Detection of HIV-1 DNA in brains of asymptomatic HIV-positive individuals and the role of cytokines in the pathogenesis of early changes

by

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ABSTRACT

While the role of human immunodeficiency virus (HIV) in producing damage to the central nervous system (CNS) is undisputed, the pathogenesis of the disorders associated with the infection still remain unclear. Suggested pathogenetic mechanisms include direct action of HIV or viral protein (gp120) or an indirect one, via products secreted by HIV-1-infected macrophages/microglia or other glial cells, one of which is represented by cytokines.

Regarding the time of the infection at which cytokines become detectable in the brain very little is known. The aim of this study was to ascertain whether 1) the presence of HIV-1 DNA and microglial hyperplasia in the brain during the pre-AIDS stages of the infection is accompanied by enhanced expression of MHC class II antigens and by presence of cytokines; 2) brain damage, including neuronal loss via apoptosis, seen in brains of AIDS patients, is present at the pre-AIDS stage.

The methods applied to the study include morphology, immunohistochemistry for detection of p24, MHC class II and cytokines, PCR and in situ end labelling.

HIV-1 DNA, but not HIV-1 p24 antigen, was detected in 17 of the 36 brains of HIV-1 positive pre-AIDS individuals. Levels of HIV-1 DNA in this group are lower than those found in AIDS group.
Microgliosis and astrogliosis are present in the majority of pre-AIDS individuals. In addition, macrophages, but not MGC, are seen in some of these cases. Elevated expression of MHC class II, tumour necrosis factor (TNF)-α, interleukin (IL)-1α, IL-4 and IL-6, and presence of apoptotic cells have been demonstrated in pre-AIDS cases.

These data demonstrate that the state of immune activation described in AIDS is already present at the pre-AIDS stage, during which cytokines may trigger the cascade of events leading to brain damage; they suggest that therapeutic strategies in HIV-1 positive individuals might have to be applied before they enter the AIDS stage.
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GLOSSARY OF ABBREVIATIONS

ADC  AIDS dementia complex
ADP  AIDS directed programme
AIDS acquired immunodeficiency syndrome
AP  alkaline phosphatase
β-APP β-amyloid precursor proteins
ARC  AIDS related complex
ARV AIDS associated retrovirus
AZT  zidovudine
BBB  blood-brain barrier
c.i.v  cerebral intraventricular
CMV  cytomegalovirus
CNS  central nervous system
CNTF  ciliary neurotrophic factor
CRF  corticotrophin releasing factor
CSF  cerebrospinal fluid
CSFs  colony stimulating factor
CTLs  cytotoxic T lymphocytes
CVLT  the California verbal learning test
DAB  3,3-diaminobenzidine
DFA  discriminant function analysis
DIG  digoxigenin
DPD  diffuse poliodystrophy
EC  endothelial cell
ELAM-1  endothelial leucocyte adhesion molecule
FGF  fibroblast growth factor
FIV  feline immunodeficiency virus
G-CSF  granulocyte colony stimulating factor
GFAP  glial fibrillary acidic protein
GM-CSF  granulocyte macrophage colony stimulating factor
gp  glycoprotein
HBCE  human brain capillary endothelial (cell)
HD  Huntington’s disease
HIV  human immunodeficiency virus
HIVE  HIV encephalitis
HIV-lep  HIV leukoencephalopathy
HTLV-III  human T-cell lymphotropic virus type III
ICAM  intercellular adhesion molecule
ICC  immunocytochemistry
IFN  interferon
IHc  immunohistochemistry
IL  interleukin
ISEL  in situ end labelling
KS  Kaposi’s sarcoma
LAV  lymphadenopathy associated virus
LPS  lipopolysaccharide
LTR  long terminal repeat
L-tropic lymphocytotropic
MCSF macrophage colony stimulating factor
Met-Enk methionine enkephalin
MGCs multinucleated giant cells
MGCE multifocal giant cell encephalitis
MHC major histocompatibility complex
MIP macrophage inflammatory protein
MRC Medical Research Council
MS multiple sclerosis
M-tropic monocytophoretic
NBT nitroblue tetrazolium chloride
NF-kB nuclear factor-kB
NGF nerve growth factor
NGFR nerve growth factor receptor
NIBSC National Institute for Biological Standards and Control
NMDA N-methyl-D-aspartate
NO nitric oxide
NSI non-syncytium inducing
O$_2$ superoxide anion
ONO$^\circ$ peroxynitrite
PAF platelet activating factor
PBM(N)C peripheral blood mononuclear(nucleated) cell
PBLs peripheral blood lymphocytes
PBS phosphate-buffered saline
PCD programmed cell death
PCR polymerase chain reaction
PDL progressive diffuse leukoencephalopathy
PGE 2 prostaglandin E2
PKC protein kinase C
PMA phorbol myristate acetate
PML progressive multifocal leukoencephalopathy
PNS peripheral nervous system
QUIN quinolinic acid
RANTES regulated-upon-activation, normal T expressed and secreted
RCA ricinus communis agglutinin
RT room temperature
SDS sodium dodecyl sulfate
SGP-2 sulphated glycoprotein-2
SI syncytium inducing
SIV simian immunodeficiency virus
SSC saline sodium citrate
TAR trans-activation response element
TBS tris-buffered saline
TDT terminal deoxynucleotidyl transferase
TGF-β transforming growth factor-β
TNF tumour necrosis factor
TNFR tumour necrosis factor receptor
TUNEL TDT-mediated dUTP-fluorescein nick end labelling
VCAM-1  vascular cell adhesion molecule 1
WBC     white blood cell
CHAPTER ONE:
REVIEW OF THE LITERATURE
1.1 Introduction

"AIDS is, now, everywhere, part of the human condition and will remain so for a very long time to come. With approximately 6,000 people becoming infected every day, and close to 20 million people already infected and sick, AIDS is one of the major tragedies of our time (Dr. Peter Piot, Executive Director, Joint United Nations programme on HIV/AIDS, 1995)."

The major routes of transmission of acquired immunodeficiency syndrome (AIDS) are through anal and vaginal intercourse, sharing of contaminated needles, transfusion of blood and blood products and from mother to fetus (Weller, 1993).

During the past 15 years, two distinct human immunodeficiency virus (HIV) epidemics have developed and expanded in different regions of the world. The first, which has spread throughout the United States and Western Europe, has involved primarily homosexual men, intravenous drug users, and people exposed to infected blood and blood products, such as haemophiliacs. This epidemic has included a relatively small number of heterosexual individuals which are presently increasing in proportion as the absolute numbers of new infections in homosexual men and drug users decrease. The second type of epidemic is seen in Sub-Saharan Africa, where HIV has spread primarily among heterosexuals as a more conventional form of sexually transmitted disease, with relatively small numbers of individuals being infected by exposure to blood or by other routes. The smaller, weaker heterosexual epidemic in the West has, at least until now, been entirely due to HIV-1 subtype B. Evidence demonstrating phenotypic differences
between HIV-1 B (in West) and non-B subtypes (in Africa) for "heterosexual transmission efficiency" is further emphasized by the pattern of HIV-1 C and E epidemic which has recently appeared in Asia (western India and southeast Asia, especially northern Thailand). In a short period of time, these viruses have caused rampant heterosexual epidemics (Essex, 1995).

As soon as the epidemic appeared, it became obvious that the central nervous system (CNS) was frequently involved in HIV-1 infection (Snider et al., 1983) as shown by the number of severe and often fatal neurological infections experienced by AIDS sufferers. It was estimated that 70-80% of AIDS patients would sooner or later suffer from some neurological disorders that, in 10% of cases, represent the first or even the sole signs of AIDS (Harrison, 1993). Individuals involved range from those who are at the stage of seroconversion to those with AIDS-related complex (ARC) or during the fully developed AIDS syndrome. It led Shaw et al (1985) to consider HIV as a neurotropic organism and the brain as the reservoir of the virus. The true incidence of neurological disease in AIDS is difficult to assess although postmortem data indicated an incidence of over 80% (Kennedy, 1993).

During the early stages of the investigation, neuropathologists kept themselves busy describing and classifying the various brain lesions, all of which, for instance, cytomegalovirus (CMV) encephalitis, progressive multifocal leukoencephalopathy (PML) and lymphoma appeared to be opportunistic infections and a consequence of immunosuppression (Scaravilli et al., 1993).
Then, in 1985, it was observed that the brains of a number of patients, showing obvious clinical signs of dementia, formerly called AIDS dementia complex (ADC, Navia et al., 1986) and, now called the HIV-1-associated cognitive/motor complex (report of a working group of the American Academy of Neurology AIDS Task Force, 1991), displayed an unusual type of leukoencephalitis, characterised by hitherto unseen multinucleated giant cells (MGCs) in a context of microglial hyperplasia and macrophages.

Though it was suspected that this new entity could represent the specific HIV-1 induced disease, conventional neuropathology could not go beyond the stage of hypothesis. The isolation of the virus from the CNS as early as in 1985 (Ho et al) gave good circumstantial evidence, not the proof, of the direct involvement of the virus. The subsequent classification made by Levy et al (1988) took this into account and a specific HIV encephalitis was included into the lists of complications of HIV-1 infection.

The arrival on the scene of specific antibodies raised against a number of HIV antigens (p24, gp 120) represented an important step forward as they demonstrated widespread presence of the virus within the CNS. However, an additional problem arose: whereas in most other viral encephalitides the aetiological agent can be seen within neurons and glial cells, usually as an intranuclear inclusion, thus giving morphological evidence of why cells suffer and die, in AIDS the virus can be identified only within cells of the microglia / macrophage lineage although, more recently, Saito et al (1994) have also demonstrated nonproductive infection in astrocytes. However, virus cannot be found within nerve cells and there is no obvious cytopathic effect of HIV on
neuroectodermal cells in vivo. Another feature of HIV in the brain is that relatively small amounts of the organism can be visualised both by immunohistochemistry and in situ hybridisation methods.

The brain is affected, as shown by a nerve cell loss (Ketzler et al., 1990; Everall et al.; Wiley et al., 1991). As the theory of brain damage as a direct effect of the virus in producing CNS lesions seemed to lose ground, various other hypotheses were put forward at this point to try and explain this apparent contradiction.

Could it be possible that this hyperplasia of mesenchymal cells could be the expression of a type of immune activation of the CNS that, in turn, could be responsible for producing substances toxic to the CNS and, at the same time, perpetuate the reaction. Suggested pathogenic mechanisms include: (1) interaction of neurotoxins from HIV-infected macrophages (Giulian et al., 1990; Pulliam et al., 1991; Griffin et al., 1991; Heyes et al., 1991) or other glial cells (Vitkovic et al., 1990; 1991); (2) nerve cell damage by the HIV-1 envelope glycoprotein (gp) 120 (Lipton, 1992); (3) induction of cytokines by gp 120 interacting with microglia/macrophages.

Among the various culprits, cytokines were considered as the most likely candidates. These proteins are by no means specific to HIV infection and can be found in a variety of abnormal conditions such as multiple sclerosis (MS). Some cytokines are directly toxic to the CNS (Merrill et al., 1992). In HIV infection, both in vivo and in vitro, data point to the role of cytokines at a number of important stages of the disorders and, indeed, cytokines were found in the cerebrospinal fluid (CSF) of these patients (Gallo
et al., 1989; Griffin et al., 1991; Grimaldi et al., 1991). However, levels in the CSF may not correlate with actual levels in the CNS as, although CSF and the brain represent two interconnected compartments, they are not in equilibrium regarding solutes. The obvious conclusion is that a reliable amount of the levels of cytokines within the CNS could be derived only from an investigation of brain tissue itself.

It has been confirmed that the CNS was in a state of immune activation, as indicated by the expression by macrophages of the major histocompatibility complex (MHC) class II antigens and that cytokines were present within the brain tissue. Enhanced expression of MHC class II antigens has been demonstrated in a number of HIV infected astrocytes, microglia and monocytes (Peudenier et al., 1991; Ennas et al., 1992), and sensory and sympathetic ganglia in HIV-1 infected subjects (Esiri et al., 1993). Expression of these antigens has been correlated not only with the various pathological changes but also with HIV encephalitis (Achim et al., 1991a and b; Kennedy and Gaines, 1992). Once activated, MHC class II positive cells can induce production of toxic proteins such as cytokines (Molina et al., 1990) which have been detected in brain in HIV infection by Tyor et al (1992). Gelbard et al (1993) and Lynn and Wong (1995) related the presence of the cytokines to neuronal loss, which Petito and Roberts (1995), Adle-Biassette et al (1995) and Gelbard et al (1995) showed takesplace through apoptosis.

It is recognized that in most individuals the initial HIV-1 infection results in a latent or chronic infection, which, before and during eventual progression towards AIDS, is accompanied by a progressive and ultimately profound immunosuppression (Mikovits et al., 1992; Pomerantz et al., 1992). Initially, however, patients remain clinically
asymptomatic, as infection is associated with vigorous virus specific immune responses, including both neutralising antibodies and cytotoxic T lymphocytes (CTLs) (Walker, 1994).

Regarding the time and mode of entry into, and spread of HIV within, the CNS, the presence of the virus in cerebrospinal fluid has been reported at an early stage (Ho et al., 1985; Resnick et al., 1988). Furthermore, examination of brains of HIV-1 positive patients, who had died prior to developing AIDS, by Gray et al (1992) showed mild changes which were initially interpreted as nonspecific because there were no MGCs and no virus could be detected by immunohistochemistry. However, these authors could not find similar changes in a control group of HIV negative drug users. Subsequently, HIV-1 proviral DNA was demonstrated in the brain tissue of these individuals which also showed excess of microglia (Sinclair et al., 1992a; Sinclair et al., 1994). These data taken together suggested that the status of immune activation previously reported by Tyor et al (1992) in AIDS may predate the AIDS stage (Sinclair et al., 1994).

In the next pages the following topics are reviewed and discussed: 1) HIV virus, 2) its latency, 3) the pathological changes and immune response of the CNS and 4) the possible pathogenetic mechanisms involved in the lesions in the CNS.

1.2 HIV virus

The acquired immune deficiency syndrome (AIDS), first recognised as a clinical syndrome by Gottlieb et al in 1981, included initially opportunistic infections and/or
neoplasia associated with immunodeficiency. Following the definition of AIDS with its characteristic loss of T helper cells, investigation into its aetiology has focused on a retrovirus that was formerly named lymphadenopathy-associated virus (LAV) (Barré-Sinoussi et al., 1983), Human T-cell lymphotropic virus type III (HTLV-III) (Popovic et al., 1984) or AIDS associated retrovirus (ARV) (Levy et al., 1984). It is now known as human immunodeficiency virus (HIV)-1 (Coffin et al., 1986).

Retroviruses are a family of enveloped RNA viruses which contain a RNA-dependent DNA polymerase (reverse transcriptase). Intermediate DNA, which can be permanently integrated within the host genome (Clements, 1985) is used in the infected cell to transcribe virion and messenger RNA.

Retroviruses can be subdivided into two types: oncogenic and non-oncogenic (Wong-Staal and Gallo, 1985). The lentiviruses (Lentiviridae) are a distinct subfamily of non-oncogenic retroviruses, which produce cellular damage or lysis rather than transformation. They may cause persistent infections and chronic diseases in their natural hosts. Typically, there is a long incubation period and progression to the disease is often gradual, hence the term slow virus or lentivirus. At present HIV-1 and -2 are the only pathogenic lentiviruses that have been identified in man. Other viruses of this group include simian immunodeficiency virus (SIV), feline immunodeficiency virus (FIV) and the "slow viruses" visna/maedi, equine infectious anaemia virus and caprine arthritis/encephalitis virus (Shaunak and Weber, 1992).

HIV-1 is slightly over 100nm in diameter. On electron microscopy it has a characteristic
dense, cylindrical nucleoid containing core proteins, two copies of single plus(+)
stranded genomic RNA, and reverse transcriptase, surrounded by a lipid envelope. The
RNA-dependent DNA polymerase, the *sine qua non* of retroviruses, is present in the
virion in association with the RNA genome (Shaw et al., 1988; Levy, 1994) (see Fig.
1.1).

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**Fig 1.1** Structure of HIV-1. See text for description. After Levy (1994).
The HIV genome is 9-10kb long and contains three principal structural genes, \textit{gag} (encoding core proteins p24 and p18), \textit{pol} (encoding for the reverse transcriptase and other enzymes) and \textit{env} (encoding for the spike (gp120) and transmembrane (gp41) glycoproteins), flanked at both the 5' and the 3' ends by a non coding long terminal repeat (LTR) region, which contains the promoter and regulatory gene product acceptor sequences (Fig. 1.2).

Other non-structural genes which encode for proteins have been identified in the HIV genome. Their function is to regulate viral replication. At least six regulatory proteins have been identified which include \textit{tat}, \textit{vpr}, \textit{vif}, \textit{vpu}, \textit{env} and \textit{nef}. Regulatory proteins affect the production not only of structural gene products, but also of regulatory gene products.

region with the interaction of the virion surface glycoprotein, gp120, with the major cell receptor (CD4), on a subset of lymphocytes and macrophages. A secondary glycoprotein, the transmembrane glycoprotein, gp41, is involved in penetration of the virus and also mediates cell-cell fusion leading to multistructured systems.

Fig. 1.2 Genomic map and protein products of HIV-1. See text for details.
In analysis of HIV genomes, the *gag* and *pol* genes have proved more stable than *env* which shows considerable variation, with up to 26 % variance between geographically distinct isolates (Wain-Hobson, 1989). The genetic diversity seen in HIV isolates, where no two viruses have identical sequences, is higher than for any other known retrovirus (Weber, 1993). Indeed, numerous variant viral forms could coexist over the time within the same patient, and it is known that "isolates" of HIV-1 actually consist of complex mixtures of genotypically distinct, albeit related, viruses (Shaw et al., 1988).

Other non-structural genes which encode for proteins have been identified in the HIV genome. Their function is to regulate viral replication. At least six regulatory proteins have been identified which included *tat*, *nef*, *rev*, *vpu*, *vif* and *vpr*. Regulatory proteins affect the production not only of structural gene proteins, but also of regulatory gene proteins, including themselves. This level of organizational complexity had not been recognized previously or ever expected in the small genomes of the animal retroviruses.

As AIDS is associated with decline of CD4 positive T lymphocytes (T helper cells) in the peripheral blood, it was postulated that the receptor for entry of HIV might be the CD4 surface molecule itself, which characterizes, and is found on, the surface of T helper cells (Dalgleish et al., 1984; Klatzmann et al., 1984). The life cycle of HIV begins with the interaction of the virion surface glycoprotein, gp120, with the major cell receptor (CD4), on a subset of lymphocytes and macrophages. A secondary glycoprotein, the transmembrane glycoprotein, gp41, is involved in penetration of the virus and also mediates cell-cell fusion leading to multinucleated syncytia.
HIV-1 can be prevented from entering CD4 positive cells by (a) recombinant soluble CD4 (Fisher et al., 1988; Hussey et al., 1988; Deen et al., 1988); (b) HIV env protein (Lasky et al., 1986) and (c) monoclonal antibodies (Dalgleish et al., 1984; Klatzmann et al., 1984).

Most human cells which express CD4 naturally or by transfection can be infected by HIV. However, cells from other species are not infected by HIV, even when human CD4 is present on the cell surface. This suggests the possible requirement of additional molecule(s) that allow the entry of HIV into the cell (Weiss, 1993). Several molecules have been considered as potential accessory factors in HIV infection, including MHC class I (Corbeau et al., 1991) and/or class II (Mann et al., 1988); galactocerebroside (Harouse et al., 1991) and the cell surface peptidase CD26 (Callebaut et al., 1993). At present it is believed that after binding to CD4, HIV-1 requires secondary molecules to effect fusion between the virus envelope and the cell membrane (Clapham et al., 1993).

Furthermore, recent evidence that cells lacking CD4 are susceptible to HIV-1 infection in vivo and in vitro (Castro et al., 1988; Chehimi et al., 1991), raises the possibility that HIV-1 infection could occur also by a mechanism independent from CD4 (Weber et al., 1989; Kunsch et al., 1989).

There is evidence suggesting that the cell types predominantly permissive of productive HIV-1 infection in the CNS are macrophages and microglia (Kure et al., 1990; 1991). More recently, using double-label immunocytochemistry (ICC) Hatch et al (1994) demonstrated infection of astrocytes in human fetal organotypic cultures. In 1994 Saito
et al and Tornatore et al (1994a) confirmed, by in situ hybridization alone or combined
with immunohistochemistry, that glial cells in brain tissue harbour a nonproductive
infection. Furthermore, using the same techniques Ranki et al (1995) found that in adults
abundant expression of HIV nef and rev proteins in astrocytes in vivo is associated with
dementia. The details will be described in 1.5.2.

Following the binding and fusion of the virus with target cells, the core nucleoprotein
complex containing the viral RNA genome enters the cellular cytoplasmic space where
the virion-associated reverse transcriptase synthesises a linear, double-stranded DNA
copy of the viral genome. Some of this DNA migrates to the nucleus where integration
can occur by co-linear insertion of the ends of the viral LTRs into the chromosomal
DNA. This integrated DNA copy of the retrovirus genome is called a provirus (O'Brien,
1994). In host cells, HIV DNA exists in three forms: unintegrated linear, unintegrated
circular forms, and the integrated form. High levels of unintegrated forms of retroviral
DNA are found in brains of AIDS patients (Pang et al., 1990) and are often associated
with superinfection and accompanying cytopathic effects. The unintegrated DNA
probably exists as the "housekeeping" repertoire of the viral genome and may be
released into the circulation with the newly formed mature virion particles during the
course of replication and release of the virus (Nandi and Banerjee, 1993). Integrated
proviral DNA becomes part of the host DNA complement, replicating as cells divide.
It is this integrative property which assures the survival of the viral genome until the
death of the host.

The next phase of the infection involves expression of integrated retroviral genes leading
to the formation of progeny viruses. Host factors synthesize and process viral RNA, and translate viral mRNA on host ribosomes in order to synthesize viral proteins. Signals for initiation of transcription of viral genes and enhancer sequences are located in the viral LTR. The host regulatory proteins involved include the cellular transcription factors Sp1 and nuclear factor-κB (NF-κB). Viral proteins are assembled following translation of the processed viral mRNA and cleavage of peptide precursors by protease. Intact infective progeny virions are released by budding through the cell membrane and can infect other permissive host cells (O'Brien, 1994). Viral protease is required for release to occur with maximum efficiency (Kaplan et al., 1994).

1.3 HIV latency (pre-AIDS)

Through the late 1980s, it was thought that most of the HIV present in asymptomatic (report from Centres of Disease Control and Prevention, 1993) individuals was latent. However, viral replication has been demonstrated throughout the course of asymptomatic and symptomatic disease by Pantaleo et al (1993a); Schnittman et al (1991); Delord et al (1992) and Piatak et al (1993). Pantaleo et al (1993a) used PCR to show that the product of this active and progressive infection of HIV accumulates in lymphoid organs and replicates actively even when there is a low viral burden and replication in peripheral blood mononuclear cells (PBMC). In early and intermediate stages of disease, there are between 5 and 10 times more infected cells in the lymphoid tissue than in PBMC whereas in advanced stages the viral burden is the same in both PBMC and lymphoid tissue. Investigations by Schmid et al (1994) demonstrated that HIV proviral DNA was not only present in CSF at all stages from asymptomatic term to overt AIDS,
but also that it was significantly higher in CSF than in blood. This work indicated that 1) there is a substantial penetration of HIV-1 into the CNS/CSF in both systemic and neurological asymptomatic disease and 2) that CNS/CSF harbour more HIV-1 than blood.

HIV-1 is characterized by variability of genome, change of tropism and phenotypes; all these features correlate with the stage of the disease. It is generally agreed that the predominant HIV-1 strains, during the early asymptomatic stage, are macrophage-monocytotropic (Schuitemaker et al., 1992) non-syncytium inducing (NSI) phenotypes (Bozzette et al., 1993; Boucher et al., 1992).

Genetic variation is the hallmark of infections by lentiviruses in general and HIV-1,2 in particular. The diversity of sequence is termed 'quasispecies' (Wain-Hobson, 1989) and is seen most in hypervariable regions (V1 to V5) of the HIV-1 gp120. The virus evades immune pressure by the continuous production of new mutations resistant to current immunological attack (Nowak, 1992). Mutations are usually located in key positions of cytotoxic T cell recognition in the immunodominant loop (Simmonds et al., 1990a). Mutations could escape neutralising antibodies (Yoshiyama et al., 1994) and may become resistant to multiple protease inhibitors which are potential therapeutic agents for HIV infection (Condra et al., 1995). The V3 region or gp41 amino terminus has the capacity to mediate syncytial formation and determines the viral tropism (Bergeron et al., 1992; Innocenti-Francillard et al., 1994; Liu et al., 1990; Sharpless et al., 1992a; Henkel et al., 1995). A single point mutation at the neutralisation epitope in the env gene could modify cellular tropism (Takeuchi et al., 1991). The variations showed tissue-specific
evolution (Epstein et al., 1991; Ball et al., 1994; Korber et al., 1994). However, Keys et al (1993) found that V3 sequences of paired HIV-1 isolates from blood and CSF cluster according to host and show variation related to the clinical stage of disease, whereas particular amino acid sequences related to tissue specificity were not identified. This finding has been confirmed by Kasper et al (1993) who, in a follow-up study of 7 infected haemophiliacs, have demonstrated that the diversification of HIV-1 strains, after infection from a unique source, is associated with the stages of infection. At the time of seroconversion high genetic homogeneity (96.5% - 100%) of HIV-1 is maintained. In the case of a patient who showed the highest decrease of CD4 positive cells, moderate genetic diversification of the virus was associated with a changed cellular tropism and the virus that originally could not be cultivated could then be isolated as a low cytopathogenic agent.

Investigations of the biological phenotype and tropism of clones of HIV-1 at different stages of infection suggested an association between progression of the disease and a viral shift from monocytotropism to T-cell-tropism (Schuitemaker et al., 1992) and conversion from non-syncytium to a syncytium inducing (SI) form (Richman and Bozzette, 1994). SI predicts decline of CD4 cells and enhanced HIV production (Kozal et al., 1994; Richman and Bozzette, 1994). In cultured cell lines, all monocytotropic variants were NSI and in vivo it has been observed that there was no progression to AIDS without evolution to SI variants (Schuitemaker et al., 1991). On the other hand, Valentin et al (1994) found that isolates with SI phenotype did not differ from NSI isolates in the ability to replicate in monocytic-derived macrophages in vitro. In another group of patients (3 pre-AIDS and 4 AIDS) Donaldson et al (1994a) found that 6 were
exclusively and one was predominantly infected by variants with NSI/macrophage-tropic phenotype, irrespective of the degree of disease progression. Furthermore, Michael et al (1993) demonstrated that the proviral genotypes from PBMC of an infected patient were differentially represented in expressed sequences. The proviral group (11 clones) was more heterogeneous than the cDNA group (9 clones). Deduced amino acid sequences showed that they were distinct in the position of N-linked glycosylation sites, and in the V3 loop. This may have implications for the pathogenesis of the disease.

Viral RNA transcripts are produced throughout the course of the infection and this production has also been confirmed in individuals with 7 or more years of non-progressive HIV infection (Pantaleo et al, 1995). A variety of intracellular mechanisms determines the levels of expression of viral regulatory and accessory genes. The degree of repression of these genes determines whether or not there is a transition to a progressive stage, i.e. AIDS. This has been demonstrated by tissue cultured latent HIV in lymphoid and monocytic cell lines. The induction of viral production was associated with a shift in HIV-1 specific mRNA from singly and multiply spliced mRNAs to the production of full length HIV-1 RNA (Laughlin et al., 1993). In asymptomatic individuals the level of multiply spliced viral RNA was higher than unspliced form in the PBMC, a condition termed "blocked early-stage latency" (Seshamma et al., 1992). The replicating ability of the virus could be associated with its phenotype. Connor and Ho (1994) showed that clones isolated just after seroconversion were slowly replicating and NSI. However, soon thereafter, SI variants appeared with rapid replication in both macrophages and CD4 positive lymphocytes. Moreover, it was suggested that the proviral copy number and RNA level could be early indicators of progression in HIV-1
infection (Verhofstede et al., 1994).

At present there is no clear understanding of the mechanisms of emergence of progression of disease from pre-AIDS to overt AIDS. Adams et al (1994) and Balboni et al (1993) showed that tat may stimulate the provirus from latency in cultured cells and that a tat mutant may inhibit the process. The expression of HIV-1 RNA by chronically infected cell lines indicated the existence of host cell specific transcriptional and post-transcriptional mechanisms of latency (Butera et al., 1994). It is possible that the genetic determinants for the changes from NSI to SI and monocyte tropism to T cell tropism may be entirely or partially responsible for the transition from latency to overt disease. In pre-AIDS, HIV harboured within the CNS is predominantly NSI and this might explain the rarity or absence of multinucleated giant cell in vivo during this stage.

1.4 HIV-1 infection of the central nervous system (CNS)

It has been reported that 40 to 50% of AIDS patients, both adults and children, have neurological symptoms which, in 10% of the cases, may be the first manifestation of the disease (Bredesen and Messing, 1983). Pathological study of up to 80% of post mortems of HIV-1 positive patients show some morphological abnormalities of the nervous system (Guarda et al., 1984; Lang et al., 1989; Anders et al., 1986; Petito et al., 1986; Budka et al., 1987).

Neurological complications appear to be due either to direct involvement by HIV and/or to the effect of opportunistic infection and malignancies (lymphomas). Neurological
disorders can be seen at any stage of the infection and some of them do not appear to be associated with any opportunistic agent. Some of these such as acute encephalopathy (Carne et al., 1985) or acute Guillian-Barré syndrome (Cornblath et al., 1987) occur early in the disease, even as early as at the time of seroconversion. Other episodes, for example aseptic meningitis with chronic headache, acute or chronic demyelinating polyneuritis, or mononeuritis multiplex have also been reported in pre-AIDS. However, the most devastating diseases are seen in patients with AIDS. These include opportunistic infections, lymphomas and the HIV-1-associated cognitive/motor complex. The last complication is characterized by cognitive, motor and behavioural dysfunction and is seen in 15-20% (30/186) of patients with fully developed AIDS (McArthur, 1987). The overall prevalence of the HIV-1-associated cognitive/motor complex in adult AIDS patients is 7.3 -11.3% (Janssen et al., 1992), but up to 30 -60% of children with AIDS manifest an analogous progressive encephalopathy (Epstein et al., 1986; Belman et al., 1988; Mintz, 1994).

Whereas there is consensus that CNS involvement in HIV infection is a late event, how often and how early the nervous system is infected during the early asymptomatic stages is still an unanswered question. Reports on abnormal changes in the CNS in HIV-1 positive pre-AIDS are reviewed briefly under three headings: evidence from neuropsychiatry and neurophysiology; analysis of CSF; and investigation in the brain.

1. Neuropsychiatry and neurophysiology

While some authors found no differences between HIV-1 positive pre-AIDS patients and
healthy controls (Janssen et al., 1989; McArthur et al., 1989; Newton et al., 1994; Connolly et al., 1994; Manji et al., 1994), others found notable deficits in nonverbal (Van-Gorp et al., 1989) or verbal memory (Wilkie et al., 1990; Peavy et al., 1994), sleep disturbances (Norman et al., 1992), cognitive (Coburn et al., 1992; Villa et al., 1993) and behavioural impairment (Stern et al., 1992).

Verbal memory was assessed by Peavy et al (1994) using the California Verbal Learning Test (CVLT) on 31 patients with symptomatic AIDS, 94 asymptomatic (pre-AIDS) HIV-1 positive and 40 HIV-1 negative control subjects. AIDS subjects were significantly impaired compared with HIV-1 negative controls. The performance of the pre-AIDS subjects fell on almost every CVLT measure. The profile by a discriminant function analysis (DFA) exhibited by the subgroup of impaired HIV-1 subjects (both AIDS and pre-AIDS) was similar to that of patients with Huntington's disease (HD), a prototype of subcortical dementia. The findings are consistent with reports of predominantly subcortical neuropathological changes associated with HIV infection. Bono et al (1990) found that, among 86 pre-AIDS cases, 25 to 50% had neuropsychological and neurophysiological abnormalities compared with HIV-1 negative controls. In addition, Villa et al (1993) reported that 19/36 (52.8%) AIDS and 10/33 (30.3%) asymptomatic HIV seropositive subjects had cognitive impairment, compared with 3.9% of HIV-1 negative controls. Furthermore, low values of CD4 positive cells, CD4/CD8 ratios and high titres of p24 antigen in the blood were found in subjects with cognitive impairment, especially in the pre-AIDS group.

Data reported by Iragui et al (1994) supported the conclusion that asymptomatic HIV
carriers manifest subclinical neurological impairment of central somatosensory function; they also concluded that the neurological impairment increases with disease progression to involve peripheral nerves and visual system. They found that abnormal rates for one or more evoked potentials were present in 18/129 (14%) asymptomatic carriers and 13/30 (43%) subjects with ARC/AIDS as compared with 1/62 (2%) seronegative controls. More recently, White et al (1995) demonstrated that asymptomatic HIV-1 positive subjects (n=23) had impaired cognitive-motor performances characterized by reduced accuracy and efficiency. Among them, those with CD4 counts >400 X 10^6/l (n=11) showed statistically significant increase in slow-wave sleep which is believed to be related to an increased immune activity (Moldofsky et al., 1986) when compared with controls (p <0.03). Those with CD4 counts <400 X 10^6/l had sleep parameters similar to controls. These data demonstrate that immune activation and cognitive-motor impairment are impaired in subjects who are at the early pre-AIDS stage of infection.

In summary, it has been suggested that memory, sleep disturbance, cognitive-motor functions are impaired even at this early stage of HIV-1 infection.

2. Detection of HIV-1 DNA, virus and other abnormalities in CSF

CSF represents the extracellular fluid of the brain (Glees, 1988). Early HIV-1 infection of the CNS has been demonstrated by numerous studies of CSF (Elovaara et al., 1991; Price et al., 1988). In 1985 Ho et al isolated HIV from CSF not only in patients with AIDS but also in one during acute aseptic meningitis with seroconversion. These results were confirmed by others (Chiodi et al., 1986; Hollander and Levy,
The identification of CSF abnormalities including pleiocytosis, elevated IgG and oligoclonal bands in individuals known to have become infected during the previous 6 to 24 months supports the hypothesis that the nervous system is an early target for HIV (McArthur et al., 1988). CSF neopterin and β2-microglobulin levels, which reflect activation of the cellular immune response in CNS, were increased at all stages of HIV infection (Bogner et al., 1992).

Sönnerborg et al (1991) detected HIV in CSF from 6/10 and 1/10 asymptomatic HIV-1 seropositive individuals by PCR and virus culture technique respectively. Schmid et al (1994) found that HIV-1 DNA was positive in blood in 30/34 (88%) and in CSF in 22/26 (85%) pre-AIDS individuals. In ARC 44/46 (96%) samples of blood and 30/31 (97%) CSF and, in AIDS, 7/7 (100%) samples of blood and 5/6 (83%) CSF were HIV-1 DNA positive. Moreover, using quantitative PCR Schmid et al (1994) demonstrated that the proviral load was significantly higher in CSF than in blood at all stages in seropositive patients (p=0.0001).

3. Investigation in the brain

In addition to the neuropathological changes described in 1.6 Sinclair et al (1992a; 1994) demonstrated by PCR the presence of HIV proviral DNA in brain of 2/8 HIV-1 positive, pre-AIDS patients, in whom there was also an increase of density of astrocytes and microglial cells. This led these authors to suggest that the status of immune activation,
existed before the manifestation of fully developed AIDS (see 1.9).

Davis et al (1992) reported that HIV-1 was isolated from blood and brain tissue 14 and 15 days respectively from a man who had accidentally received an inoculum of HIV-1 infected white blood cells. The brain showed mild perivascular cuffing and mild lymphocytic meningitis, features similar to those described by Gray et al (1992) in HIV-1 positive pre-AIDS individuals; however, there was no evidence of multinucleated giant cells. HIV-1 DNA was present in several brain areas, but not in several other organs (gut, liver, heart, lung and spleen). This is coincident with the observation by Shaw et al (1985) using blot hybridization from an AIDS patient that brain was by far the organ most heavily infected with HIV-1 compared with spleen, lymph node, liver and lung. Taken together, these data suggest that infection of brain/CSF by HIV-1 might not only take place early but also be more severe. On the other hand, observations by Donaldson et al (1994b) show that in both AIDS (5 cases) and pre-AIDS (3 cases) brains have lower level of infection than lymph node and spleen. These authors as well as Bell et al (1993) considered positive results obtained from pre-AIDS cases as due to contamination by blood in the organ as they were below 1/10 of the value in PBNC, although in their paper 1 case, among 13 pre-AIDS patients to whom PCR was applied, showed 27 copies $/10^6$ cells without paired data from PBNC; in addition among the other 14 pre-AIDS cases, for which no quantitative data of HIV-1 in both brain and PBNC were presented, one showed high level of p24 in serum with MGC in brain.

1.5 Role of macrophages, microglial cells and astrocytes in the HIV-1 infection of the brain
The CD4 positive T lymphocytes are the major reservoir of HIV in peripheral blood compartment and in the lymphoid tissues, whereas in nonlymphoid organs such as brain and the lung, local infection is predominantly sustained by mononuclear phagocytes (Poli et al., 1993). HIV-1 infection in the CNS has provoked a flurry of interests in the functions of microglia/macrophages and astrocytes and in their roles in the pathogenesis of HIV-1. It has been known for a long time that microglia and macrophages are both reservoirs and host cells of HIV-1 in the brain. Though there is evidence that under certain circumstances neurons and glial cells may be infected in vitro, there is as yet no evidence that these are important targets for HIV infection in vivo. Histological changes including presence of MGCs, white matter pallor and microglial nodules, may be relatively mild even in patients with severe cognitive dysfunction and thus may not provide a satisfying explanation for the clinical picture (Glass et al., 1993). These observations suggest that HIV-1 does not act directly on the neural cells and that alternative pathways exist in which microglia and macrophages may be involved. Utilizing in situ RT/PCR and immunohistochemistry methods Wesselingh et al (1994) found that a large number of macrophages are expressing TNF-α but only small number are infected with HIV in brains of AIDS patients with dementia. On the other hand, IL-4 was not detectable in these brains while it was present in most control tissues and in patients with AIDS but without dementia, suggesting that the lack of normal downregulatory factors (IL-4, IL-10) might be an important contributor to the increased activation of macrophages and the increased production of neurotoxins, such as TNF-α (Tyor et al., 1995). HIV-1 infected macrophages are both perpetrators and amplifiers for neurotoxic activities.
Astrocytes are involved in many activities that are critical to brain function including: neuronal migration; neurite outgrowth; maintenance of the blood-brain barrier; regulation of water, ion and amino acid neurotransmitter metabolism; energy and nutrient support of neurons; modulation of immune/inflammatory responses; phagocytic function. In addition, specific enzyme systems enable astrocytes to metabolise ammonia, glutamate, free radicals, and metals, thus protecting the brain from the toxicity of these agents (Norenberg, 1994). Moreover, recent data have demonstrated that astrocytes are also directly infected by HIV-1 (see pages 19 and 45 to 47).

1.5.1 Microglial cells and macrophages

Microglia and macrophages are cells of the mononuclear-phagocyte lineage. Intrinsic microglia and tissue macrophages within the CNS are derived from precursor cells, which circulate in the blood as monocytes, and migrate through the vessel walls into the CNS early in gestation (Ling and Wong, 1993). They are apparently randomly distributed in normal host tissues, in which they make up about 13% of normal human white matter glial cells (Hayes et al., 1987), and appear in larger numbers, under chemotactic stimuli, in inflammatory lesions (Gordon, 1986). Some of them become perivascular and subarachnoidal or are located within the choroid plexus. A variety of pathological insults result in microglia activation, as well as a complex of morphological and biochemical alterations (Mrak et al., 1995). 'Activation' is defined as the process by which macrophages and monocytes modify their phenotype and acquire new functional activities in response to environmental stimulation (Varesio et al., 1995). A microglial cell response consists of an increase in size and in number of cells and in length of their
processes (Esiri, 1993). Sometimes ramified microglia are referred to as "resting" microglia and ameboid (rod-shaped) microglia are referred to as "activated" microglia. Ameboid microglia are mobile and adhesive cells (Dickson et al., 1993). A potential marker for microglial activation is the expression of major histocompatibility complex (MHC) class II antigens. Ferritin has also been associated with the reactive microglia and HIV-1 RNA (Yoshioka et al., 1992). Macrophages can derive both from circulating monocytes as well as activated microglia. It is unclear to what extent intrinsic microglia contribute to the perivascular macrophage production and whether perivascular cells too can enter the parenchyma and differentiate into ramified intrinsic microglia in the adult (for review, see Dickson et al., 1994). Both resting and reactive microglia as well as macrophages show histochemical evidence of strong binding of the lectin *Ricinus Communis agglutinin* (RCA)-1 (Murabe and Sano, 1982; Mannoji et al., 1986).

Activated microglia have been demonstrated in HIV-1 infected brains in both grey and white matter and the activation was not correlated with the presence of HIV-1 antigen in the brain region. These activated microglial cells could be those, among other cells, producing cytotoxic factors which, in turn, cause brain damage (Weis et al., 1994).

There are two main ways by which activated microglia and macrophages can act as cytotoxic cells in the brain.

The first involves direct cell-to-cell contact as demonstrated by coculture of human embryonic neurons, astrocytes and HIV-1 infected U937 monocyctic cells (Tardieu et al., 1992). HIV-1 infected monocyctic cells can induce toxicity on neurons, as well as on
astrocytes after cell-to-cell contact (Genis et al., 1992; Epstein and Gendelman, 1993). Cocultures of HIV-1 infected monocytes and astrocytes release high levels of cytokines and arachidonate metabolites leading to neuronal toxicity. However, when HIV-1 infected monocytes were cocultured with other cells (but not astrocytes), or when HIV-1 infected monocyte fluids or viral particles instead of HIV-1 infected monocytes were used on astrocytes, these cells failed to induce cytokines and neuronotoxins, suggesting that the neuronal toxicity associated with HIV CNS disorders is mediated, in part, through cytokines and arachidonic acid metabolites, produced during cell-to-cell interaction between HIV-1 infected brain monocyte/macrophages and astrocytes (Genis et al., 1992). The role of CR3 (on monocytes) and intercellular adhesion molecule (ICAM)-1 (on neurons and astrocytes), proteins known to be ligands for each other, has been investigated. It has been confirmed that adhesion between monocytes and neurons or astrocytes was 80% inhibited by mAbs against the CR3 determinant or against ICAM-1, but not others [anti-vascular cell adhesion molecule 1 (VCAM-1), anti-CD44, anti-endothelial leukocyte adhesion molecule 1 (ELAM-1)]. The expression on neurons of ICAM-1, on astrocytes of ICAM-1 and VCAM-1 was up-regulated by exogenous TNF-α, IL-1α and IFN-gamma. The adhesion between monocytes and CNS cells is a further inducer of IL-1α and TNF-α which were greatly increased after the adhesion (Héry et al., 1995).

