
Normal		
1	Parietal convex	-
2	Cerebellum	-
5	Frontal	-
	Basal ganglia	-
	Cerebellum	-
6	Basal ganglia	-
7	Pons	-
Non-HIV drug users		
9	Medulla	+/-
	Occipital	+/-
	Frontal	-
10	Occipital	-
	Temporal hippocampus	-
	Parietal convexity	-
8	Frontal	-
	Medulla	-
Pre-AIDS		
13	Cerebellum	-
15	Parietal parasagittal	-
16	Pons	-
	Cerebellum	-
	Mid brain	-
17	Occipital	-
18	Occipital	-
AIDS no CNS pathology		
21	Basal ganglia	+/-
	Pons	+/-
AIDS HIVE		
30	Pons	+
	Cerebellum	+
	Basal ganglia	+/-
	Parietal convexity	+/-
29	Mid brain	+
	Medulla	+
	Temporal hippocampus	+

Case number	Area of brain	Parenchymal fibrinogen staining
	Parietal parasagittal	+
34	Temporal hippocampus	-
	Occipital	-
	Pons	+
AIDS infiltrate		
25	Medulla	+/-
	Basal ganglia	+
24	Temporal hippocampus	+/-
	Occipital	-
	Medulla	-
26	Mid brain	-
	Medulla	-
	Basal ganglia	-
27	Frontal	-
	Mid brain	+
	Pons	+
	Thalamus	+
	Temporal hippocampus	+
	Parietal convexity	-
	Basal ganglia	-
AIDS PCNSL		
38	Thalamus #	-
	Temporal hippocampus	+
	Mid brain	+
	Basal ganglia	+
39	Occipital #	-
41	Cerebellum	+
	Basal ganglia	+
	Thalamus	+
	Medulla	+
Non-HIV viral encephalitis		
43	Parietal Convexity	+/-
	Cerebellum	+/-
	Thalamus	+
	Frontal	-
Key: + Atleast one blood vessel with leakage of fibrinogen - No leakage of fibrinogen # Sections from lymphoma case which did not contain lymphoma		

Appendix E

Table E1: PCR primer sequences, probe sequences and band sizes

PCR primers:

Human gene / primers	Primer sequence (5' – 3')	Expected band size (Base pairs)
Bam W 3'	AGG ACC ACT TTA TAC CAG GG	298
Bam W 5'	CTT TAG AGG CGA ATG GGC GC	
B-globin 3'	ACA CAA CTG TGT TCA CTA GC	110
B-globin 5'	CAA CTT CAT CCA CGT TCA CC	
FR3 5'	ACA CGG C(CT)(GC) TGT ATT ACT GT	~100
VH1 5'	GCGAATTCATCGATAYTCACCATGGACTGG	~300
VH2 5'	GCGAATTCATCGATAACCACCATGGACATAC	~300
VH3 5'	GCGAATTCATCGATAAGRACTCACCATGGAG	~300
VH4 5'	GCGAATTCATCGATAAGAACATGAARCACTG	~300
VH5 5'	GCGAATTCATCGATGTCCTCCACCATCATG	~300
VH6 5'	GCGAATTCATCGATATCCAGACAATGTCTGTC	~300
JH (3' PRIMER FOR FR3 AND VH1-6)	CTG ACC AGG GTN CCT TGG CCC CAG	
IL-2 3'	AGG TAA TCC ATC TGT TCA GA	266
IL-2 5'	ACT CAC CAG GAT GCT CAC AT	
IL-4 3'	ACG TAC TCT GGT TGG CTT CCT TCA CAG GAC AG	344
IL-4 5'	CGG CAA CTT TGA CCA CGG ACA CAA GTG CGA TA	
IL-6 3'	CAT CCA TCT TTT TCA GCC AT	190
IL-6 5'	ATG TAG CCG CCC CAC ACA GA	
IL-10 3'	AGC TAT CCC AGA GCC CCA GAT CCG ATT TTG G	328
IL-10 5'	AAG CTG AGA ACC AAG ACC CAG ACA TCA AGG GG	
INF γ 3'	ACC GAA TAA TTA GTC AGC TT	356
INF γ 5'	AGT TAT ATC TTG GCT TTT CA	

Table E2: Probe sequences:

Human gene	Probe sequence (5' – 3')
β actin	GGA TAG CAA CGT ACA TGG CT
Bam W	TGA CTT CAC CAA AGG TCA GG
IL-2	CCC TGG GTC TAA AGT GAA AG
IL-4	GTC TGT TAC GGT CAA CTC GG
IL-6	TCT TGT TAC ATG TCT CCT TTC TCA G
IL-10	CAG GTG AAG AAT GCC TTT AAT AAG GTC CAA GAG AAA GGC ATC TAC AAA GCC ATG AGT GAG TTT GAC ATC
INF γ	GCT ACA TCT GAA TGA CCT GC