The second way is that microglia and macrophages release potentially cytotoxic substances (Banati et al., 1993). The major secretory products of microglial cells are free oxygen radicals, proteolytic enzymes, arachidonic acid metabolites and inflammatory cytokines (IL-1, IL-6, TNF-α) (see 1.10.1 and 1.10.2). In particular,
colony stimulating factors (CSFs) seem to regulate both the proliferation of microglia (Raivich et al., 1991) as well as their differentiation into macrophages (Suzumura et al., 1990). Stanley et al (1994) found that in HIV-infected individuals microglia was abundant, activated and intensely immunoreactive for IL-1α, which in turn increases the synthesis and processing of β-amyloid precursor protein (β-APP) and promotes proliferation and activation of astroglia. In addition, astrogliosis was accompanied with increased expression of IL-1α and S100β (a small soluble calcium-binding protein that is synthesized and released by astroglia). Overexpression of IL-1α and S100β may then induce gliosis, growth of dystrophic neurites and calcium-mediated neuronal cell loss in AIDS.

The mechanism of microglial infection is unclear because microglia have low levels of CD4 antigen expression (Funke et al., 1987). On the other hand, CD4 can be detected easily in rodent microglia, where cellular activation is associated with up-regulation of CD4 (Perry and Gordon, 1987). Possible mechanisms could involve non CD4 mediated binding of virus to target cell membranes (Harouse et al., 1991) such as galactosyl ceramide or a derivative (Bhat et al., 1991), or antibody mediated uptake, as microglia can express immunoglobulin Fc receptors (Takeda et al., 1988). As already mentioned (see page 27), two groups have recently reported that entry of HIV-1 into target cells requires cell-surface CD4 and additional host cell cofactors. The principal cofactor for entry of primary macrophage-tropic strains of HIV-1 has been identified to be CC-CKR-5, a receptor for the β-chemokines regulated-upon-activation, normal T expressed and secreted (RANTES), macrophage inflammatory protein (MIP)-1α and MIP-1β (Deng et al.; Dragic et al.; 1996). Elevated expression of MIP-1α and MIP-1β mRNA has been
demonstrated in brain of HIV-1 infected patients by RT-PCR and RT-\textit{in situ} PCR (Schmidtmayerova et al., 1996).

Changes in the microglial and macrophage population due to HIV-1 infection have been examined in HIV-1 infected individuals before the onset of AIDS. Almost all such cases show some degree of microglial and macrophage alteration and some show well-defined nodules or clusters of reactive microglial cells (Esiri et al., 1989) (see 1.6.2). Another feature in the brain of these cases is the presence of sparse cuffs of perivascular lymphocytes and macrophages, surrounding veins and in the leptomeninges (Esiri, 1993).

1.5.2 Astrocytes

Another response to brain injury is proliferation of astrocytes. Astrocytes are important in maintaining normal levels of extracellular potassium, ammonium, and glutamate and thus were attributed a neuroprotective function (Barres, 1991; Hansson, 1988). Astrocytes are the most numerous cellular element in brain. They outnumber neurons by ten to one and occupy about one-third of the volume in the cerebral cortex (Norenberg, 1994). Astrocytes contain receptors to most neurotransmitters and neuropeptides (Murphy and Pearce, 1987) which provide key mechanisms for intercellular communication. Their loss could lead to a secondary degeneration of neurons or their processes (Petito and Roberts, 1995). Astrocytes, like microglia, become activated in response to various pathological insults. This activation is marked by hypertrophy and results in expression of structural protein (GFAP), adhesion molecules, antigen presenting capabilities including MHC antigens and cytokines (Mrak
et al., 1995). The GFAP gene which encodes an intermediate filament cytoskeletal protein (Balcarek and Cowan, 1985) is expressed predominantly in astrocytes (Bignami et al., 1972) and is widely used as astrocyte marker. HIV-1 envelop gp120 causes astrocytic, but not neuronal, alteration and/or death as well as decreased expression of GFAP leading to the hypothesis that AIDS dementia may partially involve a perturbation of astrocyte function, which further impairs neuronal function (Pulliam et al., 1993).

Gliosis, the most frequent and important cellular reaction to brain insults, has been demonstrated to correlate with the increase in size of astrocytes in white matter rather than either with the density of astrocytes or the intensity of GFAP staining (da-Cunha et al., 1993b). IL-1β-activated astrocytes reveal a marked decrease of mRNA and protein of GFAP which was accompanied by the change in cell shape in vitro. Activated astrocytes displayed intense GFAP and vimentin immunoreactivity in the small perikarya and processes. In contrast, the large, flat astrocytes in control culture showed diffuse pale immunoreactivity of GFAP and vimentin. Observation by Liu et al (1994) on appearance of GFAP in activated astrocytes and astrocytes in control culture suggest that increase in immunoreactivity was related to factors such as redistribution of epitope, rather than increase in total protein content. Ammonia has been found to reduce stabilisation of GFAP mRNA by up to 85% without inhibiting total RNA synthesis (Neary et al., 1994). IL-1β stimulates acquisition of the GD3 positive phenotype of human fetal astrocytes and inhibits cell division (Lee et al., 1995).

It has been customary to date, to assume that the only cells harbouring HIV in the CNS were those of microglial and macrophages lineage. HIV has been shown to be present
in MGC, blood-derived monocytes/macrophages, microvascular endothelial cells, and in resident microglial cells (Koenig et al., 1986; Wiley et al., 1986; Kure et al., 1990; Moses and Nelson, 1994; Bagasra et al., 1996). Astrocyte infection, either in vivo or in vitro, has only rarely been reported (Wiley et al., 1986; Cheng-Meyer et al., 1987; Dewhurst et al., 1987; Sharpless et al., 1992b). More recently, however, Tornatore et al (1994a) and Saito et al (1994) have reported nef expression by astrocytes in paediatric AIDS patients (4/12 and 2/6, respectively). The latter authors found nef mRNA and protein in up to 20% of astrocytes. In addition, Ranki et al (1995) demonstrated nef protein in astrocytes in 7/14 adult AIDS patients and that expression of HIV nef and rev proteins is associated with dementia (6/7 with nef positive astrocytes had suffered from moderate to severe dementia).

Tornatore et al (1991) found that astroglial cells transfected with HIV-1 genome rapidly transcribed the viral genome producing high levels of p24 protein and infectious virions. Thereafter this expression progressively diminished and a persistent phase of infection developed during which neither virus nor viral p24 protein could be demonstrated. In addition, the persistently infected glial cells could be stimulated to produce viral p24 protein if either TNF-α or IL-1β was added to the medium, suggesting that astrocytes may serve as an undetected reservoir for HIV-1 and disseminate the virus to other susceptible cells in the brain upon being triggered by some cellular or biochemical signal. Furthermore, by applying regulatory protein probes Tornatore et al (1994b) demonstrated a shift from transcripts coding for both structural and regulatory proteins to those coding only for the latter, suggesting that rev function (to stabilize the longer, structural protein-encoding transcripts) is impaired (Conant et al., 1994). Moreover,
work by Nath et al (1995) has shown that human fetal astrocytes can be infected by HIV although only the lymphocytotropic (L-tropic) variants [but not the monocytotropic (M-tropic)] is capable of doing so; similarly, McKnight et al (1995) showed that when a laboratory isolate of HIV-1 is selected to grow in astrocytes, it loses its ability to grow in macrophages. As the L-tropic variants increase proportionally over the M-tropic with the progression of the infection (Schuitemaker et al., 1992) it could be postulated that invasion of astrocytes could be regarded as a sign that the illness is entering the final (AIDS) stage. On the other hand, the results of Hatch et al (1994) demonstrated that both L- and M-tropic isolates infect microglia and astrocytes in human fetal organotypic cultures. It is not known whether during the pre-AIDS stage glial cells are infected.

Astrocyte infection may lead to neuronal dysfunction through loss of supporting growth factors, excitotoxicity due to deregulation of neurotransmitter reuptake and loosening of the blood-brain barrier permitting further seeding of HIV-1 in the CNS. HIV-1 infected macrophages initiate inflammatory processes and the processes could be amplified through cell-cell interaction with astrocytes (Blumberg et al., 1994). Investigation on the binding site for HIV-1 gp120 on human fetal astrocytes suggested that neither CD4 nor galactocerebroside was involved in this binding (Ma et al., 1994).

1.6 Neuropathology of human immunodeficiency virus infection

1.6.1 AIDS

Neuropathological changes observed in the brains of AIDS sufferers include those
produced by HIV and those associated with opportunistic infections and lymphomas. The following description will deal only with the primary changes produced by HIV.

HIV was first discovered in lymph nodes by Barré-Sinoussi et al (1983) and in peripheral blood lymphocytes by Gallo et al (1984); subsequently Shaw et al (1985) demonstrated its presence in the nervous system by Southern analysis and in situ hybridization. These results were followed by the description of multinucleated giant cells (MGC) (the most characteristic neuropathological feature in AIDS) in the brain of HIV-1 positive patients by Sharer et al (1985). MGC had been previously illustrated by Horoupian et al (1984), without any comment on their specific features.

MGC with the characteristic features (Gray et al., 1988) are considered to be unique to AIDS (Petito et al., 1986) and the hallmark of HIV infection in the brain (Budka, 1986). MGC demonstrated the capacity of HIV-infected cells for fusion in the nervous system, analogous to the formation of syncytia in infected permissive cell cultures (Popovic et al., 1984). These giant cells are 15 to 25 \( \mu \text{m} \) in diameter (Sharer et al., 1985; Budka 1986); they can occur together with other cells in a microglial nodule, in isolated clusters scattered throughout the parenchyma or, most frequently, around blood vessels in the leptomeninges and in brain (De Girolami et al., 1992). MGC may appear as 'macrophage-like' with a large volume of well demarcated cytoplasm containing peripheral lipid vacuoles or as smaller 'microglia-like' multinucleated cells with prominent processes, scanty cytoplasm and elongated nuclei clustered at the centre of the cell (Scaravilli et al., 1993). They may have only 2-3 nuclei, but the larger ones have 20-30 or even more. They have been found in the lesions of HIV-myelitis (Maier et al.,
1989) and mediastinal node of one man with AIDS (Gray et al., 1990). However, they have not been associated so far with peripheral neuropathy in HIV infection (Esiri, 1993).

In some respects MGC in the CNS in AIDS resemble morphologically those found in chronic infective granulomas. They are found also in measles encephalitis (Esiri et al., 1982). In these conditions their origin has not been fully investigated. MGCs found in HIV infected CNS have the capacity to function like macrophages in host defense against infection (Schlesinger et al., 1984). They show evidence of strong binding to the lectin *Ricinus Communis* agglutinin (Mannoji et al., 1986) and the HIV envelop protein *gpl20* is known to effect fusion of these type of cells by a direct action on their cell membranes.

It has been demonstrated that macrophages and macrophage-derived MGC are capable of synthesizing HIV RNA and may be considered both the main reservoir and the vehicle of spread of the virus (Koenig et al., 1986). Various techniques have demonstrated the presence of HIV genome or protein in their cytoplasm (Koenig et al., 1986; Sharer et al., 1986; Wiley et al., 1986; Budka et al., 1987; Vazeux et al., 1987). Ultrastructural studies have also shown viral particles either free in the cytoplasm or in cytoplasmic cisterns (Koenig et al., 1986; Budka et al., 1987; Gray et al., 1987). Nuclear bridges have also been occasionally observed in MGC, suggesting that amitotic nuclear division might account for the formation of multinucleated cells in addition to cell fusion, (Mizusawa et al., 1988). On the other hand, HIV protein has been demonstrated immunohistochemically in brains of AIDS patients in the absence of MGC (Gabuzda,
1986; Vazeux et al., 1987), suggesting that MGC represent a highly characteristic, but not essential, feature of HIV infection of the nervous system. The differences in brain sampling may affect the number of MGC in AIDS brains (Scaravilli et al., 1993). Immunohistochemistry for HIV antigens should be a valuable diagnostic aid in some brains in which MGC are rare (Budka, 1993).

Direct involvement of CNS by HIV produces a form of leukoencephalitis. Lesions, which predominate in the hemispheric white matter and deep grey nuclei, with sparing of the gyral white matter and corpus callosum, include myelin pallor, astro- and microgliosis with variable number of MGCs. According to whether myelin pallor is focal, usually perivascular, or diffuse, two types of encephalitis have been described. Indeed, they are variation of the same process and some cases with both appearances are seen. Both pathological conditions occur in the later stages of the AIDS infection and consistently demonstrate relatively large amounts of HIV products.

1). HIV encephalitis (HIVE) is synonymous with multifocal giant cell encephalitis (MGCE) (Budka 1986, 1989), subacute encephalitis with multinucleated cells (Petito et al., 1986), multinucleated cell encephalitis (Price et al., 1988) and giant cell encephalitis (Michaels et al., 1988). It is characterized by multiple 'microgranulomatous' foci irregularly disseminated predominantly in white matter, but also in smaller numbers in the grey, usually in a perivascular position. These foci consist of microglia/macrophages and MGC, cells show production of HIV. Reactive astrogliosis is also a feature.

2). HIV leukoencephalopathy (HIV-lep) is synonymous with progressive diffuse
leukoencephalopathy (PDL) (Kleihues et al., 1985). It is characterized by diffuse histopathological triad of myelin reduction, reactive astrogliosis and infiltration of mono- and multi-nucleated microglia/macrophages with HIV production involving the white matter (Budka, 1993).

HIVE and HIV-lep may co-exist in the same brain. The observation of microvascular pathology including thickening of the walls, increased cellularity, and enlargement and pleomorphism of endothelial cells in HIV-lep as well as the lesions of HIVE, suggested that the lesions may follow the entry of HIV-1 infected haematogenous cells through the vessel wall and that altered vascular permeability may lead to damage to myelin and axons in HIV-lep (Smith et al., 1990). Cytokines including TNF secreted by HIV-1 infected macrophages may influence capillary endothelium (Feuerstein et al., 1994).

The incidence of encephalitis with MGC varies in different series: from 42/149 (28%, Petito et al., 1986), 38/100 (38%, Budka et al., 1987), 21/135 (15%, Lang et al., 1989), 52/135 (38.5%, Gray et al., 1991a) to 40/100 (40%, Scaravilli et al., 1993). This may reflect an actual yearly increase in incidence of MGC encephalitis, as observed by the latter author, 6/22 (27%) patients who died before 1987 have MGC; subsequently the incidence increased to 21/48 (41%) in 1987-8 and 13/30 (43%) in 1989. This may also reflect changes in survival time related to improved diagnosis and better treatment of opportunistic infections (Scaravilli et al., 1993). The characteristic microglial nodules and multinucleated cells can occasionally be seen in spinal cords (HIV myelitis). Myelitis was present in 5% of the 178 spinal cords examined by Petito (1993) and in 8% of the 26 spinal cords reported by Grafe and Wiley (1989). Zidovudine (AZT) treatment
significantly reduces the occurrence of productive HIV infection of the brain in AIDS if continued until death. Discontinuing AZT therapy may favour the occurrence of HIV encephalitis (Gray et al., 1994; Mæhlen et al., 1995). The main problems of AZT are reversible anaemia which results in about 30% of patients not tolerating long-term use, and the development of drug resistance which may be associated with clinical failure of the drug (Peto, 1992).

Although cortical abnormalities first described in 1987 by Budka have received for some time relatively little attention, Ciardi et al (1990) found increased expression of GFAP and microglial hyperplasia in neurologically asymptomatic AIDS patients; in addition, cortical thinning and reduced number of nerve cells were reported in 1990 by Ketzler et al and confirmed by Everall et al and Wiley et al in 1991. Subsequently, Gray et al (1991b) reported the neuropathology in one AIDS patient with HIV encephalopathy showing predominant cortical changes including severe neuronal loss and only minimal changes in white matter and basal ganglia, suggesting that HIV encephalopathy is not necessarily limited to white matter lesions. Furthermore, detection of HIV proviral DNA in the cortex of AIDS patients both with and without obvious abnormalities (Sinclair et al, 1992b), suggests that HIV may be involved in the pathogenesis of cortical abnormalities.

HIV-1 associated (possibly HIV-1 induced) central nervous system changes include also lymphocytic meningitis (usually very discrete), vacuolar myelopathy [incidence in adult patients at postmortem ranging from 18 to 30% (Petito, 1993)], multifocal vacuolar leukoencephalopathy and diffuse poliodystrophy (DPD) (Budka, 1993) and mineralization
of blood vessel walls (Scaravilli et al., 1993).

1.6.2 pre-AIDS

The time and mode of entry and spread of the virus in the CNS remain unanswered questions. A neuropathological study by Esiri et al (1989) of HIV-1 infected haemophiliacs who died from intracranial haemorrhage and cirrhosis of the liver before the onset of AIDS (7 cases) or at the AIDS stage (4 cases) showed that not only AIDS but also pre-AIDS cases suffered some degree of microglial and macrophage alteration. Neuropathological disorders included, in addition to acute and/or old haemorrhage, in 3 out of 4 AIDS cases, microglial nodules in 2 (one was with myelin pallor also); anoxic foci in 1 whereas among 7 pre-AIDS patients, mild lymphocytic meningitis / encephalitis, or both were found in 2, myelin pallor in 2. Two of these 4 showed also microglial nodules. The other 3 cases showed MGC encephalopathy in 1 and acute and chronic haemorrhage was present only in 2. Examination by Gray et al (1992) of 11 brains of HIV-1 positive individuals who had died from overdose or a gunshot prior to developing AIDS, showed lymphocytic meningitis, myelin pallor, reactive gliosis and microglial proliferation. These changes had been previously considered as nonspecific (probably related to the drug addiction) as there was no evidence of encephalitis and HIV antigen could not be detected by immunohistochemistry. However, they could not be seen in a control group of HIV-negative drug users (11 cases who died from same causes, i.e. 10 overdose and 1 gunshot). Similar changes have been found by others (Lenhardt et al., 1988; Bell et al., 1993). It is notable that meningitis which has been observed by all authors mentioned above occurs in the pre-AIDS stages of HIV infection.
but is rare in AIDS. However, association of leptomeningitis with HIV-1 specific neuropathology has been found in a few AIDS brains with prominent collections of HIV producing cells in the leptomeninges. This suggests that, at least in some cases, HIV pathology may spread to the leptomeninges (Budka, 1993). Furthermore, Santosh et al (1995) reported that the brain of a pre-AIDS case who died from road traffic accident showed features of HIV associated early leukoencephalopathy. These early brain changes, including leptomeningitis, myelin pallor and gliosis are considered secondary to vascular inflammation and opening of the blood-brain barrier (Gray et al., 1993).

1.7 level of HIV-1 DNA in the PBMC and CNS during different stages of disease

HIV causes a chronic disease in man. During the early period after primary infection there is widespread dissemination of virus and a decrease in the number of CD4 T cells in peripheral blood. An immune response to HIV ensues, with a decrease in detectable viraemia followed by a prolonged period of clinical latency (see Fig. 1.3. Pantaleo et al., 1993b). It has been suggested that evaluation of proviral copy number could be an early indicator of AIDS progression (Verhofstede et al., 1994; Lefrere et al., 1992; Chevret et al., 1994).
Fig. 1.3 Typical course of HIV infection. See text for description. The courtesy of Dr Fauci (Pantaleo et al., 1993b).
The polymerase chain reaction (PCR) technique has been applied in DNA and RNA research since 1985 and developed for the quantitative evaluation and for cloning and sequencing purposes. The goal of quantitative PCR is to deduce, from the final amount of PCR product, the initial number or related levels of target molecules among samples. In HIV research, it has been performed mostly on PBMC (Aoki et al., 1990; Bieniasz et al., 1993; Wood et al., 1993; Jurriaans et al., 1992; Escaich et al., 1992; Simmonds et al., 1990b). It has also been employed on tissues such as lymphoid organs, lung, liver (Sei et al., 1994; Cao et al., 1992; Donaldson et al., 1994b), semen and saliva (Liuzzi et al., 1995). Only a few data regarding brain are available (Pang et al., 1990; Achim et al., 1994) and the results were obtained using either ^32^P-labelled primer (probe) or fresh frozen tissues (Cao et al., 1992; Pang et al., 1990; Achim et al., 1994).

In addition to PCR having a critical role in the quantification of HIV-1 DNA (Stieger et al., 1991; Piatak et al., 1993; Poznansky et al., 1991), nested or hemi-nested PCR was often applied in view of its advantages: it eliminates nonspecific products which could interfere with quantitation and it increases sensitivity of PCR, the threshold of detection is lowered (Haff, 1994; Zimmermann et al., 1994; Yourno and Conroy, 1992; Simmonds et al., 1990b; Ferré, 1994).

1.7.1 PBMC and CD4 positive T cells

Quantitative analysis of HIV-1 DNA in asymptomatic carriers by PCR has been performed using PBMC (Lee et al., 1991; Bush et al., 1993; Ferré et al., 1992) and CD4 positive T lymphocytes (Oka et al., 1990; Brinchmann et al., 1991). Comparison
between data in these individuals and AIDS patients has shown that the titres of HIV were significantly higher in AIDS (O'Shea et al., 1991) and that viral load has an inverse correlation to the number of CD4 positive cells. When selected individuals with low CD4 cell counts (below 50/mm³) (they were considered as AIDS patients regardless of whether they were asymptomatic or symptomatic) were taken into consideration, no significant difference in viral load was found between the two groups (Jacques et al., 1994).

Because good correlation between the proviral DNA in PBMC and the viral RNA levels in plasma was observed in a group of asymptomatic patients with a CD4 positive T-cell count >200X10⁶/l (Verhofstede et al., 1994) and because HIV RNA was expressed during all stages of infection (Piatak et al., 1993), measuring proviral copy numbers by PCR in early stages of HIV-1 infection was considered a useful means of predicting progression to AIDS by Chevret et al (1994).

1.7.2 Cerebrospinal fluid (CSF) & brain

Little is known about HIV-1 proviral DNA load in brain, particularly regarding the early stages of disease. One group (Bell et al., 1993) believes that HIV-1 DNA detected in pre-AIDS cases is a contamination from residual blood within brain. However, more recently, a quantitative study by Schmid (1994) demonstrated that, at all stages, HIV-1 proviral load in seropositive patients was significantly higher in cerebrospinal fluid (CSF) than in blood [median 25 vs. 0.6 copies/1,000 CD4 positive cells (p = 0.0001)]; in addition the load was higher in the blood and CSF of subjects with more advanced
systemic disease and with neurological signs of HIV-1 infection.

A summary of published data on the load of HIV-1 DNA in PBMC, CD4 positive cells and RNA in plasma is quoted in the Table 1.1.

Table 1.1 List of HIV-1 DNA load in PBMC, CD4+ cells and RNA in plasma in the published literature

<table>
<thead>
<tr>
<th>Source</th>
<th>AIDS</th>
<th>pre-AIDS</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBMC</td>
<td>1/4,606</td>
<td>1/10,714</td>
<td>Lefrere et al., 1992</td>
</tr>
<tr>
<td>PBMC</td>
<td>450-10,516/10^6PBMC (mean=2,403)</td>
<td>50-2,570/10^6PBMC (mean=779)</td>
<td>Escaich et al., 1992</td>
</tr>
<tr>
<td>PBMC</td>
<td>1/700-3,300</td>
<td>1/6,000-80,000</td>
<td>Simmonds et al., 1990b</td>
</tr>
<tr>
<td>PBMC</td>
<td>200-4,000/10^6PBMC (mean=1,245)</td>
<td>2-1,000/10^6PBMC (mean=213)</td>
<td>Lee et al., 1991</td>
</tr>
<tr>
<td>CD4+</td>
<td>1/10 cells</td>
<td>1/10,000 cells</td>
<td>Wood et al., 1993</td>
</tr>
<tr>
<td>CD4+</td>
<td>1/100-10,000</td>
<td></td>
<td>Poznansky et al., 1991</td>
</tr>
<tr>
<td>CD4+</td>
<td>18-2,857/10^4 CD4</td>
<td></td>
<td>Oka et al., 1990</td>
</tr>
<tr>
<td>CD4+</td>
<td>1/2,500-26,000 (media,1/12,000)</td>
<td></td>
<td>Brinchmann et al., 1991</td>
</tr>
<tr>
<td>CD4+</td>
<td>10.7/1,000 cells</td>
<td>0.09/1,000 cells</td>
<td>Schmid et al., 1994</td>
</tr>
</tbody>
</table>

The proviral load in patients, all stages, was significantly greater in CSF than blood [media 25 vs. 0.6 copies/1,000 CD4+ cells (p=0.0001)].

<table>
<thead>
<tr>
<th>Source</th>
<th>AIDS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>100-22,000,000 copies /ml</td>
</tr>
<tr>
<td>Plasma</td>
<td>10^9-8X10^9 /ml</td>
</tr>
</tbody>
</table>

Piatak et al., 1993
Scadden et al., 1992
1.8 Programmed cell death (PCD) and HIV infection

HIV infection leads to the progressive collapse of two systems within the human body: the immune system, and the central nervous system. AIDS results from the loss, in each organ, of a selective cell population: CD4 positive T cells in the immune system, leading to immune incompetence; neurons in the brain, leading to dementia (Ameisen, 1995).

The asymptomatic phase of the disease is characterized by the inability of CD4 positive T cells to proliferate as shown in vitro by the response to MHC class II restricted recall antigens (Shearer and Clerici, 1991). This functional impairment is followed by the decline of CD4 positive T cells and the development of AIDS.

A number of virological and immunologic mechanisms have been proposed to account for the CD4 positive T cell defect and depletion, including syncytium formation between infected and non-infected cells, selective infection and destruction of memory T help cells, inappropriate immune killing of uninfected cells, and autoimmune responses (Fauci, 1988; Banda et al., 1992; Habeshaw et al., 1992). Recently, new developments in this area of research have suggested that the loss of CD4 cells in HIV-1 infected patients is associated with lymphocyte activation. Activation, however, does not result in cell proliferation, but rather in cell death, through a mechanism known as programmed cell death (PCD) (Wyllie et al., 1980; 1984).

PCD is characterized by disappearance of individual cells and is an active cell suicide mechanism. It represents the endpoint of a genetically determined programme which
requires de novo gene expression and protein synthesis. The morphological characteristics of PCD have been called apoptosis. Apoptosis differs from necrosis which is an accidental event triggered by factors external to the dying cells. Moreover, whereas in cell necrosis the event involves large groups of cells and there is loss of membrane integrity, swelling and degeneration of cell organelles and eventually lysis, in apoptosis single cells appear to die: organelles are normal and nuclei show dense chromatin, which is degraded into single and multiple oligonucleosomes, clumped against the nuclear membrane. The DNA is cleaved in the internucleosomal linker region, where it is relatively weakly associated with histone H1, and electrophoretic separation of DNA of apoptotic cells reveals a "ladder" pattern of bands averaging about 200, 400, 600bp, etc., corresponding to oligonucleosomal fragments (Arends et al., 1990; Walker and Sikorska, 1994). This fragmentation of DNA is enzymatic and generally occurs after activation of a calcium-dependent endogenous endonuclease (Arends et al., 1990).

1.8.1 T lymphocytes

Apoptosis has been recognized as a possible way by which immune cells (Cameron et al., 1994; Schnittman and Fauci, 1994; Carbonari et al., 1994; Howie et al., 1994; Lu et al., 1994; Martin et al., 1994; Sandstrom et al., 1993; Shearer et al., 1993; Lewis et al., 1994) are eliminated in AIDS, and more recently it has been detected among CD4 and CD8 lymphocytes in HIV-1 positive individuals at the asymptomatic stage of the infection (Groux et al., 1992; Jaleco et al., 1994; Meyaard et al., 1992; 1994). A study by Gougeon et al (1993a) quantified the apoptotic peripheral blood lymphocytes (PBLs) from 29 asymptomatic HIV-1 infected individuals and 28 seronegative controls. Up to
25% of patients' cells were apoptotic after stimulation with ionomycin, compared to 9% in cultures from seronegative donors. The apoptotic process could be accelerated by increasing the intracellular Ca^{++} mobilization which was supposed to activate the endogenous endonuclease; the latter destroys the chromatin structure and induces apoptosis. Cyclosporin A, a powerful suppressor of the immune system and an inhibitor of activation, and Zn^{++} ions, known to inhibit endonuclease and DNA fragmentation in PCD induced cell death, reduce the ionomycin-induced DNA fragmentation to control level. A similar mechanism, whereby infected cytotoxic T-lymphocytes are eliminated, has been reported in HTLV-1 infection (Umehara et al., 1994).

An observation by Gougeon and Montagnier (1993) suggested that the T cell tropism of the activator used to induce apoptosis in patients' lymphocytes will determine which subpopulation will die: ionomycin (Gougeon et al., 1993a,b) and anti-CD3 antibodies (Meyaard et al., 1992) activate the death of both CD4 and CD8 positive T cells, whereas superantigens induce preferentially the death of CD4 positive T cells (Groux et al., 1992).

T cell PCD may exert a beneficial role in the control of viral infection as seen during acute benign Epstein-Barr virus induced mononucleosis in children (Uehara et al., 1992). However, correlation between T cell PCD and AIDS pathogenesis suggests that, in HIV infection, this deletional mechanism is not beneficial and may contribute to CD4 positive T cell depletion and to development of AIDS (Gougeon et al., 1993b).

1.8.2 Neurons
Apoptosis of these cells is thought to be implicated not only during developmental but also in the post-maturation stages of the peripheral nervous system (PNS) and CNS neurons, as recent studies have suggested that apoptotic neuronal death occurs in the mature nervous system and that it may be even involved in a number of human diseases such as neuro-degenerative disorders including Alzheimer's and Parkinson's disease or in ageing (Chen et al., 1995).

Neuropathological studies in AIDS have revealed that brains of patients suffering from the AIDS dementia complex with the typical encephalitis show, in addition to the well known features, variable amounts of neuronal loss in cortical and subcortical regions (Everall et al., 1993; Ketzler et al., 1990; Wiley et al., 1991). Quantitative studies showed that the closer to the microglial nodules, the greater the neuronal loss is. However, this effect disappears 300 μm away from the nodule, suggesting the possible diffusion of neurotoxins from the nodule (Masliah et al., 1994a). It has been postulated that, in AIDS, cell loss would take place through a process of apoptosis and indeed, apoptosis has been unquestionably demonstrated in the CNS of adult (Adle-Biassette et al., 1995; Petito and Roberts, 1995) as well as paediatric AIDS patients (Gelbard et al., 1995).

The regulation of apoptosis is rather complex. Reports showing that some genes induce apoptosis (Rabizadeh et al., 1993; Itoh et al., 1991), and other genes inhibit it (Mah et al., 1993; Zhong et al., 1993), suggest a parallel between the system that modulates the propensity of cells to undergo neoplastic transformation - oncogenes and tumour suppressor genes - and the system that modulates the propensity of cells to undergo
apoptosis - presumably, necrogenes and cellular death suppressor genes. Among them expression of \textit{bcl}-2 oncogene, which functions as death suppressor gene, is associated with a marked inhibition of neuronal cell death (Bredesen, 1994). \textit{Mcl-1} (Kozopas et al., 1993), which has some similarity to \textit{bcl}-2, and \textit{bcl}-x (Boise et al., 1993), are also thought to be involved in the inhibition of apoptosis in the nervous system whereas p53, a tumour suppressor gene induces the occurrence of apoptosis (Yonish-Ronach et al., 1991).

Neurotrophic factors and cytokines are also thought to be involved in apoptosis. Nerve growth factor (NGF) and its low-affinity receptor p75 \textit{NGF receptor (NGFR)} (Itoh et al., 1991), TNF and TNF receptor (TNFR) I (a widely expressed gene whose product may induce apoptosis or necrosis when bound by its ligand, TNF) appear to be involved in cell death. In contrast, TNFR II does not induce cell death when bound by TNF (Tartaglia et al., 1991). As the possible mechanism is still incompletely understood it would be interesting to know by which mechanism the binding of TNFR I by TNF induces cell death (Clement and Stamenkovic, 1994). TNF binds to TNFR I and releases arachidonic acid intracellularly. Reaction oxygen molecules increase intracellularly, possibly because of both arachidonic acid metabolism and a poorly understood block at mitochondrial complex III (Lancaster et al., 1989). The second member of the TNFR superfamily shown to induce cell death is \textit{fas} antigen, which is a cell surface protein that belongs to the TNFR and NGFR and can mediate apoptosis (Itoh et al., 1991). However, it has not been demonstrated to be expressed in the CNS (Watanabe-Fukunaga et al., 1992). Among other neurotrophic factors ciliary neurotrophic factor (CNTF) is found in astrocytes and is known to protect oligodendrocytes from death induced by TNFs
(apoptosis) (Louis et al., 1993).

1.9 Immune response of CNS to HIV-1 infection

HIV-1 infection results in a virus-induced immunosuppression characterized by loss of cell-mediated immunity, depletion of CD4 positive cells, loss of core antibody, and an increased viral burden (Moss et al., 1994).

An important aspect of HIV disease is that it comprises complex pathogenic processes that are multifactorial and multiphasic, including a paradoxical state of immune activation overlapping with immunodeficiency.

The CNS is relatively isolated from systemic immune responses in the absence of disease, and there are several differences between CNS and the majority of peripheral sites. Firstly, classic antigen-presenting cells, the dendritic cells that reside in almost all tissues of body, are absent from the brain. Secondly, MHC antigens, which have a key role in generating and propagating the immune response, are not expressed at detectable levels in healthy, inactivated CNS tissue, apart from endothelial cells. Finally, blood proteins and cells are excluded from CNS under normal conditions by the blood-brain barrier (BBB) (Sharief, 1995).

HIV-1 mediated disease in brain is dependent on CNS immune activation. Microglia, macrophages and astrocytes are cells implicated in the immune regulatory and effector function. They are potent HIV antigens presenting cells and are necessary for T cell
activation. Activation of T helper cells specific for viral antigens is critical for antibody production and for generation of cytotoxic cells during the immune response to HIV (Manca et al., 1994). The role of these cells is discussed in 1.5.

Immune response phenomenon has been demonstrated in brains of AIDS and HIV-1 positive pre-AIDS individuals. Priming of macrophages occurs due to local interferon (IFN)-gamma or IFN-gamma-like effects induced by virus. MHC class II and superoxide anions are increased in brain macrophages and astrocytes (Tyor et al., 1992). Neopterin [(6-D-erythro-trihydroxypropyl)pterin], a product of guanosine triphosphate metabolism and a marker for cell-mediated immune stimulation (Fuchs et al., 1988), is produced by monocytes/macrophages and increases after stimulation by IFN-gamma (Huber et al., 1984). Neopterin might modulate the effects of toxic oxygen intermediates which are known to be cytotoxic and thus neopterin may act to enhance macrophage cytotoxicity (Weiss et al., 1993) (see 1.10.2). CSF neopterin, IgG, IgG synthesis, IgG index, and \( \beta_2 \)-microglobulin are increased in neurologically asymptomatic HIV-1 positive individuals and have an inverse correlation with blood CD4 positive T cell count (Lucey et al., 1993). Elevated CSF neopterin 1 suggests that microglia/macrophage activation occurs during the relatively early pre-AIDS stages (Lucey et al.; Hagberg et al., 1993). In addition, the highest CSF and brain quinolinic acid (QUIN), N-methyl-D-aspartate (NMDA) receptor agonist levels occur in condition of macrophage infiltration and gliosis within the CNS (Wiley et al., 1992; Heyes et al., 1991; Martin et al., 1992). QUIN increases significantly in CNS of children with AIDS compared with controls \( (p<0.001) \). The concentrations were also higher in patients with encephalopathy than those without encephalopathy \( (p<0.01) \) and in patients who died compared with those
who are still alive. These data suggest that CSF QUIN could be a mediator of neurological dysfunction and an additional marker of neurological disease (Brouwers et al., 1993). More recently, however, Giulian et al (1996) found that HIV-1 infection did not produce an increase of QUIN from mononuclear cells. Although elevations of QUIN have been found in the CSF of HIV-1 infected individuals, Giulian et al (1996) believe that the increases were likely attributable to entry through damaged BBB. In contrast, a neurotoxic amine, NTox, was produced by blood monocytes and by brain mononuclear phagocytes infected with HIV. NTox can be extracted directly from brain tissues infected with HIV-1.

In conclusion, a bidirectional circuit exists between the CNS and the immune system since activation of the immune system results in the elaboration of cytokines and inflammatory mediators; these mediators induce hypothalamic corticotrophin releasing factor (CRF), which stimulates the release of the immunosuppressive molecules (Black, 1994).

1.10 Possible neuropathogenetic mechanisms of HIV-1 infection

Whilst fatal immunosuppression is the ultimate outcome, it is shown that HIV-1 infection is a syndrome of immune deregulation in which abnormal immune activation may also occur (Oldstone, 1994) (see 1.9).

It has been mentioned above that the pathogenesis of the changes within the CNS in AIDS, including nerve cell loss, could be produced directly by the virus and/or viral
products (Dreyer et al., 1990; Kaiser et al., 1990; Sabatier et al., 1991; Werner et al., 1991) or induced indirectly via products secreted by HIV-infected macrophages / microglia. The latter mechanism seems the more likely and it has been hypothesised that the damage can be induced via production of cytokines.

1.10.1 Cytokines

Cytokines are among the most potent factors mediating intercellular communication and interaction. These proteins are by no means specific of HIV infection and can be found in normal brain (Breder et al., 1988) as well as in a variety of abnormal conditions (Hofman et al., 1989). The complex network of cytokines involved in inflammatory and immunoregulatory responses has an important role in several components of the pathogenesis of HIV infection and AIDS. Firstly, expression and production of several cytokines are deregulated in HIV-infected patients. Secondly, several cytokines have an important role as growth factors for AIDS-associated malignancies, namely B-cell lymphomas and Kaposi's sarcoma (KS) (Ensoli et al., 1992); this topic however will not be discussed further in this thesis. Finally, multiple cytokines can directly regulate the replicative capacity of HIV at multiple levels of its life cycle. The fact that cytokines are produced and secreted by the same cells that are potential targets of HIV infection, provides an example of how infected cells can contribute to the autoregulation of virus expression and spread. In HIV infection, both in vivo and in vitro, data appears to show that the role of cytokines could be exerted at various stages of the disorder (Vitkovic et al., 1994): entry of the virus into the brain tissue (see 1.10.3), up- and down-regulation of HIV expression in the brain and induction of astrogliosis and myelin pallor.
TNF-α is used as the reference cytokine in the majority of the studies dealing with the role of cytokines as regulators of viral production and spreading, due to its demonstrated mechanism of activation of HIV expression. Both TNF-α and IL-1β have also been thought to be neurotoxic (Gendelman et al., 1994). Induced TNF-α and IL-1β may lead to autocrine feedback loops involving further productive viral replication and cytokines including IL-6 and granulocyte macrophage colony stimulating factor (GMCSF). Cytokines, such as TNF-α (Reddy et al., 1988; Lahdevirta et al., 1988; Mintz et al., 1989); IL-1 (Arditi et al., 1991; Scott-Algara et al., 1991); IL-6 (Breen et al., 1990; Honda et al., 1990; Birx et al., 1990; Rautonen et al., 1991) have been found to be elevated in the plasma, serum and CSF (Grimaldi et al., 1991; Mastroianni et al., 1992; Gallo et al., 1989; Laurenzi et al., 1990; Perrellia et al., 1992; Griffin et al., 1991) of HIV-1 infected patients.

Indeed cytokines have been detected in the CNS during HIV infection (Griffin et al., 1991; Grimaldi et al., 1991; Lahdevirta et al., 1988; Tyor et al., 1992). In an investigation of brain tissue in AIDS, Tyor et al (1992) reported presence of various cytokines in the white matter. Within the CNS, activated astrocytes and microglia are the main source of cytokines, although IL-1 and transforming growth factor-β (TGF-β) can be secreted also by oligodendrocytes. Their functions have been extensively described and the literature on the subject has been recently reviewed by Vitkovic et al (1994) and Benveniste (1994).

Local production of IL-1 in the brain (da-Cunha et al., 1993c; Poli et al., 1994; Genis et al., 1992; Epstein and Gendelman, 1993) together with that of other proinflammatory
cytokines and factors, is hypothesized as contributing to the HIV-induced pathological effects in this organ. IL-1β is a selective and potent activator of human astrocytes \textit{in vitro} (Liu et al., 1994) (see 1.5.2).

At molecular level, induction of virus expression by TNFs (α and β) and IL-1 has been correlated with activation of the cellular transcription factor NF-kB (Osborn et al., 1989; Swingler et al., 1992; Griffin et al., 1989; Duh et al., 1989; Okamoto et al., 1989). It is known that TNFs interacting with their p55 receptor on the cell surface, other than p75 receptor, mediate the TNF-associated cytotoxic effects leading to activation of NF-kB (Kruppa et al., 1992).

Report by Vitkovic et al (1994) indicated that TNF-α is specifically increased in AIDS brain and that the magnitude of the increase is associated with the severity of dementia. IL-6 immunoreactivity (Tyor et al., 1992) and mRNA (Wesselingh et al., 1993) have been detected in AIDS autopsy brains. IL-6 appears to exert both transcriptional and post-transcriptional effects on virus expression in cultured cells. It does not usually affect the constituent levels of HIV transcription, and it does not activate NF-kB itself, whereas it potently increases the inductive effect of TNF-α and IL-1 on both viral transcription and virion production (Poli et al., 1990; 1994).

With regards to the role of IL-4 in HIV replication, it appears that, similarly to IL-10, TGF-β and IFN-gamma, it may exert different effects depending on the experimental condition (Schuitemaker et al., 1992). IL-10 mRNA and receptor mRNA have been detected in both microglia and astrocytes \textit{in vitro}, suggesting that IL-10 is produced in
the CNS and plays a role as an inhibitor regulator in the CNS cytokine network (Mizuno et al., 1994). Low concentration of IL-10 is synergistic with multiple cytokines (TNF-α, IL-6) in enhancing HIV production in cells of monocytic lineage while high concentrations of it completely block the production of endogenous TNF-α and IL-6 from acutely infected monocyte-derived macrophages (Weissman et al., 1995). IL-4 induces proliferation and activation of microglia but suppresses their induction of MHC class II antigens expression (Suzumura et al., 1994). On the one hand, Boyle et al (1993) detected IL-4 mRNAs by in situ hybridization in patients’ lymph nodes, although at levels not higher than those found in reactive lymph nodes obtained from HIV-1 seronegative individuals. In contrast, messages for this cytokine could not be detected from PBMC and cells from lymphoid tissue by PCR by Graziosi et al (1994). The latter study is consistent with the reports by others (Re et al., 1992; Cayota et al., 1992; Maggi et al., 1994). Furthermore, it has also been demonstrated that exposure of CD4 positive T cell to gp120 leads to a decreased ability to secrete IL-2 and IL-4 (Chirmule et al., 1992). Lower levels of IL-4, as well as IL-1, have been detected in the brains of AIDS patients with dementia compared with those without dementia, whereas TNF-α exhibited the opposite pattern (Wesselingh et al., 1993).

In the brain, activated macrophages and microglia are the major cellular source for TNF-α, IL-1 and IL-6, although other cell types such as astrocytes can also be stimulated to secrete these cytokines. In addition, oligodendrocytes could produce IL-1 and endothelial cells produce TNF-α and IL-6 (Benveniste, 1994).

Perhaps most relevant to neuropathogenesis of HIV-1 infection is the ability of TNF-α
to mediate myelin and oligodendrocyte damage *in vitro* (Selmaj and Raine, 1988), and its ability to cause cell death of brain-derived oligodendrocytes in rat (Robbins et al., 1987) and man (Wilt et al., 1995) *in vitro*. A dual role of TNF-α - enhancing viral replication by activated microglia and damaging oligodendrocytes - has been demonstrated *in vitro* by Wilt et al (1995) recently using culture of purified human microglia and oligodendrocytes derived from adult human brain. It has been postulated and demonstrated by Masliah et al (1994b) that cytokines released by macrophages/microglia within the brain are involved in the modulation of neuronal survival and death. They examined the distribution of cytokine receptors in the human brain and found that IL-1β and TGF-β1 receptors increase by 35% (average) in intensity of labelling in cases with moderate HIVE compared to cases without HIVE or with severe HIVE while the receptor of IL-2 was not altered in HIVE. Once cytokines bind to receptors, a cascade of intracellular events including the activation of protein kinase C (PKC) and calcium release and phosphorylation of specific proteins (Yamato et al., 1990) is produced. Similar findings were also reported by Ban (1994) and Sawada et al (1995) in mouse. They demonstrated that both neurons and glial cells did express IL-1 receptors (Ban, 1994) at the site of astrogliosis induced by a local mechanical injury and IL-2 receptor in microglia which were activated by lipopolysaccharide (LPS) (Sawada et al., 1995).

Cytokines are potent inducers of cytokine production. IL-1 induces the production of IL-6, TNF-α, colony stimulating factors (CSFs), and IL-1 itself whilst astrocytes produce three colony stimulating factors upon stimulation with TNF-α: granulocyte macrophage colony stimulating factor (GM-CSF), granulocyte colony stimulating factor (G-CSF), and
macrophage colony stimulating factor (M-CSF) (Aloisi et al., 1992; Malipiero et al., 1990; Tweardy et al., 1991). IL-1 and TNF-α in combination with IL-6 and GM-CSF could account for many clinical and histopathological findings in AIDS nervous system disease (Merrill and Chen, 1991). TNF-α and IL-1 induced IL-6 mRNA and its biological activity in astrocytes, but not in microglia (Sawada et al., 1992).

Other cytokines such as TGF-β1 and IL-13 (Denis and Ghadirian, 1994; Montaner et al., 1993), and IFN-α (Fernie et al., 1991) are thought to inhibit HIV replication. The former has been identified in the brains of patients with AIDS (Wahl et al., 1991) and they may play an important role as a negative regulator of HIV expression in infected macrophages/microglia by either autocrine or paracrine manner (for review, see Benveniste, 1994).

1.10.2 Virus, viral proteins and other neurotoxic factors

Recent evidence supported the existence of HIV-1 or immune-related toxins that lead indirectly to the injury of neurons via interactions between macrophage/microglia, astrocytes and neurons. HIV-1 infected monocyteid cells, after interacting with astrocytes, secrete neurotoxic substances (Giulian et al., 1990; Pulliam et al., 1991). These substances may include eicosanoids, i.e. arachidonic acid and its metabolites, as well as platelet-activating factor (PAF). Others include nitric oxide (NO), superoxide anion (O₂⁻) and the N-methyl-D-asparate (NMDA) agonist, cysteine. These factors can lead to increased glutamate release or decreased glutamate re-uptake. In addition, IFN-gamma stimulation of macrophages induces release of the NMDA-like agonist,
quinolinate. HIV-1 infected or gp120 stimulated macrophages also produce cytokines, as described above (see 1.10.1).

It has been found that PAF is increased in the CSF of patients with AIDS and is correlated with their degree of neurological impairment. The nanomolar concentration of PAF produced by HIV-1 infected human macrophages, when these cells are cultured in the presence of astrocytes, is toxic to either rat or human neurons cultured from cerebrocortex or retina (Gelbard et al., 1994).

gp120 by itself is not neurotoxic, i.e. it does not have any direct effects on neurons (Sharer, 1992). It can however induce neurotoxicity either by directly enhancing neurotoxic factor release from macrophage/microglia, or by promoting glutamate-induced neurotoxicity (Choi, 1988). gp120 kills neurons in a nitric oxide dependent manner in primary cortical culture at low picomolar concentrations. gp120 neurotoxicity also requires calcium and glutamate and is blocked by glutamate receptor antagonist.

The tat protein of HIV-1 is a potent activator of transcription directed by the viral long terminal repeat. The basic domain of the lentiviral tat protein has been demonstrated responsible for damages in mouse brain by infusion of tat peptides in the lateral ventricle or in the grey matter by either systemic (hippocampus) or local (thalamus) injection. An inflammatory process characterized by the formation of an edema and invasion of macrophage accompanied by reactive astrogliosis led to the loss of neurons in grey matter. TNF-α, IL-1α and β, IL-6 RNA have been detected by probing. Blockade of TNF-α by pentoxifylline treatment led to the decrease of IL-1 and inducible nitric oxide

The neurotoxins - NO, O$_2$ and ONOO$^-$, as a group, potentiate NMDA receptor-mediated neurotoxicity. It has been proposed that NO interacts with the superoxide anion (O$_2^-$) to form peroxynitrite (ONOO$^-$) which is an extremely reactive and toxic molecule (Dawson et al., 1993). Injury to the astrocyte by cytokines, HIV or HIV gene products results in increased production of Na$^+$/H$^+$ antiport potassium conductance (Benos et al., 1994) and ultimately glutamate release from astrocytes. In turn, glutamate over-excites neurons leading to an increase in intracellular Ca$^{++}$, neuronal injury and further release of glutamate amplifying these cellular interactions. The final pathway of neurotoxic action may be blocked by NMDA antagonists (Bernton et al., 1992). Calbindin immunoreactive neurons in the neocortex were significantly reduced in HIVE (p < 0.001). The loss of these neurons was correlated with viral burden (p < 0.001), suggesting the differential vulnerability of these neurons in HIVE (Masliah et al., 1995)

1.10.3 Blood-brain barrier (BBB) and HIV-1 infection
The term blood-brain barrier (BBB) is generally applied to describe the overall interfaces between circulating blood at one side, and the extracellular as well as the CSF space at the other side (Felgenhauer, 1986). The BBB consists, in part, of the microvascular endothelium and associated astrocyte foot processes, found in close apposition to the abluminal side of the vascular endothelial cells (EC) and the basal lamina.

Impairment of the BBB is another important pathogenetic feature that contributes to brain damage in HIV-1 infection. A common histological observation in HIV encephalitis is the localization of HIV proteins within the cells of monocyte/macrophage lineage, suggesting that HIV can enter the CNS as cell-associated virus (Hurwitz et al., 1994).

To gain entry into the CNS, through the BBB, (1) HIV may directly infect endothelial cells (EC) and pass into the CNS as cell-free virus; (2) it can enter the CNS either cell free or cell associated virus through cytokine or other factors disrupted BBB; (3) HIV-1 enters the CNS via infected monocytes or lymphocytes and the entry is facilitated by the expression of adhesion molecules by the EC and astrocyte components of the BBB. The function of adhesion molecules has been studied recently by Hurwitz et al (1994) using a tissue culture model of BBB. Results demonstrate that astrocytes upregulate the expression of ICAM-1 (a pan-leucocyte adhesion protein) by EC; TNF-α stimulated EC/astrocytes or astrocytes culture express the adhesion proteins IG9, ICAM-1, vascular cell adhesion molecule 1 (a lymphocyte and monocyte adhesion molecule) (VCAM-1) and E-section (a lymphocyte and neutrophil adhesion protein) (Lasky, 1992) and finally, EC bind more HIV-1 infected monocytes than uninfected monocytes. IL-1 has also been demonstrated to alter the BBB by inducing other cytokines and adhesion molecules. It
likely stimulates the expression of adhesion molecules VCAM-1 and endothelial leucocyte adhesion molecule (ELAM-1) on endothelial cells and possible ICAM-1 in astrocytes (Frohman et al., 1989). These molecules have been detected in brains of HIV-1 infected humans and SIV-infected monkeys (Sasseville et al., 1992). TGF-β1 induced in astrocytes of the BBB could also facilitate this entry as nanogram of TGF-β1 in circulation thus providing a necessary signal attracting monocytes/macrophages, including those that are latently infected with HIV, into parenchyma (da-Cunha et al., 1993c).

Moreover, Moses and Nelson (1994) have demonstrated that human brain capillary endothelial (HBCE) cells are permissively infected by HIV. This is in keeping with results of several other groups (Wiley et al., 1986; Koenig et al., 1986; Stoler et al., 1986; Ward et al., 1987; Rostad et al., 1987). This infection is noncytolytic and is mediated by a CD4 and GalCer lacking independent mechanism. T cell tropic but not brain-derived macrophage tropic HIV strains selectively infect brain endothelium suggesting that T cell tropism is important for HIV entry through the BBB either by infection of HBCE cells or via entry of HIV-1 infected leucocytes. Brain endothelial cells have also been identified as a target cell for SIV in the CNS of rhesus macaques (Lackner et al., 1991). Recently, Nottet and Gendelman (1995) propose that HIV-1 penetrates the BBB inside differentiating macrophages, which become immune-activated once inside the brain, and secrete high levels of neurotoxins. Chronic, subclinical disease results by astrocyte regulation of macrophage effector functions. Ultimately, endogenous control mechanisms break down, leading to motor and mental impairments in some affected subjects.
Taken together, the data supported the hypothesis that the BBB may participate in the entry of HIV infected monocytes into the CNS and that adhesion molecules may contribute to the extravasation of HIV infected monocytes into the CNS parenchyma.

1.11 Summary

Elucidating the pathogenesis of HIV-1 induced lesions in the CNS must take into account that the virus cannot be found within nerve cells; the track is further complicated by the fact that relatively small amounts of organism can be visualised both by immunohistochemistry and in situ hybridization methods. These findings, and the lack of obvious cytopathic effect by HIV on neuroectodermal cells in vivo, have led to a number of pathogenetic mechanisms being proposed. One widely regarded hypothesis takes into consideration the state of immune activation of the brain, as indicated by the expression by macrophages of MHC class II antigens. Enhanced expression of these antigens has been demonstrated in a number of HIV infected cells (astrocytes, microglia, monocytes) as well as in sensory and sympathetic ganglia of HIV-1 infected subjects. Expression of MHC class II antigens has been correlated with the AIDS dementia complex as well as with HIV encephalitis. Once activated, MHC class II positive cells can produce toxic proteins such as cytokines. Indeed cytokines have been detected in the CNS during HIV infection.

The point remains that, to date, it is not clear when (and how) the virus enters and how commonly HIV is present in the brain at early stages of infection before development of AIDS. This is important to elucidate these points as they may show when to start
feeling worried and initiate treatment.

1.12 Aims of the present study

The aim of this study was to ascertain 1) whether the presence of HIV provirus DNA and microglial hyperplasia in the brain during the pre-AIDS stages of the infection is accompanied by enhanced expression of MHC class II antigens and by presence of cytokines, 2) which may suggest that brain damage could take place even at this early stage of the infection; finally 3) whether levels of HIV-1 DNA in brain correlate with stages of the infection and might be a predictor of progression of disease.
CHAPTER TWO:
MATERIALS AND METHODS
2.1 Subjects

Brain samples of 63 individuals were examined by histochemistry for detection of RCA and immunohistochemistry (IHC) for astrocytic density, HIV-1 p24, MHC class II and cytokines as well as by PCR to detect HIV-1 DNA. Samples included 18 AIDS cases and 9 normal controls chosen from the series of the Department of Neuropathology, Institute of Neurology, University of London. Cases with no neuropathological abnormalities as well as those with a neuropathological diagnosis of HIVE, HIV-lep were selected for study. The normal controls included five which did not belong to any risk group for HIV-1 infection and in which no neuropathological abnormalities of any type as well as four HIV-1 negative drug addicts. Of the 36 HIV-1 seropositive asymptomatic cases, 23 were obtained from Professor F Gray, Department of Neuropathology, Hôpital Henri Mondor, France; 13 were obtained from the Edinburgh Medical Research Council (MRC) AIDS Brain Bank, through the courtesy of Dr. Jeanne Bell. The cause of death of the 36 pre-AIDS individuals was recorded as accidental and included drug overdose in 31 and suicide in 5.

Among these 63 individuals 33 HIV-1 DNA positive on PCR including 16 AIDS (6 demented and 10 undemented) and 17 HIV-1 positive pre-AIDS cases were further quantitatively examined.

In a pilot study the PCR technique used to detect HIV-1 DNA in frozen tissue was adapted for paraffin embedded specimens; 20 AIDS brains, from which both fresh frozen and paraffin specimens were available, were randomly chosen from the series of the
Department of Neuropathology, Institute of Neurology, London. The frontal poles of brain were removed and frozen whilst the rest of the brain was fixed in 10% buffered formalin for a variable length of time (Table 3.5). For the purpose of this study, paraffin embedded tissue from sites adjacent to the frozen sample was used.

All the specimens used for morphology, IHC, in situ end labelling (ISEL) and PCR (pilot study exclusively) were formalin fixed and paraffin embedded. Anterior frontal lobe block was selected from each case. For morphology, IHC and ISEL, all sections were cut at 7 microns, mounted on silane (3-aminopropyl triethoxysilane) coated slides and air dried at 37°C. All sections were examined blindly. The delay between death and post mortem was recorded and was between 20 and 36 hours for all the cases. Fixation time for the AIDS and control brains ranged from 3 weeks to 5.5 months; for the HIV-1 positive pre-AIDS brains it did not exceed 3 weeks.

2.2 Methods

2.2.1 Morphological examination

Routine histological examination included staining with haematoxylin and eosin and van Gieson methods in all HIV-1 positive AIDS, pre-AIDS and HIV-1 negative controls. Luxol fast blue / cresyl violet, Glees and Marsland's silver stain, histochemical and immunohistochemical and in situ hybridisation methods for opportunistic infections were carried out on selected blocks from each case of AIDS, to exclude other aetiologies that might not have been morphologically obvious.
2.2.2 Histochemistry and immunohistochemistry (IHC)

2.2.2.1 Markers for macrophages / microglia and astrocytes

Monoclonal anti-GFAP (see Table 2.1) and the biotinylated lectin *Ricinus communis agglutinin* (RCA)-120 (Vector Labs, UK) were used as astrocyte and macrophage / microglia markers respectively.

2.2.2.2 Antibodies

The details of antibodies applied in the study are listed in Table 2.1, together with optimal dilutions and other technical information. To identify HIV antigen, polyclonal anti-p24, which recognizes the *gag*-associated protein p24, was used. A monoclonal anti-human MHC class II (HLA-DR) antibody (BioGenex, UK) was used to identify MHC class II expression, and polyclonal anti-human interleukin 1α (IL-1α, Cistron, USA), interleukin 4 (IL-4) and interleukin 6 (IL-6) as well as tumour necrosis factor-α (TNF-α, Genzyme, UK) were used for detection of cytokines.
Table 2.1 Summary of the antibodies employed in the study

<table>
<thead>
<tr>
<th>Primary antibodies</th>
<th>Source</th>
<th>Dilution employed</th>
<th>Supplier</th>
<th>Effect of cytokine on HIV replication (Poli and Fauci, 1992b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-p24</td>
<td>Rabbit</td>
<td>1:400</td>
<td>DuPont Diagnostics, UK</td>
<td></td>
</tr>
<tr>
<td>Anti-GFAP</td>
<td>Rabbit</td>
<td>1:400</td>
<td>Dako Ltd, UK</td>
<td></td>
</tr>
<tr>
<td>Anti-HLA-DR (MHC II)</td>
<td>Mouse</td>
<td>1:5</td>
<td>BioGenex, UK</td>
<td></td>
</tr>
<tr>
<td>Anti-TNF-α</td>
<td>Rabbit</td>
<td>1:25</td>
<td>Genzyme, UK</td>
<td>inductive</td>
</tr>
<tr>
<td>Anti-IL-1</td>
<td>Rabbit</td>
<td>1:15</td>
<td>Cistron Technology Inc, USA</td>
<td>inductive</td>
</tr>
<tr>
<td>Anti-IL-4</td>
<td>Rabbit</td>
<td>1:25</td>
<td>Genzyme, UK</td>
<td>inductive/suppressive</td>
</tr>
<tr>
<td>Anti-IL-6</td>
<td>Rabbit</td>
<td>1:20</td>
<td>Genzyme, UK</td>
<td>inductive</td>
</tr>
</tbody>
</table>
2.2.2.3 IHC

IHC was performed as described by An et al (1994) and Sinclair et al (1994). The sections were deparaffinized by washing 3 times with xylene, treated with 1% H$_2$O$_2$ in methanol (to block endogenous peroxidase), followed by rehydration and washing in tap water and phosphate-buffered saline (PBS). After blocking of non-specific immunoglobulin binding with 5% normal swine serum for 10 min at room temperature (RT) in Tris-buffered saline (TBS), the sections were incubated with primary antibody at 4°C overnight. After further rinsing in TBS and incubation with biotinylated secondary antibodies for 30 min at RT and a 30 min reaction with an avidin-peroxidase complex at RT, the reaction was finally developed with 3,3-diaminobenzidine (DAB) and sections were counterstained with Meyer’s haematoxylin. Procedures for p24 and cytokine detection were applied with a little modification from the above protocol. During the procedure, following the deparaffinisation and blocking of endogenous peroxidase, sections were heated for 5 min twice in a microwave oven at high power in 0.1M sodium citrate buffer pH 6.0. Microwave antigen retrieval represented a technical advance within IHC that greatly increased the range of antibodies which can be used to study formalin fixed, paraffin embedded tissues (Cuevas et al., 1994).

2.2.3 Quantitative assessment of density of microglia / macrophages and astrocytes

Quantitative measurements of the density of RCA-120 and GFAP positive cells were made by counting cells in a defined area on a tissue section as described by Ciardi et al (1990). Counting was performed using a Zeiss microscope with a 40 x objective and a
graticule was fitted in the eyepiece of the microscope to define an area over the section. The area of the field defined by the graticule was 1 mm$^2$, corroborated using a slide micrometer. Cells were counted in seven fields and the mean of these counts was the number of cells per mm$^2$. In both gray and white matter RCA-120 and GFAP positive cells with a recognisable nucleus were counted (endothelial cells which are also stained by RCA-120 were not taken into account). Fields can be selected at random when the tissue is homogenous, and the cells to be counted are distributed evenly throughout the tissue. Normally, ramified microglial cells appear uniformly dispersed and are present in nearly equal numbers in the gray and white matter (Dickson et al., 1993). However, in HIV-1 infection hyperplasia of microglia / macrophages is prominent in the white matter. The density of microglia / macrophages has been counted in gray and white matter separately. In contrast to microglia, astrocytes appear to have a nonuniform distribution and in particular, in tissues with a complex structure, such as the cerebral cortex. GFAP positive astrocytes have been counted in the white matter, subpial and middle area of cortex and expressed as cells per mm$^2$ respectively.

A semi-quantitative method was also devised in pre-AIDS individuals from ± to ++ based on the morphology of RCA-120 and GFAP positive cells, i.e. their size and the length of cell processes, and indicated as ±, + or ++ on each section, in which there is microglia infiltrate or gliosis (see Table 3.1). RCA-120 positive cells were defined as ±: RCA positive cells rare; +: ramified microglia with slender processes; ++: ramified microglia with enlarged nucleus and thickened processes. Scoring system for GFAP immunoreactivity was based on appearance of the majority of GFAP positive astrocytes in each section. ± indicates that positive cells were rare; +: a small amount
of ill-defined cytoplasm or a few short processes; ++ moderate amount of cytoplasm or processes of moderate length. Cells with a macrophages like morphology were simply classified as macrophages.

2.2.4 Semi-quantitative assessment of expression of MHC II and cytokines

A semi-quantitative scoring system for evaluation of expression of MHC class II antigens and cytokines was devised which took into account density of positive cells and scored the results applying a scale ranging from 0 to 3. Accordingly, absent or minimal expression either of MHC class II or cytokines were labelled 0 to 1; moderate 2 and high 3. Alternatively, 1, 2 and 3 indicate the presence of less than 10, between 10 and 20 and more than 20 cells, respectively, per high-power field (x250).

The specificity of the immunostaining for cytokines was verified using phorbol myristate acetate (PMA) activated human cell line (U937; monocyte) and lymph nodes obtained from a patient with AIDS as it is known that the macrophages derived from stimulated monocytes (Harris et al., 1993) and HIV infected lymph nodes (Aggarwal and Gutterman, 1992) express TNF-α, IL-1α, 4 and 6.

2.2.5 In situ end labelling (ISEL)

2.2.5.1 Positive control

Three specimens of neuroblastoma were used as positive controls to optimise conditions
for ISEL and in each procedure using ISEL.

2.2.5.2 Optimisation of conditions for ISEL

As the specimens used in this investigation were formalin fixed, permeation and digestion by proteinase was a preliminary essential step for the success of this technique. Concentrations of proteinase K (Boehringer Mannheim, U. K.) ranging from 1-100 μg/ml as well as incubation times ranging from 10 to 30 min were used to determine the optimal concentration of the enzyme. Initially, incubation was fixed at 37°C for 10 min. A series of dilutions containing proteinase K at concentrations of 1, 10, 20, 50, 100 μg/ml was used on a positive control. After an optimised concentration of proteinase K has been selected, sections were digested at that concentration of proteinase K for 10, 20 and 30 min respectively to select the optimised time for digestion.

2.2.5.3 Detection of DNA fragmentation using DNA polymerase 1 and terminal deoxynucleotidyl transferase (TDT)

All 54 HIV-1 positive patients, 9 negative and 3 surgical tumour control specimens were investigated by ISEL with DNA polymerase 1. Moreover, to test the validity of the ISEL with DNA polymerase, 2 cases with HIVE, 2 HIV-1 positive non-AIDS, 2 negative and 2 surgical positive control were also studied by ISEL with terminal deoxynucleotidyl transferase (TDT).

2.2.5.3.1 ISEL of fragmented DNA by DNA polymerase 1 (nick translation)
Paraffin embedded sections were deparaffinized and rehydrated, then treated with 0.3% Triton X-100 in PBS for 15 min at RT for detergent permeabilization, followed by proteinase K (1 μg/ml) digestion in 100 mM Tris-HCl pH8.0, 50 mM EDTA at 37°C for 10 min; digestion was stopped by washing with PBS. Sections were incubated at 37°C with the end labelling buffer (50 mM Tris-HCl pH7.5, 5 mM MgCl_2, 10 mM 2-mercaptoethanol, 0.005% BSA) containing 0.03 mM dATP, dCTP, dGTP and 0.02 mM dTTP (Promega, UK), 0.01 mM digoxygenin (DIG)-11-dUTP (Boehringer Mannheim) and 50 U/ml Klenow DNA polymerase 1 (Boehringer Mannheim) for 120 min. After blocking with buffer 1 (0.1 M Maleic acid, 0.15 M NaCl Ph 7.5) containing 2% normal goat serum and 0.3% Triton X-100 for 30 min at RT, sections were incubated with anti-DIG antibody conjugated with alkaline phosphatase (AP) (Boehringer Mannheim) diluted 1:500 in buffer 1 containing 1% normal goat serum at 4°C overnight. After washing with buffer 1, colorimetric detection was followed. Slides were stained with nitroblue tetrazolium chloride (NBT) / X-phosphate in buffer 2 (100 mM Tris-Hcl pH9.5, 50 mM MgCl_2, 100 mM NaCl) and then counterstained with nuclear fast red.

2.2.5.3.2 TDT-mediated dUTP-fluorescein nick end labelling (TUNEL)

Nuclear DNA fragmentation was detected by ISEL using TDT (Gold et al., 1994). Boehringer Mannheim's In situ cell death detection kit was used to detect apoptotic neurons in 2 HIV-1 positive cases with AIDS, 2 HIV-1 positive pre-AIDS individuals showing a positive reaction by end labelling with DNA polymerase 1 as well as in 2 positive and 2 negative controls. Cell permeabilization and proteinase K digestion were carried out as described above (see 2.2.5.3.1). End labelling procedures were carried
out according to the protocol recommended by the supplier. Briefly, sections were incubated with the TUNEL reaction mixture containing TDT and fluorescein-dUTP. During the incubation period (37°C for 120 min), TDT catalyses the additional fluorescein-dUTP at free 3'-OH groups in single and double stranded DNA. After washing, the label incorporated at the damaged site of the DNA is marked by an anti-fluorescein antibody conjugated with AP. Colorimetric detection was as above (2.2.5.3.1).

The details of procedures of ISEL are shown in Appendix B.

2.2.5.4 Double labelling with ISEL and lectin (RCA-120)

The lectin RCA-120 was applied to reveal cells of the microglia / macrophage lineage, on slides previously labelled with ISEL method.

2.2.6 PCR

2.2.6.1 Preparation of DNA

2.2.6.1.1 Extraction of DNA from fresh frozen tissue

DNA was extracted by the methods of Pang et al (1990) and Sinclair and Scaravilli (1992b). Each block (around 0.5 g) of semi-frozen frontal lobe containing both white and gray matter was stripped of leptomeninges and diced into small pieces. Tissue was
digested overnight with proteinase K (final concentration of 0.25 mg/ml in proteinase K buffer) at 55°C with gently shaking. Subsequently DNA was purified by the standard method of Sambrook et al (1989). DNA was extracted with phenol, phenol / chloroform and chloroform and purified by precipitation with ethanol.

2.2.6.1.2 Extraction of DNA from formalin fixed paraffin embedded tissue

The technique for DNA preparation from formalin fixed, paraffin embedded tissue was described by An et al (1994). As leptomeninges in AIDS and pre-AIDS cases may include discrete numbers of lymphocytes, some of which may contain HIV, the area of the paraffin section including the meninges was removed from the specimen to be used for PCR with a disposable scalpel for each case examined. Ten sections (10 µm) were cut and placed in a 1.5 ml Eppendorf tube providing a yield of about 30 µg of DNA. The paraffin was removed by 3 washes with xylene for 15 min followed by 100% ethanol for 5 min. The tissue was dried at 95°C for approximately 30 min, then digested for 24 to 48 hours at 55°C with proteinase K at a final concentration of 5 mg/ml. 50 µl of proteinase K stock solution (50 mg/ml) might be added to the tube after an overnight digestion if necessary. Extraction of DNA in the supernatant was purified by a standard method with phenol / chloroform and chloroform, followed by a centrifugation through Microcon-30 (Amicon UK) using an Eppendorf centrifuge at 13,000g for 10 min. The initial volume of 0.5 ml was finally reduced to about 5-20 µl (pure DNA). DNA was recovered and quantified by spectrophotometer (see Appendix C).

2.2.6.2 Primers
All the sequences of primers are written from 5' to 3':

Human β-globin primers [MRC, AIDS Directed Programme (ADP), National Institute for Biological Standards and Control (NIBSC)]-

ADP894.1: ACA CAA CTG TGT TCA CTA GC;
ADP894.2: CAA CTT CAT CCA CGT TCA CC.

A 110bp (position 14-123) fragment is flanked by primers ADP894.1 and ADP894.2.

HIV-1 primers 3855/1 and 3855/2 [Perkin Cetus Elmer (ILS Ltd, London, UK)] have been used in pilot PCR study.

3855/1 (SK145): AGT GGG GGG ACA TCA AGC AGC CAT GCA AAT;
3855/2 (SK431): TGC TAT GTC AGT TCC CCT TGG TTC TCT.

A 142bp (position 1366-1507) fragment is flanked by primers 3855/1 and 3855/2.

HIV-1 primers pol 1, pol 2 and pol 3 were used in the nested PCR for detection of HIV-1 DNA in the brains as well as semi-quantitative analysis.

pol 1 (3181-3203): CAG GAA AAT ATG CAA GAA TGA GG;
pol 2 (3324-3302): CCC ATG TTT CCT TTT GTA TGG GT;
pol 3 (3228-3247): CAA TTA ACA GAG GCA GTG CA with 5’ digoxigenin (DIG) -11-dUTP end labelled.

A 144 bp fragment was amplified at the first round of PCR by pol 1 and pol 2. The second round of PCR was amplified by pol 3 with 5’ digoxigenin (DIG) -11-dUTP end labelled and pol 2. A 97 bp fragment is flanked by pol 2 and pol 3.
2.2.6.3 PCR analysis

2.2.6.3.1 Pilot study

Human single copy gene β-globin was used as a DNA positive control and only samples that were clearly positive were used for HIV PCR. Ten molecules of non-infectious plasmid containing a completely rearranged HIV sequence (provided by UK MRC, ADP, PCR Reference Center) were used as the HIV positive control. DNA from brain tissue of patient not in any HIV risk group was used as a negative control.

50 μl of the reaction mixture contained 0.5 μM of each primer (ADP894.1 and 894.2), 0.2 mM of dATP, dCTP, dGTP and dTTP and 2.5 units amplitaq DNA polymerase in PCR buffer (10 mM Tris-HCl, pH8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.001% gelatin). Forty cycles of PCR were performed with 1 μg of extracted template DNA, each cycle consisting of thermal denaturation at 94°C for 1 min, primer annealing at 60°C for 2 min and extension at 72°C for 3 min for β-globin gene and, using 3855/1 and 3855/2 and annealing at 55°C for HIV-1.

Amplified β-globin products were visualised by ethidium bromide staining of agarose gel electrophoresis. HIV products were further detected by Southern blot hybridisation to ascertain their specificity. To detect the 142bp PCR product (amplified by HIV-1 primers 3855/1 and 3855/2) modified Saluz and Jost "filling in" method (1986) was applied. Oligonucleotides were provided by the MRC ADP NIBSC. Their sequences are:

- gag (30 mer): 5'-CAT CAA TGA GGA AGC TGC AGA ATG GGA TAG;
An annealing mix containing 10 µl of the 30 mer, 10 µl of the 10 mer and 2.5 µl of the oligonucleotide labelling buffer (500 mM NaCl, 100 mM Tris-HCl pH 7.5, 100 mM MgCl₂, 10 mM dithiothreitol) was prepared and incubated at 75°C for 2 min, at room temperature for 20 min and on ice for 10 min. This was followed by addition of 1 µl each of 10 mM dATP, dGTP and dCTP. 2 µl of this mix was labelled by addition of 0.5 µl DIG-11-dUTP, 0.5 µl 1 mM dTTP and 1 µl Klenow (Boehringer Mannheim, UK) and incubated for 1 hour at RT. The probe was precipitated by ethanol.

Nylon membranes (Boehringer Mannheim, UK) were prehybridized for more than 1 hour at 42°C in hybridization buffer [5 x Saline-Sodium Citrate (SSC), 50% formamide, 0.1% N-lauroylsarcosine, 0.02% sodium dodecyl sulfate (SDS) and 2% blocking reagent] and then hybridized overnight at 42°C with 200 ng/ml of labelled probe. They were then washed in 4 changes 0.2 x SSC, 0.1% SDS at RT for 1 hour.

Colorimetric detection with NBT and X-phosphate was performed according to the Boehringer Mannheim protocol (Boehringer Mannheim, 1993). After incubation with anti-DIG-AP at RT for 30 min and a post-incubation wash, the filter was incubated in substrate for development of colour.

2.2.6.3.2 Detection of HIV-1 DNA from patients with pre-AIDS, HIVE and AIDS without detectable neuropathological changes

Nested PCR was used to detect HIV sequences in paraffin embedded brain tissues of all
63 cases. The primers used in this study were designed to amplify a sequence of HIV pol gene. 50 μl of the reaction mixture contained 0.3 μM of each primer (either pol 1 and pol 2 or pol 2 and pol 3), 0.2 mM of dATP, dCTP, dGTP and dTTP, and 2.5 units of Biotaq DNA polymerase in PCR buffer [16 mM (NH₄)₂SO₄, 67 mM Tris-HCl (pH8.8), 3 mM MgCl₂, 0.01% Tween-20]. Thirty two cycles of first round of PCR were performed with 1 μg extracted DNA, each cycle consisting of thermal denaturation at 94°C for 1 min, annealing and extension at 63°C for 2 min. In the second round of PCR 28 to 30 cycles were performed with 2.5 μl of PCR product obtained from first round amplification in the 50 μl of reaction. Thus 97 bp DIG labelled PCR product was obtained after the second round amplification. PCR product was analyzed by electrophoresis and Southern transfer followed by chemiluminescence.

2.2.6.3.3 PCR positive control

Human β-globin gene was amplified in all samples to confirm that the quality and quantity of extracted human DNAs were suitable for the amplification. Amplified β-globin product was visualised by ethidium bromide staining of agarose gel electrophoresis.

2.2.6.4 Semi-quantitative PCR

2.2.6.4.1 Standard DNA

A series of dilution containing 1, 10, 100 and 1,000 copies of HIV-1 plasmid DNA
(MRC, ADP, PCR Reference Kit) was supplemented with normal human genomic DNA to the concentration of 1 μg per reaction. These dilutions run parallel with samples at each PCR amplification.

2.2.6.4.2 Optimised conditions for quantification

Nested PCR was used. In order to obtain exponential phase in both first and second round of amplifications, standard DNAs were amplified for different cycles. In the first round of PCR, 28, 30, 32, 34 and 36 cycles were applied whilst 12, 14, 16, 18 and 20 cycles were applied in the second round of amplification.

2.2.6.4.3 Detection of levels of HIV-1 DNA from HIV-1 positive individuals with and without symptoms

From the results of the amplification 28 cycles were chosen for the first round and 18 for the second. Each cycle consists of thermal denaturation at 94°C for 1 min, annealing and extension at 63°C for 2 min. A 144 bp fragment was amplified in the first round of PCR by pol 1 and pol 2. A 97 bp fragment was obtained from the second round of PCR applying pol 3 [with 5’ DIG-11-dUTP end labelled] and pol 2. A series of dilutions of known copy number of HIV DNA was amplified parallel as external standard in each amplification. After 28 cycles of the first round amplification 2.5 μl of PCR product was amplified for 18 cycles using primers pol 2 and DIG-11-dUTP labelled pol 3. Following electrophoresis and Southern transfer (VACU-AID, Hybaid, UK) amplified DNA was
detected by chemiluminescence (CSPD, Boehringer Mannheim) and densitometry (see Appendix D). The value represented mean of two or three tests.
CHAPTER THREE:

RESULTS
3.1 Neuropathology

The brains of 18 AIDS patients were examined: 10 showed changes characteristic of HIV leukoencephalopathy (Fig. 3.1a) / encephalitis (Fig. 3.1b). Both types will be labelled as HIVE in this thesis; the other 8 showed no obvious neuropathological lesions. The main HIV-related neuropathological abnormalities of encephalitis were predominantly in the hemispheric white matter and consisted of multiple small foci, predominantly perivascular, of microglia, macrophages and astrocytes and included a number of multinucleated giant cell (MGCs). MGCs were localised predominantly in nodules of microglial cells and in the perivascular region in places forming clusters. Fig. 3.1c shows reactive astrocytes, macrophages and MGCs. One large MGC is at the centre with a few nuclei forming a circle at the edge of the cell; some nuclei are joined by nuclear bridges (arrow) are seen. MGC (arrow) in Fig. 3.1d is located in the perivascular region. Microglia and macrophages were forming, in place, loose irregular nodules in HIVE. These foci were separated from each other, giving the area a moth-eaten appearance (Fig. 3.1b). These features have been called HIV encephalitis (HIVE). These changes are usually most severe in the anterior regions of the hemispheres but are found also in the deep grey nuclei and brain stem. Areas of complete loss of myelin appear diffusely pale and the whole process has been named HIV-leukoencephalopathy (HIV-lep, Fig. 3.1a). The difference between HIVE and HIV-lep is that lesion patterns in the former is multifocal and inflammatory, whereas in the latter, lesion appears as a diffuse damage to the white matter featuring myelin loss, reactive astrogliosis, macrophages and MGCs.
Abnormalities in the 36 HIV-1 positive pre-AIDS group included astrogliosis in 34, microgliosis (31 cases), discrete meningitis (11 cases, see Fig. 3.2) and macrophages in 4 cases. Fig. 3.2 shows thick meninges with small cell infiltration. Microgliosis and astrogliosis are discussed in 3.2. Hypoxic changes appearing as angulated somata and shrunken nuclei of some neurons with uniformly eosinophilic cytoplasm were seen in 14 cases. There was one completely normal brain. The neuropathology of the 36 pre-AIDS individuals is summarized in Table 3.1. Cases No. 1 to 17 were HIV-1 DNA positive by PCR whereas cases 18 to 36 were PCR negative. There was no correlation between microgliosis and astrogliosis and presence of HIV-1 DNA whereas a correlation could be established between the presence of meningitis and presence of HIV-1 DNA (see Table 3.1, 8 out of 17 HIV-1 DNA positive compared with 4 out of 19 HIV-1 DNA negative cases, p = 0.0753, Fisher).

3.2 Density and morphology of microglia/macrophages and astrocytes

Lectin. RCA-120 positive cells were increased in number in most AIDS brains with HIVE as well as in a number of pre-AIDS brains when compared with normal controls (see Table 3.2). Amongst normal controls there was no difference between 5 normal brains which had no any risk of HIV-1 infection and brains of 4 HIV-1 negative drug addicts. The means of RCA-120 positive cells were 219 ± 122 (standard deviation, SD) and 175.4 ± 93.3 in HIVE and pre-AIDS groups, respectively.

Fig. 3.3 shows the results of RCA-120 staining in various groups. Fig. 3.3a refers to RCA-120 staining in normal control. In normal brains only a few scattered ramified
microglial cells with thin, faint cell processes can be seen, whereas RCA-120 positive cells showed increase in size and number of branched processes in most of the AIDS brains with HIVE (Fig. 3.3b) and in a number of pre-AIDS brains (Fig. 3.3c). The difference between these two HIV-1 positive groups was the presence in the former, of numerous macrophages and some MGC (Fig. 3.3b). On the other hand, in AIDS brains without detectable neuropathological changes, there was only a slight increase in number of microglial cells (mean = 81.4±28.5) (whose size was only moderately increased) in addition to a few scattered macrophages (Fig. 3.3d). Comparison of results of histochemistry and the densities per mm² of both microglia and macrophages in the various groups are shown in Fig. 3.4 and Table 3.2.

GFAP. The density of GFAP-positive astrocytes was significantly increased only in the HIVE group as compared with normal controls. On the other hand, there was no statistically significant difference between AIDS without CNS disease, pre-AIDS and normal control cases which includes HIV-1 negative drug addicts (see Table 3.2 and Fig. 3.4). However, when the size of cells was considered, this parameter was significantly increased in each of the three HIV-1 seropositive groups compared with control (3.5a). In particular, 34/36 pre-AIDS individuals showed slightly or severely swollen astrocytes (Fig. 3.5c), whereas the appearance of astrocytes in severe cases could be similar to that seen in HIVE (Fig. 3.5b). A difference in the distribution of astrocytes between HIVE and pre-AIDS groups is that, in the former, both white and grey matter were variously involved whereas in the latter obvious astrogliosis was seen only in white matter. In brains of AIDS without neuropathological disorders astrocytes showed similar appearance as seen in pre-AIDS group (Fig. 3.5d) .
Fig. 3.1 Photograph (a) and photomicrographs (b, c & d) of brain sections showing the appearances of HIV-lep (a), HIVE (b) and the histological appearances of leukoencephalitis (c & d). (b) - (d) are shown in the next page. (a) shows that myelin is diffusely pale in HIV-lep; (b) shows multiple foci of myelin pallor in the white matter of HIV encephalitis; (c) shows reactive astrocytes and two MGCs. One is large, irregularly round and shows some of the nuclei linked by internuclear bridges (arrow). In (d) there is an increased number of glia as well as the presence of MGC (arrow) localised to the perivascular region. (a) Luxol fast blue (LFB) and haematoxylin & eosin (H & E). (x5); (b) LFB. (x38); (c & d) H & E. (x500)
Fig. 3.3. Photomicrograph showing lymphoid nodes in a pre-AIDS case. Lymphoid nodes are slightly thickened by discrete lymphocytic infiltration. H & E. (x140)
Fig. 3.2 Photomicrograph showing leptomenigitis in a pre-AIDS case. Leptomeninges are slightly thickened by discrete lymphocytic infiltration. H & E. (x144)
Table 3.1 Summary of the neuropathological changes in 36 HIV-1 positive pre-AIDS individuals

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Meningitis</th>
<th>Microglia infiltrate</th>
<th>Gliosis</th>
<th>Hypoxic changes</th>
</tr>
</thead>
<tbody>
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<td></td>
<td></td>
<td>Morphology</td>
<td>RCA-120/mm²</td>
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</tr>
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<td>1</td>
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<td>-</td>
<td>64</td>
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<tr>
<td>2</td>
<td>+(L)</td>
<td>-</td>
<td>42</td>
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<tr>
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<td>-</td>
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<td>++</td>
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<td>4</td>
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<tr>
<td>6</td>
<td>-</td>
<td>+(W,G)(M)</td>
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<td>-</td>
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<tr>
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<td>-</td>
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<tr>
<td>8</td>
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<td>+</td>
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<td>10</td>
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<td>+(W)</td>
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<td>++</td>
<td>283</td>
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</tr>
<tr>
<td>12</td>
<td>-</td>
<td>±(W)</td>
<td>133</td>
<td>+ + (W), ±(G)</td>
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<td>±</td>
<td>231</td>
<td>+(W)</td>
</tr>
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<td>14</td>
<td>-</td>
<td>+</td>
<td>166</td>
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<td>+</td>
<td>+(W)(M)</td>
<td>143</td>
<td>+ + (W), +(G)</td>
</tr>
<tr>
<td>Case No.</td>
<td>Meningitis</td>
<td>Microglia infiltrate</td>
<td>Gliosis</td>
<td>Hypoxic changes</td>
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<td>---------</td>
<td>----------------</td>
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<td></td>
<td></td>
<td>Morphology</td>
<td>RCA-120/mm²</td>
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<td>18</td>
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<td>±</td>
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<td>170</td>
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<td>+(W), ±(G)</td>
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<td>+ (W)</td>
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<td>±(W)</td>
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<td>+</td>
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<td>±</td>
<td>128</td>
<td>+ + (W), ±(G)</td>
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<td>30</td>
<td>+</td>
<td>±(W)</td>
<td>142</td>
<td>+</td>
</tr>
<tr>
<td>31</td>
<td>-</td>
<td>±</td>
<td>241</td>
<td>±(W), +(G)</td>
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<tr>
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<td>±</td>
<td>+ + (W), +(G)</td>
<td>266</td>
<td>+ +</td>
</tr>
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<td>33</td>
<td>-</td>
<td>+ + (W), +(G)(M)</td>
<td>365</td>
<td>+ (W), ±(G)</td>
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<td>-</td>
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<td>+ + (W), +(G)</td>
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<td>+ (W)</td>
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<td>36</td>
<td>-</td>
<td>+</td>
<td>141</td>
<td>+ + (W), ±(G)</td>
</tr>
</tbody>
</table>

W = White matter; G = Grey matter; L = Lymphocyte; M = Macrophage; 
- = absent; ± = slight; + = moderate; ++ = high
Table 3.2 Density of RCA-120 positive and GFAP positive cells in pre-AIDS, HIV encephalitis (HIVE) and AIDS without CNS disease

<table>
<thead>
<tr>
<th>Group</th>
<th>Cases</th>
<th>RCA 120</th>
<th>GFAP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean±SD</td>
<td>Median</td>
</tr>
<tr>
<td>Control</td>
<td>9</td>
<td>38±30.1</td>
<td>40</td>
</tr>
<tr>
<td>HIVE</td>
<td>10</td>
<td>219.2±122</td>
<td>205</td>
</tr>
<tr>
<td>AIDS</td>
<td>8</td>
<td>81.4±28.5</td>
<td>80</td>
</tr>
<tr>
<td>pre-AIDS</td>
<td>36</td>
<td>175.4±93.3</td>
<td>153.5</td>
</tr>
</tbody>
</table>

HIVE = HIV encephalitis
AIDS = AIDS no neuropathology
Fig. 3.3 Photomicrographs showing brain sections stained with the lectin RCA-120. In normal brain (a) the few RCA-120 positive cells are ramified microglia with thin and faintly staining cell processes. (b) shows that in HIVE RCA-120 positive cells are increased in number and size and appear as macrophages and some MGC, only a few small microglial cells are seen (arrow); in pre-AIDS (c) increased (in number and in size) microglial cells are seen; and in AIDS without neuropathological lesions (d) the density of RCA-120 positive cells and their size are moderately increased. RCA and haematoxylin. (x350)
Fig. 3.4 Distribution of the density of RCA-120 and GFAP positive cells in pre-AIDS, HIVE and AIDS without CNS disease and in normal control.

AIDS = AIDS without neuropathological changes
Fig. 3.5a-d Photomicrographs showing brain sections stained with GFAP. The density of GFAP positive cells is increased only in HIVE group (b) compared with control (a). However, hypertrophy of GFAP positive cells is seen in all three HIV-1 positive groups (b,c,d). The proportion of swollen astrocytes is high in HIVE (b), moderate in pre-AIDS (c) and low in AIDS without neuropathology (d). GFAP and haematoxylin. (x350)
3.3 Expression of MHC class II and cytokines

3.3.1 Expression of MHC class II antigens and cytokines: comparison between groups (control, HIVE and AIDS without CNS disease vs pre-AIDS)

Elevated expression of MHC class II antigens and cytokines was demonstrated in all three HIV-1 positive groups. Group comparison was made by presenting the percentages of cases that scored low, moderate and high of MHC class II, TNF-α, IL-1α, IL-4, IL-6 in the white matter of each group (see Fig. 3.6 - Fig. 3.10). The expression of MHC class II and cytokines as represented by scores was high in HIV-1 positive patients (taken as a single group) compared with HIV-1 negative individuals; there was no obvious difference observed between normal and drug addicts. Interestingly, pre-AIDS patients showed higher scores for MHC class II, TNF-α and IL-1α than AIDS without neuropathological changes and higher scores for TNF-α and slightly higher for IL-4 than AIDS brains with HIVE. The grey matter was examined separately using the same scoring system described above (see materials and methods). Data for MHC II were comparable to those found in the white matter in both in AIDS and pre-AIDS brains. Regarding the cytokines, the scores obtained for the AIDS and pre-AIDS groups were considerably lower than those found in the white matter and in the latter group could be regarded as minimal.
Fig. 3.6 - 3.9 Graphs showing percentage of cases showing low, moderate and high levels of the expression of TNF-α (3.6), IL-1α (3.7), IL-4 (3.8) and IL-6 (3.9) in pre-AIDS, HIVE, AIDS without CNS disease and in normal control.

AIDS = AIDS without neuropathological changes
Mean score:  
pre-AIDS = 1.7;  
HIVE = 2.7;  
AIDS = 0.6

Fig. 3.10 Graph showing percentage of cases showing low, moderate and high levels of the expression of MHC class II antigens in pre-AIDS, HIVE, AIDS without CNS disease and in normal control.

AIDS = AIDS without neuropathological changes
3.3.2 Statistical analysis

Statistics using Mann-Whitney U test (preplanned group comparison without adjustment) (see Table 3.3) showed that the expression of MHC class II antigens was significantly higher in HIVE group than in pre-AIDS ($p = 0.004$) and in pre-AIDS group than in normal control and AIDS without neuropathology ($p = 0.051$ and $0.041$, respectively).

Regarding the cytokines, comparison between pre-AIDS with the other three groups (normal control, HIVE and AIDS without neuropathological changes) showed that they are not significantly (AIDS without neuropathology, $p = 0.056$) or significantly different (controls and HIVE, $p = 0.016$ and $0.015$, respectively) in the expression of TNF-α; highly significantly (controls, $p = 0.0001$), significantly (HIVE, $p = 0.015$) or slightly different (AIDS no neuropathology, $p = 0.08$) in expression of IL-1α. Except for the observation that expression of IL-4 and IL-6 in normal controls is lower than that found in pre-AIDS, there is only slight difference between pre-AIDS and two AIDS groups in the expression of IL-4 and 6.

Table 3.3 Group comparison vs pre-AIDS

<table>
<thead>
<tr>
<th></th>
<th>MHC II</th>
<th>TNF-α</th>
<th>IL-1α</th>
<th>IL-4</th>
<th>IL-6</th>
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<tbody>
<tr>
<td>Normal control</td>
<td>p=0.051</td>
<td>p=0.016</td>
<td>p=0.0001</td>
<td>p=0.057</td>
<td>p=0.027</td>
</tr>
<tr>
<td>HIVE</td>
<td>p=0.004</td>
<td>p=0.015</td>
<td>p=0.015</td>
<td>p=0.392</td>
<td>p=0.906</td>
</tr>
<tr>
<td>AIDS without neuropathology</td>
<td>p=0.041</td>
<td>p=0.056</td>
<td>p=0.08</td>
<td>p=0.463</td>
<td>p=0.259</td>
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3.3.3 Results: HIV-1 positive pre-AIDS individuals

Individual scores of the 36 pre-AIDS individuals are illustrated in Table 3.4 and include results of PCR. There are correlations between presence of HIV-1 DNA and expression of MHC II and cytokines. In 17 cases with positive PCR for HIV-1 DNA, moderate to high levels (scores 2 or 3) of expression have been found in 76% (13 out of 17) cases for MHC II, 59% for TNF-α, 76% for IL-1α, 65% for IL-4 and 71% for IL-6 compared with 47%, 42%, 63%, 37% and 26% in HIV-1 PCR negative individuals respectively. Statistical analysis using Fisher test showed that the expression of MHC II, TNF-α, IL-1α and IL-4 was higher in HIV-1 DNA positive cases than in HIV-1 DNA negative individuals (p = 0.058, 0.16, 0.197 and 0.068, respectively) whereas the expression of IL-6 was significantly higher (p = 0.008) in HIV-1 DNA PCR positive cases than HIV DNA negative ones.
Table 3.4 Summary of the results of PCR and IHC of HIV-1 positive pre-AIDS individuals

<table>
<thead>
<tr>
<th>Case No.</th>
<th>PCR</th>
<th>MHC II</th>
<th>TNF-α</th>
<th>IL-1α</th>
<th>IL-4</th>
<th>IL-6</th>
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<td>3</td>
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<td>+</td>
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<td>3</td>
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</tr>
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<tr>
<td>Case No.</td>
<td>PCR</td>
<td>MHC II</td>
<td>TNF-α</td>
<td>IL-1α</td>
<td>IL-4</td>
<td>IL-6</td>
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</tr>
</tbody>
</table>

0 = absent; 1 = low; 2 = moderate; 3 = high; 
- = negative; + = positive
3.3.4 Types of cell expressing MHC II and cytokines

Examples of cells immunostained for MHC class II antigen in pre-AIDS and AIDS brains are shown in Fig. 3.11a,b. In pre-AIDS (a) microglial cells and possibly some astrocytes were stained and signals were located in cytoplasm and the cell processes; HIVE (b) macrophages and some astrocytes showed positive immunoreactivity for MHC II.

Regarding the types of cells immunostained with the various antibodies against cytokines, TNF-α stained microglia/macrophages, pericytes and a small number of astrocytes; in pre-AIDS (Fig. 3.12a) the majority of immunostained cells were microglial cells in addition to a few astrocytes, whereas in HIVE (Fig. 3.12b) macrophages were also positive. IL-1 was seen in microglia/macrophages, astrocytes and some endothelial cells and in (d) an occasional glial cell. Expression of IL-4 was higher in pre-AIDS (e) than in AIDS (f) and appeared in microglia and astrocytes; IL-6 was seen in microglia, astrocytes and possibly some endothelial cells. Examples of the various cell types immunostained with the range of the different cytokine antibodies in the same groups are illustrated in Fig. 3.12a-h.
Fig. 3.11 Photomicrographs showing the expression of MHC class II antigen in the brains of HIV-1 positive pre-AIDS (a) and AIDS (b) individuals. In (a) the majority of positive cells are microglia whereas in (b) also positive macrophages are seen. MHC class II and haematoxylin. (X600)
Fig. 3.12 Photomicrographs showing the results of immunostaining with antibodies against cytokines. (a), (c), (e) and (g) represent pre-AIDS, (b), (d), (f) and (h) AIDS cases, (e) - (h) are shown in the next page. (a) & (b) illustrate TNF-α; (c) & (d) IL-1α; (e) & (f) IL-4; (g) & (h) IL-6. Counterstained with haematoxylin. (X900)
3.4.3. Apoptotic cells in brains of AIDS and pre-AIDS patients

No apoptotic cells were seen in any brain slide stained with control method. The ISFL method revealed some immunostained cells in the white matter of the negative controls and
3.4 Apoptotic cells in brains of AIDS and pre-AIDS cases

3.4.1 Effects of fixation on in situ end labelling (ISEL)

Digestion by 1μg/ml of proteinase K at 37°C for 10 min showed good sensitivity and minimized the intensity of the background. Increasing the concentration of proteinase K above this optimal level did not improve the sensitivity. Using this concentration, apoptotic cells could be demonstrated in brain tissue samples fixed in buffered formalin for periods ranging from 2 weeks to 5.5 months.

3.4.2 Other conditions for ISEL

Many other factors can also affect the sensitivity of detection of apoptosis. For example, the 1:3 ratio of dig-dUTP:dTTP recommended by Boehringer Mannheim (1989) for random primer directed synthesis and used by An et al (1992) in PCR amplification has also confirmed its suitability to improve sensitivity when compared with the use of dig-dUTP alone in the reaction. On the other hand, incubation with anti-DIG antibody at 4°C overnight provided the best results when compared with incubation either at RT for 2-3 hours or at 37°C for 30 min (data not shown).

3.4.3 Apoptotic cells in brains of AIDS and pre-AIDS patients

No apoptotic cells were seen in any brain slide stained with routine methods. The ISEL method revealed only an occasional cell in the white matter of the negative controls and
these could be identified as glial cells. Apoptotic cells were identified by *in situ* end labelling for free-3’-OH ends of cleaved DNA in 11 specimens; these included 6 of 10 HIVE cases, 1 of the 8 AIDS without neuropathological changes and 4 of the 36 HIV positive pre-AIDS cases. All 3 positive control specimens of neuroblastoma showed numerous isolated apoptotic cells.

A representative case of a patient with HIVE is shown in Fig. 3.13a and b. In Fig. 3.13a cortical neurons with apoptotic nuclei are shown. Nuclei are virtually of normal size. In Fig. 3.13b a perivascular cell with an apoptotic nucleus is seen. Fig. 3.13c and d show apoptotic cells in grey (c) and white matter (d) of a pre-AIDS case. In Fig. 3.13c a large neuron with an apoptotic nucleus is shown in detail whereas in Fig. 3.13d there are several apoptotic glial cells in white matter.

In the HIV positive pre-AIDS group, all but 1 ISEL positive case were also HIV-1 DNA positive on PCR. Except for the positive nuclei, apoptotic cells did not differ morphologically from negative cells; in particular the size and shape of the nuclei were unremarkable, the outlines of their cell bodies were identifiable and the cytoplasm did not appear dissimilar from that of negative cells.

With regard to the distribution of apoptotic cells, in the HIVE cases they were found both in the grey matter, where they were almost exclusively nerve cells (Fig. 3.13a), and in the white matter (Fig. 3.13b). In the cortex their density varied from area to area, did not seem to correlate with the areas of vascular vulnerability and appeared to be distributed throughout all the layers.
In the HIV-1 positive pre-AIDS cases, apoptotic cells were found in the white matter in all four cases (Fig. 3.13d), whereas in two, neurons were also stained (Fig. 3.13c). In the cortex, ISEL positive cells appeared scattered throughout all the layers, in places forming clusters of 3 - 4 cells. As far as the cortex is concerned, although the total population of apoptotic neurons was not quantified in these tissue sections, the density of ISEL-positive neurons was smaller in pre-AIDS than in AIDS (both with and, in the single case, without encephalitis) brains.

Using double labelling with ISEL and RCA-120, a number of cells in the white matter in HIVE cases showed double staining (see Fig. 3.14), whereas in the HIV-1 positive pre-AIDS brains only scattered apoptotic cells appeared RCA-120 positive, suggesting that, in the latter group, dying cells were predominantly glial in nature. In addition, some of their morphological features suggested an oligodendroglial nature. A number of endothelial cells were also seen to be stained by these methods. Moreover, numerous apoptotic bodies would be seen in tumour specimens which were ISEL positive whereas areas of massive necrosis in the tumour (control) samples did not show the typical positive features. Fig. 3.14a and b show double staining using ISEL and RCA-120 of white matter of an AIDS brain. In Fig. 3.14a two microglia/macrophages show apoptotic nuclei (blue) and brown cytoplasm (RCA-120) whereas in Fig. 3.14b one apoptotic cell is RCA-120 negative.

In this study, detection of cells by ISEL using DNA polymerase 1 and TDT confirmed that both enzymes were suitable for this technique and that the latter did not appear more sensitive than the former.
Fig. 3.13a-d Photomicrographs showing apoptotic cells as revealed by the ISEL method in the grey (a & c) and white matter (b & d) of HIV encephalitis (a & b) and HIV-1 positive pre-AIDS brains (c & d). In (a) neurons and in (b) one glial cell with apoptotic nuclei are seen. A large neuron with an apoptotic nucleus is present in (c) and several apoptotic glial cells are seen in (d). ISEL and nuclear fast red. (X552)
Fig. 3.14a,b Photomicrographs showing double staining using RCA-120 and ISEL methods. ISEL shows a blue nuclear staining and RCA-120 positive appear brown. In (a) two apoptotic cells are also RCA-120 positive whereas in (b) one cell with an apoptotic nucleus is RCA-120 negative. ISEL, RCA-120 and nuclear fast red. (X950)
3.5 Detection of p24 antigen and HIV-1 DNA by IHC and PCR

3.5.1 Pilot study by PCR: comparison between results using fresh frozen and paraffin embedded specimens

The neuropathological changes observed in the brain tissue of the 20 patients with AIDS, from which both frozen and formalin fixed, paraffin embedded are available, in this study are summarised in the Table 3.5. HIV encephalitis was present in 7 cases; other neuropathological findings included cytomegalovirus encephalitis (3 cases), progressive multifocal leukoencephalopathy (1 case), cryptococcosis (2 cases), lymphoma (1 case), tuberculosis (1 case); 3 cases did not show any abnormalities. Nine cases, including all those with neurological evidence of HIV encephalitis, were HIV p24 positive.

Sixteen cases resulted PCR positive on frozen specimens. These included all those with immunohistochemical evidence of HIV. Of these, 15 were also positive when paraffin embedded material was used. Southern blot confirmed the positive results. None of the cases which was positive in paraffin samples gave negative results when frozen material was examined (An et al., 1994). Among the 20 cases, delay of post mortem ranged between 17 hours to 4 days and fixation time between 1 to 8 months. One (No. 19) with the longest delay of post mortem and fixation time showed PCR positive on both frozen and paraffin specimens. Fig.3.15a,b show the results of PCR amplification in 3 cases using both frozen and paraffin samples. Case 1 (lanes A&B) was positive in both; case 2 (lanes C&D) was the single case positive only with frozen material and case 3 (lanes E&F) was an example of the 4 cases negative on both frozen and paraffin embedded
Material.

Table 3.5 Summary of the results of PCR detection with pathological and immunohistochemical findings

<table>
<thead>
<tr>
<th>No.</th>
<th>Sex</th>
<th>Age</th>
<th>Delay of PM (Days)</th>
<th>Fixation (Mths)</th>
<th>Neuropathological diagnosis</th>
<th>HIV p24</th>
<th>PCR</th>
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<td>+</td>
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<tr>
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</tr>
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NK, Not known
HIVE, HIV encephalitis
CMVE, Cytomegalovirus encephalitis
PML, Progressive Multifocal Leukoencephalopathy
FPL, Focal Pontine Leukoencephalopathy
Fig. 3.15 Results of PCR amplification on frozen and paraffin specimens in 3 cases. a) Electrophoresis and b) Southern blot hybridization. In lanes A, C and E DNA was extracted from fresh frozen samples; In lanes B, D and F it was extracted from paraffin material; Lane G: HIV plasmid DNA positive control; Lane H: normal brain negative control; Lane I in a): DNA molecular weight marker.
3.5.2 p24 antigen in brains of individuals with pre-AIDS and AIDS

Among the 63 cases studied, immunohistochemistry with anti-p24 antibody was positive in 7 of the 10 cases showing HIVE. No positive staining was seen in the other 8 AIDS, in all the pre-AIDS and in control brains. Fig. 3.16a-c show the staining of p24 in cases with HIVE. In Fig. 3.16a two MGCs with several nuclei clustered at the centre of the cells show p24 staining in their cytoplasm. Fig. 3.16b show p24 positive macrophages and in Fig. 3.16c one microglial cell is positive for p24.

3.5.3 Detection of HIV-1 DNA from cases with pre-AIDS, HIVE and AIDS without CNS disease

All the control cases were negative. HIV-1 DNA was detected in all (10 cases) HIVE, in 5 out of 8 AIDS without neuropathological changes and in 17 out of 36 HIV-positive pre-AIDS patients. Results of PCR in pre-AIDS individuals are shown in detail in Table 3.3. Fig. 3.17a,b illustrate an example of the results of PCR by both electrophoresis (a) and chemiluminescence (b).
Fig. 3.16a-c Photomicrographs showing staining of HIV-1 p24 in cases with HIVE. In (a) two MGCs with nuclei clustered at the centre of the cells show p24 staining at the periphery of the cytoplasm; (b) shows p24-positive macrophages and in (c) one microglial cell shows positive for p24. Haematoxylin and p24. (X912)
Fig. 3.17a,b Results of PCR shown by electrophoresis (small arrow) and chemiluminescence (large arrow). Lanes (A-H) represent pre-AIDS; (I-L) AIDS cases; (M, N) positive controls; (O) negative control; (P) DNA molecular weight marker.
3.6 Quantification of HIV-1 DNA

3.6.1 Standard curve for semi-quantitative PCR

A linear relationship between the logarithm (log) of the amount of PCR product and the log of the initial amount of sample DNA could be obtained following 28 cycles in the first round and further 18 to 20 cycles of amplification in the second round of PCR (see Fig. 3.18). The effect of the numbers of cycles on the amplification by PCR was investigated. As shown in Fig. 3.19, PCR products obtained after the first round of PCR using known copies of HIV-1 plasmid DNA as initial templates were followed by further 14, 16 or 20 cycles of amplification. Thus 18 cycles were chosen for the second round of PCR for quantitative purpose.

3.6.2 Sensitivity of the technique

The sensitivity of detection of HIV-1 DNA by this technique, i.e. when the exponential phase could be obtained by the conditions mentioned above (3.6.1), is in range of 1-10 to 1,000 copies/150,000 cells, assuming that 1μg DNA corresponded to 150,000 cells (6.6pg DNA per diploid cell) (Simmonds et al., 1990b). The results of PCR in 9 cases of HIV-1 positive pre-AIDS (4 cases) and AIDS (5 cases) individuals are shown in Fig. 3.20a,b.

3.6.3 Correlation between level of HIV-1 DNA and stage of disease
The levels of viral DNA in brain of AIDS (mean = 169.2, median = 135, range: 5 - 540, interquartile range: 90 - 180) and HIV-1 positive pre-AIDS individuals (mean = 55.8, median = 45, range: 5 - 215, interquartile range: 15 - 70) are listed in Table 3.6 and represented in Fig. 3.21. Results indicated as 5 - 10 were considered positive (see discussion). The results show that levels of HIV-1 DNA in brains of the AIDS group were higher than those found in asymptomatic individuals. As shown in Table 3.6 and Fig. 3.21 most copy numbers of HIV-1 DNA in pre-AIDS group are less than 100 (with three exceptions) whereas in AIDS group majority of cases show levels between ≈ 100 to 300 copies (except two have 540 copies and four have less than 100 copies). There is a significant difference between AIDS and pre-AIDS cases (P < 0.012, Mann Whitney U test). However, no correlation between levels of HIV-1 DNA and either neuropathological disorders or HIV-associated dementia is seen.
Fig. 3.18 shows a linear relationship between the logarithm (log) of the amount of PCR product and the log (copies) of the initial amount of sample DNA obtained.
Fig. 3.19 Effect of cycle numbers of amplification by nested PCR. A to E, F to J and K to O illustrate the chemiluminescence results when 14, 16 and 20 cycles were amplified, following 28 cycles of amplification in the first round of PCR, respectively. A, F, K: negative control with normal human DNA only; B, G, L: 1 copy of HIV-1 DNA; C, H, M: 10 copies; D, I, N: 100 copies; E, J, O: 1,000 copies.
Fig. 3.20 (a and b) A semi-quantitative detection of HIV-1 DNA in specimens of pre-AIDS (A to D) and AIDS (E to I) cases is shown. J to L illustrate a standard HIV-1 DNA, J: 200 copies; K: 100 copies and L: 10 copies. a) represents an agarose gel electrophoresis stained by ethidium bromide; b) represents the result of chemiluminescence.
Table 3.6 Summary of the neuropathological findings and the results of level of HIV-1 DNA in brains of AIDS and pre-AIDS individuals

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Neuropathology</th>
<th>Dementia</th>
<th>Copies/150,000 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIDS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>HIVE</td>
<td>Yes</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>HIVE</td>
<td>No</td>
<td>150</td>
</tr>
<tr>
<td>3</td>
<td>HIVE</td>
<td>Yes</td>
<td>540</td>
</tr>
<tr>
<td>4</td>
<td>HIVE</td>
<td>No</td>
<td>65</td>
</tr>
<tr>
<td>5</td>
<td>HIVE</td>
<td>Yes</td>
<td>5-10</td>
</tr>
<tr>
<td>6</td>
<td>HIVE</td>
<td>No</td>
<td>540</td>
</tr>
<tr>
<td>7</td>
<td>HIVE</td>
<td>No</td>
<td>150</td>
</tr>
<tr>
<td>8</td>
<td>HIVE</td>
<td>Yes</td>
<td>210</td>
</tr>
<tr>
<td>9</td>
<td>HIVE/ focal pontine leukoencephalopathy</td>
<td>Yes</td>
<td>5-10</td>
</tr>
<tr>
<td>10</td>
<td>HIV leukoencephalopathy</td>
<td>No</td>
<td>120</td>
</tr>
<tr>
<td>11</td>
<td>No abnormalities</td>
<td>Yes</td>
<td>5-10</td>
</tr>
<tr>
<td>12</td>
<td>No abnormalities</td>
<td>No</td>
<td>180</td>
</tr>
<tr>
<td>13</td>
<td>No abnormalities</td>
<td>No</td>
<td>162</td>
</tr>
<tr>
<td>14</td>
<td>No abnormalities</td>
<td>No</td>
<td>105</td>
</tr>
<tr>
<td>15</td>
<td>No abnormalities</td>
<td>No</td>
<td>280</td>
</tr>
<tr>
<td>16</td>
<td>No abnormalities</td>
<td>No</td>
<td>90</td>
</tr>
<tr>
<td>Case No.</td>
<td>Neuropathology</td>
<td>Copies/150,000 cells</td>
<td></td>
</tr>
<tr>
<td>---------</td>
<td>--------------------------------------------------------------------------------</td>
<td>---------------------</td>
<td></td>
</tr>
<tr>
<td>pre-AIDS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>Meningitis(Lym), Microglia(+W), Gliosis(+W)</td>
<td>120</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>Microglia(+W, ±G), Gliosis(+/++W)</td>
<td>10</td>
<td></td>
</tr>
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<td>19</td>
<td>Meningitis, Microglia(+W), Gliosis(+/++W, ±G)</td>
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<td>Microglia(+/++), Gliosis(+W)</td>
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</tr>
<tr>
<td>21</td>
<td>Microglia(+W), Gliosis(+/++W, ±G)</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>Microglia ±, Gliosis(+/++W, +G), Hypoxic changes</td>
<td>5-10</td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>Microglia(+W), Gliosis(+W, +G), Hypoxic changes</td>
<td>5-10</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>Meningitis, Microglia(+W), Gliosis(+/++W, +G), Hypoxic changes</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>Meningitis(focal), Microglia ±, Gliosis(+W), Hypoxic changes</td>
<td>70</td>
<td></td>
</tr>
<tr>
<td>26</td>
<td>Meningitis(Mac), Gliosis(+W, ±G), Hypoxic changes</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>27</td>
<td>Microglia(+W, ±G), Gliosis(+/++W)</td>
<td>75</td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>Microglia(+W), Gliosis(+W), Hypoxic changes</td>
<td>160</td>
<td></td>
</tr>
<tr>
<td>29</td>
<td>Microglia infiltrate+/++(Mac)</td>
<td>5-10</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>Menigitis, Gliosis(+)</td>
<td>5-10</td>
<td></td>
</tr>
<tr>
<td>31</td>
<td>Meningitis(Lym), Gliosis(+W, ±G)</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>32</td>
<td>Meningitis, Microglia (+, Gliosis(+W)</td>
<td>45</td>
<td></td>
</tr>
<tr>
<td>33</td>
<td>Microglia(+/++W, +/++G), Hypoxic changes</td>
<td>27</td>
<td></td>
</tr>
</tbody>
</table>

HIVE = HIV encephalitis;
Lym = lymphocyte;
Mac = macrophage;
W = white matter;
G = grey matter
± = absent to slight;
+ = moderate;
++ = high
Fig. 3.21 Distribution of levels of HIV-1 DNA in AIDS and pre-AIDS groups.
CHAPTER FOUR:

DISCUSSION
4.1 Neuropathological changes and microglial reactivation in brains of HIV-1 positive pre-AIDS cases

Neuropathological changes, observed in the brains of 10 out of 18 cases in the AIDS group and which included myelin pallor, infiltration by macrophages, increased numbers of microglial cells and of GFAP positive cells and MGCs, fulfil the criteria for the diagnosis of HIV encephalitis as previously described by Budka (1991). In the brains of the other 8 cases in the same group there were no obvious neuropathological abnormalities except in one in which the white matter showed a discrete increase in number of GFAP positive astrocytes.

Findings in HIV-1 positive pre-AIDS group were discrete leptomeningitis, myelin pallor as well as micro- and astro-gliosis. Leptomeningitis, usually not a feature of HIVE, was present in 11 among the 36 cases of this group. A correlation between the presence of meningitis and HIV-1 DNA could be established although the difference was not significant (p=0.0753). Meningitis is a relatively common finding in HIV-1 positive asymptomatic individuals and confirms the finding of perivascular inflammation observed by Gray et al (1992) in half (6 of the 11) of their cases. Early HIV infection of leptomeninges has been demonstrated in pre-AIDS individuals. Infected individuals go through an early acute phase during which there is detectable virus and host cell reaction in the CSF and a subsequent phase in which virus is reduced or absent whilst the local production of HIV-1 antibodies continues (Price et al., 1988). Evidence in early SIV encephalopathy confirms that infected cells in the brains are located mainly in perivascular and meningeal compartments (Hurtrel et al., 1991). The above data suggest
that meningitis may be one of the early (or acute) appearances of HIV-1 infection in brain. Considering the abundance of blood vessels in meninges it seems to be a reasonable explanation that HIV or viral infected cells enter brain parenchyma through a compromised blood-brain barrier.

Regarding the lack of MGCs in the brain of pre-AIDS subjects, this might be explained by the observation that, at the early stage of HIV infection, virus harboured within the CNS might be the non syncytium-inducing (SI) variant as demonstrated in the blood (Schuitemaker et al., 1991).

With regard to the microglial hyperplasia, seen in 31 of the 36 cases, increased numbers of cells of microglia/macrophage lineage are a prominent feature of HIV-associated disorders of the CNS during late AIDS (Genis et al., 1992) as well as early pre-AIDS stages (Esiri et al., 1989; Gray et al., 1992); it is known that these cells are susceptible to infection by HIV and may serve as its reservoir (Ho et al., 1994). Latent infection of monocytes/macrophages is an important mechanism by which HIV escapes immune surveillance and enters the CNS (Achim et al., 1991b). A semi-quantitative evaluation of white matter microgliosis, based on the density of cells as well as their size and the length of cell processes, showed that microgliosis is present in all HIV-1 seropositive groups. The highest increase of microglial cells and macrophages is seen in cases with HIV encephalitis when compared with pre-AIDS individuals and those with AIDS without obvious neuropathological changes. Morphologically, amoeboid (rod) microglial cells were seen in most AIDS brains with HIVE as well as in a number of pre-AIDS. In brains of AIDS without neuropathological changes their sizes are only moderately
increased compared to the two formers. The increase in density of microglial cells in pre-AIDS group indicates that a cellular response to the virus in CNS is already present at this early stage of the infection; moreover, the higher density of microglia in pre-AIDS group compared to AIDS cases without neuropathological changes suggests that the increase is temporary (Sinclair et al., 1994). This is in keeping with an observation by Pantaleo et al (1993a) and Embretson et al (1993) that HIV-1 infection is active and progressive in lymphoid tissue during the clinically latent stage of disease. Dendritic cells in lymph nodes trap HIV at early stage of infection and this is followed by decrease in viral load possibly due to immune response. This might explain why the density of microglia was higher in pre-AIDS group than that in AIDS without neuropathological changes.

Regarding the density of GFAP positive astrocytes, it is higher in HIVE group than in any of the other groups. However, comparison of cell size in the different groups shows that this is higher in the 3 HIV positive than in normal controls. The difference of appearances of astrocytes in HIVE and pre-AIDS groups is that, in the former, activated astrocytes are located in both white and grey matter whereas in the latter, obvious astrogliosis is found in white matter only.

4.2 Detection of HIV-1 proviral DNA in brains of patients at pre-AIDS stage

HIV-1 DNA was detected in brain of a number of HIV-1 positive pre-AIDS individuals (17 of the 36, = 47%). This confirms and extends previous results by Sinclair et al (1994) who demonstrated that HIV-1 DNA in 2 of the 8 brains of pre-AIDS individuals
and confirms that entry of HIV-1 into CNS takes place at the early stage of the infection. These results are at variance with those of Donaldson et al (1994b) who believe that HIV-1 DNA detected in pre-AIDS brains to be due to contamination of residual blood in the tissue.

The possibility that positive PCR findings could result from HIV harboured in infected cells contained in the intraparenchymal or meningeal blood vessels must be considered. As mentioned earlier (see 2.2.6.1.2), the meninges were removed from the paraffin embedded section used for PCR; as for the hypothesis that virus is present in cells trapped in intraparenchymal vessels, the three possible sources of contamination from the blood are (1) PBMC, (2) CD4 positive cells and (3) HIV-1 DNA/RNA in plasma, an unconfirmed possible source suggested by Nandi and Banerjee (1993). The possibility of contamination from blood has been calculated and details are shown in Appendix A assuming (1) that distribution of blood was even throughout the whole body at the time of death; (2) that blood flow in the brain had normal rate of 50-55ml per 100g brain per minute and hence blood flow in the whole brain per minute = 50 X 14 (1,400g weight of organ / 100g) = 700ml = 700,000mm$^3$ (Adams and Victor, 1985) and (3) that the white blood cells (WBC) count was the normal level (7.4 X 10$^3$ / mm$^3$) (Dittmer, 1961).

Data from the literature show that only between 1 / 1,000 and 1 / 500,000 peripheral blood mononuclear cells (Lee et al., 1991) and between 1 / 2,500 and 1 / 26,000 CD4 cells (Brinchmann et al., 1991) carry the virus in seropositive pre-AIDS individuals. As only less than one paraffin section (about 30µg of DNA could obtained from 10 sections) per case was used for PCR studies, this amount of tissue could not conceivably contain
a number of cells high enough to give a positive result, taking into account the threshold of sensitivity of our PCR technique.

Infected cells contain on average 1 to 2 provirus copies (Brinchmann et al., 1991). Therefore, as shown in Appendix A, the possibility of contamination from blood within brain tissue would be about $1.185 (0.025 + 0.33 + 0.83)$ copies /per PCR reaction, assuming 1 copy of HIV-1 DNA per infected cell and no more than $1.54 (0.05 + 0.66 + 0.83)$ copies whilst we assumed 2 copies per infected cell. In the formula in Appendix A 1 of the 1,000 PBMC, 1 of the 100 CD4 T cells and $10^4$ copies / ml reflect the values higher than average levels (see Table 1.1 in Chapter 1).

The application of quantitative PCR to the investigation of HIV-1 DNA in post mortem brain could yield accurate qualitative and quantitative data about the pre-AIDS. Thus, it is obviously necessary and useful to analyze quantitatively the levels of HIV-1 DNA in brains of, particularly, HIV-1 positive pre-AIDS individuals and to compare them with those in brains of AIDS patients. The result might be able to answer two questions. The first is whether PCR proved positive results of HIV-1 DNA in pre-AIDS cases in this study reveals viral DNA harboured in cells in parenchyma of brain rather than those in infected cells in blood flow. As calculated in Appendix A if levels of HIV-1 DNA is $\geq 2$ copies / reaction then the excess (copy number - 2) are considered to be obtained from brain tissue. The second concerns whether or not levels of HIV-1 DNA in brains correlate with the stage of the infection and are suitable predictor of progression of disease.
Results using nested semi-quantitative PCR demonstrated that, with regard to the first question, levels of HIV-1 DNA are higher than 2 copies / PCR reaction (see Table 3.6 in chapter 3) and represent HIV-1 DNA within parenchyma of these brains, thereby indicating that HIV-1 enters brains at an early pre-AIDS stage (An et al., 1996b). This is confirmed by the finding by Schmid et al (1994) who demonstrated that proviral load in patients, at all stages, was significantly higher in the CSF than in the blood. Results also suggest that CNS is not only involved early but also that levels of HIV-1 DNA in the brains of HIV-1 positive pre-AIDS group, although lower than those found in group with AIDS, are already considerably high. Among the 17 HIV-1 DNA positive cases in pre-AIDS group, half (9 of the 17, = 53%) showed moderate (> 10 to < 100) copy numbers of HIV-1 DNA. In the other half, 3 showed high copies (> 100) of HIV-1 DNA and 5 had low copies (5-10). There was neither CD4 cell count nor any other clinical information available, because it was impossible in most of these cases to know before death even whether they were HIV-1 positive, which made further analysis difficult. Therefore, it cannot be excluded that whilst some cases were at the stage of infection just preceding AIDS, others might just have been at the stage immediately following seroconversion and we cannot even exclude that some cases would have become nonprogressors.

It has been demonstrated that in PBMC (Escaich et al., 1992; Simmonds et al., 1990b; Lee et al., 1991) and CD4 positive T cells (Wood et al., 1993) HIV-1 DNA was higher in patients with AIDS than in those with pre-AIDS.

Regarding the second question whether or not levels of HIV-1 DNA in brains correlate
with the stage of the infection and are a suitable predictor of progression of disease, this study has further demonstrated that higher copy numbers were found in brains of the group of patients with more advanced disease than in those of asymptomatic individuals. However, when the results of single individuals were considered, some degree of overlapping could be noted: indeed, high numbers of copies were present in some asymptomatic cases whereas some AIDS patients showed low copy numbers. Regarding the relatively high level of HIV-1 DNA found in three asymptomatic cases (No. 17, 20 and 28), this does not rule out the possibility that, as all members of this group were 'normal' and died of causes unrelated to AIDS (suicide, drug overdose etc) without CD4 count available, they could have reached a stage of the infection just preceding AIDS. On the other hand, no plausible reason could be produced for the low copy number found in some AIDS cases. On the basis of these results it is concluded that whereas the use of HIV-1 DNA load in CNS is a valuable indicator of progression of the disease, its application should be restricted to investigation of large series and not to single cases.

Regarding the AIDS control group, it has been noted that there was no correlation between levels of viral DNA and presence or absence of either neuropathological changes or dementia; this would suggest that the presence of HIV in the brain is a necessary but not sufficient condition for the development of HIV encephalitis and dementia. Glass et al. (1995) found that HIV-associated dementia correlates better with the presence of macrophages and microglia than with the presence and amount of HIV-infected cells in the brain, suggesting that HIV-associated dementia is due to indirect effects of HIV infection of the brain including infection by different neurovirulent strains.
of HIV-1 and/or neurotoxic factors. This interpretation could also apply to the lack of correlation between presence of encephalitis and levels of HIV-1 DNA in brain.

4.3 Immune activation in brains of pre-AIDS individuals

In this study increased density of microglia was associated, in pre-AIDS brains, with enhanced expression of MHC class II molecules. The mean score [1.7] in these brains was lower than in cases associated with HIVE [2.7], but was higher than that found in AIDS brains without associated pathology [0.6] (see Fig. 3.10, Table 3.3). Fig. 3.10 shows the proportion of cases with low, moderate and high scores, respectively. Twenty two of the 36 (61%) cases in pre-AIDS group and 9 of the 10 (90%) cases of HIVE group showed moderate to high scores (p = 0.004) whereas in AIDS without neuropathological changes only 2 of the 8 (25%) cases have moderate to high scores (p < 0.05), the others are scoring low. In addition, in the pre-AIDS group expression of MHC class II was found to be higher in HIV-1 DNA-positive than in HIV-1 DNA negative brains (p = 0.058, Fisher). The mean score of expression of MHC class II antigens in HIV-1 DNA positive was 1.94 a value considerably higher, though statistically not significantly so, than in negative cases [1.4]. Alternatively, in HIV-1 DNA positive individuals a low score was found in 4, moderate in 8 and high in 5 cases whereas in HIV-1 DNA negative ones, low score was found in 10, moderate in 7 and high in 2 cases only.

The key role played by MHC class II in the process of antigen presentation by antigen-presenting cells (dendritic cells, macrophages and B-cells) to CD4 positive T
cells is known (Pupo et al., 1991). Brinkmann et al. (1993) investigated \textit{in vitro} and \textit{in vivo} microglial cells of rhesus monkeys infected by SIV and found that isolated cells were latently infected independently of the presence of neuropathological lesions. In addition, though only a small percentage of these cells were productively infected \textit{in vivo}, the majority of them expressed MHC class II molecules, indicating a previous state of activation acquired \textit{in vivo}. The elevated expression of MHC II in AIDS has been previously confirmed by several groups (Tyor et al., 1992; Achim et al., 1991b; Kennedy and Gairns, 1992). Regarding MHC II in pre-AIDS, it has been demonstrated that CD4 positive T cells in asymptomatic HIV-1 infected individuals are functionally abnormal before these cells are depleted. The cells are programmed for death, are non-responsive after antigenic stimulations and fail to produce IL-2 (TH1 cytokine). These different T cells abnormalities are explained by the effects of HIV on antigen-presenting cells. Alteration of the functions of the antigen-presenting cell may program T cells for activation induced death (Meyaard et al., 1993).

The demonstration of the increased expression of MHC class II antigens in brains of individuals at the pre-AIDS stage lends support to previous hypothesis (Sinclair et al., 1994) that a status of immune activation, observed in AIDS by Tyor et al. (1992), already exists in brain during the asymptomatic stages preceding AIDS. At this stage, viral specific immune responses (shown by the presence of MHC class II molecules), including both neutralising antibodies and cytotoxic T cells, help inhibit virus replication (Walker, 1994). However, after this latent period, in which the balance seems to be in favour of the host, new viral variants, derived from the high rate of HIV mutagenesis, arise; they in turn stimulate the immune system, induce new cycles of viral replication.
and new virulent mutants, leading to the final collapse of the immune system (Caetano, 1991). During this complex pathogenic processes a paradoxical state of immune activation overlapping with immunodeficiency can be observed, called 'immune disequilibrium'. Immune-related toxins may lead indirectly to the injury or demise of neurons.

In most subjects infected with HIV-1, clinical or laboratory evidence of immunodeficiency develops within 10 years of seroconversion. However, in a small percentage of infected individuals, there is no progression of disease and CD4 positive T cells counts remain stable for more than a decade. These subjects have been termed 'long-term survivors' or 'long-term nonprogressors'. They have a vigorous virus-inhibitory CD8 positive lymphocyte response and a strong neutralizing-antibody response (Cao et al., 1995); their viral load is low but viral replication persists (Pantaleo et al., 1995). As a result of effective host immunity, these subjects, initially infected with a virulent strains of HIV-1, become eventually infected with a more-attenuated viral quasispecies. Anti-HIV-1 CD8 positive cytotoxic T lymphocytes have been thought to play an important role in controlling HIV-1 replication and preventing disease in long-term nonprogressors (Rinaldo et al., 1995). However, it is not yet clear why and how these subjects can keep their intact humoral and cellular immune responses for a long time lacking of disease whereas others can not. This phenomenon shows us, at least, a ray of hope indicating that it is possible to live with the virus for prolonged periods without harm.

4.4 Elevated expression of cytokines and their role in the pathogenesis of early
Regarding the time of the infection at which cytokines become detectable in the brain, as shown by their presence in the CSF, very little is known, as most investigations have only compared levels in AIDS patients with and without dementia. Moreover, data reflecting levels in the CSF may not correlate with the actual levels of cytokines within the parenchyma of brain. Indeed, although CSF and brain tissue represent two interconnected compartments, they are not in equilibrium regarding solutes (Pardridge, 1991). Results at the AIDS stage are conflicting as Perrella et al (1992) did, whereas Weller et al (1991) did not, find evidence of TNF-α. On the other hand, Vitkovic et al (1994) detected slightly elevated levels of TNF-α in the CSF in HIV infection, compared with HIV-negative individuals, but no difference between demented and non-demented subgroups, confirming previous data by Tyor et al (1992). Results of the latter are at variance with those by Perrella et al (1992) and Grimaldi et al (1991), who reported higher TNF-α levels in demented than non-demented AIDS patients.

In this study elevated expression of MHC class II antigens and cytokines TNF-α, IL-1α, IL-4, IL-6 has been demonstrated in HIV-1 positive patients compared to HIV-1 negative controls. The study has revealed also, for the first time, that expression of IL-1α, IL-4, IL-6 and TNF-α in formalin fixed, paraffin embedded brain tissue is elevated in HIV-1 positive pre-AIDS compared with HIV-1 negative individuals as well as HIV-1 positive brains without neuropathological changes (An et al., 1996a).

Regarding the patterns and levels of expression of cytokines, scores of most of these as
well as of MHC class II antigens in brains of AIDS without neuropathological disorders were usually lower than those showed in pre-AIDS and HIVE groups. The highest levels of IL-1α were found in patients with HIVE, whereas pre-AIDS individuals showed only moderate levels of this cytokine and little was present in AIDS brains without neuropathology. Levels of IL-6 were similar in HIVE and pre-AIDS groups, but were higher than those seen in AIDS group without neuropathological changes. However, the pre-AIDS group showed higher scores of TNF-α and IL-4 than AIDS with HIVE. Results showed that scores for IL-4, an anti-inflammatory cytokine (Lucas and Hohlfeld, 1995), were lower in HIVE than in pre-AIDS group. In HIV infection HIV-1 gp120 has been demonstrated to decrease ability of CD4 positive cells to secrete IL-4 (Chirmule et al., 1992). A double effect (inductive / suppressive) of IL-4 in HIV replication has been demonstrated (Poli and Fauci, 1992b). Regarding the expression of TNF-α, a number of groups have reported an increased expression of TNF-α in brain of patients who had HIV-associated dementia (Wesselingh et al., 1993; Glass et al., 1993). Data obtained from brains of patients with HIVE in this study were in keeping with those found by Pulliam et al (1994) who found that production of TNF-α by HIV-infected macrophages in AIDS was relatively low. There were statistical difference (TNF-α, IL-1α, IL-4) or significantly difference (IL-6) between correlation of the presence of HIV-1 DNA and expression of cytokines in pre-AIDS individuals.

Positive cells included microglia and astrocytes (for all the cytokines), pericytes (TNF-α) as well as endothelial cells (IL-1α, IL-6). Although both grey and white matter were involved, most positive cells were found in the latter.
In conclusion, the finding of presence of HIV-1 DNA in brains of pre-AIDS individuals as well as immune activation, as revealed by elevated expression of MHC class II antigens associated with production of cytokines, enables us to propose the following possible explanation for some of the neuropathological changes previously reported in pre-AIDS individuals (Gray et al., 1992). Functions of cytokines in pathogenesis of HIV-1 infection in the CNS is summarised in Fig. 4.1. HIV-1 infected macrophages/microglia secrete cytokines and neurotoxins such as platelet-activating factor (PAF), arachidonic acid and metabolites and QUIN. Cytokines enhance HIV-1 replication and the replicated viral product - HIV-1 gp120 further activates uninfected macrophages to secrete these neurotoxins. Cytokines and neurotoxins may have a direct effect on neurons. Interaction of macrophages with astrocytes could lead to gliosis. Reactivated astrocytes further alter and lack the ability of maintaining normal neurotrophic factors [nerve growth factor (NGF), fibroblast growth factor (FGF)], resulting in increase of Ca^{2+} and adhesion molecules (ICAM, VCAM). Cell activation processes may be mediated by these cell-to-cell signalling molecules. Furthermore, adhesion molecules will upregulate cytokines, and through feedback loop, finally, lead to neuronal injury or death. As from the works of Selmaj and Raine (1988) and Wilt et al (1995) it is known that TNF-α has a direct effect on oligodendrocytes and that it mediates myelin and oligodendrocyte damage in vitro, we can hypothesise that the myelin pallor described by Gray et al (1992), and confirmed in my study can be correlated with the presence of TNF-α. TNF-α is known to act through a mechanism of apoptosis (Selmaj et al., 1991).

A more worrying possibility is that cytokines could, at this early stage, trigger the cascade of events by which IL-1 can stimulate the production of itself as well as of other
cytokines such as TGF-β1 (da-Cunha and Vitkovic, 1992; da-Cunha et al., 1993a), IL-6 and TNF-α (Dinarello, 1992). Production of IL-1, TGF-β1 and TNF-α by various cell types within the CNS, in turn, activates HIV replication (Poli and Fauci, 1992a; Vitkovic et al., 1991), thus creating a vicious circle that perpetuates the damage. Furthermore, cytokines are low molecular weight proteins that can act both locally or at sites remote from their site of origin (Giulian and Lachman, 1985) and may have long lasting effects (Dickson et al., 1993). Admittedly, the amounts of these proteins found within cells in the cortex in our cases were not large; nevertheless we cannot exclude that they may diffuse from the adjacent white matter where their effect on myelin, and possibly axons, is a strong possibility.

Data from this study demonstrated that immune activation and, in particular, elevated expression of cytokines are present already in brains of HIV-1 positive pre-AIDS individuals. As it is known that overexpression of cytokines, especially those proinflammatory cytokines such as TNF-α and IL-1, can be one of mechanisms of pathogenesis of CNS disorders in HIV-1 infection, another question arose, i.e. is brain damage already taking place during asymptomatic infection of HIV-1?
Fig. 4.1 Diagram showing the functions of cytokines in pathogenesis of HIV-1 infection in the CNS.
4.5 Apoptosis in brains of pre-AIDS individuals

In order to ascertain whether nerve cell loss was occurring at this early stage of the infection two methods were at our disposal; the use of stereology and the visualisation of DNA damage leading to apoptosis using one of the techniques now available. Stereology has yielded good results in the AIDS brains. However, as we were anticipating only minimal (if any) cell loss, we concluded that the former technique would not be able to detect subtle decreases and opted for the latter.

Apoptosis has been associated with specific and characteristic morphological and chemical changes and it is known that the cycle of the former may be limited to a few minutes only (Kerr et al., 1987), and that after this time apoptotic bodies are rapidly phagocytosed (Wyllie et al., 1980). One biochemical event that is almost exclusively associated with apoptosis is the internucleosomal degradation of genomic DNA. The initiation of DNA cleavage occurs prior to morphological changes (Wyllie, 1980) and it has been suggested to be the first irreversible event leading to the inevitable cellular demise (Arends et al., 1990). As I could not detect in my material apoptotic bodies I undertook in situ end labelling (ISEL) method in the hope that it could yield results.

I was aware of the fact that formalin fixation, especially over long periods, may produce false negative results of apoptosis using ISEL (Davison et al., 1995). As the specimens used in this investigation were formalin fixed, I submitted the specimens to pre-digestion by proteinase to permeate the section. Using optimised conditions, this technique enabled me to detect apoptosis initially in surgical material (tumours) and subsequently in post
mortem brain tissue samples fixed in buffered formalin for periods ranging from 2 weeks to 5.5 months. Results of this study confirm that the ISEL technique can be successfully applied to paraffin sections (Wijsman et al., 1993) for the detection of DNA fragmentation (Ansari et al., 1993; Gavrieli et al., 1992; Gold et al., 1994; Migheli et al., 1994). Furthermore, they show that the two methods applied, DNA polymerase 1 and TDT, have the same degree of sensitivity and can be used interchangeably.

Presence of apoptotic nerve cells in AIDS has been reported recently in brains of adults (Adle-Biassette et al., 1995; Petito and Roberts, 1995) and of children (Gelbard et al., 1995). The results of the present study demonstrate, for the first time, that apoptotic cells also occur in brain tissue during the asymptomatic stage of the HIV infection. However, unlike Petito and Roberts (1995) we, as well as Adle-Biassette et al (1995) and Gelbard et al (1995), could not find evidence of apoptotic bodies in routinely stained sections. Apoptotic cells were detected in the white matter in four cases, two of which showed also apoptotic cortical neurons. As far as the cortex is concerned, although no cell count was done, the density of ISEL positive neurons appeared lower in pre-AIDS than in AIDS (both with and in the single case without encephalitis) brains. The absence of quantitative study in the papers mentioned above (Adle-Biassette et al., 1995; Petito and Roberts, 1995; Gelbard et al., 1995) makes any comparison of the severity of cell loss between their and our AIDS cases impossible except for confirming that AIDS cases without encephalitis showed lower numbers of positive cells than those with the disorder (Gelbard et al., 1995). Among the cells in the white matter undergoing apoptosis, a number of glial, and some RCA-120 positive microglial cells were identified. The latter finding is not surprising as apoptosis has appeared to be a mechanism by which activated
microglial cells are gradually eliminated following CNS injury as observed by Gehrmann and Banati (1995) in rat. Although a further characterisation (double staining) of the glial cells was not attempted for technical reasons, some of them could be identified as oligodendrocytes on morphological grounds. The finding of neuroectodermal cells other than neurons taking part in apoptosis is in keeping with the observations by Petito and Roberts (1995) who reported cell death both in neurons and astrocytes. Furthermore, Louis et al (1993) demonstrated that mature oligodendrocytes can die by apoptosis in response to toxic signals in vitro. No apoptotic cells other than neurons are mentioned by Gelbard et al (1995) and Adle-Biassette et al (1995).

Various pathogenetic theories have been put forward to explain the loss of neurons in AIDS patients. A mechanism triggered by HIV envelope glycoprotein has been reported for CD4 cells (Laurent-Crawford et al., 1993), cortical cell cultures (Muller et al., 1992) and in the cerebral cortex of rat (Bagetta et al., 1995). Investigation by Bagetta et al. (1995) demonstrated that, after cerebral intraventricular (c.i.v.) injection of recombinant HIV-1 gp120, brain of rats showed DNA fragmentation in neurons in the neocortex, thus suggesting that apoptosis is the mechanism through which neurons of the neocortex are disposed of. It has been observed also that HIV gp120 and gp41 heterodimer complex and CD4 receptor are necessary for the induction of apoptosis in a synchronous infection of HIV in cultures. HIV-producing cells are also potent effector cells capable of inducing apoptosis in uninfected CD4 expression cells (Hovanessian, 1994).

Cytokines (Kizaki et al., 1993; Mangan and Wahl, 1991; Ohno et al., 1993) and some cellular toxins have been considered to play a role in apoptosis. Induction of apoptosis
of lymphocytes has been correlated with some cellular toxins, such as prostaglandin E2 (PGE 2), produced by HIV infected macrophages (Mastino et al., 1993), sulphated glycoprotein-2 (SGP-2) (Michel et al., 1992) and lipid hydroperoxides (Sandstrom et al., 1994). Indeed, tumour necrosis factor can cause DNA fragmentation (Obeid et al., 1993) and can activate latent HIV. On the other hand, apoptosis of T cells from HIV-1 infected patients is partly or entirely suppressed by IL-2 or a mixture of IL-1α and IL-2 (Gougeon et al., 1993a; Lewis et al., 1994).

In the present study, three of the four cases showing apoptotic cells were found to be positive for HIV-1 DNA by PCR, thus emphasising the links between presence of the virus and cell damage. In view of the close relationship between presence of HIV-1 and toxic factors (Tyor et al., 1992) and between the latter and cell loss in AIDS brains (see Chapter 1 1.8), I suggest that the same mechanisms described in full blown AIDS may operate in the HIV-1 positive pre-AIDS brains in which elevated expression of MHC II antigen and of various cytokines has been proved. Abnormalities found in brains of pre-AIDS individuals in this study were less severe in grey than in the white matter, which is in keeping with the appearances in brains of AIDS patients.

The demonstration that a number, albeit small, of individuals at the pre-AIDS stage show apoptotic neurons (An et al., 1996c), combined with the findings described above, gives further support to the opinion that brain damage is taking place during the early stages of HIV infection and suggests that therapeutic strategies in HIV-1 positive individuals might have to be applied before they enter the AIDS stage.
AZT is extensively used in the treatment of HIV infection. It is still debated whether AZT should be offered to asymptomatic HIV-1 infected individuals in the hope of delaying or even preventing progression to AIDS. Results obtained by several groups are conflicting. AZT treatment appeared to protect SIV-infected newborn macaques against rapid onset of AIDS (Van-Rompay et al., 1995). Zaretsky (1995) analyzed the data of two studies and demonstrated that there was no benefit from AZT treatment in terms of progression to AIDS for those who are asymptomatic with CD4 positive cell count $>500/\text{mm}^3$, due perhaps to the toxic effects of AZT on the more intact immune system of these patients. This is supported by McKallip et al (1995) who found that AZT, when present at the time of thymocyte differentiation or during the response of T cells to antigens in vivo, can mediate significant inhibition of such functions, suggesting that AZT may affect the immune response to HIV antigens. In addition, AZT therapy combined with other agents may now become the preferred choice of therapy to delay disease progression in patients with asymptomatic or mildly symptomatic HIV infection (see review, Rachlis, 1990). Methionine enkephalin (Met-Enk), which has been reported to be immunostimulatory, acts in combination to improve the efficacy of AZT in reducing progression of murine retrovirus-induced disease (Specter et al., 1994) and, more recently, Goldstein et al (1995) reported that progression rates from asymptomatic to AIDS or death were reduced by application of thymopentin in AZT-treated asymptomatic HIV-1 infected subjects with 200 - 500 CD4 cells / mm$^3$. Results were evaluated in a double-blind, randomized, placebo-controlled trial. A similar result was demonstrated by Collier et al (1996) when saquinavir, a HIV protease inhibitor, was given with two antiretroviral agents (AZT and zalcitabine) to 297 patients who had CD4 positive counts of 50 to 300 cells per cubic millimetre and included 111 (37%)
symptomless and 153 (52%) symptomatic non-AIDS patients. This three drugs combination reduced HIV-1 replication, increased CD4 positive cell counts, and decreased levels of activation markers in serum more than did treatment with AZT and either saquinavir or zalcitabine.

4.6 Methodological aspects

Regarding the techniques used in this study, the PCR method has been adapted to amplify HIV-1 DNA from formalin fixed, paraffin embedded brain tissue; this has made retrospective study possible as the frozen samples, especially those of HIV-1 positive pre-AIDS individuals are in limited supply. Moreover, by using fixed material, risk of contamination and infection become virtually impossible. A pilot study demonstrated that this method offered almost the same sensitivity as that which used frozen tissue (An et al., 1994). Semi-quantitative nested PCR using chemiluminescence has also been introduced. It displays advantages in its sensitivity and specificity as well as lack of possible contamination of radioisotope. In this study using a single round semi-quantitative PCR, the exponential phase was obtained when 20 - 40 to 2,000 copies were amplified (Davison et al., 1996). The double rounds of PCR, in which the phase is between < 10 to 1,000 copies, makes this technique more reliable for the quantification of samples with low copy of HIV-1 DNA (An et al., 1996b).

Immunohistochemistry is now a routine technique widely used in histopathology laboratories and is performed in tissue routinely fixed with 10% formaldehyde. Whereas the recommended duration of fixation for post mortem brain was 3 weeks, there is now a tendency to shorten these times as it is known that prolonged fixation can reduce the intensity of the immunostaining and needs vigorous antigen retrieval procedures (Taylor et al., 1994). Of the brain tissue used in this work, all the pre-AIDS cases were fixed for approximately 3 weeks whereas the AIDS and HIVE cases were fixed for periods varying from 3 weeks to 5.5 months. In this respect it should be pointed out that: 1) in
a previous study (Sinclair, PhD Thesis, 1992) it was observed that fixation time did not affect the intensity of GFAP and RCA-120 staining in HIVE cases examined up to 10 months. I am aware, however, that different antigens can react differently to the same fixation as shown by the lack of reaction with anti-cytokine antibodies in fixed tissue unless antigens are unmasked by microwave treatment. 2) one of the HIVE cases in my own series in which fixation time was the longest (5.5 months) produced high expression of MHC II and moderate expression of IL-1 (IL-4, IL-6 and TNF-α were low). On the other hand, in another case of HIVE which was fixed for 3 weeks only, MHC II was high, IL-1 was moderate whereas TNF-α, IL-6 and IL-4 were completely negative. Although more strict fixation times are recommended in this type of study, the two examples quoted above suggest that the intensity of the immunostaining obtained in my work could reflect in general the amount of antigen contained in the tissue.

The specificity of the reaction has been checked in a limited number of samples by applying normal rabbit serum in parallel with antibodies for each cytokine. No staining was observed. A further check of specificity i.e. the omission of the primary antibody was not done by myself; however in the laboratory where I did my work this is a common practice and all the reagents I used had been previously used and checked by the MLSO in charge of immunohistochemistry. The results were invariably negative.

Apoptosis was originally defined only by specific morphological criteria. Its characteristic morphological features are chromatin condensation, nuclear fragmentation with subsequent formation of an apoptotic body. In addition to morphological identification, two other methods have been made available more recently for the study of this event, in situ end labelling (ISEL) and gel electrophoresis, both of which identify cleaved DNA. During apoptosis, DNA fragmentation proceeds through an ordered series of stages, starting with the production of large (300kb-1mb) fragments, that are then degraded to fragments of moderate length (30-50kb). These fragments are further degraded to smaller fragments and then into the small oligonucleosome fragments (∼200bp) in some but not all cells (Walker et al., 1993).
In my work ISEL positive cells have been found in HIV-1 positive cases. However, apoptotic bodies were not seen in the same cases. Similar findings were reported by Gelbard et al (1995) and Adle-Biassette et al (1995). As a number of reports from the literature suggest that morphological (apoptotic body formation) and biochemical (fragmentation of chromatin) processes occurring during apoptosis could take place simultaneously, our results clearly do not fit this pattern. However, this could be interpreted as follows: 1) the light microscopic recognition of apoptosis mainly depends on the detection of discrete well-preserved apoptotic bodies. Convoluted budding cells are rarely seen in paraffin sections of immersion fixed tissue. Apoptotic bodies that have undergone significant degradation following their phagocytosis are also difficult to recognize. 2) the chromatin condensation could be correlated only when DNA digestion reached about 50kb or nuclear fragmentation with DNA laddering (Ghibelli et al., 1995). Therefore, it is likely that apoptotic body and obvious morphological changes could not be seen by light microscopy in the cells which have only large and moderate length of degraded DNA fragments. Furthermore, in post mortem material it may be difficult to recognise the early stages of nuclear irregularity which might represent stage 1 (see Ghibelli et al., 1995) of apoptosis.

One likely problem in the study of apoptosis using fixed tissue sections is the possible occurrence of false positive results due to DNA damage taking place during the fixation. False negative results have also been found to occur (Davison et al., 1995); their likelihood was correlated with the length of fixation resulting in overcrosslinking. Proteinase K digestion has therefore become an essential step, as an optimised digestion gives good sensitivity and less background. At the same time, it is also important to increase the sensitivity by optimising the conditions, such as period of incubation with antibody, proportion of dig-dUTP and dTTP, as I did in my study. All these criteria were taken into consideration and applied to this study.

Finally, multiple statistical tests using Mann-Whitney U-test (two tailed) have been used in my study. This non-parametric test was chosen because the values did not have a
normal distribution. It should be emphasized that this test carries potential difficulties. The dividing line between the 'likely' and 'unlikely' classes is usually defined in terms of a probability \( p \) and 0.05 was chosen as the threshold for statistical significance. However, it should be kept in mind that this is a generally accepted, albeit arbitrary, criterion.

In comparison with pre-AIDS, there are significant differences in the expression of MHC II in HIVE \( (p=0.004) \), TNF-\( \alpha \) in normal control \( (p=0.016) \) and HIVE \( (p=0.015) \), IL-1\( \alpha \) in normal control \( (p=0.0001) \) and HIVE \( (p=0.015) \), and IL-6 in normal control \( (p=0.027) \). Additionally, some differences found are borderline in statistical terms. These are the expression of MHC II in normal control \( (p=0.051) \) and AIDS without neuropathological change \( (p=0.041) \), TNF-\( \alpha \) in AIDS without neuropathological change \( (p=0.056) \), and IL-4 in normal control \( (p=0.057) \). Finally, there is no difference in the expression of IL-1\( \alpha \) in AIDS without neuropathological change \( (p=0.08) \), IL-4 in HIVE \( (0.392) \) and AIDS without neuropathological change \( (p=0.463) \), IL-6 in HIVE \( (p=0.906) \) and AIDS without neuropathological change \( (p=0.259) \) (see Table 3.3).

4.7 Overview

In undertaking the work discussed in this thesis I hoped that the results obtained could answer some questions. Firstly, how frequently does HIV-1 enter the brain during the early stages of HIV-1 infection and do levels of HIV-1 DNA in infected cells of parenchyma of these brains correlate with the stages of infection? Secondly, regarding the immune response in brains of individuals at this stage, are immune activation (revealed by elevated expression of MHC class II antigens) and cytokines already present, in addition to the reactivation of microglial cells? Which cells are involved in the responses to the virus in the brain?

Examination of brains of asymptomatic HIV-1 positive individuals who died prior to the
development of AIDS demonstrated that invasion of the CNS by HIV-1 occurs early after primary infection, an opinion that now has been generally accepted (Tardieu, 1996) and that invasion induces an immunological process including a perivascular inflammatory T-cell reaction and leptomeningitis (Gray et al., 1996). I have now further demonstrated an immune activation of brain parenchyma with hyperplasia of microglial cells, upregulation of MHC class II antigens and production of cytokines.

Cytokines are peptides that bind to specific receptors and provide a number of signals. Most cytokines are extremely "pleiotropic", are produced by a variety of cell types and act on various cells in different ways. Cytokines have been divided into two classes: $T_{h2}$ cells ("good") and $T_{h1}$ cells ("bad") as well as regulatory ("good") or proinflammatory ("bad") cytokines (Lucas and Hohlfeld, 1995). It is therefore, not surprising that the cytokine network is extremely complex, and our understanding of it is far from complete.

Cytokines participate in normal physiologic events within the CNS. At the time of infection and inflammation, their role is emphasized by dysregulation of their expression and production and that of their receptors within the CNS, with consequent effects on brain function. Cytokines have been implicated in the pathogenesis of many CNS disease, including HIV-1 infection, multiple sclerosis (MS), and their role continues to be intensely studied. Investigation on cytokines have become a central topic within MS research. It has been suggested that cytokines play an essential role in the pathophysiology of MS, both by regulating autoimmune responses and by mediating myelin damage.
How cytokines exert effects on the brain is still a subject of much research; cytokines detected in the CNS may be produced by cells within the CNS or, alternatively, they may have passed from the circulation into the CNS. Cytokines have been described in CNS endothelial cells of rodents (Gutierrez et al., 1993), thus suggesting that they are able to cross the blood-brain barrier. Moreover, it is known that cytokines are active adhesion molecules and play a direct role in adhesion, a necessary step for cells to pass through the blood-brain barrier. Cytokines have been demonstrated not only by immunocytochemical studies which can be applied to localise cytokines in the CNS, but also by in situ hybridisation to determine their sites of synthesis within the brains of HIV-1 infected cases (Wesselingh et al., 1994) and MS (Woodroofe, 1995). Production of cytokines could be initiated by stimulation of local viral infection, such as HIV-1 infection in the CNS. It is generally accepted that subsequently cytokines can trigger a cascade of events including feedback loop involving themselves leading to brain damage.

As cytokines are involved in the pathogenesis of many CNS diseases, the restoration of proinflammatory cytokine homeostasis by naturally occurring cytokine inhibitors and inhibitory cytokines seems a promising way to treat chronic inflammatory disease (Burger and Dayer, 1995). A better understanding of the behaviour of each factor (cells and mediators), however, is a prerequisite to guarantee the safety of such treatment. The cytokine inhibitors usually act by hampering the binding of the cytokine to the specific cell surface receptor. Soluble receptors are able to bind cytokines and thus compete with the membrane receptors. The production of proinflammatory cytokines, such as IL-1 and TNF-α, seems to be highly dependent on the direct cell-to-cell contact between stimulated cells and other cells; therefore, coordinating inhibitions of biologic activity
of these cytokines and induction and production of anti-inflammatory cytokines might provide new specific immunotherapies to prevent tissue destruction.

4.8 Suggestions for further study

1) The characteristic changes (local productive HIV infection revealed by immunohistochemistry and electron microscopy; presence of MGC) seen in brain of patients with AIDS have never been described in the pre-AIDS (Budka, 1993). Unfortunately, many of these studies relied on structural protein probes which are not suitable for their demonstration in cells having a restricted infection of HIV-1. It is still unclear whether HIV-1 existing in the CNS during the early pre-AIDS stage is only latent as proviral DNA, or whether the replication of HIV-1 is restricted to regulatory genes. Indeed Seshamma et al (1992) observed that during the period of viral latency regulatory transcripts rather than full length HIV-1 RNA predominate in PBMC. If this were to apply to the brain, it would explain why p24 and p41 (which represent viral structural proteins) could not be detected immunohistochemically in pre-AIDS brain. Therefore, an investigation on the expression / replication of HIV-1 and their localization using both structural and regulatory proteins and/or mRNA markers would be of great interest for the understanding of HIV-1 in the CNS in the early pre-AIDS stage following infection.

2) Regarding the levels of HIV-1 DNA in the AIDS group, correlation was found neither between levels of viral DNA and presence or absence of neuropathological changes; nor between viral DNA levels and HIV-associated dementia. This would suggest that other
factor(s) in addition to the presence of HIV itself might be necessary to produce HIV encephalitis and dementia. These factors include the genotype of the virus and the condition of the host. Regarding the former, further studies, to examine whether there are specific sequence differences in brain-derived HIV-1 clones between patients with and without HIVE and between those with and without HIV-associated dementia, are necessary to better understand the pathogenetic mechanism involving viral strain. As for the latter, the existence of some individuals who, despite having HIV viraemia, remain carriers without progressing to the AIDS status (Pantaleo et al., 1995) tell us how important individual variability can be in this type of infection.

3) Elevated expression of cytokines such as TNF-α, IL-1α, IL-4 and IL-6 has been demonstrated in brains of HIV-1 pre-AIDS individuals. However, a further investigation on the receptor of these cytokines in the brain would be helpful to understand their involvement in the CNS disease in HIV-1 infection. Masliah et al (1994b) found that cytokine receptors are altered during HIV infection. IL-1β and TGF-β1 receptors have been found in dendritic processes of neurons in the neocortex. In cases with moderate HIVE, an average 35% increase was observed compared to cases without HIVE or with cases with severe HIVE.

4.9 Conclusions

The study has demonstrated in brain of HIV-1 positive pre-AIDS individuals that:

1. Neuropathological changes including infiltration of microglial cells, presence of macrophages and meningitis;
2. HIV-1 DNA in 17 of the 36, but not p24;

3. Levels of HIV-1 DNA (median = 45, mean = 55.8, range: 5-125) are lower than those found in brain of AIDS cases (median =135, mean =135, range: 5-540);

4. Elevated expression of major histocompatibility complex class II antigens, cytokines such as TNF-α, IL-1α, IL-4 and IL-6;

5. Apoptotic cells in white matter in 4 of the 36, 2 have also shown apoptotic neurons in the grey matter;

By showing presence of HIV provirus DNA in a large proportion of pre-AIDS brains, together with the identification of a condition of immune reaction, the existence of detectable amounts of cytokines as well as evidence of apoptosis, these results emphasise the potential risk brain tissue, and nerve cells in particular, are exposed to and indicate the need for therapeutic interventions from the very early stages of the infection.
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APPENDIX A

Calculations showing the possibility of contamination of HIV-1 DNA from residual blood within brain

The possibility of contamination from blood could be calculated assuming blood distribution to be even throughout the whole body at the time of death; that blood flow has a normal rate of 50-55 ml per 100 g brain per minute [50X14(1,400 g weight of organ/100 g)=700 ml=700,000 mm³] (Adams and Victor, 1985) and that white blood cells (WBC) count is at normal level (7.4X10⁹/mm³) (Dittmer, 1961).

1. As PBMC represent 4% of circulating white blood cells, the number of PBMC infected by HIV-1 in 10 mg of brain =

\[
\frac{7,400\text{WBC/mm}^3 \times 4\% \times 700,000\text{mm}^3}{1,000^3 \times 1,400\text{g} \times 60(\text{seconds})} = 0.025 \text{ cell;}
\]

2. Assuming a CD4 count of 400 cells/mm³ in the pre-AIDS patients, thus the number of HIV-1 infected CD4 positive T cells in 10 mg of brain =

\[
\frac{400\text{CD4 cell/mm}^3 \times 700,000\text{mm}^3}{100^3 \times 1,400\text{g} \times 60(\text{seconds})} = 0.33 \text{ cell;}
\]
3. Assuming HIV-1 DNA copies are $10^4$ copies/ml in the pre-AIDS patients and the copies of HIV-1 DNA from plasma in 10 mg of brain =

\[ 10^4 \text{copies/ml} \times 700 \text{ml} \times 1,400 \text{g} \times 60 \text{(seconds)} = 0.83 \text{ copies} \]

\[ \text{Total} = 0.025 + 0.33 + 0.83 = 1.185 \text{ copies (assuming that 1 copy of HIV-1 DNA per infected cell)}; \text{ or} \]

\[ 0.025 \times 2 + 0.33 \times 2 + 0.83 = 1.54 \text{ copies (assuming that 2 copies per infected cell).} \]

\[ ^1 \text{Usually, 1-2 \mu g of DNA obtained from 10 mg of brain tissue.} \]
\[ ^2 \text{This figure refers to the number of PBMC infected in an asymptomatic individual (Lee et al., 1991).} \]
\[ ^3 \text{The figure refers to the number of CD4 positive T cell infected in an AIDS patient (Poznansky et al., 1991).} \]
\[ ^4 \text{This figure refers to the copy number of HIV-1 RNA in plasma in an AIDS patient (Scadden et al., 1992).} \]
APPENDIX B

Detection of DNA fragmentation using DNA polymerase 1 and terminal deoxynucleotidyl transferase (TDT)

REAGENTS

PBS

10 mM phosphate-buffered saline pH7.4
138 mM NaCl
2.7 mM KCl

Proteinase K buffer

100 mM Tris-HCl pH8.0
50 mM EDTA

Buffer 1

100 mM Tris-HCl pH7.5
150 mM NaCl

Buffer 2

100 mM Tris-HCl pH9.5
100 mM NaCl
50 mM MgCl₂

Colour solution

4.5 μl NBT
3.5 μl X-phosphate
10μl of 2.5% levamisole solution
buffer 2 till 1 ml
End labelling buffer (for polymerase 1)

50 mM Tris-HCl pH 7.5
5 mM MgCl₂
10 mM 2-mercaptoethanol
0.005% BSA

30X dNTP mix

1 mM dATP
1 mM dCTP
1 mM dGTP
0.65 mM dTTP
0.35 mM DIG-11-dUTP

10X Enzyme solution (kit contents)

terminal deoxynucleotidyl transferase (TDT) in storage buffer

Label solution

nucleotide mixture in reaction buffer

Converter-AP

anti-fluorescein antibody, conjugated with AP

METHODS

I ISEL using DNA polymerase 1 (nick translation)

1. Deparaffinise specimen
   a. Heat slides to 60°C in oven for 15 min.
   b. Rinse slides 3 times in xylene, 2 min each.
2. Rehydrate specimens by immersing in serial graded ethanol washes (100%, 100%, 95%, 70%, 50%) 2 min each.

3. Detergent permeabilisation
   a. Immerse slides in PBS for 2 min.
   b. Immerse slides in 0.3% Triton X-100 in PBS 15 min at RT.
   c. Wash slides 2 times in PBS, 5 min each.

4. Proteinase K digestion
   a. Incubate slides in proteinase K solution (1μg/ml) at 37°C for 10 min.
   b. Wash in PBS for 5 min.

5. Dehydrate slides by immersing in serial graded ethanol washes (50%, 70%, 95%, 100%) 2 min each.

6. Prepare ISEL reaction mixture and apply 100μl to each slide, cover with parafilm, each 100μl contain:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X buffer</td>
<td>10 μl</td>
</tr>
<tr>
<td>30X dNTP</td>
<td>3.3 μl</td>
</tr>
<tr>
<td>Klenow</td>
<td>5ο</td>
</tr>
<tr>
<td>dH₂O till</td>
<td>100 μl</td>
</tr>
</tbody>
</table>

7. Incubate slides in humid chamber at 37°C for 120 min.

8. Wash with PBS 2 times, 5 min each.


10. Incubate slides in buffer 1 containing 2% normal goat serum and 0.3% Triton X-100 for 30 min at RT.

11. Dilute anti-DIG-AP conjugated antibody 1:500 with buffer 1 containing 1% normal goat serum and 0.3% Triton X-100.

12. Apply 100μl of diluted antibody to the slides and incubate in a humid chamber at 4°C overnight (about 12 hours).
13. Wash slides for 10 min with shaking in buffer 1.
14. Wash slides for 10 min with shaking in buffer 2.
15. Apply 200μl colour solution to each slide and incubate slides in humid chamber in absence of light for 10 min or longer as needed.
16. Stop reaction by washing slides with water.
17. Counter stain with fast nuclear red for 5 min and then wash with water.
18. Dehydrate as step 5, then 2 min in histoclear.
19. Mount coverslip with ralmount.

II TDT-mediated dUTP-fluorescein nick end labelling (TUNEL)

1-5. are same as 1 through 5 in I.
6. Prepare TUNEL reaction mixture by adding 50μl of 10X enzyme solution to 450μl label solution to obtain 500μl TUNEL reaction mixture.
7-10. are same as 7 through 10 in I.
11. Apply 100 μl Converter-AP solution onto each slide.
12. Incubate in a humid chamber at 4°C overnight.
13-19. are same as 13 through 19 in I.
APPENDIX C

Extraction of DNA from formalin fixed, paraffin embedded brain specimens

REAGENTS

Proteinase K buffer

10 mM Tris-HCl pH 7.4
1 mM EDTA
0.5% SDS

Proteinase K stock solution 50 mg/ml sterile distilled water

Equilibration of phenol (100 g Molecular biology grade, sigma)

1. Remove from freezer and warm to room temperature.
2. Melt at 68°C in water bath.
3. Add hydroxyquinoline to a final concentration of 0.1%.
4. Add 100 ml 0.5 M Tris-HCl pH 8.0, stir 15 min then aspirate the upper (aqueous) phase.
5. Add 100 ml 0.1 M Tris-Hcl pH 8.0, stir and aspirate the upper phase then repeat 2 to 3 times until the pH of the phenolic phase is > 7.8.
6. Add 10 ml 0.1 M Tris-Hcl pH 8.0 containing 0.2% β-mercaptoethanol.

Preparation of chloroform:isoamyl alcohol

Add 2 ml isoamyl alcohol to 48 ml chloroform.
Preparation of phenol:chloroform:isoamyl alcohol (25:24:1)

Mix equal volume of equilibrated phenol and chloroform:isoamyl alcohol.

METHODS

1. Cut 10 μm section and remove part of tissue containing meninges with disposable scalpel and forceps, change blades for each block.
2. Put 5 to 10 sections in one Eppendorf tube.
3. Dewax by washing 3 times with xylene and once with 100% ethanal.
4. Dry tissue at 95°C for 30 min.
5. Add proteinase K solution to a final concentration of 5 mg/ml in proteinase K buffer.
6. Digest for 24 hours at 55°C in water bath with gently shake.
7. Add fresh proteinase K solution to tissue suspension and continue digestion for another 24 hours if tissue is still visible.
8. Extract DNA by standard method, i.e. add an equal volume of phenol: chloroform and mix the contents of the tube until an emulsion forms.
9. Centrifuge the mixture at 12,000g for 10 min.
10. Use a pipette to transfer the aqueous phase to a fresh tube. Discard the interface and organic phase.
11. Repeat steps 8 through 10 until no protein is visible at the interface of the organic and aqueous phases.
12. Add an equal volume of chloroform and repeat steps 9 and 10.
13. Remove the spare liquid by spin through a Microcon-30 column (30,000
molecule weight cut off) at 13,000g for 10 min.

14. Recover the purified, concentrated DNA by placing sample reservoir upside down in a new tube then spin 2 min at 1,000 rpm.

15. Quantify DNA using spectrophotometer.
APPENDIX D

Semi-quantitative PCR

REAGENTS

PCR reaction buffer (50 \( \mu \)L)

- 16 mM \((\text{NH}_4)_2\text{SO}_4\)
- 67 mM Tris-HCl pH 8.8
- 0.01% Tween-20
- 0.3 \( \mu \)M of each primer
- 0.2 \( \mu \)M of dATP, dCTP, dGTP, dTTP
- 3 mM MgCl\(_2\)
- 2.5\(^{th}\) BioTaq polymerase

10 mM dNTP

- 10 mM dATP
- 10 mM dCTP
- 10 mM dGTP
- 10 mM dTTP

Blocking stock solution

Blocking reagent is dissolved in buffer 1 to a final concentration of 10% and autoclaved.

Buffer 1

- 0.1 M Maleic acid
- 0.15 M NaCl pH 7.5

Buffer 2

blocking reagent in buffer 1 at a final concentration of 1%.
Buffer 3

100 mM Tris-HCl pH9.5
50 mM MgCl₂
100 mM NaCl

Washing buffer

buffer 1 with 0.3% Tween 20

2X SSC

0.3 M NaCl, 30 mM Na-citrate pH7.0

CSPD solution

200 μl of CSPD in 20 ml buffer 3

METHODS

I Semi-quantitative PCR

1. Preparation of master mix for the first round of PCR, each 50 μl contain:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X PCR buffer</td>
<td>5 μl</td>
</tr>
<tr>
<td>50 mM MgCl₂</td>
<td>3 μl</td>
</tr>
<tr>
<td>10 mM dNTP</td>
<td>1 μl</td>
</tr>
<tr>
<td>primer pol 1</td>
<td>5 μl</td>
</tr>
<tr>
<td>primer pol 2</td>
<td>5 μl</td>
</tr>
<tr>
<td>Taq</td>
<td>0.5 μl (2.5%)</td>
</tr>
<tr>
<td>dH₂O</td>
<td>26.5 μl</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>45 μl</strong></td>
</tr>
<tr>
<td><strong>template</strong></td>
<td><strong>5 μl (1 μg of DNA)</strong></td>
</tr>
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</table>
2. Run samples for 28 cycles using Thermal Cycler as below:

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Duration</th>
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<tbody>
<tr>
<td>95°C</td>
<td>5 min</td>
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<tr>
<td>94°C</td>
<td>1 min</td>
</tr>
<tr>
<td>63°C</td>
<td>2 min</td>
</tr>
<tr>
<td>72°C</td>
<td>7 min</td>
</tr>
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</table>

3. Preparation of master mix for second round of PCR, each 50 μl contain:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X PCR buffer</td>
<td>5 μl</td>
</tr>
<tr>
<td>50 mM MgCl₂</td>
<td>3 μl</td>
</tr>
<tr>
<td>10 mM dNTP</td>
<td>1 μl</td>
</tr>
<tr>
<td>primer pol 3 (5'-DIG labelled)</td>
<td>5 μl</td>
</tr>
<tr>
<td>primer pol 2</td>
<td>5 μl</td>
</tr>
<tr>
<td>Taq</td>
<td>0.5 μl</td>
</tr>
<tr>
<td>dH₂O</td>
<td>29 μl</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>47.5 μl</strong></td>
</tr>
<tr>
<td><strong>PCR product</strong></td>
<td><strong>2.5 μl</strong></td>
</tr>
</tbody>
</table>

4. Run samples for 18 cycles as step 2.

II Performing a Southern transfer on the Vacu-AID

1. Run 10 μl PCR product from second round of PCR on 2% agarose electrophoresis gel.

2. Carefully measure the size of the gel to be blotted. Cut an aperture 5-10 mm smaller than the gel in a rubber mask.

3. Cut a piece of 3 mm filter paper 2-4 cm larger that the gel. Pre-wet with 2X SSC. Cut the nylon membrane (positive charged) slightly longer that the gel and place on top of the 3 mm paper.

4. Carefully locate the rubber mask over the membrane and filter paper so as to position the aperture exactly over the membrane.

5. Locate top manifold and clamp into place.
6. Flood the aperture in the rubber mask with a small quantity of transfer buffer and carefully position the agarose gel exactly over the aperture. Remove all air bubbles if there is any.

7. Apply vacuum at 80 cm of water pressure and transfer buffer to the top of the gel. Blotting proceeds for 90 min.

8. Fix DNA onto nylon membrane by baking membrane at 120°C for 30 min.

III Chemiluminescence

1. Rinse membrane briefly in washing buffer.

2. Incubate for 30 min in buffer 2.

3. Dilute anti-DIG-AP conjugate to 75 mU/ml (1:10,000) in buffer 2.

4. Incubate membrane for 30 min in 20 ml antibody solution.

5. Wash 2 X 15 min with washing buffer.

6. Equilibrate 2-5 min in buffer 3.

7. Incubate membrane for 5 min in 20 ml CSPD solution.

8. Let excess liquid drip off and blot membrane briefly on Whatman 3 mm paper. Do not let membrane dry completely.

10. Seal damp membrane in a hybridisation bag and incubate for 5-15 min at 37°C to enhance the luminescent reaction.

11. Expose for 15-25 min at room temperature to X-ray film.
IV Densitometry

Scan the X-ray film using the molecular Dynamics Densitometer.

V Standard curve

Standard curve is made according to the value of area or volume obtained from a series of dilutions of standard DNA and linear relationship between the log of the amount of PCR product and the log of the initial amount of sample DNA is represented in log/log XY chart.
List of supplementary material submitted for the degree of Doctor of Philosophy

Published papers


Abstracts


PCR detection of HIV proviral DNA (gag) in the brains of patients with AIDS: comparison between results using fresh frozen and paraffin wax embedded specimens

S F An, A Ciardi, F Scaravilli

Abstract

Aims—To adapt the polymerase chain reaction (PCR) technique of HIV detection to paraffin wax embedded brain tissue and to compare the results with those obtained using frozen tissue.

Methods—HIV antigen and HIV proviral DNA were detected in specimens of frontal lobe using immunohistochemistry and PCR, respectively. DNA was extracted from fresh tissue using standard methods whereas the technique for extracting DNA from paraffin wax embedded tissue was partly modified.

Results—Twenty cases were examined. HIV DNA was detected in 16 cases in frozen specimens. Of these, 15 were also positive when paraffin wax embedded material was analysed.

Conclusions—This study shows that HIV proviral DNA can be detected in formalin fixed, paraffin wax embedded brain tissue by PCR. The results obtained from paraffin wax embedded specimens showed a similar degree of reliability to those from fresh frozen brain. Factors such as fixative, fixation time, and delay in performing post mortem examinations did not seem to influence PCR amplification as positive results were obtained with specimens left in fixative for up to eight months, as well as in cases where post mortem examinations had been delayed for up to four days.

 IMMUNOHISTOCHEMISTRY

The primary antibody HIV p24 (Du Pont Diagnostics, Stevenage, UK), which detects the HIV core protein p24, was used to screen paraffin wax embedded sections of anterior frontal lobes from all cases in this study. Briefly, paraffin wax embedded sections were dewaxed and endogenous peroxidase was blocked with 0.03% hydrogen peroxide in methanol. Incubation for one hour with primary antibody (HIV p24) was followed by incubation with biotinylated rabbit anti-mouse (30-60 minutes) and peroxidase conjugated avidin (30 minutes). 3,3'-Diaminobenzidine was used as a substrate to reveal the peroxidase.

DNA was extracted as previously described by Pang et al. Briefly, blocks of semi-frozen frontal lobes were stripped of leptomeninges and diced into small pieces. Tissue was digested overnight with proteinase K (final concentration 0.25 mg/ml in 10 mM TRIS-HCl, pH 7.4, 1 mM EDTA, and 0.5% sodium dodecyl sulphate (SDS)) at 56°C. DNA was subsequently purified using standard methods.

Paraffin wax embedded sections (10 μm) were cut and placed in a 1.5 ml Eppendorf
### Results of PCR detection with pathological and immunohistochemical findings

<table>
<thead>
<tr>
<th>Case No</th>
<th>Sex</th>
<th>Age (years)</th>
<th>Delay of PM (days)</th>
<th>Fixation (months)</th>
<th>HIV</th>
<th>PCR</th>
<th>p24</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>31</td>
<td>1</td>
<td>5</td>
<td>HIV encephalitis</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>29</td>
<td>1</td>
<td>5</td>
<td>No abnormalities</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>59</td>
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<td>-</td>
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<td>M</td>
<td>53</td>
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<td>+</td>
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<td>+</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>42</td>
<td>3</td>
<td>5</td>
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<td>6</td>
<td>M</td>
<td>25</td>
<td>2</td>
<td>3</td>
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<td>-</td>
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<td>8</td>
<td>M</td>
<td>47</td>
<td>3</td>
<td>1</td>
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<td>-</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>M</td>
<td>28</td>
<td>2</td>
<td>2</td>
<td>Cryptococcosis</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>M</td>
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<td>+</td>
<td>+</td>
</tr>
<tr>
<td>13</td>
<td>M</td>
<td>25</td>
<td>1</td>
<td>4</td>
<td>CMV encephalitis</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>14</td>
<td>M</td>
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<td>+</td>
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<td>+</td>
</tr>
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<td>+</td>
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<td>4</td>
<td>6</td>
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<td>-</td>
<td>-</td>
</tr>
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<td>52</td>
<td>4</td>
<td>8</td>
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<td>-</td>
<td>-</td>
<td>-</td>
</tr>
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<td>20</td>
<td>M</td>
<td>52</td>
<td>2</td>
<td>1</td>
<td>No abnormalities</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

PML = progressive multifocal leucoencephalopathy; CMV = cytomegalovirus; FPL = focal pontine leucoencephalopathy; F = frozen tissue; P = paraffin wax embedded tissue.

**PCR ANALYSIS**

Human single copy gene β-globin was used as a DNA positive control and only clearly positive samples were used for HIV PCR analysis. Ten molecules of non-infectious plasmid containing a completely rearranged HIV sequence (provided by UK Medical Research Council (MRC), AIDS Directed Programme (ADP), PCR Reference Centre) were used as the HIV positive control. DNA from brain tissue of patients not in any HIV risk group was used as a negative control. All the primer sequences are written from 5' to 3'. Two human β-globin primers (MRC, ADP, National Institute for Biological Standards and Control (NIBSC)) were used: ADP894-1, ACA CAA CTG TGT TCA CTA GC; ADP894 2, CAA CTT CAT CCA CGT TCA CC. A 110 base pair (position 14–123) fragment is flanked by primers ADP894-1 and ADP894-2. Two HIV primers 3855-1 and 3855-2 (Perkin Cetus Elmer UK) were used: 3855-1 (SK145), AGT GGG GGG ACA TCA AGC AGC CAT GCA AAT; 3855-2 (SK431), TGC TAT GTC AGT GCC CCT TGG TCC TCT. A 142 base pair (position 1366–1507) fragment is flanked by primers 3855-1 and 3855-2.

Of the reaction mixture, 50 μl contained 0.5 μM of each primer, 0.2 mM of dATP, dCTP, dGTP, and dTTP, and 2.5 units of AmpliTaq DNA polymerase in PCR buffer (10 mM TRIS-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, and 0.001% gelatin). Forty cycles of PCR were performed with 0.5–1 μg extracted template DNA, each cycle consisting of thermal denaturation at 94°C for one minute, primer annealing at 55°C for two minutes, and extension at 72°C for three minutes.

Human β-globin gene amplification was similar to HIV amplification except that the annealing temperature for β-globin gene amplification was 60°C and 0.1 μM of primers were used in the PCR reaction. Amplified products were visualised by ethidium bromide staining of agarose gel electrophoresis. HIV products were visualised by ethidium bromide staining of agarose gel electrophoresis. HIV products were visualised by ethidium bromide staining of agarose gel electrophoresis.
were further detected by Southern blot hybridisation to ascertain their specificity.

**SOUTHERN BLOT HYBRIDISATION**

To detect the 142 base pair PCR product, modified Saluz and Jost’s filling in methods were applied as described before. Oligonucleotides were provided by the MRC, ADP, and NIBSC. Their sequences are:

- gag (30-mer), 5’-CATCAAAGGAGCTGCAAGATGTAG; (10-mer), 5’-TCCCATTCTG.

Briefly, an annealing mix containing 10 μl of the 30-mer, 10 μl of the 10-mer, and 25 μl of the oligonucleotide labelling buffer was prepared and incubated at 75°C for two minutes, at room temperature for 20 minutes and on ice for 10 minutes. This was followed by incorporation of digoxigenin-11-dUTP and precipitation of the probe by ethanol.

Nylon membranes (Boehringer Mannheim, UK) were prehybridised for more than one hour at 42°C in hybridisation buffer (5 x saline-sodium citrate (SSC), 50% formamide, 0.1% N-lauroylsarcosine, 0.02% SDS, and 2% blocking reagent) and then hybridised overnight at 42°C with 200 ng/ml labelled probe. They were then washed in 0.2 x SSC and 0.1% SDS at room temperature for one hour with four changes.

Colorimetric detection using nitroblue tetrasodium and X-phosphate was performed according to the Boehringer Mannheim protocol. Briefly, after incubation with antidigoxigenin-alkaline phosphatase at room temperature for 30 minutes and a postincubation wash, the filter was incubated in substrate to permit colour development.

**Results**

Figure 1 illustrates the results of PCR amplification with the oligonucleotide probe for the HIV gag gene in a variety of samples of the PCR reference kit. The result of this test shows that 10 molecules of HIV-1 DNA gave a clear signal after Southern blot hybridisation. There were no false positive results following either PCR amplification or Southern blot hybridisation.

The neuropathological changes observed in the brain tissue of the 20 patients with AIDS included in this study are summarised in the table. HIV encephalitis was present in seven cases; other neuropathological findings included cytomegalovirus encephalitis (three cases), progressive multifocal leucoencephalopathy (one case), cryptococcosis (two cases), lymphoma (one case), tuberculosis (one case), and non-specific changes (four cases); three cases did not show any abnormalities. Nine cases, including all those with neurological evidence of HIV encephalitis, were HIV p24 positive.

Specimens were regarded as positive or negative whenever PCR results were consistently positive or negative (PCR amplifications were repeated three times). Samples that were positive in only one or two of three tests (cases 12 and 17–20 in the table) were defined as indeterminate, and underwent another five tests, on the basis of which they were reclassified as positive (four cases) or negative (one case).

Sixteen cases were positive on analysis of frozen specimens. These included all those with immunohistochemical evidence of HIV. Of these, 15 were also positive when paraffin wax embedded material was analysed. Southern blot hybridisation confirmed the positive results. None of the cases positive on analysis of paraffin wax embedded samples gave negative results when frozen tissue was examined.

Figure 2 shows the results of PCR amplification in three cases using both frozen and paraffin wax embedded samples. Case 1 (lanes A and B) was positive in both; case 2 (lanes C and D) gave a positive result with frozen material only; and case three (lanes E and F) is an example of the four cases with negative results on analysis of both frozen and paraffin wax embedded material.

**Discussion**

In this study we have shown that HIV proviral DNA can be detected by PCR in brain tissue...
PCR detection of HIV proviral DNA in AIDS

of patients with AIDS using formalin fixed, paraffin wax embedded tissue. The HIV proviral DNA was chosen as it is highly conserved and specific sequence of the HIV gag region. As in a previous study,\(^2\) gag primers produced more reliable results than pol or env primers, and highly conserved areas have been identified in the HIV-1 genome in long terminal repeat sequences and in the gag gene.\(^3\) Moreover, as the region spanning the gag sequence is thought to be the last to be synthesised during reverse transcription, positive results using the gag region reflect completely or almost completely synthesised viral DNA.\(^4\)

In a previous investigation using PCR\(^5\) specimens were classified as positive, negative, or indeterminate depending on whether most of the tests were positive; all tests were positive; or one or two only were positive, respectively. In the present study each indeterminate case underwent five additional tests and samples were defined as positive when at least three of the latter gave positive results.

Of the 20 cases in this study, 16 were positive on analysis of frozen samples; of the latter, 15 were also positive when paraffin wax embedded material was studied. On the other hand, none of those which were negative on analysis of frozen samples gave positive results on analysis of paraffin wax embedded sections. Positive cases on analysis of both frozen and paraffin wax embedded material included the seven with morphological and immunohistochemical evidence of HIV encephalitis. A possible explanation for the single negative result (fig 2) could be the presence of an inhibitor of Taq polymerase.\(^6\) Although the human β-globin gene was successfully amplified in this case, the degree of inhibition may not have been uniform for the different sets of primers.\(^7\)

Several investigators have drawn attention to the effects of various fixatives and fixation times on PCR results.\(^8\) \(^9\) In this study the specimens had been kept in 10% buffered formalin (the most widely used fixative in neuropathology) for one to eight months, and the results show that even relatively long fixation times are compatible with successful amplification of HIV. However, DNA extracted from paraffin wax blocks will probably need to be concentrated using a microconcentrator instead of ethanol precipitation (data not shown).

The PCR technique has been used to amplify specific DNA from paraffin wax embedded human tissue in a variety of disorders. Human papillomavirus was identified by Brandwein et al.,\(^10\) Nawa et al.,\(^11\) Ohta and Ikeda,\(^12\) Saltzstein et al.,\(^13\) Van-Bommel et al.,\(^14\) and Kellokoski et al.;\(^15\) an adenovirus, cytomegalovirus, and herpes simplex virus (HSV) 1 and 2 were detected by Vesey et al.,\(^16\) whilst Epstein-Barr virus (EBV) was detected by Niederwieser et al.\(^17\) and Morehead et al.,\(^18\) and hepatitis C virus by Bresters et al.\(^19\) Central nervous system (CNS) tissue specimens were used by Woodall et al.\(^20\) for the identification of enterovirus RNA; Nicoll et al.\(^21\) detected HSV1 DNA in the brains of patients with herpes encephalitis and Lager et al.\(^22\) detected EBV in the brains of patients with primary biliary cirrhosis. Meier et al.\(^23\) examined EBV and HIV in 12 AIDS related CNS lymphomas and detected EBV in six of the 12 tumours but could not detect HIV; Lai-Goldman et al.\(^24\) detected EBV in various organs of patients with AIDS but not in the brain. In this study we have shown not only that HIV proviral DNA can be detected by PCR in paraffin wax embedded tissue from the CNS but also that the results have the same degree of reliability as those obtained using fresh frozen material.

Supported by a grant from the Brain Research Trust and MRC (SFA and FS), and the European Concerted Action on AIDS (FS). AC is a recipient of a grant from the Istituto Superiore di Sanità of Italy.

We thank Dr S Lucas and Mr M Karsenas for providing the brain tissue, and Mr A Beckert for the detection of the HIV-1 core protein p24. We are grateful to the Medical Research Council, AIDS Directed Programme repository, and the National Institute of Biological Standards and Control for providing primers and probes.


Clinical staging system for AIDS patients

Sir—Mocroft et al have shown that information on past diagnoses of AIDS can be correlated with CD4 T-cell counts to assess the risk of mortality for people with AIDS. However, it is not clear how this system could be used to measure reductions in the risk of mortality during antiretroviral treatment. For example, a patient with a history of two severe disease episodes in the course of AIDS counts to assess the risk of mortality for people with AIDS. However, if this patient were then treated with an antiretroviral agent and the CD4 count rose above 100 cells/μL, would the score remain at 100: the score does not improve if CD4 counts rise and no further AIDS exacerbations were diagnosed, the score would remain at 100: the score does not improve if CD4 counts rise and no further AIDS defining events are diagnosed. In this example, the inference to be drawn from the proposed staging system is that HIV-1 infection is progressive and irreversible, and that treatments which reduce viral burden, allowing recovery in the CD4 count and a lowering of the incidence of AIDS, will not then reduce the risk of mortality below the pre-treatment level.

Analysis of the ACTG 019 trial has shown that recency of CD4 counts is more predictive of the risk of progression to AIDS, and that treatment-related rises in CD4 count may lead to reductions in the risk of progression and, possibly, of mortality. Reanalyses of the cohort studies from Mocroft et al could help to address this question. If CD4 counts and/or AIDS diagnoses were to become less important factors in determining mortality risk, if they occurred before a certain time (eg, 2 or 3 years previously), this would suggest, from a statistical viewpoint, that the course of HIV-1 infection is not irreversible, and that sustained improvements in CD4 counts, and an absence of new AIDS diagnosis, may predict a lower risk of mortality.

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Author’s reply

Sir—Hill raises two points about our recent study which derived a staging system for AIDS patients.1 The first concerns treatment and the use of minimum CD4 count in the staging system. While we accept that treatment often raises CD4 counts, the degree to which such improvement translates into better survival prospects is debatable.2,3 In our original derivation of the score, we tried modelling the CD4 count in different ways, such as latest CD4 count, mean of last two counts etc, but these different CD4 models were studied to have little effect on the final model. If new therapies for patients with AIDS can induce substantial rises in the CD4 count, which improvement translates into clear survival benefits, then the score and staging system we have suggested could be remodelled to take account of the most recent CD4 count.2,4

Concerning the second issue raised by Hill, we have studied mortality risk while ignoring AIDS-defining diseases and CD4 counts (the ‘memory’ period). If a model incorporating such a limited memory fits no worse than the original one used to derive the score and staging system, which assumed unlimited memory, it would imply that the risk of death is not affected by events which happened before the beginning of the memory period. The fit of the models is measured by changes in the value of the log likelihood (−2LogL), where the lower the value of this statistic, the worse the fit of the model. A model fitted with a memory period of 3 years fits no worse than the model we originally used (−2LogL=127·2 and 126·9 respectively), while a model fitted with a memory period of two years has an appreciably poorer fit (−2LogL=121·7). As the memory period is reduced further to one year, the fit of the model continues to deteriorate (−2LogL=94·2). This suggests that events occurring as much as two or three years ago remain relevant for assessing the risk of death in patients with AIDS.

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Programmed cell death in brains of HIV-1-positive pre-AIDS patients

Sir—Neuropathological studies have revealed that a number of brains of AIDS patients show characteristic HIV encephalitis and, in addition, neuronal loss. More recently, neuronal cell loss has been interpreted as taking place via a process of programmed cell death (apoptosis) and has been visualised by the in-situ end-labelling (ISEL) technique.1 Whilst it is well accepted that the brains of AIDS patients harbour HIV, there is no consensus about the time of entry of the virus into the brain parenchyma, despite early reports demonstrating it in the cerebrospinal fluid. As for the mechanism of brain damage, and of the neuronal loss in particular, a widely accepted opinion attributes the damage to an indirect effect of HIV, probably mediated by cytokines.

In a previous investigation, we found that the brains of a number of HIV-positive pre-AIDS individuals, who had died of causes unrelated to the disease, showed morphological abnormalities such as lymphocytic meningitis, myelin pallor and gliosis. Moreover, when their brains were examined by PCR it was found that a number of them contained HIV-1 proviral DNA.2 Subsequently, we were able to confirm the presence of HIV-1 DNA in 17 out of 36 brains of HIV-1 positive asymptomatic patients and to demonstrate elevated expression of major histocompatibility complex (MHC) class II antigen and cytokines such as TNF-α, IL-1α, IL-4, IL-6 (unpublished). These results confirm a hypothesis that the condition of immune activation, previously described in AIDS brains,3 was already present at this early stage and suggested that nerve-cell damage might also be taking place. Therefore we undertook an investigation of the same 36 pre-AIDS brains by ISEL. As positive controls, 18 AIDS (10 HIV encephalitis and 8 neuropathologically normal) brains were used; 5 HIV-negative brains from people without neurological disease served as negative controls.

Apoptotic cells were detected in 6/10 HIV encephalitis, 1/8 AIDS without central nervous system (CNS) disease and
New chlamydial antigen as a serological marker in HIV infection

Sir—pgp3 is a protein encoded by the pCT plasmid of Chlamydia trachomatis, it is immunogenic in chlamydial infections and antibodies to it can be detected by ELISA (pgp3-ELISA). 80% of patients with sexually-transmitted diseases (STDs) who were seropositive for C. trachomatis surface antigens by reference micro-immunofluorescence test with whole purified bacteria (CtMIF+) also had antibodies to pgp3 in their serum; 50 CuVUF-negative sera from patients with respiratory changes and PCR detection of HIV provirus DNA in brains of asymptomatic HIV-positive patients. J Neuropathol Exp Neurol 1994; 53: 43–50.


### Table: Percentage of pgp3ELISA positivity in different groups of patients

<table>
<thead>
<tr>
<th>Patients (no)</th>
<th>CtMIF+</th>
<th>pgp3+</th>
<th>Prevalence of pgp3+ in CtMIF+ patients</th>
<th>Prevalence of pgp3+ in CtMIF- patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy (50)</td>
<td>0%</td>
<td>0%</td>
<td>11% (3/27)</td>
<td>83% (17/21)</td>
</tr>
<tr>
<td>NGU (40)</td>
<td>32% (13/40)</td>
<td>32% (13/40)</td>
<td>77% (15/19)</td>
<td>81% (26/32)</td>
</tr>
<tr>
<td>PID (40)</td>
<td>67% (26/39)</td>
<td>72% (26/37)</td>
<td>81% (26/32)</td>
<td>89% (26/29)</td>
</tr>
<tr>
<td>HIV+ (50)</td>
<td>30% (15/50)</td>
<td>30% (15/50)</td>
<td>85% (26/30)</td>
<td>83% (26/31)</td>
</tr>
<tr>
<td>HIV+ (22)</td>
<td>32% (7/22)</td>
<td>32% (7/22)</td>
<td>86% (15/19)</td>
<td>81% (15/19)</td>
</tr>
</tbody>
</table>

HIV sera were stored at the Biobanque de Picardie, Amiens and were obtained from Hospital Clinics in Amiens and from Prof P Delamansis (Oru, Nice). CtMIF=microimmunofluorescence test with C trachomatis elementary bodies; NGU=non-gonococcal urethritis; PID=pelvic inflammatory disease; angiotis/stenisty.

### Genetic diversity and HIV detection by polymerase chain reaction

Sir—Numerous HIV-1 and HIV-2 strains have been isolated from various geographic sites and nucleic acid sequence analyses have identified at least eight distinct HIV-1 subtypes. Recently, fully sequenced HIV strains isolated from Cameroonian patients (isolates AN770 and MVP5180) have been classified as HIV-1 (subtype O) despite their high degree of nucleotide divergence. Charnay et al suggested categorising HIV-1 into two groups, M (major) and O (outlier) comprising the ten viral isolates so far characterised in Belgium, Germany, and France. The polymerase chain reaction (PCR) is useful in detecting neonatal HIV-1 infection, and in quantifying HIV load. Amplicor (Roche Diagnostics Systems, Branchburg, NJ, USA) is a qualitative DNA probe that uses nucleic acid cross-reactions could be attributed to possible autoimmune responses, which should be, in this case, common to HIV and chlamydial infections.

- There is a true anti-chlamydia humoral response with an atypical profile: ie, the response to the normally immunodominant surface antigens of C. trachomatis would be absent or undetectable while antibodies to pgp3 would be detected.

Our data suggest that pgp3 ELISA could provide a particularly effective way to screen HIV and STD patients for studies on Chlamydia-HIV interaction in clinical cases. Detection of asymptomatic chlamydial infections is particularly important in the case of HIV patients, not only because immuno-stimulation by any new antigen is thought to increase the probability of progress towards AIDS, but also because C. trachomatis has been recently shown to interact specifically with polymorphonuclear leucocytes and increase HIV replication. 

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Programmed cell death in brains of HIV-1-positive AIDS and pre-AIDS patients

Abstract Neuropathological studies have revealed that the brains of HIV-1-infected AIDS patients show the typical encephalitis and, in addition, neuronal loss. More recently, this neuronal cell loss has been thought to take place via programmed cell death (apoptosis) which has been demonstrated by an in situ end labelling (ISEL) technique. In this study 54 brains of HIV-1-positive patients were investigated by the ISEL technique to investigate whether apoptosis is also present in the brains of patients at the asymptomatic stage. Of these, 10 patients suffered from HIV encephalitis (HIVE), 8 had AIDS without neuropathological disorders and 36 were HIV-1-positive pre-AIDS patients. Apoptotic cells were detected in 6 of the 10 HIVE, 1 of the 8 AIDS without central nervous system (CNS) disease and 4 of the 36 asymptomatic individuals. A difference seen between the AIDS and pre-AIDS cases was that, in the latter, apoptotic cells were found in the white matter in all 4 cases, while only 2 of these 4 showed apoptotic neurons. The presence of apoptotic cells in a number, albeit small, of brains of HIV-1-positive pre-AIDS individuals, combined with abnormalities described previously in the same group of patients gives further support to the opinion that brain damage already occurs during the early stages of HIV infection.

Key words Asymptomatic • HIV • In situ end labelling (ISEL) • Neuronal cell loss • Programmed cell death (apoptosis)

Introduction

Programmed cell death (apoptosis) is characterised by the disappearance of individual cells and represents the endpoint of a genetically determined programme which requires de novo gene expression and protein synthesis. It differs from necrosis which is an accidental event triggered by factors external to the dying cells. Morphologically, whereas in cell necrosis there is loss of membrane integrity, swelling and degeneration of cell organelles and eventually lysis, in apoptosis organelles are normal and nuclei show dense chromatin clumped against the nuclear membrane. Apoptosis is thought to be implicated not only during developmental but also in the post-maturation stages of peripheral and central nervous system (PNS and CNS) neurons, as recent studies have suggested that apoptotic neuronal death occurs in the mature nervous system and that it may be even involved in a number of human diseases including neuro-degenerative disorders such as Alzheimer's and Parkinson's disease or in ageing [7].

Neuropathological studies in AIDS have revealed that the brains of patients suffering from the AIDS dementia complex with the typical encephalitis show, in addition to the well-known features, variable amounts of neuronal loss in cortical and subcortical regions [8, 19, 44]. However, as no firm morphological evidence of cell death had been reported, it was initially postulated that a discrete and morphologically undetectable process of apoptosis could exist leading to cell loss. Indeed, apoptosis had been already considered as the possible mechanism of T cell (especially CD4+ T cells) elimination [5, 6, 13, 17, 24, 26, 29, 35, 38], and depletion of CD8+ T cells [22]; moreover, recently, neuronal cell loss by apoptosis has been unquestionably demonstrated in the CNS of adult [11, 34] as well as young AIDS patients [10].

In a previous investigation [40], it was found that the brains of a number of HIV-1-positive pre-AIDS individuals, who had died of causes unrelated to the disease, showed morphological abnormalities including lymphocytic meningitis, myelin pallor and gliosis. When these brains were...
examined by polymerase chain reaction (PCR) it was found that a number of them contained HIV-1 proviral DNA [39, 40]. Subsequently, we were able to confirm the presence of HIV-1 DNA in large proportion of brains of HIV-positive patients at the asymptomatic stage and, in addition, to demonstrate elevated expression of major histocompatibility complex (MHC) class II antigens and cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1α, IL-4 and IL-6 (in press).

As the status of immune activation, revealed by the up-regulation of MHC class II and the presence of cytokines, is comparable to that seen in AIDS in which it is implicated in the mechanism of neuronal cell death, we undertook the present study with the aim of investigating whether neuronal cell death is also evident at the early stages of infection. A positive answer to this question would confirm that cell damage is indeed taking place well before patients enter the AIDS stage of the disease.

Materials and methods

Subjects

Brain samples of 54 HIV-1-positive individuals were examined: they included 18 full-blown AIDS patients and 36 HIV-1-seropositive asymptomatic patients (of these 23 formed part of a previously studied series of one of the authors, F. G.); 13 brains were obtained from the Edinburgh MRC AIDS Brain Bank, courtesy of Dr. Jeanne Bell. In addition 14 HIV-negative control brains were examined. Specimens were taken from the frontal lobe in 45 cases, whereas in 9 the temporal lobe was examined. The delay between death and post mortem was between 20 and 36 h. All the specimens used for in situ end labelling (ISEL) were formalin-fixed and paraffin-embedded. Fixation times for the AIDS and control cases ranged from 3 weeks to 5.5 months; those of HIV-1-positive pre-AIDS cases did not exceed 3 weeks. Surgical sections from three medulloblastomas (a tumor with a high percentage of apoptotic cells) were used as a quality control.

Detection of DNA fragmentation using DNA polymerase 1 and terminal deoxynucleotidyl transferase (TDT)

All 54 HIV-1-positive patients, 14 negative and 3 surgical control specimens were investigated by ISEL with DNA polymerase 1. To test the validity of the ISEL, with DNA polymerase 2, 2 HIV, 2 HIV-1-positive non-AIDS, 2 negative control and 1 tumor cases were also studied by ISEL with TDT.

Neuropathological changes were examined using routine techniques. In addition, p24 and glial fibrillary acidic protein (GFAP) antibodies and the lectin Ricinus communis agglutinin (RCA)-120 were applied to reveal HIV, glial cells and cells of the microglia/macrophage lineage, respectively.

ISEL of fragmented DNA using DNA polymerase 1 (nick translation)

Paraffin-embedded sections were deparaffinised, rehydrated and treated with 0.3% Triton X-100 in PBS for 15 min at room temperature (RT) for detergent permeabilisation, followed by proteinase K (Boehringer Mannheim, UK; 1 µg/ml) digestion in 100 mM TRIS-HCl pH 8.0, 50 mM EDTA at 37°C for 10 min; digestion was stopped by washing with PBS. Sections were incubated at 37°C with the end labelling buffer (50 mM TRIS-HCl pH 7.5, 5 mM MgCl₂, 10 mM 2-mercaptoethanol, 0.005% BSA) containing 0.03 mM dATP, dCTP and dGTP and 0.02 mM dTTP (Promega, UK), 0.01 mM digoxigenin-11-dUTP (Boehringer Mannheim) and 50 U/ml Klenow DNA polymerase 1 (Boehringer Mannheim). After blocking with buffer 1 (0.1 M maleic acid, 0.15 M NaCl pH 7.5) containing 2% normal goat serum (NGS) and 0.3% Triton X-100 for 30 min at RT, sections were incubated with anti-digoxigenin conjugated with alkaline phosphatase (AP; Boehringer Mannheim) diluted 1:500 in buffer 1 containing 1% NGS at 4°C overnight. After washing with buffer 1, staining was developed in NBT/BCIP in buffer 2 (100 mM TRIS-HCl pH 9.5, 50 mM MgCl₂, 100 mM NaCl). Sections were then counterstained with nuclear fast red.

TDT-mediated dUTP-fluorescein nick end labelling (TUNEL)

Nuclear DNA fragmentation was also detected by ISEL using TDT [11]. In situ cell death detection kit (Boehringer Mannheim) was used to visualise apoptotic neurons in 2 HIV-1-positive cases with AIDS, 2 HIV-1-positive pre-AIDS individuals who showed positive end labelling with DNA polymerase 1, and 2 positive and 2 negative controls. Cell permeabilisation and proteinase K digestion were carried out as described above. End labelling procedures were carried out according to the protocol recommended by the manufacturer. Briefly, sections were incubated with the TUNEL reaction mixture containing TDT and fluorescein-dUTP. During the incubation period, TDT catalyses the addition of fluorescein-dUTP to free 3'-OH groups in single-and double-stranded DNA. After washing, the label incorporated at the damaged site of the DNA is marked by an anti-fluorescein antibody conjugated with AP. Substrate reaction was as above.

Results

Neuropathological examination of the 18 AIDS cases revealed HIV encephalitis (HIVE and/or HIV lep) in 10 of them. In all these cases both grey and white matters showed variable amounts of astrocytic gliosis and increased numbers of activated microglia, in addition to macrophages and multinucleated giant cells (MGC). Myelin stain showed patchy or diffuse pallor and immunostaining with p24 revealed positive cells in 7 cases. In the 8 cases without encephalitis, pathological changes were limited to discrete glial hyperplasia and none of them was p24 positive. In the cortex of some AIDS patients (both with and without encephalitis), shrunken and eosinophilic neurons (features characteristic of anoxia) could be found among normal-looking cells.

In the brains of HIV-1-positive pre-AIDS patients, a discrete increase in astrocytic cells was seen in a number of cases, with only few cells containing obvious GFAP-positive material. On the other hand, a variable degree of microglia infiltration was seen in 31 cases and except for occasional macrophages, no MGCs were seen; immunostaining with p24 was negative.

No apoptotic cells or bodies were seen in any brain slide stained by routine methods. The ISEL method revealed only rare cells in the white matter of the negative controls and these could be identified as glial. Apoptotic cells were found in 11 specimens; these derived from 6 of the 10 HIVE, 1 of the 8 AIDS without neuropathological changes and 4 of the 36 HIV-positive pre-AIDS cases. All three medulloblastoma specimens showed numerous isolated apoptotic cells.

In situ cell death detection kit (Boehringer Mannheim) was used to visualise apoptotic neurons in 2 HIV-1-positive cases with AIDS, 2 HIV-1-positive pre-AIDS individuals who showed positive end labelling with DNA polymerase 1, and 2 positive and 2 negative controls. Cell permeabilisation and proteinase K digestion were carried out as described above. End labelling procedures were carried out according to the protocol recommended by the manufacturer. Briefly, sections were incubated with the TUNEL reaction mixture containing TDT and fluorescein-dUTP. During the incubation period, TDT catalyses the addition of fluorescein-dUTP to free 3'-OH groups in single-and double-stranded DNA. After washing, the label incorporated at the damaged site of the DNA is marked by an anti-fluorescein antibody conjugated with AP. Substrate reaction was as above.
Fig. 1 Photomicrographs showing apoptotic cells as revealed by the in situ end labelling method in the grey (A, C) and white matter (B, D) of HIV encephalitis (A, B) and HIV-1-positive pre-AIDS brains (C, D). A–D × 246

In the HIV-positive pre-AIDS group, all but one ISEL-positive cases were also HIV-1 DNA positive using PCR. Except for a positive nucleus, apoptotic cells did not differ morphologically from ISEL-negative cells; in particular the outlines of their cell body were identifiable and cytoplasm did not appear dissimilar to that of negative cells.

With regard to the distribution of apoptotic cells, in the HIVE cases they were found in both the grey matter, where the cells involved were almost exclusively nerve cells (Fig. 1 A), and in the white matter (Fig. 1B). In the cortex their density varied from area to area, did not seem to correlate with the areas of vascular vulnerability and appeared to be distributed throughout all the layers.

In the HIV-1-positive pre-AIDS cases, apoptotic cells were found in the white matter in all four cases (Fig. 1 D) whereas, in two, neurons were also stained with the method (Fig. 1C). In the cortex, ISEL-positive cells appeared scattered throughout all the layers, in places forming clusters of three to four cells.

Using double labelling with RCA-120, a number of cells in the white matter in HIVE cases showed double staining, whereas in the HIV-1-positive pre-AIDS brains only scattered apoptotic cells appeared RCA-120 positive, suggesting that in the latter group dying cells were predominantly glial in nature. Their consistent negative staining with GFAP and their morphology suggested an oligodendroglial nature. A number of endothelial cells were also seen to be stained by these methods. When the slides stained by the ISEL method were compared with consecutive H&E-stained sections, we could confirm that eosinophilic neurons with small nuclei and damaged chromatin, representing acutely anoxic neurons (a relatively common finding in terminally ill AIDS patients) were constantly ISEL negative. Moreover, numerous apoptotic bodies could be seen in tumor specimens which were ISEL positive, whereas areas of massive necrosis in the tumor (control) samples did not show the typical positive features.

In our study, detection of cells by ISEL using DNA polymerase 1 and TDT confirmed that both enzymes were suitable for this technique and that the latter did not appear more sensitive than the former.

Discussion

The results of the present investigation confirm that the ISEL technique can be successfully applied to paraffin sections [43] for the detection of DNA fragmentation [3, 9, 11]. Furthermore, they show that the two methods applied, DNA polymerase 1 and TDT, have the same degree of sensitivity and can be used interchangeably. However, formalin fixation, especially over long periods, is known to produce false-negative results (unpublished observation). As the specimens used in this investigation were
formalin-fixed, proper digestion by proteinase to permeate the section became an essential step for the success of this technique. Concentrations of proteinase K ranging from 1 to 100 μg/ml were used to obtain an optimal concentration for the enzyme. Digestion using 1 μg/ml proteinase K at 37°C for 10 min resulted in good sensitivity and decreased the intensity of the background. Increasing the concentration of proteinase K did not improve the sensitivity. Using this protocol, we were able initially to visualize apoptotic cells in surgical material (tumor) and subsequently in brain tissue samples fixed in buffered formalin for periods ranging from 2 weeks to 5.5 months.

Many other factors can affect the sensitivity of detecting apoptosis. The 1:3 ratio of digoxigenin (dig)-dUTP: dTTP recommended by Boehringer Mannheim [4] for random primer-directed synthesis and used by An et al. [2] in PCR amplification has also been confirmed to improve sensitivity when compared with the use of dig-dUTP alone. On the other hand, incubation with anti-dig antibody at 4°C overnight provided the best results when compared with incubation either at RT for 2–3 h or at 37°C for 30 min.

Apoptosis has been recognized as a possible mechanism by which immune cells [5, 14, 37] are eliminated in AIDS, and more recently it has been detected among CD4 and CD8 lymphocytes in HIV-positive individuals at the asymptomatic stage of the infection [13, 15, 16, 18, 25, 29]. A similar mechanism, whereby infected cytotoxic T lymphocytes are eliminated, has been reported recently in adult [1, 34] and young brains [10]. Our results demonstrate, for the first time, that apoptotic cells were detected in the white matter, while in two of these cases the brain also showed apoptotic cortical neurons. As far as the cortex is concerned, although no cell count was done, the density of ISEL-positive neurons was smaller in pre-AIDS than in AIDS (both with and in the single case without encephalitis) brains. The absence of such a study in the reports mentioned above [1, 10, 34] makes any comparison of the severity of cell loss between their and our cases of AIDS impossible, except for confirming that AIDS cases without encephalitis show lower numbers of positive cells [10]. Among the cells in the white matter taking part in apoptosis, a number of glial, and some microglial, cells were identified. Although a further characterization (double staining) of the glial cells was not attempted for technical reasons, some of them could be identified as oligodendrocytes on morphological grounds. The finding of neuroectodermal cells other than neurons taking part in apoptosis is in keeping with the observations of Petito and Roberts [34], who reported cell death in both neurons and astrocytes in AIDS. Furthermore, Louis et al. [23] demonstrated that mature oligodendrocytes can die by apoptosis in response to toxic signals. No apoptotic cells other than neurons were described by Gelbard et al. [10] and Adle-Biassette et al. [1].

Various pathogenetic theories have been put forward to explain nerve cell loss in AIDS patients. A mechanism triggered by the HIV envelope glycoprotein has been described for CD4 cells [21] and cortical cell cultures [31]. In addition, induction of apoptosis of lymphocytes has been correlated with some cellular toxins, such as prostaglandin E2, produced by HIV-infected macrophages [27], sulfated glycoprotein-2 [30] and lipid hydroperoxides [36]. Cytokines have also been considered in apoptosis [20, 25, 33]. Indeed, TNF can cause DNA fragmentation [32] and can activate latent HIV. On the other hand, apoptosis of T cells from HIV-1-infected patients is partly or entirely suppressed by IL-2 [12, 22].

In our study, three of the four cases showing apoptotic cells were found to be positive for HIV-1-DNA by PCR (in press), thus emphasising the links between the virus and cell damage. In view of the close relationship between the presence of HIV-1 and toxic factors [41] and between the latter and cell loss in AIDS brains (see discussion above), we suggest that the same mechanisms described in full-blown AIDS may operate in the HIV-1-positive pre-AIDS brains in which an elevated expression of MHC II antigens and various cytokines have been detected in our laboratory (in press).

The demonstration that a number, albeit small, of individuals at the pre-AIDS stage show apoptotic cells including cortical neurons gives further support to the opinion that brain damage is taking place during the early stages of HIV infection and suggests that therapeutic strategies in HIV-1-positive individuals might have to be applied before they enter the AIDS stage.

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References


Regional cerebral glucose metabolism and postmortem pathology in Alzheimer’s disease

Received: 22 May 1995 / Revised, accepted: 13 October 1995

Abstract In four patients with an antemortem diagnosis of probable Alzheimer’s disease (AD) regional cerebral glucose metabolism (rCMRGl) was studied prospectively by positron emission tomography (PET) and compared with postmortem semiquantitative neuropathology. The interval between the last PET study and autopsy was 1.3±0.8 years. In comparison with age-matched controls, the AD patients showed predominant temporoparietal hypometabolism spreading to other cortical and subcortical regions during serial PET scans. All patients had neuropathological findings typical for AD. There was a significant relationship between rCMRGl and density of senile plaques (SP) in one patient (\( r_s = -0.86, P < 0.05 \)). SP were distributed quite homogeneously in all regions examined. Neurofibrillary tangles (NFT) were concentrated focally in the hippocampus-amygdala-entorhinal complex. In the context of widespread developing cortical hypometabolism, the predilection of NFT for involvement in limbic areas suggests a disruption of projection neurons as the pathogenetic process of cortical dysfunction.

Key words Alzheimer's disease • Regional cerebral glucose metabolism • Semiquantitative neuropathology • Neurofibrillary tangles • Senile plaques

Introduction

Numerous studies of dementia of the Alzheimer type (DAT) with positron emission tomography (PET) using (18F)fluoro-2-deoxy-D-glucose (18-FDG) have demonstrated a characteristic metabolic pattern with hypometabolism most pronounced in temporoparietal and frontal association areas [3, 7, 13, 16, 17, 20, 27, 30] and relative preservation of primary sensorimotor and visual cortex, basal ganglia, thalamus, brain stem and cerebellum. Data obtained from cortical biopsy [10, 24] and autopsy [4, 11, 34, 40, 43] specimens suggest a relationship between cognitive impairment and certain neuropathological changes, i.e., neocortical plaques [4, 11, 24, 43], loss of large neocortical pyramidal cells [34], and loss of synapses [10, 40]. To date only a few studies comparing cross-sectional assessment of regional cerebral glucose metabolism (rCMRGl) and postmortem regional neuronal pathology [9, 12, 14, 25, 27, 28] have been published and these results are controversial. Some of them are based on preliminary or single-case data. Additionally, in the end-stage of Alzheimer’s disease (AD) whole brain CMRGl is often low, equalizing regional differences of rCMRGl. We, therefore, thought it important to study additional patients with proven AD and to investigate antemortem longitudinal changes of rCMRGl in relation to regional neuropathological measures. We examined four cases with a clinical diagnosis of probable AD according to NINCDS-ADRDA Criteria [29]. In all patients we compared rCMRGl to regional neuropathological changes at autopsy. In three patients antemortem brain glucose metabolism was also investigated longitudinally.

Materials and methods

Patients

During the last 3 years, four patients (two men with presenile onset and two women with senile onset of disease) from our probable AD sample died and underwent postmortem examination. Antemortem diagnosis of probable AD was based on commonly used diagnostic criteria [29]. The degree of mental deterioration was quantified based on the Mini-Mental-State Examination (MMSE) of Folstein et al. [15] and on the Global Deterioration Scale (GDS) of Reisberg et al. [36]. Patients’ brain glucose metabolism was investigated by PET using 18-FDG. One to six PET studies were
Investigation on the expression of major histocompatibility complex class II and cytokines and detection of HIV-1 DNA within brains of asymptomatic and symptomatic HIV-1-positive patients

Abstract Among the various mechanisms proposed to explain the pathogenesis of cerebral lesions in human immunodeficiency virus (HIV)-induced encephalitis, a cytokine-mediated action has found most favour. Indeed, elevated expression of cytokines such as interleukin (IL)-1 and tumour necrosis factor-α (TNF-α), thought to be neurotoxic, has been found in AIDS patients. As a previous study had demonstrated the presence of HIV proviral DNA in brain tissue of a number of HIV-positive non-AIDS patients, we undertook this present investigation using morphological, immunohistochemistry (IHC) and polymerase chain reaction (PCR) methods to detect the expression of major histocompatibility complex (MHC) class II molecules, the presence of HIV-1 proviral DNA and of the cytokines TNF-α, IL-1α, IL-4 and IL-6 in brains of the same group of individuals. The study included brains of 36 asymptomatic HIV-1-positive cases and the results were compared with those of AIDS patients either affected by HIV encephalitis (n = 8) or exempt from any neuropathological changes (n = 10) as well as of normal controls (n = 5). Results show that: HIV proviral DNA could be detected by PCR in 17 out of the 36 brains from HIV-positive pre-AIDS cases; most (15 of 17) of PCR-positive brains showed minimal to severe expression of MHC class II antigen; and cytokines could be detected predominantly within white matter even at this early stage. The data demonstrated that the state of immune activation described in AIDS is already present at the pre-AIDS stage and suggest that the presence of cytokines may already trigger the cascade of events leading to brain damage.

Key words Asymptomatic • Cytokines • Human immunodeficiency virus • Major histocompatibility complex class II • Polymerase chain reaction

Introduction

Several cerebral disorders have been described in patients suffering from AIDS. Among these, the subacute encephalitis, which represents the pathological counterpart of the AIDS dementia complex, is characterised by myelin pallor, astrogliosis and hyperplasia of microglial cells [52].

Up to now, microglial cells, as well as macrophages and multinucleated giant cells (MGCs), were the only cells within the central nervous system (CNS) in which human immunodeficiency virus (HIV) antigens could be visualised, as revealed by the use of specific antibodies [34, 47, 57]. More recently, however, Saito et al. [51] have also demonstrated infection in astrocytes. Elucidating the pathogenesis of HIV-1-induced lesions in the CNS must take into account that the virus cannot be found within nerve cells and is further complicated by the fact that relatively small amounts of the organism can be visualised both by immunohistochemistry (IHC) and in situ hybridisation methods. These findings, and the lack of an obvious cytopathic effect of HIV on neuroectodermal cells in vivo, have led to a number of pathogenetic mechanisms being proposed. One widely regarded hypothesis takes into consideration the state of immune activation described in AIDS is already present at the pre-AIDS stage and suggest that the presence of cytokines may already trigger the cascade of events leading to brain damage.
It has been recognised that, in most individuals, the initial HIV-1 infection results in the establishment of a latent or chronic infection, which, before and during eventual progression towards AIDS [37, 46, 48], is accompanied by a progressive and ultimately profound immunosuppression. Initially, however, as infection is associated with vigorous virus-specific immune responses, including both neutralising antibodies and cytotoxic T lymphocytes (CTLs) [64], patients remain clinically asymptomatic.

Regarding the time and mode of entry and spread of HIV in the CNS, the presence of the virus in the cerebrospinal fluid (CSF) at an early stage of infection has been reported [29, 49, 56]; furthermore, examination of brains of HIV-positive patients, who had died prior to developing AIDS, showed mild changes [24] interpreted as being nonspecific as there were no MGCs and no virus could be detected. Subsequently, however, HIV-1 proviral DNA was also demonstrated in the brain tissue, which also showed excess of microglia, of these individuals [58, 59]. This suggested that the status of immune activation previously reported in AIDS [60] may predate the AIDS stage [59].

The aim of this paper was to ascertain whether the presence of HIV provirus DNA and microglial hyperplasia in the brain during the pre-AIDS stages of the infection was accompanied by enhanced expression of MHC class II antigens and by presence of cytokines which may suggest that brain damage could take place even at this early stage of the infection.

### Materials and methods

#### Subjects

Brain samples of 59 individuals were examined. They included 18 full-blown AIDS patients and 5 normal controls chosen from the series of the Department of Neuropathology, Institute of Neurology, University of London. Of the 18 AIDS brains, 10 showed evidence of HIV encephalitis (HIVE), which is accompanied by expression of cytokines [60]. Of the 36 HIV-1-seropositive asymptomatic patients, 23 were part of the series of one of the authors (F. G.); 13 brains were obtained from the Edinburgh MRC AIDS Brain Bank, courtesy of Dr. Jeanne Bell. The cause of death of the AIDS patients was drug overdose (n = 30) and suicide (n = 5). One person was found dead at the site of a road traffic accident. The delay between death and postmortem was recorded as between 20 and 36 h for all the cases. Fixation time for the AIDS and control brains ranged from 3 weeks to 5.5 months; for the HIV-1-positive pre-AIDS brains it did not exceed 3 weeks. All the specimens used for morphology, histochemistry and polymerase chain reaction (PCR) were formalin-fixed and paraffin-embedded.

#### Immunohistochemistry

The details of antibodies applied in the study are listed in Table 1.

<table>
<thead>
<tr>
<th>Primary antibodies</th>
<th>Source</th>
<th>Dilution employed</th>
<th>Supplier</th>
<th>Effect of cytokine on HIV replication</th>
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<tr>
<td>Anti-p24</td>
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<td>1:400</td>
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<td>Anti-GFAP</td>
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<td>Citron Technology, USA</td>
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<tr>
<td>Anti-IL-4</td>
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<td>Anti-IL-6</td>
<td>Rabbit</td>
<td>1:20</td>
<td>Genzyme, UK</td>
<td>Inductive</td>
</tr>
</tbody>
</table>

Regarding the time and mode of entry and spread of HIV in the CNS, the presence of the virus in the cerebrospinal fluid (CSF) at an early stage of infection has been reported [29, 49, 56]; furthermore, examination of brains of HIV-positive patients, who had died prior to developing AIDS, showed mild changes [24] interpreted as being nonspecific as there were no MGCs and no virus could be detected. Subsequently, however, HIV-1 proviral DNA was also demonstrated in the brain tissue, which also showed excess of microglia, of these individuals [58, 59]. This suggested that the status of immune activation previously reported in AIDS [60] may predate the AIDS stage [59].

The aim of this paper was to ascertain whether the presence of HIV provirus DNA and microglial hyperplasia in the brain during the pre-AIDS stages of the infection was accompanied by enhanced expression of MHC class II antigens and by presence of cytokines which may suggest that brain damage could take place even at this early stage of the infection.

The details of antibodies applied in the study are listed in Table 1. To identify HIV antigen, polyclonal anti-p24, which recognises the gag-associated protein p24 was used. Monoclonal anti-glia fibrillary acidic protein (GFAP) and the biotinylated lectin Ricinus communis agglutinin (RCA)-120 (Vector Labs., UK) were used as astrocyte and macrophage/microglia markers, respectively. The density of GFAP- and RCA-120-positive cells was assessed as previously described [11]. Briefly, the density in both grey and white matter of GFAP- and RCA-120-positive cells with a recognisable nucleus was assessed by recording all positive cells (except endothelial cells which are RCA-120 positive) in seven high-power consecutive fields (x 400) and the mean value/mm² of each case calculated. In Table 2 these values were also expressed semiquantitatively using labels (−) to (+++).

A monoclonal anti-human MHC class II (HLA-DR) antibody (BioGenex, UK) was used to identify MHC class II expression and polyclonal anti-human interleukin (IL)-1α, IL-4 and IL-6 as well as tumour necrosis factor-α (TNF-α) (Genzyme, UK) were used for detection of cytokines.

HIC was performed as described previously [5, 59]. Procedures for p24 and cytokines detection were applied with a slight modification from the published protocol. Briefly, the sections were deparaffinised by washing three times with xylene, treated with 1% H₂O₂ in methanol (to block endogenous peroxidase), washed in tap water and phosphate-buffered saline (PBS). Sections were heated for 5 min twice in a microwave oven at high power in 0.1 M sodium citrate buffer pH 6.0. After blocking of non-specific immunoglobulin binding with 5% normal swine serum for 10 min at room temperature (RT) in TRIS-buffered saline (TBS), the sections were incubated with primary antibody at 4°C overnight. After further rinsing in TBS and incubation with biotinylated secondary antibodies for 30 min at RT and a 30-min reaction with avidin-peroxidase complex at RT, the reaction was finally developed with 3,3'-diaminobenzidine (DAB) and sections were counterstained with Meyer's haematoxylin. A semiquantitative system for evaluation of expression of MHC class II antigens and cytokines was devised which took into account the density, size and number of processes of positive cells and scored the result as a single figure applying a scale ranging from 0 to 6. Accordingly, absent or minimal expression either of MHC class II or cytokines were labelled 0 to 1, moderate 2 to 3 and high 4 to 6. The specificity of the immunostaining for cytokines was verified using a phosphomystate acetate (PMA)-activated human cell (U937; monocye) line and lymph nodes obtained from patients with AIDS, as it is known that both the macrophages derived from stimulated monocytes [27] and HIV-infected lymph nodes [4] express TNF-α, IL-1α, IL-4 and IL-6.
Table 2 Neuropathological changes in 36 HIV-1 positive pre-AIDS individuals (W white matter, G grey matter, L lymphocyte, M macrophage)

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<thead>
<tr>
<th>Case no.</th>
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<th>Gliosis</th>
<th>Hypoxic changes</th>
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<td>+/++ (W), ± (G)</td>
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<td>+ (W), ± (G)</td>
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<td>36</td>
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<td>+/++ (W), ± (G)</td>
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</table>

Polymerase chain reaction analysis

Nested PCR was used to detect HIV sequences in paraffin-embedded brain tissues of all 59 cases studied. As leptomeninges in pre-AIDS cases may include discrete amounts of lymphocytes, some of which may contain HIV, the area of the paraffin section including meninges was removed from the specimen to be used for PCR with a scalpel whose blade was replaced for each case examined. For each test one paraffin section per case was used. The technique for DNA preparation from formalin-fixed, paraffin-embedded tissue has been described in a previous report [5]. The primers used in this study were designed to amplify a sequence of HIV pol gene. Of the reaction mixture, 50 μl contained 0.3 μM of each primer, 0.2 mM of dATP, dCTP, dGTP and dTTP, and 2.5 U Biotaq DNA polymerase in PCR buffer [16 mM (NH4)2SO4, 67 mM TRIS-HCl (pH 8.8), 3 mM MgCl2, 0.01% Tween-20]. Thirty-two cycles of the first round of PCR were performed with 1 μg extracted DNA, each cycle consisting of thermal denaturation at 94°C for 1 min, annealing and extension at 63°C for 2 min. A 144-bp fragment was amplified at the first round of PCR by pol 1 (3181–3203, 5'-CAG GAA AAT ATG CAA GAA TGA GA) and pol 2 (3324–3302, 5'-CCC ATG TTT CCT TTT GTA TGG GT). The second round of PCR was amplified by pol 3 (3228–3247, 5'-CAA TTA ACA GAG GCA GTG CA) with 5' digoxigenin (Dig)-11-dUTP end-labelled and pol 2. In this round of PCR 28–30 cycles were performed with 2.5 μl of PCR product obtained from first round amplification in the 50 μl of reaction. Thus, a 97-bp Dig-labelled PCR product was obtained after the second-round amplification. The PCR product was analysed by electrophoresis and Southern transfer followed by chemiluminescence (CSPD, Boehringer Mannheim, UK) detection. Human β-globin gene was amplified in all HIV-1 PCR-negative and some -positive individuals to confirm that the quality and quantity of extracted human DNA were suitable for the amplification. Amplified β-globin products were visualised by ethidium bromide staining of agarose gel electrophoresis.

Results

Neuropathological changes

Among the 18 AIDS patients, 10 showed changes characteristic of HIV encephalitis/leukoencephalopathy (both labelled HIVE in this report), while the remaining 8 showed
Table 3 Results of PCR, IHC and Lectin in HIV-1 positive pre-AIDS patients compared with other groups. Results represent mean values for each group (PCR polymerase chain reaction, IHC immunohistochemistry, HIVE HIV encephalitis, RCA ricinus communis agglutinin, MAC macrophages, MIC microglial cells)

<table>
<thead>
<tr>
<th>Group</th>
<th>PCR</th>
<th>MHC II</th>
<th>RCA-120/mm²</th>
<th>TNF-α</th>
<th>IL-1α</th>
<th>IL-4</th>
<th>IL-6</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Mac</td>
<td>Mic</td>
<td></td>
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<tr>
<td>Normal control</td>
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<td>37.2</td>
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<td>172</td>
<td>1.8</td>
<td>2.6</td>
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</table>

Case no. (pre-AIDS) | PCR | MHC II | RCA-120/mm² | TNF-α | IL-1α | IL-4 | IL-6 |
<table>
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<tr>
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no obvious neuropathological lesions. The main HIV-related pathological abnormalities were predominantly in the hemispheric white matter and included myelin pallor, diffuse astrogliosis, microglial nodules and MGCs. Abnormalities in the HIV-positive pre-AIDS group in this study included discrete meningitis in 11 cases, presence of microglia (31 cases) and an occasional macrophage (1 case) and astrogliosis in 34 cases; 1 case showed a completely normal brain. The neuropathology of the 36 pre-AIDS individuals is summarized in Table 2.

IHC and lectin staining

IHC was positive in 7 of 10 cases showing HIVE. No staining was seen in the other 8 AIDS patients or in any of the pre-AIDS or control brains.
Fig. 1 Photomicrographs showing the expression of major histocompatibility complex class II antigen in the brains of HIV-1 positive pre-AIDS (A) and AIDS (B) individuals by immunohistochemistry. In A the majority of positive cells are microglia, while in B also macrophages and multinucleated giant cells (inset) were positive. X 360

Lectin

RCA 120-positive cells were increased in size and showed many branched processes in all AIDS brains with HIVE as well as in 18 of the 36 pre-AIDS brains. The two groups differed, however, by the presence, in the former, of numerous macrophages and some MGCs. On the other hand, in AIDS brains without neuropathological changes, there was a slight increase in number of microglial cells (whose size was only moderately increased) in addition to a few scattered macrophages. The results of IHC and the densities of both microglia and macrophages in the various groups are shown in Table 3.

Cytokines and MHC class II antigen

The mean scores of MHC class II, TNF-α, IL-1α, IL-4 and IL-6 for each group and the individual scores of 36 pre-AIDS subjects are shown in Table 3. They refer to observations made in the white matter. The expression of MHC class II and cytokines, as represented by the mean scores, was high in HIV-1-positive patients (taken as a single group) compared with HIV-1-negative individuals; interestingly, pre-AIDS patients showed higher scores for TNF-α and IL-4 than AIDS brains with HIVE (whereas counts of RCA-120-positive cells were elevated in all three HIV-1-positive groups). Regarding the cell types that immunostained with the various antibodies, TNF-α appeared to be expressed in microglia/macrophages, pericytes and a number of astrocytes; IL-1 in microglia/macrophages, astrocytes and some endothelial cells; IL-4 in microglial and astrocytes, and IL-6 in microglia, astrocytes and possibly some endothelial cells. Examples of cells immunostained for MHC class II antigen in pre-AIDS and AIDS brains are shown in Fig. 1; examples of the various cell types immunostained with the range of the different cyto-
Fig. 2 A–H Photomicrographs of brain sections immunostained with antibodies against cytokines. A, C, E, G represent pre-AIDS, B, D, F, H AIDS cases. A, B illustrate TNF-α; C, D IL-1α; E, F IL-4; as showed in Table 3, the expression of IL-4 is higher in pre-AIDS (E) than in AIDS (F) cases; G, H IL-6. Cells represented in the photographs include microglia (B, C), glial cells (A, D, G) and an endothelial cell (F). (TNF tumor necrosis factor, IL interleukin) × 600.

The grey matter was examined separately using the same scoring system described above. Data for MHC class II molecules were comparable to those found in the white matter in both in AIDS and pre-AIDS brains. For
the cytokines, the scores obtained for the AIDS and pre-AIDS groups were considerably lower than those found in the white matter and for the pre-AIDS could be regarded as minimal.

Polymerase chain reaction

All the control cases were negative. HIV-1 DNA was detected in all \((n = 10)\) HIVE cases, in 5 out of 8 AIDS patients without CNS disease and in 17 out of 36 HIV-positive pre-AIDS patients. Results of PCR of pre-AIDS individuals are shown in detail in Table 3. Figure 3 illustrates the results of PCR by both electrophoresis and chemiluminescence. Lanes A–H represent 8 randomly chosen pre-AIDS cases, while lanes I–L are the results of similarly chosen AIDS cases.

Discussion

PCR findings in this study revealed the presence of HIV-1 DNA in the brains of a large proportion of HIV-1-positive patients at the asymptomatic stage. This is keeping with previous results [59] and confirms that entry of HIV-1 into CNS takes place at an early stage of the infection. The improved results obtained using chemiluminescence emphasise the superiority of this method over the traditional technique. These results are at variance with those published by Donaldson et al. [16] who could find no evidence of HIV-1 positive proviral DNA in pre-AIDS brains. The possibility that positive PCR findings could result from HIV harboured in lymphocytes contained in the intraparenchymal or meningeal blood vessels must be considered. As mentioned in Materials and methods, the meninges were removed from the paraffin-embedded section used for PCR; as for the virus being present in cells trapped in intraparenchymal vessels, data from the literature show that only between 1 in 1,000 and 1 in 500,000 peripheral blood mononuclear cells [36] and between 1 in 2,500 and 1 in 26,000 CD4 cells [8] carry the virus in seropositive pre-AIDS individuals. As only one paraffin section per case was used for PCR studies, this amount of tissue could not conceivably contain a number of cells high enough to give a positive result, taking into account the threshold of sensitivity of our PCR technique.

Neuropathological examination in patients with HIVE revealed changes (infiltration by macrophages, increase numbers of microglial cells, gliosis, myelin pallor and MGCs) identical to those previously reported [9], while MGCs were absent in HIV-1-positive pre-AIDS patients; on the other hand meningitis, usually not a feature of HIVE, was present in 11 cases of the latter group. Most HIV-1-positive pre-AIDS patients in this study were drug addicts and all had died from causes unrelated to the HIV infection. We can assume that these individuals were at an early latent stage of disease, a phase at which HIV, harboured within the CNS, could be the non-syncytium-inducing (SI) variant [53], thus possibly explaining the absence of MGCs.

Our results using the lectin RCA-120 revealed large numbers of macrophages and microglia in AIDS brains but also confirmed previous findings [59] of high values of microglia in all but 2 pre-AIDS patients. Increased numbers of cells of microglia/macrophage lineage are a prominent feature of HIV-associated disorders of the CNS [21]; it is known that these cells are susceptible of infection by HIV and may serve as its reservoir [30]. Latent infection of monocytes/macrophages is an important mechanism by which HIV escapes immune surveillance and enters the CNS [2].

Increased density of microglia was associated, in the pre-AIDS brains in our study, with enhanced expression of MHC class II molecules; the mean score (2.2) was lower than in cases associated with HIVE (4.0), but was more than twice that found in AIDS brains without associated pathology (1.0). These results regarding microglial cells in pre-AIDS and AIDS with and without encephalitis are in keeping with those by Sinclair et al. [59]. Moreover, in the pre-AIDS group a more elevated expression was found in most (15/17) HIV-1 DNA-positive than in -negative brains. Among the pre-AIDS individuals the mean score of expression of MHC class II antigens of the HIV-1 DNA-positive cases was 2.65, a value considerably higher, although statistically not significantly so, than PCR-negative cases (1.8).

Other investigators have previously confirmed increased expression of MHC II in AIDS [2, 33, 60]. By showing increased expression during the pre-AIDS stages, our data lend support to our previous hypothesis [59] that a status of immune activation, demonstrated in AIDS by Tyror et al. [60], already exists during the asymptomatic stages preceding AIDS. At this stage, viral-specific immune responses (shown by the presence of MHC class II molecules), including both neutralising antibodies and cy-
toxic T cells, help inhibit virus replication [64]. However, after a latency period, in which the balance seems to be in favour of the host, new viral variants, due to the high rate of HIV mutagenesis, arise; they in turn stimulate the immune system, induce new cycles of viral replication and new virulent mutants which eventually lead to the final collapse of the immune system [10].

It has been suggested that the pathogenesis of the changes within the CNS in AIDS, including nerve cell loss, could be produced directly by the virus [17, 32, 50, 66] or induced indirectly via products secreted by HIV-infected macrophages/microglia [23, 25, 28] or other glial cells [61, 62]. However, as neurons are not directly infected by HIV-1, the latter mechanism seems the more likely and it has been hypothesised that the damage can be induced via production of cytokines. These proteins are by no means specific for HIV infection and can be found in normal brain [7, 13] as well as in a variety of abnormal conditions [31]. In HIV infection, both in vivo and in vitro data point to their role at three important stages of the disorder [63]: entry of the virus into the brain tissue, up- and down-regulation of HIV expression in the brain and induction of astroglialosis and myelin pallor.

In a recent investigation of brain tissue in AIDS, Tyor et al. [60] reported the presence of various cytokines in the white matter. Within the CNS, activated astrocytes and microglia are the main source of cytokines, although IL-1 and transforming growth factor-β (TGF-β) can also be secreted by oligodendrocytes. Their functions have been extensively described and the literature on the subject has recently been reviewed by Vitkovic et al. [63] and Benveniste [6].

Regarding the time of the infection at which cytokines become detectable in the brain, as shown by their presence in the CSF, very little is known, as most investigations have only compared levels in AIDS patients with and without dementia. Moreover, data reflecting levels in the CSF may not correlate with the actual levels of cytokines within the parenchyma. Indeed, although CSF and brain tissue represent two interconnected compartments, they are not in equilibrium regarding solutes [40]. Results at the AIDS stage are conflicting, as Perrella et al. [41] did, whereas Weller et al. [65] did not find evidence of TNF-α. On the other hand, Vitkovic et al. [63] detected slightly elevated levels of TNF-α in the CSF in HIV infection, compared with HIV-negative individuals, but no difference between demented and non-demented subgroups, confirming previous data by Tyor et al. [60]. The latter results are at variance with those by Perrella et al. [41] and Grimaldi et al. [26], who reported higher TNF-α levels in demented than in non-demented AIDS patients.

The present study has revealed, for the first time, that expression of IL-1α, IL-4, IL-6 and TNF-α in formalin-fixed, paraffin-embedded brain tissue is elevated in HIV-positive pre-AIDS compared with HIV-1-negative individuals and HIV-1-positive brains with no neuropathology. Positive cells included microglia and astrocytes (for all the cytokines), pericytes (TNF-α) as well as endothelial cells (IL-1α, IL-6). Although both grey and white matter were involved, most positive cells were found in the latter. The pattern and level of expression showed that TNF-α and IL-4 were surprisingly higher than in the two AIDS subgroups (in which their presence within pericytes compensated for the lower numbers of microglia). On the other hand, the highest levels of IL-1α were found in patients with HIVE, whereas pre-AIDS individuals showed only moderate levels, and little was present in AIDS brains without neuropathology. Levels of IL-6 were similar in the HIVE and pre-AIDS groups, compared with that without neuropathology in which they were low.

In the light of the results described above, some of the neuropathological changes previously reported in pre-AIDS individuals [24] may now find an easier explanation. Among these, myelin pallor can be correlated with the presence of TNF-α, which is known to have a direct effect on oligodendrocytes and which, in vitro [54], mediates myelin and oligodendrocyte damage. Moreover, TNF-α is known to act through a mechanism of apoptosis [55].

A more worrying possibility is that cytokines could, at this early stage, trigger the cascade of events by which IL-1 can stimulate the production of itself as well as of other cytokines such as TGF-β1 [12], IL-6 and TNF-α [15]. Production of IL-1, TGF-β1 and TNF-α by various cell types within the CNS activates, in turn, HIV replication [44, 62], thus creating a vicious circle that perpetuates the damage. Furthermore, cytokines are low molecular weight proteins that can act both locally or at sites remote from their site of origin [22] and may have long-lasting effects [14]. As cytokines have been implicated in the process of neuronal apoptosis in AIDS [3, 20, 42], it was not surprising to find nerve cell loss already in the pre-AIDS stage (An et al., in press). Admittedly, the amounts of these proteins found in the cortex in our cases were not large. However, by showing the presence of HIV provirus DNA in a large proportion of pre-AIDS brains, together with the identification of a condition of immune reaction, the existence of detectable amounts of cytokines and evidence of apoptosis, our results emphasise the potential risk that brain tissue, and nerve cells in particular, are exposed to and indicate the need for therapeutic interventions from the very early stages of the infection.

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Clinical and histopathological analysis of proliferative potentials of recurrent and non-recurrent meningiomas

Abstract Proliferative potentials of meningiomas from 127 patients were examined immunohistochemically using the anti-Ki-67 monoclonal antibody, MIB-1, on paraffin sections, and the correlation among MIB-1 staining index (SI), histopathological finding, and clinical course of the disease was analyzed retrospectively. The mean MIB-1 SI of 50 male patients with meningioma was 5.5%, whereas that of 77 female patients was 2.7%. Higher MIB-1 SI were observed for younger patients. These age- and sex-related differences in MIB-1 SI were statistically significant. The patients were assigned to one of three groups: those with non-recurrent meningioma (n = 73); those with recurrent meningioma in whom the specimens obtained during the initial surgery were used to calculate the MIB-1 SI (n = 21); and those with recurrent meningioma for whom the specimens obtained during the surgery for recurrent tumors were used to calculate the MIB-1 SI (n = 33). The mean MIB-1 SI in these patients were 1.6%, 3.6%, and 8.8%, respectively, and there were statistically significant differences among these three groups. Statistical analyses reveal that meningiomas with a MIB-1 SI of 3% or more have a significantly high tendency for recurrence during the clinical courses, especially within the first 10-year follow-up periods. Moreover, there is statistically significant correlation between MIB-1 SI and recurrence in each Simpson's grade. The time interval to the next recurrence for recurrent meningiomas is associated with the proliferative potential represented by the MIB-1 SI, and a correlation equation has been proposed to predict the date of the next recurrence. Analyses on cellularity of meningiomas revealed no statistically significant difference in cellularity between non-recurrent and recurrent meningiomas. There was no statistically significant relationship between cellularity and MIB-1 SI of meningiomas. In conclusion, examination on proliferative potentials of meningiomas using MIB-1 SI is very important for biological and histopathologic analyses and the prediction of future recurrence.

Key words Meningioma • MIB-1 • Ki-67 • Proliferative potential • Recurrence

Introduction Meningiomas are common, usually benign tumors of the meninges arising from the arachnoid cells. They are considered to be a heterogeneous group of tumors which is categorized into 14 subtypes with three grades of malignancy [15]. Clinical and biological features of recurrent and non-recurrent meningiomas have been studied by several investigators [1, 4, 6, 7, 9, 11, 12, 16–18, 25]. Recent studies have shown that the recurrence of meningiomas appears to be also modulated by their proliferative potentials [2, 5, 10, 20], which have been studied using bromodeoxyuridine (BrdU) labeling index (LI) [10], proliferative cell nuclear antigen (PCNA) [13], argyrophilic nucleolar organizer region (AgNOR) count [2, 5, 20], and Ki-67 staining index (SI) [8, 21]. The BrdU LI study, which detects S phase cells alone [10, 22], is inappropriate for retrospective studies, since it requires intravenous injection of BrdU prior to surgery or incubation of cultured tumor cells with BrdU. Immunohistochemical staining of the Ki-67 antigen is capable of detecting all proliferative cells [8], and has been applied for evaluating proliferative potentials of various tumors using the frozen sections; however, this is also inappropriate for retrospective studies. Recently, an anti-Ki-67 monoclonal antibody, MIB-1, has become available for the analysis of proliferative potentials of meningiomas.
Patients with normal anti-GM1 antibody titres also respond to immune modulating therapy. There are three possible reasons for the normal anti-GM1 antibody titres observed in our patients: firstly, the polyneuropathies of unknown origin investigated might not be immune mediated; secondly, these polyneuropathies might be immune mediated but induced by antibodies directed against epitopes other than those of gangliosides; and thirdly, the assay applied was of low sensitivity and specificity and may not have recognised disease specific anti-GM1 antibodies. Other authors, however, have detected raised anti-GM1 antibody titres using the EL-GM1 assay and, hopefully, the specificity of anti-GM1 antibody testing will be increased by analysing GM1 target epitopes other than Gal(β1-3)GalNAc. In conclusion, our results suggest that quantification of anti-GM1 antibody titres is of little help in the diagnosis of polyneuropathies of unknown origin.


Quantification of HIV DNA in the brain by PCR: differences between fresh frozen and formalin fixed tissue

F Davison, S F An, F Scaravilli

Abstract
HIV-1 DNA extracted from frozen and formalin fixed brain tissue can be detected using PCR. This work has been extended by amplifying, using semiquantitative PCR, HIV DNA extracted from frontal lobe tissue of 16 patients with AIDS (eight positive and eight negative for p24 antigen). DNA was amplified using HIV-1 pol gene digoxigenin labelled primers and detected by chemiluminescence and densitometry. Cloned standards were amplified in parallel for quantification. HIV DNA levels detected in frozen tissue showed a correlation with p24 positivity and the severity of the histological diagnosis. This correlation was less clear in the formalin fixed material.

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Keywords: HIV, PCR, frozen tissue, formalin fixed tissue.

About 30% of adult patients and 50% of paediatric patients with AIDS are likely to develop symptoms encompassed by the term HIV associated dementia complex (HIV-ADC) and it is probable that these percentages would be greater if patients did not succumb to earlier complications. The HIV-ADC includes a wide range of neurological problems in which the pathogenetic processes involved are not fully understood. There are two likely pathogenetic mechanisms, both of which may be contributing to disease. Firstly, the direct cytopathic effects of viral infection may be responsible for the cell destruction and loss of neurons which are normally observed in HIV-ADC. This type of pathogenesis may be important during the terminal stages of AIDS. However, neurons in vivo show no evidence of HIV infection while microglia and macrophages are typically infected and the formation of multinucleated giant cells (MGC) is a diagnostic feature of HIV encephalitis (HIVE) and HIV leucoencephalopathy (HIV lep). Secondly, damage is the indirect consequence of HIV infection and is probably macrophage mediated. Neuropathological changes are the result of secretion of neurotoxic factors including arachidonic acid, cytokines and toxic oxygen metabolites. Several reports have im-

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complicated viral proteins in the pathogenetic process—for example, the viral tat protein and gp 120, the latter being expressed in transgenic mice which showed some HIV-ADC-like symptoms. Recently, it was suggested that neuronal loss was due to apoptosis occurring after the local release of neurotoxic factors during HIV infection. All of these possibilities may well be involved in the pathogenesis. An important consideration in attempting to understand the pathogenesis of HIV-ADC in the light of these possibilities is whether or not there is a link between viral load and the extent of neuropathology. This has been noted in previous reports where there was correlation between increasing pathology and the amount of virus present in the tissue. It was these observations which were of interest for the present study, particularly the link between viral load and the more severe diseases, such as HIVE and HIV lep. We have chosen to estimate the amount of HIV in the brain in patients with AIDS by measuring levels of HIV DNA. This DNA represents a total of integrated provirus and extrachromosomal viral DNA, which is often present in relatively high amounts in the brain. Viral particles (containing RNA only) are normally found in the brain tissues at low levels so the HIV DNA represents the consequence of latent rather than active infection.

We also wished to assess the effects of formalin fixation on the tissue being examined by PCR quantification. Postmortem brain material is typically fixed in 4% formaldehyde solution (formalin) for long periods of time (that is, weeks or months). This procedure has the advantage of rendering the tissue non-infectious, preventing deterioration and preserving the morphology. However, formalin crosslinks proteins and nucleic acids, which can make their analysis more difficult. Determination of the molecular weight for DNA extracted from frozen tissue shows that it is normally intact, whereas the molecular weight of DNA from formalin fixed material is typically less than 500 base pairs (bp) (our unpublished observations). This may cause problems for some DNA studies. However, in a previous study comparing amplification in frozen and formalin fixed brain material, we showed that the fixation process only slightly compromised the ability to detect HIV DNA by PCR.

**Methods**

Frozen and formalin fixed frontal lobe tissue from postmortem brain material of 16 patients with AIDS was studied. The samples selected for quantification had previously been shown to be HIV DNA positive by PCR and the quantification studies were done without prior knowledge of the histological details of each sample. Negative controls, consisting of PCR reagents only, were included with all batches of samples being tested to ensure no contamination was present. Histological examination of the brain tissue from each patient was done on a routine basis to determine the type of HIV encephalopathy.

**IMMUNOHISTOCHEMISTRY**

p24 HIV antigen screening was done routinely on all sections as described previously.

**EXTRACTION OF DNA**

Formalin fixed tissue samples were dewaxed with three 15 minute xylene washes followed by five minutes in ethanol. Small blocks of brain tissue from each source were finely diced before being placed in DNA extraction buffer. DNA was prepared by two methods. (1) Standard DNA extraction procedures using proteinase K digestion (0.25 mg/ml 56°C, overnight) followed by chloroform/phenol purification and ethanol precipitation. For the formalin fixed tissue it was necessary to increase the proteinase K concentration to 5 mg/ml to extract sufficient DNA. (2) A technique using guanidium isothiocyanate to dissolve tissue was found to produce slightly better quality DNA, especially from formalin fixed brain so this latter technique was eventually used on a routine basis. The purified DNA was finally quantified by spectrophotometry.

**PCR AND DNA QUANTIFICATION**

A semiquantitative PCR technique was used to estimate the number of HIV DNA copies in each sample. The primers used amplified a 143 bp fragment from the HIV pol gene; the upstream primer was 5' labelled with digoxigenin. The amplified DNA incorporated the digoxigenin, which enhanced detection and enabled the amount of reaction product to be quantified. The amplification reaction included identical amounts of brain DNA (normally 1 pg) and reagents as specified by the manufacturer (Bioline, UK) under the following conditions: denaturation, five minutes at 94°C; annealing/extension, two minutes at 62°C; for 30–32 cycles followed by five minutes extension at 72°C. This protocol maintained the reaction in the exponential phase which is essential for quantification, as during this phase there is a direct correlation between the quantity of target DNA and the amount of amplified product.
containing digoxigenin. Serial dilutions of cloned HIV DNA were run in parallel with brain DNA samples to permit quantification within a range of 20–2000 copies. After amplification, aliquots of reaction sample were separated by electrophoresis in 2% agarose and transferred to nylon membranes (Boehringer). The membrane bound digoxigenin was then reacted with a chemiluminescent substrate (Boehringer), detected by autoradiography and quantified by densitometry (Molecular Dynamics). The amplified standards were used to plot the HIV copy number against densitometry values to produce a standard curve. From this curve, the number of HIV copies in each brain sample was extrapolated. The number of HIV copies was then expressed relative to the amount of total brain DNA in each reaction.

Results
The patients’ diagnoses and the results are given in table 1. Patients 1–9 were ranked together because their diagnoses reflected a uniform type of pathology. These were HIVE and HIV del which are associated with the presence of MGC and p24 antigen positivity. Immunostaining of the tissues for p24 antigen was usually observed with HIVE, but two non-HIVE cases (patients 14 and 15) were also positive. There was a perifocal pattern of staining in these two patients. This was due to local boosting of HIV levels around the region of pathology which is often seen when there is coinfection with another virus, such as DNA viruses, and did not represent typical distributions of p24 antigen seen in HIVE. It was possible to detect HIV DNA in all patient samples from both frozen and formalin fixed tissues. However, in many cases the levels of HIV DNA were barely detectable and therefore were not quantified and were designated as <10 copies. Increasing the number of PCR cycles confirmed that these cases were positive (data not shown). The 1 μg of total DNA used in each case represents an analysis of approximately 150,000 cells. Plots of cloned standards of HIV DNA against densitometer values produced straight line graphs in the range of 10–2000 copies from which test samples values were extrapolated (data not shown).

Discussion
The results from frozen tissue suggested that there was a slight, direct correlation between viral load and a more severe pathology—that is, HIVE and HIV del. In particular, patients with considerably higher HIV DNA levels—for example, patients 1 and 2, would be expected to have HIVE. However, a converse conclusion that patients with HIVE will always have a greater HIV DNA load was not indicated by our data. The precise significance of brain HIV DNA load in relation to pathogenesis is not clear, although it may represent a latent infection with intermittent expression of viral antigens at low levels with consequent immune mediated cytopathology. It is not known whether or not these antigens and HIV nucleic acid are being assembled into intact virions which may reinfest local cells. The cellular localisation of HIV is restricted mainly to macrophages and MGC, both of which are thought to be reservoirs of infection in the brain. In situ DNA studies will help to define more precisely this localisation and determine whether or not other cell types are involved.

The quantification of HIV DNA in formalin fixed material resulted in lower values than in frozen material in most cases, with limited agreement between the two, especially in patients 1 and 2 where HIV DNA levels were high. In those samples where there was more HIV DNA in the formalin fixed tissue this was possibly due to local fluctuations within the brain tissue under analysis. The ability to detect and quantify DNA from formalin fixed tissue will be largely determined by the size of the fragment to be amplified. In this study the amplified pol gene fragment was 143 bp, which was significantly smaller than the average size of DNA fragments extracted from formalin fixed tissue of approximately 500 bp. However, as the reduction in molecular weight of extracted chromosomal DNA is likely to be a random process it is inevitable that some of the target sequences will no longer be present. The discrepancy between the results of frozen and formalin tissue may also be due to differences in the fixation times of the samples and to vagaries in the fixation process. Although quantification of HIV DNA in formalin fixed tissue using PCR was feasible, the results must be interpreted with caution and are likely to be lower than those from fresh material.

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Myelofibrosis presenting as chronic cholecystitis

P M Geddy, K R Wedgwood

Abstract
A 61 year old man presented with abdominal pain typical of chronic cholecystitis of one month’s duration. Pallor was noted on examination and investigation uncovered myelofibrosis and a small gallstone. Cholecystectomy relieved the pain and pathological examination of the gall bladder showed widespread myeloid metaplasia. This is the first reported case of myelofibrosis presenting as chronic cholecystitis.

(Pathology)
An opened gall bladder, measuring 7 × 2.5 × 1.5 cm, was submitted for pathological examination and fixed in 10% buffered formalin. A few adhesions were present and the mucosal surface was unremarkable. The gall bladder wall was diffusely thickened and grey, measuring 0.4 to 0.6 cm in depth. Histological examination of the neck, body and fundus revealed extensive myeloid metaplasia. This completely replaced the normal lamina propria and adventitia, and extended between smooth muscle bundles in the muscularis. Epithelium and smooth muscle were spared. The infiltrate was composed mainly of immature myeloid and erythroid cells, with dysplastic megakaryocytes, often forming small clusters (fig 1). The fibrous stroma was rich in reticulin fibres. The appearances were similar to those in the bone marrow trephine biopsy specimen. Immunostaining for CD68 (Dako, High Wycombe, UK; 1:50) and factor VIII (Dako; 1:50) confirmed the presence of immature myeloid cells and megakaryocytes, respectively. Immunostaining for CAM 5.2 (Becton Dickenson, San Jose, California, USA; 1:1), vimentin (Dako; 1:100),

Figure 1 Megakaryocytes in the adventitia immunostained by factor VIII. (Immunoperoxidase, × 40.)
Neuropathology of Early HIV-1 Infection

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Early HIV-1 invasion of the central nervous system has been demonstrated by many cerebrospinal fluid studies; however, most HIV-1 carriers remain neurologically unimpaired during the so called “asymptomatic” period lasting from seroconversion to symptomatic AIDS. Therefore, neuropathological studies in the early pre-AIDS stages are very few, and the natural history of central nervous system changes in HIV-1 infection remains poorly understood. Examination of brains of asymptomatic HIV-1 positive individuals who died accidentally and of rare cases with acute fatal encephalopathy revealing HIV infection, and comparison with experimental simian immunodeficiency virus and feline immunodeficiency virus infections suggest that, invasion of the CNS by HIV-1 occurs at the time of primary infection and induces an immunological process in the central nervous system. This includes an inflammatory T-cell reaction with vasculitis and leptomeningitis, and immune activation of brain parenchyma with increased number of microglial cells, upregulation of major histocompatibility complex class II antigens and local production of cytokines. Myelin pallor and gliosis of the white matter are usually found and are likely to be the consequence of opening of the blood brain barrier due to vasculitis; direct damage to oligodendrocytes by cytokines may also interfere. These white matter changes may explain, at least partly, the early cerebral atrophy observed, by magnetic resonance imaging, in asymptomatic HIV-1 carriers. In contrast, cortical damage seems to be a late event in the course of HIV-1 infection. There is no significant neuronal loss at the early stages of the disease, no accompanying increase in glial fibrillary acid protein staining in the cortex, and only exceptional neuronal apoptosis. Although HIV-1 proviral DNA may be demonstrated in a number of brains, viral replication remains very low during the asymptomatic stage of HIV-1 infection. This makes it likely that, although opening of the blood brain barrier may facilitate viral entry into the brain, specific immune responses including both neutralising antibodies and cytotoxic T-lymphocytes, continuously inhibits viral replication at that stage.

Introduction

Involvement of the central nervous system (CNS) is common in the acquired immune deficiency syndrome (AIDS). It has been shown that 30-60% of the patients have neurological symptoms (71) which may be the first manifestation of the disease in 10% of the cases, and represent the main cause of disability and death in that population. In addition, neuropathological studies have demonstrated that 80-100% of AIDS patients have pathological abnormalities in the CNS (46, 47, 57). These include lesions due to direct infection of the nervous tissue by the human immunodeficiency virus (HIV): HIV encephalitis/leukoencephalopathy both referred to as HIVE in this paper, opportunistic infections and lymphomas related to the immunodeficiency syndrome, and changes secondary to systemic complications of the disease (48). Most of these complications occur late in the course of HIV-1 infection, usually at the terminal stage of AIDS. The neuropathology of full blown, symptomatic AIDS, established in many large autopsy series (6, 14, 46, 66, 79, 93), has been described in great detail (43) and is now universally accepted (15). In contrast, the natural history of the changes occurring in the CNS during the earliest stages of HIV-1 infection remains poorly understood.
Seroconversion may be clinically silent, however 50 to 70% of patients experience a transient "acute HIV syndrome" which may include neurological symptoms, approximately 3 to 6 weeks following primary infection. It is followed by a phase of clinical latency which commonly lasts 8-10 years until the onset of the symptomatic AIDS, the so called "asymptomatic" period. This is, however, a somewhat confusing term as these patients may develop a variety of conditions and, on the other hand, patients with low CD4 count may remain asymptomatic, thus "asymptomatic" individuals may have very different levels of immunodeficiency (55). For obvious reasons, neuropathological studies are uncommon at the early stages of HIV-1 infection. They include either exceptional observations of fatal acute encephalopathy during which HIV-1 seropositivity is discovered, or autopsy studies of asymptomatic HIV-1 seropositive individuals who died accidentally or from an other pathological condition. Studies of CNS changes in AIDS patients who died without neurological signs and definite neuropathology, also give information on the further stages of the disease, preceding the onset of HIV-1. In addition, experimental simian immunodeficiency virus (SIV) and feline immunodeficiency virus (FIV) infections, have provided interesting insight into the understanding of the natural history of HIV infection of the CNS (61, 62).

CNS changes at the time of seroconversion.

Early invasion of the CNS by HIV-1, suggested by occasional clinical observations of transient symptomatic meningoencephalopathy (59), encephalopathy (13, 17, 75) or myelopathy (26) coinciding with seroconversion for the virus, has been supported by many cerebrospinal fluid (CSF) studies. Intrathecal synthesis of HIV-1 specific antibodies (40, 87) and direct virus isolation from the CSF (59), found in asymptomatic individuals at the time of, or subsequent to, seroconversion, indicate that the virus enters the CNS during the initial stage of systemic infection. This was confirmed in a case of iatrogenic HIV-1 infection in whom the virus was isolated from brain 15 days after accidental HIV-1 inoculation, 1 day after the virus was recovered from blood (25).

Three clinicopathological cases of fulminating encephalopathy revealing HIV-1 infection have been reported, one in a seronegative patient in whom HIV was cultured from CSF (63) and the others in two non-immunocompromised patients in whom the neurological signs led to the discovery of HIV-seropositivity (45). In all these cases, acute periventricular inflammatory changes were present in the cerebral white matter. In the case of Jones et al. (63), the neuropathological features resembled acute haemorrhagic leukoencephalopathy including variably well defined areas of demyelination in the hemispheric white matter with a possible perivascular distribution and good preservation of axons, and petechial haemorrhages. A comparable case was reported in a 31-year old Carribean male (31); however, although the possibility of an acute encephalopathy coinciding with HIV-1 primary infection was discussed, serodiagnostic evidence was not available. In the 2 other cases (45), the lesions resembled recent plaques of multiple sclerosis (MS) forming well circumscribed foci in the white matter of the cerebral hemispheres, brainstem and cerebellum. Pathological changes included extensive myelin loss with numerous lipid laden macrophages expressing major histocompatibility complex (MHC) class II antigens, preservation of axons and nerve cell bodies, reactive astrocytosis and mononucleated perivascular inflammatory infiltrates. In one patient, these abnormalities were associated with changes resembling acute demyelinating perivenous encephalitis. In none of the 3 cases were multinucleated giant cells found and immunostaining for HIV was negative.

These cases show some similarities with 3 of the 7 cases with MS-like illness occurring with HIV infection reported by Berger et al. (11) in whom HIV seropositivity was demonstrated at the time of the neurological disease or within 3 months of its onset, thus implicating the virus in the aetiology of the disorder. None had abnormal CD4 count, all of them presented with remitting relapsing episodes and, in one case, cerebral biopsy revealed features characteristic of recent plaques of MS.

These observations suggest that, in exceptional instances, primary HIV-1 infection may induce an immunopathological process in the CNS similar to that proposed for Guillain-Barré-type polyradiculoneuropathies associated with HIV seroconversion (77). This process could induce a CNS demyelinating disease of variable severity to present as acute haemorrhagic leukoencephalopathy (63), acute demyelinating perivenous encephalomyelitis or acute MS-like leukoencephalopathy (45). More chronic lesions might also appear with remitting relapsing episodes (11). Finally an acute transient aseptic meningitis (59), encephalopathy (13, 17, 75) or myelopathy (26) might also occur. These observations support the view that in genetically susceptible patients, who acquired MS as a systemic "trait", a number of non specific pathogens, including viral infection, producing an alteration of the blood brain barrier (BBB), may act as a trigger or a facilitator in the development or enlargement of MS lesions in the CNS (80).

Neuropathological studies in HIV-1 asymptomatic carriers.

Despite persistent CSF abnormalities (7, 16, 20, 21, 28, 29, 73, 74, 84, 87), most HIV-1 carriers remain neurologically unimpaired in the so called "asymptomatic" preAIDS stage. However, a number of psychometric, radiological and electrophysiological studies suggest that neurological abnormalities are present in these patients (65, 81, 82, 86, 99-101),
although they are not constantly found (36, 55, 70) and their significance is questionable (81, 82).

Lenhardt et al. (68) described neuropathological changes in an asymptomatic 30-year-old male, known to be HIV positive for 3 months, who denied homosexuality or drug addiction and died from a motor vehicle accident. Two of us (FG, MD) and others examined the brains of HIV-positive asymptomatic individuals who died from unnatural causes. In an early study, we examined 11 HIV-seropositive individuals, mainly drug addicts, who died from heroin overdose and compared them with seronegative individuals with similar causes of death (51). Subsequently (56), we compared the results of post mortem brain MRI and neuropathological studies in 7 additional asymptomatic HIV-seropositive individuals, 8 seronegative controls with similar cause of death and 6 patients who died from AIDS in the absence of focal cerebral opportunistic infection or tumour. We have now collected 32 cases including 30 intravenous drug addicts and 2 homosexual men. Twenty-nine drug addicts died from heroin overdose, 1 committed suicide by hanging herself, 1 homosexual committed suicide by ingestion of benzodiazepine and the second homosexual died from gunshot injury. Serology was performed post mortem by ELISA. For each case information was obtained from the family; none had had medical problem or neurological deficit, only 3 patients were known to be HIV-positive. A complete post mortem was carried out in each case and none had any lesions suggestive of AIDS or AIDS-related complex (ARC). This supports the view that most of the cases were at a preAIDS stage although CD4 counts were not available. Twenty-five seronegative cases with similar causes of death, collected in the same institution have been examined according to the same protocol to serve as controls. A comparable study of drug addicts (8 seropositive and 16 seronegative) who died from heroin overdose collected in Forensic Medicine was performed by Gosztonyi et al. (41). In Edinburgh, one of us (JB) and others examined 23 drug addicts who died suddenly from overdose, traumatisms, liver failure, septicemia, pneumonitis, meningitis, with transmural infiltration of inflammatory cells but there was no necrosis of the vessel wall, no granulomas and no leucocytosis. In the seronegative cases, vascular inflammation was absent or mild; neither transmural vascular inflammation nor meningitis was detected in this group. Perivascular cuffs were mainly composed of lymphocytes and monocytes/macrophages. These latter usually expressed MHC class II antigens in their cytoplasm (Fig. 1E). Lymphocytic infiltrates were predominantly T-cells with relatively few B cell present (Fig. 1F, G). T-lymphocytes were mostly UCHL1-positive and many were CD3-positive (10). Further investigation (JB) of these lymphocytic infiltrates using cryostat sections has recently demonstrated that they are composed almost exclusively of CD8 lymphocytes and that very few CD4 cells are present.

Myelin pallor was also more frequent and more marked in the seropositive cases than in the controls and severe changes were only observed in these cases. It was diffuse, ill defined, and involved the deep white matter, tending to spare the gyral white matter and compact myelin pathways such as corpus callosum, internal capsule, optic radiations, descending tracts in the brainstem, hilus of dentate nuclei, and intracerebral parts of cranial nerves (Fig. 2). Myelin loss with lipid laden macrophages was occasionally more obvious around blood vessels. Axons were usually spared. Axonal damage was found only in the most severely affected areas. The significance of myelin pallor, in this material, may have been questionable. However, in the seropositive brains, it is unlikely to be due only to autopsy delay or terminal cerebral edema, as it was associated with glial reactions. Semiquantitative evaluation of white matter astrocytosis, according to the density of astrocytes, size of the cytoplasm and intensity of glial fibrillary acid protein (GFAP) positivity, showed that it was significantly more severe in HIV-positive cases. Reactive protoplasmic astrocytosis with marked enlargement of the perivascular processes predominated in perivascular areas. Morphometric study performed on this material (IE, PL) confirmed a significant increase in the number of white matter astro-
**Figure 1:** Vascular inflammation in HIV-seropositive asymptomatic individuals who died accidentally. (A) Lymphocytic meningitis (H&E, x 100). (B) Intraparenchymal mononuclear cuffs around small vessels predominantly veins (H&E) with transmural infiltration (x 100), in the subependymal region (x 40), and basal ganglia (x 40). (E) Immunocytochemistry demonstrating that the perivascular cuffs are mainly composed of lymphocytes and monocytes/macrophages. Macrophages express MHC class II antigens in their cytoplasm: (E) (HLA-DR, M 775 Dako revealed by an alkaline phosphatase-anti alkaline phosphatase (APAAP) technique, x 160). Lymphocytic infiltrates are predominantly T-cells: (F) (UCHL1, M 755 Dako, APAAP x 160), with relatively few B cells present: (G) (CD20, M 755 Dako, APAAP x 160).

**Figure 2:** White matter changes in a HIV-seropositive asymptomatic drug addict who died from heroin overdose (Loyez stain for myelin). (A) Coronal section of the left cerebral hemisphere, just behind the splenium of corpus callosum. Diffuse, ill-defined myelin pallor involving the deep white matter tending to spare the gyral white matter and optic radiations. (B) Horizontal section of brainstem/cerebellum at the level of mid pons. Diffuse myelin pallor relatively sparing the white matter of the folia, corticospinal tracts and hilus of dentate nuclei.

cytes. Associated microglial proliferation with occasional microglial nodules was better demonstrated by immunocytochemistry (ICC) (3, 96). Upregulation of MHC class II antigens and expression of cytokine such as TNF-a, IL-1a, IL4 and IL-6 was subsequently demonstrated (3), indicating immune activation.

These white matter changes may be involved in the cerebral atrophy which occurs early in HIV-1 infection as suggested by radiological studies (53, 86) and confirmed in a recent combined radiological and pathological study (56). In a morphometric study (FG, FC) on the cases of this later study using a point grid (Table 1), the ratio of the number of points in the cerebral parenchyma to that of points in the ventricles and sulci, in selected coronal sections of the left cerebral hemisphere, was higher in seronegative controls than in seropositive asymptomatic cases, and in the latter it was higher than in AIDS patients. This confirmed that seropositive asymptomatic cases were more atrophic than the seronegative controls.
and less atrophic than AIDS patients. In contrast, the ratio of the number of points in the cerebral cortex to that of points in the white matter was similar or even higher in seropositive asymptomatic cases than in seronegative controls, whereas it was lower in AIDS patients in whom neuronal loss in the cerebral cortex has been demonstrated by many morphometric studies (34, 36, 49, 64, 104, 105). This suggest that cortical atrophy, if present, is not obvious at that stage and cannot be the only factor of early cerebral atrophy.

Table 1

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<th>month of death</th>
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<th>G/W</th>
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Non-drug-addict HIV-seronegative control

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AIDS cases without focal cerebral changes

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n°RP: autopsy case number at Raymond Poincare hospital, Garches
G/W: ratio of the number of points in the cerebral cortex to that of points in the white matter
(G+W)/V: ratio of the number of points in the cerebral parenchyma to that of points in the ventricles and sulci, in 2 selected coronal sections of the left cerebral hemisphere, through the rostrum of corpus callosum, and through the splenium of corpus callosum.

Changes in the cerebral cortex have been subsequently described. Using the same stereological method used to demonstrate neuronal loss in the frontal cortex of AIDS patients (36), Everall et al. evaluated neuronal density in the superior frontal gyrus of 14 HIV-positive cases of our series and compared it with that of 15 HIV-negative controls. Although the values appeared slightly lower in the seropositives, there was no significant difference in the neuronal density of the frontal cortex between the two groups (35). Moreover there was no difference between our cases and non-drug-addict controls. This suggests that neuronal loss occurs later in the course of HIV infection and is only a characteristic of symptomatic AIDS.

This is in agreement with ICC studies of astrogliosis performed in the asymptomatic HIV-1 seropositive cases of our series as well as in cases at later stages of the disease (96) which did not show any obvious astrogial reaction in the cerebral cortex of non-AIDS either HIV-positive or HIV-negative patients. In contrast, there was marked astrogliosis in AIDS patients, with or without HIVE. Astrogliosis reaction was particularly severe in cases with HIVe confirming previous ICC and morphometric studies (23, 103).

The absence of significant neuronal loss in asymptomatic HIV-positive individuals is also consistent with recent studies of neuronal apoptosis in the brains of HIV infected individuals using in situ end labelling (ISEL) and electrophoresis of DNA extracted from cerebral cortex (Z, 5, 69) which show that apoptotic neurons are constantly (Z, 69) or at least frequently (5) observed in AIDS cases and are absent or rare in HIV-positive asymptomatic individuals.

Contrasting with the absence or rarity of neuronal damage and accompanying astrocytic proliferation in the early stages of HIV infection, there is already obvious microglial activation at that stage, in the cerebral cortex, as in the white matter. Using lectin Ricinus communis agglutinin (RCA)-120, a significant increase in density of microglial cells was observed in the cerebral cortex of HIV-positive non-AIDS cases (96); this was associated with upregulation of MHC class II antigens comparable to that in the white matter; in contrast, cytokines expression was considerably lower in the cerebral cortex than in the white matter and could be considered minimal (3).

Productive infection of the CNS is very low at the asymptomatic stage of HIV-1 infection. This contrasts with lymphoid organs in which high viral load has been found during the latent phase of HIV-1 infection (28), as in experimental studies of SIV (60) and FIV (8) infections.

Immunostaining for HIV-1 proteins has been repeatedly negative in all the studies. HIV-1 proviral DNA was detected by polymerase chain reaction (PCR) in about half of asymptomatic virus carriers in one study (96, 97) with comparable results on frozen and paraffin embedded material (4). Low positive results were also found in 7 of 13 cases of the Edinburgh series using quantitative PCR; however these findings were considered consistent with contamination by residual infected blood in the brain tissue (10, 28). In the study of the Institute of Neurology of London (3, 96, 97), the low sensitivity of the method (10 mol of HIV-1 DNA per mg genomic DNA) which was purposely reduced tenfold by addition of 1 mg genomic DNA brain tissue DNA, made it very unlikely that the low number of circulating infected cells could be the only source of false-
positive results (97). This was confirmed in a recent virological study reporting the positive PCR amplification of HIV-1 genome from 2 asymptomatic HIV-1 carriers (27). The discrepancies between the 2 studies are more likely to be due to recruitment bias as the cases studied in Edinburgh (10, 28) were in general at a later stage than those studied at the Institute of Neurology of London (3, 96, 97). Nevertheless, both studies agreed that the virus might be present in the brain at the pre-AIDS stages of HIV-1 infection but that replication could be continuously suppressed by destruction of infected cells by cytotoxic T lymphocytes (28).

Neuropathological studies at later stages of HIV-1 infection, preceding HIV encephalitis

Changes comparable to those in asymptomatic patients, although less inflammatory, have been described at later stages of HIV-1 infection. Comparative neuropathological study of haemophiliacs and non haemophiliacs with HIV-1 infection provided useful information on the natural history of brain changes in AIDS (32, 33). As the course of HIV-1 infection in haemophiliacs may be cut short by death from haemophilia-associated diseases, these patients often die earlier than non-haemophiliacs with HIV-1 infection. Indeed they have fewer CNS opportunistic infections and lower prevalence, at death, of HIVE. In contrast, they show a higher prevalence of non-specific features, comparable to those observed in asymptomatic cases, suggestive of low-grade meningo-encephalitis. These include mild perivascular lymphocyte cuffing, scattered microglial nodules not associated with demonstrable CMV antigens, and mild, diffuse myelin pallor with mild astrocytosis, and microglial reaction. Subpial astrocytosis in cerebral cortex is also common. HIV proteins are seldom detected by ICC in occasional perivascular macrophages or microglia (33).

Astrocytosis of white matter and mild pallor of myelin staining in the absence of inflammation, multinucleated giant cells and brain atrophy were observed in a homosexual man with early ARC and HIV-1 related dementia (85). ICC study confirmed astrocytosis in the white matter and increased number of microglial cells with a few scattered macrophages. However, microglial proliferation and activation was weaker in these cases than in those with HIVE and also than in asymptomatic preAIDS cases (3). In the cortex of these cases, microglial proliferation and activation were also weaker than in asymptomatic HIV-1 positive cases (3, 96). In contrast there was obvious astrocytic proliferation which was not observed in asymptomatic individuals either seropositive or negative (96). The latter finding is in keeping with morphometric studies of neuronal density in AIDS patients which demonstrated that neuronal loss occurs in AIDS patients whether they had HIVE or not (36, 64).

Whereas high loads of virus are constantly found in the CNS of AIDS patients with HIVE, in that of AIDS patients without neuropathology (3, 96), or even with opportunistic infection or lymphoma (28), the virus load may remain low (27). Thus the onset of immunodeficiency does not seem to be sufficient to convert a latent infection into a productive one. Moreover, analysis of env V3 sequences from HIV-1 infected patients at different stages of the disease revealed the presence of a homogeneous virus population in the brain, at every clinical stage of the disease (27); this suggests that, although there is an increase in the late stage of the disease, HIV-1 replication in the brain is restrained until the terminal phase of HIVE. Additional factors are likely to interfere with productive HIV infection of the CNS. The emergence of virus variants with increased replicative capacity in brain cells has been hypothesized but has not been demonstrated (20). A bidirectional potentiation of HIV and other opportunists, particularly herpes viruses, has been proposed and seems likely in a few cases (9, 44).

Early experimental SIV or FIV encephalopathy.

Comparison of early HIV changes with those in early SIV and FIV infection is of interest for the understanding of the natural history of HIV infection of the CNS. SIV is a retrovirus closely related to HIV both in its pathogenicity and genetic structures. In Rhesus monkeys, it induces an immunodeficiency syndrome and an encephalitis very similar to HIV in human (88). Early brain changes were studied in Rhesus macaques after intravenous (18, 60, 94) and intracerebral inoculation (12). Monkeys inoculated intravenously and sacrificed within 3 months following inoculation, had mild but detectable changes including astrogliosis, microglial nodules, perivascular infiltrates and more rarely, meningitis. Productive SIV encephalitis with multinucleated giant cells could not be seen in animals in the first months following inoculation except in animals presenting no antibody response against SIV or only a very weak response. Similar changes were found in animals
infected intracerebrally; this route of inoculation did not lead to preferential infection of the brain around the inoculation point providing evidence that resident microglia have a low susceptibility to SIV infection (12).

In intravenously SIV infected monkeys, viral genome was detected by in situ hybridization (ISH) initially in the leptomeninges, and subsequently in the perivascular brain parenchyma (94). Combined ISH and ICC showed that infected cells were mainly perivascular and expressed macrophages markers suggesting that infected monocytes/macrophages crossing the BBB are the main source of entry of virus into the CNS (18). During the so-called "latent phase" of infection only a few infected cells with a low level of viral replication were observed in the brain parenchyma. This contrasted with immunosuppressed animals at the terminal stage of the disease and, to a lesser extent, with animals at the initial viremic phase; those have not yet developed an antiviral response, suggesting that there is an active immunological control of viral replication in the CNS during the latent period (18). A recent correlative study of neuropathological changes, cytokine mRNA production, and virus load at different stages of SIV infection performed by some of us (DB, FG, BH), concluded that brain infection occurs early, remains constant and low during primary infection and asymptomatic period, and increases in some but not all the animal in the symptomatic period. The finding by Sasseville et al. (89) of elevated vascular cell adhesion molecule-1 in monkeys with SIV encephalitis suggests that expression of this molecule may influence monocyte and lymphocyte recruitment to the CNS and the development of SIV encephalitis. In our study (DB, FG, BH) we found a correlation between viral loads, lesions, and local production of IL1 and IL6 mRNA in the brain. Local production of TGFβ1 mRNA was associated with severe encephalopathies. Local production of TNFα in the brain correlated better with the viral load in lymph nodes than the viral load in brain.

FIV is a lentivirus belonging to the same subfamily as HIV and SIV to which it resembles in both its molecular and pathogenetic properties. FIV infection progresses in 3 phases: a primary infection lasting several weeks with viraemia, generalized lymphadenopathy, fever neutropenia and mild lymphopenia, a long asymptomatic phase lasting several years and a terminal stage with immunosuppression during which encephalopathy may occur (76). Hurtrel et al. (62) examined FIV infected cats, either naturally infected, or inoculated. Lesions were similar in naturally infected cats and experimentally infected cats with short survival (below 12 months). They included low grade leptomeningitis, perivascular mononuclear cuffs, mild cortical and white matter gliosis and white matter pallor with frequent perivascular pattern. Interestingly, these changes were more frequent and severe in cats co-infected with feline leukaemia virus and feline infectious peritonitis virus.

Tentative interpretation of the significance of early brain changes and their relationship to HIV-1 infection.

The perivascular inflammation is different from the necrotizing angitis sometimes observed in drug addicts. This latter involves larger visceral arteries and seems to be related to methamphetamine injection (24). The perivascular inflammation also differs from granulomatous (90, 107) or necrotizing (102) cerebral vascular lesions which have been described, in rare instances, in patients with AIDS or ARC and some of which were subsequently related to varicella zoster virus infection (22, 44). The distribution and microscopical features are rather comparable to the vasculitis observed in peripheral nerve and muscle of HIV-infected patients, mainly at the early stages of the disease (19, 39).

The perivascular inflammation does not seem to be due to productive HIV-1 infection as it is never associated with multinucleated giant cell and the viral load remains very low. It seems more likely to be an expression of an immunological process with T-cell reaction induced in the CNS, peripheral nervous system and skeletal muscles, as in other organs, following primary infection with HIV-1. It is remarkable that perivascular inflammation predominates in areas through which the virus may enter the brain by haematogenous route. In particular, the frequent observation of perivascular cuffs in the choroid plexus and subependymal regions, support the hypothesis that these might be important sites of entry into the brain for viruses (37, 54, 91).

It seems likely that the vasculitis may be responsible for opening of the BBB. Although it has been shown that activated lymphoblasts and monocytes easily gain access to the CNS without any disruption of the BBB (58, 67), this latter may facilitate entry of the virus into brain parenchyma. On the other hand, T-cell reaction may participate in the immunological control of viral replication.

The significance and pathogenesis of the white matter changes are also unclear and they may not be due to a single cause. The topography and perivascular predominance of myelin pallor, in the HIV positive cases, resembles that of HIV leukoencephalopathy at the origin of which an abnormality of the microvasculature has been proposed (92, 98). It has also been suggested that myelin pallor observed by MRI in AIDS patients was not due to myelin destruction, as myelin basic protein was not identified in these areas, but rather to oedema with a BBB defect (83). Recently Petito et al. (78) demonstrated that a diffuse BBB leak is present at time of autopsy, in about 50% of patients with AIDS. They suggested that it could not only facilitate viral entry into the
brain, but contribute also to the diffuse myelin pal­
lor and gliosis common to all patients with AIDS. It is also possible that, in early HIV-1 infection, the white matter changes result from opening of the BBB due to vasculitis, and that consequent fibrous thickening of the vessel walls participate in persistent BBB leak at later stages of the disease (98). However, other factors may be involved in persistent BBB defect, particularly cytokines such as TNF-α which has been shown to mediate BBB damage in HIV infection of the CNS (95) and was demonstrated by ICC in microglia, macrophages and pericytes in the white matter of asymptomatic HIV-1 positive individuals, at higher level than in AIDS patients (3). TNF-α could also induce direct damage to oligodendrocytes as it has been recently demonstrated that it may induce programmed cell death in oligodendrocytes in vitro (106). This would be in keeping with recent observa­tion of apoptotic glial cells in brain tissue of asymptom­atic HIV-1 positive individuals, some of which could be identified as oligodendrocytes on morpho­logical grounds (5).

Neuronal loss does not seem to be a predominant feature in asymptomatic HIV-1 positive cases. However, rare apoptotic neurons were demonstrated by ISEL in 1 of 4 cases in a first study (2) and 2 of 36 case in a second one (5). As none of the cases had CD4 counts, it is possible that these few patients, although asymptomatic, were already at an early stage of AIDS, when apoptosis of neurons is frequent­ly or even constantly found (2). It is also understandable that the course of HIV-1 infection is more pro­gressive and programmed cell death already starts in the asymptomatic pre-AIDS stage. As a matter of fact, the status of immune activation in the brain, revealed by upregulation of MHC class II and the presence of cytokines, is comparable at that stage to that seen in AIDS (1, 3) in which it is implicated in the mechanism of neuronal cell death (52). Moreover the presence of HIV-1 proviral DNA was also demonstrated in the brains of the asymptomatic cases in which apoptotic neurons were identified (5).

Pathogenetic hypothesis

A number of clinical, biological and experimental observations indicate that, in addition to the sys­temic infection, invasion of the CNS by HIV-1 occurs at the time of primary infection (20). It it followed by an inflammatory T-cell reaction in the CNS with vasculitis and leptomeningitis. This reaction is usually asymptomatic, only characterized by an aseptic meningitis at CSF examination. In rare cases, it may be excessive causing a demyelinating disease of vari­able severity and neurological symptoms which are usually transient. There is also immune activation of brain parenchyma with increased number of microglial cells, upregulation of MHC class II anti­gens and local production of cytokines.

The inflammatory T-cell reaction is transient; at later stages of the disease, in immunodeficient patients, vasculitis and aseptic leptomeningitis are no longer observed. In contrast, fibrous thickening and mineralisation of the vessel wall are frequent, likely to correspond to the healing phase following vascular injury. Microglial activation usually persists, however its intensity may decrease in AIDS patients without neuropathology to increase again in those with HIVE.

Myelin pallor and gliosis of the white matter are frequent in early HIV-1 infection and persist at the later stages of the disease. They may explain, at least partly, the early cerebral atrophy observed by MRI in asymptomatic HIV-1 carriers. The white matter changes are likely to be the consequence of opening of the BBB due to vasculitis and subsequent persist­ent BBB leak due to sequelar abnormalities of the microvasculature. Direct damage to myelin and oligodendrocytes by cytokines may also interfere.

In contrast, cortical damage seems to be a late event in the course of HIV-1 infection. There is no significant neuronal loss at the early stages of the disease, no accompanying increase in GFAP staining and only exceptional neuronal apoptosis.

Although HIV-1 proviral DNA may be demonstrated in a number of brains, viral replication remains very low during the asymptomatic stage of HIV infection. This makes it likely that, although opening of the blood brain barrier may facilitate viral entry into the brain, viral specific immune responses including both neutralizing antibodies and cytotoxic T-lymphocytes, continuously inhibit viral replication. Viral replication may remain low at later stages, in AIDS patients without HIVE, suggesting that the onset of immunodeficiency is not sufficient by itself to convert a latent infection into a productive one, and that additional factors are likely to play a role at the onset of HIVE.

References


While the relationship between HIV infection and tentatively localized to microglia/monocyte/macrophages predominantly CD8+ with few if any CD4 cells encephalitis (HIVE) characterized by parenchymal approximately 30% of AIDS patients have HIV infection.

Variety of viral products (e.g., gpl20) and cellular factors (e.g., platelet activating factor, quinolinic acid, nitric oxide) produced in response to infection (4).

Editorial

Neuroinvasion and the Neuropathogenesis of HIV infection.

In addition to causing immunologic dysfunction, infection with human immunodeficiency virus 1 (HIV-1) often causes neurologic disease. At autopsy, approximately 30% of AIDS patients have HIV encephalitis (HIVE) characterized by parenchymal and perivascular infiltrates of microglia, macrophages and multinucleated giant cells (17, 22). Lymphocytes are also present in these lesions but are predominantly CD8+ with few if any CD4+ cells (20). HIV antigens and nucleic acid have been consistently localized to microglia/monocyte/macrophages in these lesions with less convincing evidence of infrequent infection of astrocytes, oligodendrocytes, endothelial cells and possibly neurons (29, 33).

While the relationship between HIV infection and HIVE is quite clear, the relationship between HIVE and the clinical syndrome (AIDS dementia complex) is controversial (31). Furthermore, while cells of the monocyte/macrophage lineage contain most of the detectable virus in the CNS and productive infection of neurons is not evident, neuronal damage in HIVE is extensive (5, 15, 32). This has focused attention on indirect mediators of neuronal injury including a variety of viral products (e.g., gp120) and cellular factors (e.g., platelet activating factor, quinolinic acid, nitric oxide) produced in response to infection (4).

Whether one or all of these factors are involved in neuronal damage is unclear. This work and the attendant controversies are derived primarily from studies of AIDS patients in the late stages of disease.

The neuropathology and pathogenesis of early infection of the CNS by HIV have been relatively neglected. In this issue of Brain Pathology, Gray and colleagues provide a detailed review of what is known of early HIV-1 infection and associated neuropathology. Using these data in conjunction with results of studies of acute infection in the nonhuman primate (simian immunodeficiency virus, SIV) and feline (feline immunodeficiency virus, FIV) models of AIDS, the authors then propose a pathogenetic hypothesis of early infection.

Their hypothesis is based on the important observation that HIV rapidly enters the CNS. While several studies have shown that HIV is frequently present in the cerebrospinal fluid (CSF) early in infection (2, 8), there is little data on neuropathology or viral localization at early time points. The best data come from an iatrogenic case of HIV-1 infection in which virus was isolated from the brain and localized to perivascular mononuclear cells 15 days after accidental infection, one day after virus was isolated from the blood (3). Similar data have been obtained by several groups using the SIV macaque model. In these studies virus has been consistently isolated from the CSF and localized to perivascular macrophages/microglia by 14 days postinoculation (1, 12, 27). These studies indicate that SIV and HIV are very neuroinvasive early in infection, and raise two important questions: 1) What is the mechanism(s) of HIV/SIV neuroinvasion? and 2) If HIV and SIV routinely invade the CNS within days of infection why do only a portion of HIV-infected humans and SIV-infected monkeys progress to HIVE (or SIVE)?

One commonly proposed mechanism of HIV/SIV neuroinvasion is the Trojan horse theory (18). According to this theory, latently infected monocytes carry virus across the intact blood-brain barrier (BBB) where they mature into perivascular macrophages/microglia and actively produce virus within the intrathecal compartment. Before addressing the mechanistic aspects of this theory it is important to first consider the biology of microglial turnover and leukocyte recruitment to the CNS. Microglia and macrophages are thought to be derived from a common bone marrow progenitor cell, and both are capable of antigen presentation (7). Studies in rodents indicate that most parenchymal microglia arise in the CNS during gestation, express few of the antigens associated with monocyte/macrophages and persist with very low turnover and limited capacity for cell division (14). In contrast, meningeal and perivascular macrophages/microglia have a more activated phenotype and are virtually indistinguishable from macrophages elsewhere in the body. In addition, it has been demonstrated that replacement of perivascular microglia occurs continuously via recruitment from the circulating monocyte pool through an

References:


intact BBB (7, 14). Thus, HIV-infected monocyte/macrophages may gain access to the CNS parenchyma in the absence of concurrent inflammation. Indeed, in the SIV/macaque model, SIV neuroinvasion occurs by 14 days postinoculation in the absence of any significant breaks in the BBB (27). Furthermore, studies in the Lewis rat have demonstrated that recruitment of perivascular macrophages/microglia can be accelerated during CNS inflammation, while resident microglia are only rarely replaced by hematogenous cells (13). While the original Trojan horse theory applied specifically to monocyte/macrophages, T cells may also be involved. In the rat it has been demonstrated that T lymphoblasts, but not mature T cells, randomly enter the CNS. However, in the absence of specific antigen recognition they exit within 1 to 2 days (6). Thus, both HIV-infected T lymphoblasts and monocytes may be potential sources of initial HIV-1 infection of the CNS. What role, if any, these normal or augmented processes of cell trafficking to the CNS have in HIV neuroinvasion and subsequent development of HIV-induced CNS disease is unknown and needs to be examined.

Mechanisms governing recruitment of leukocytes to the cerebral perivascular space from the systemic circulation are not fully characterized but presumably include leukocyte and endothelial adhesion molecules and chemotactant (e.g., chemokine) stimuli (for reviews see 16, 28). Three families of adhesion molecules have been characterized: 1) the immunoglobulin superfamily (e.g., ICAM-1 and VCAM-1), 2) integrins (e.g., VLA-4 and LFA-1), and 3) selectins (E-selectin, P-selectin, and L-selectin). Combinatorial use of leukocyte and endothelial adhesion molecules in conjunction with chemotactant stimuli generates distinct “area codes” for specific leukocyte subpopulations to migrate to specific tissues. Of particular importance in the activation and directed migration of leukocytes is a group of structurally-related, low molecular weight, chemotactic, and proinflammatory proteins, termed chemokines. These proteins are induced in various cell types (including endothelial cells and leukocytes) and are distinct from classical chemotactic agents in that these molecules affect the migration of specific subsets of leukocytes (16). Together these data suggest that primary HIV neuroinvasion occurs as a result of selective recruitment and retention of mononuclear cells in the CNS via the action of specific chemotactants and adhesion molecules. Evidence to support this has been obtained in the SIV macaque model where VCAM-1 expression is increased early in infection and uniformly present in the CNS of macaques with SIVE (23, 24). Furthermore, neuroinvasion by SIV has been associated with intrathecal immune activation which could upregulate VCAM-1 expression (23, 27).

While inflammation and breaks in the BBB ultimately occur and contribute to the pathogenesis of HIVE, these breaks are unlikely to play a major role in primary neuroinvasion. This assertion is based not only on results of Smith et al (27) which demonstrated neuroinvasion of SIV without a break in the BBB, but also from direct intracerebral (IC) injection of SIV in macaques (9, 26). It was assumed that IC inoculation would increase the rapidity and incidence of SIVE by bypassing the BBB; however, it did not. Thus, while it is generally agreed that neuroinvasion by HIV and SIV is necessary for the development of HIV/SIVE, neuroinvasion alone is insufficient.

This raises the second major question: Why do only a portion of HIV-infected humans and SIV-infected monkeys progress to HIVE (or SIVE)? Convincing explanations are lacking but those that exist fall into the broad categories of either host or viral determinants. Several groups have evidence to suggest that specific changes in the envelope gene of HIV are associated with tissue specific tropism (11, 19). These studies, however, have all involved terminal AIDS patients and have been unable to associate the appearance of specific viral variants with neurologic disease. Similarly, certain strains of SIV have been reported to cause an increased incidence of SIVE (25, 26). Moreover, high levels of virus replication in the CNS and SIVE have been associated with specific sequence changes in env (10). While these findings are promising and deserve further study there is also evidence that the host immune system plays a major role in controlling viral infection in the CNS. In macaques infected with SIV there is a very early burst in viral replication in both the periphery and CNS that decreases as the host immune system responds (12, 21). In the CNS as in the periphery both SIV-specific cytotoxic lymphocytes and antibody are involved (27, 30). As the disease progresses and the immune system fails, however, only a portion of the animals develop SIVE. Thus the answer to why only a portion of HIV-infected humans and SIV-infected monkeys progress to HIVE (or SIVE)? is likely to be complex involving viral determinants, the host immune response and the appropriate expression of adhesion molecules and chemokines to cause recruitment and retention of mononuclear cells in the CNS. Clearly there is much to be done to elucidate the pathogenesis of this complicated disease.

References
14 F. Gray et al: Neuropathology of Early HIV-1 Infection


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EDITORIAL

HIV and brain pathology: where do we go from here?

In this issue, Gray et al present an extensive review of most neuropathological and other aspects of early HIV infection. The question of early HIV seeding to the brain and its effects is crucial in our knowledge of this infection but poorly understood. Thus such a review is most welcome. Gray and her co-authors cover changes at seroconversion, during the asymptomatic period, and at later stages before HIV encephalitis develops. Their study shows an impressive number of asymptomatic cases gathered from geographically distinct areas.

Despite the usefulness of this review, one cannot overlook that we are in an area where many data are still fragmentary. They are necessarily fragmentary because longitudinal studies of brain pathology in HIV-infected individuals are not available. We like to think that changes observed in autopsies of persons dying at early stages of the infection are harbingers of subsequent more prominent pathology; however, we cannot be sure that this is true. Experimental SIV infection, as considered in Françoise Gray’s review, is expected to give more insight in such a situation. We have to assume that this animal model reflects what is going on in the human. Given these uncertainties which equally pertain to brain changes in the later phase of the infection, a critical mind might become depressed how little progress we have made on HIV and the brain.

Ten years ago several seminal papers described the presence of HIV in the brain and its association with a new type of pathology. According to my bias, little has moved since. It became clear that, in contrast to most other virus-mediated conditions, clinicopathological correlation of important morbidity such as AIDS dementia, myelopathy or neuromyopathy is not clear-cut but rather a complicated scenario which is co-orchestrated by several players (viral, immune, neurotoxic, metabolic and vascular mechanisms) in direct or indirect ways. We hear the various tunes but do not perceive any harmony yet.

Being a clinical neuropathologist, it is sad for me to realize that even the most sophisticated assessment of tissue changes in persons dying with HIV is unlikely to resolve the puzzle of functional, structural, and pathogenetic correlation. What we need first is some sort of balancing on HIV and the brain; papers like that by Gray et al are a good starting point. However, what is much more urgently needed is an imaginative strategy by which this type of research can go on. Hopefully, this might become a major incentive for neuropathology in the last four years of the Decade of the Brain.

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Seventeen asymptomatic individuals positive for human immunodeficiency virus type 1 (HIV-1) and 16 patients with acquired immunodeficiency syndrome (AIDS), all with polymerase chain reaction evidence of HIV-1 DNA, were selected for quantitative analysis to correlate the levels of HIV-1 DNA in brain tissue with the stage of infection. The AIDS patients either were clinically asymptomatic or presented various abnormalities. Neuropathological lesions were assessed by morphological and immunohistochemical methods. To determine the level of HIV-1 DNA, semiquantitative nested polymerase chain reaction was applied using a digoxigenin-labeled primer and chemiluminescence. Serial dilutions of standard HIV DNA were run in parallel with brain DNA samples. Among the 16 AIDS brains studied, 9 showed changes characteristic of HIV encephalitis/leukoencephalopathy while 1 showed focal pontine leukoencephalopathy and 6 showed no obvious neuropathological lesions. Abnormalities in pre-AIDS individuals included meningitis, microgliosis, and astrogliosis.

Copy numbers of HIV-1 DNA in the brains of AIDS patients were higher than those in asymptomatic individuals (median, 135 vs 45 copies/150,000 cells \( p < 0.012 \)). However, there was some degree of overlapping between the two groups, with some AIDS patients showing low figures while 3 asymptomatic patients had high copy numbers. This suggests that the use of HIV-1 DNA load in the central nervous system as an indicator of progression of the disease should be restricted to large series and not single patients.

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Human immunodeficiency virus (HIV) causes a chronic disease in humans. During the early period after primary infection there is widespread dissemination of virus and a decrease in the number of CD4 T cells in peripheral blood [1]. An immune response to HIV ensues, with a decrease in detectable viremia followed by a prolonged period of clinical latency [1] that could last up to 7 years or even longer [2]. On the other hand, at least during the terminal stages of the infection, viral loads increase and are accompanied by the development of HIV-specific lesions, including those in the nervous system, based on a dose-effect relation [3]. It has therefore been suggested that the evaluation of proviral copy numbers could be an early indicator of AIDS progression [4-7].

Polymerase chain reaction (PCR) technique has been applied in DNA and RNA research since 1985 and developed for quantitative evaluation, cloning, and sequencing purposes. The goal of quantitative PCR is to deduce, from the actual amount of PCR product, the initial number of target molecules existing in the sample. In HIV-1 research this technique has been applied to peripheral blood mononuclear cells (PBMCs) [8-13] and also to tissues such as lymph nodes, spleen, lung, and liver [14, 15]. Only a few data regarding brain tissues in patients with AIDS are available to date [16, 17]; in particular, little is known regarding the early stages of infection. Sinclair and colleagues [18] and An and coworkers [19] detected HIV-1 DNA by PCR in the brains of a proportion (2/8 and 17/36, respectively) of HIV-1-positive asymptomatic individuals. These results are at variance with those of Bell and associates [20] who are of the opinion that HIV-1 DNA detected in the brain of these individuals corresponds to provirus within the blood included in the specimen. However, more recently, Schmid and colleagues [21] demonstrated that HIV-1 proviral load in seropositive patients, at all stages, was significantly greater in cerebrospinal fluid (CSF) than in blood. In addition, data from our laboratory, using a chemiluminescence technique show that some of the signals obtained from HIV-1-positive pre-AIDS individuals are as strong as those from AIDS patients. Although this requires confirmation by quantitative analysis, the levels of HIV-1 DNA in the brain in the early stages may indeed be high.

Previous PCR work on the brain used either \( ^{32}P \)-labeled primers (probe) or fresh-frozen tissue [16, 17].
Recently, we successfully amplified [22] and evaluated semiquantiatively [23] HIV-1 DNA from formalin-fixed, paraffin-embedded brain tissue. The purpose of this study was to investigate the amount of HIV-1 DNA in the brains of AIDS and HIV-1–positive pre-AIDS individuals by amplifying HIV-1 DNA using digoxigenin (DIG)-labeled primers and detection by chemiluminescence and densitometry.

Materials and Methods

Subjects
Brain tissues from 33 HIV-1–positive individuals with PCR evidence of HIV-1 DNA were examined. They included 16 AIDS patients chosen from the series of the Department of Neuropathology, Institute of Neuropathology. Six of them had clinical evidence of dementia and the remaining 10 were asymptomatic. A report of the morphological, immunohistochemical, and PCR findings from the 17 HIV-1–positive asymptomatic patients [11 obtained from Professor F. Gray, Department of Neuropathology, Hôpital Henri Mondor, France; 6 obtained from the Edinburgh MRC AIDS Brain Bank, courtesy of Dr. Jeanne Bell], included in the present study, has been published [19]. The tissue that was taken from the frontal lobe of all brains and included cortical gray matter and subcortical white matter was fixed in formalin and embedded in paraffin. Histological examination was done applying routine morphological and immunohistochemical techniques.

Polymerase Chain Reaction

DNA Extraction. DNA was extracted as previously described [22]. Briefly, 10 paraffin-embedded sections (10 μm thick) were cut. The area of the paraffin section containing the meninges was removed using a disposable scalpel, which was replaced for each brain examined. The sections were deparaffinized by washing with xylene and then 100% ethanol, followed by drying at 95°C. Tissue was digested with proteinase K at a final concentration of 5 mg/ml in 10 mM Tris–hydrochloric acid, pH 7.4, 1 mM ethylenediaminetetraacetic acid (EDTA), and 0.5% sodium dodecyl sulfate (SDS). Digestion took place at 55°C for 48 hours. The supernatant was extracted three times with a 1:1 mixture of phenol and chloroform, and finally once with chloroform. Subsequently, supernatant was centrifuged through a Microcon-30 filter (Amicon, Gloucs, UK) using a Eppendorf centrifuge at 13,000g for 10 minutes. Purified DNA was quantified using a spectrophotometer and 1 μg of extracted DNA was then amplified.

Primer 5'-CGA GAA CAT TGG GTA TGG GT-3', and 5'-DIG-DIG-11-dUTP end labeled) and pol 2. A 97-bp fragment was amplified using primers pol 2 and DIG-dUTP–labeled pol 3. Following electrophoresis and Southern transfer, amplified DNA was detected as 97-bp band. Human β-globin DNA was amplified from each sample as control.

Results

Of the 16 AIDS brains studied 9 showed changes characteristic of HIV encephalitis/leukoencephalopathy, both labeled in the paper as HIV+1 (1 had in addition cytomegalovirus encephalitis); of the remaining 7 brains, 1 showed focal pontine leukoencephalopathy while the other 6 had no obvious neuropathological lesions. The main HIV-related pathological abnormalities in individuals with AIDS were predominantly in the hemispheric white matter and included myelin pallor, diffuse astrogliosis, microglial nodules, and multinucleated giant cell (MGC). Abnormalities in the HIV-1–positive pre-AIDS group in this study included discrete meningitis in 8, presence of microglial nodules, and astrogliosis in 15 (Table 1).

The levels of viral DNA in the brains of AIDS (median; 135; range: 5–540; interquartile range: 90–180) and HIV-1–positive pre-AIDS (median: 45; range: 5–215; interquartile range: 15–70) individuals are listed in Table 1 and represented in Figure 1. Results indicated as 5 to 10 were considered positive (see Discussion). The difference between the AIDS and pre-AIDS group was significant (p < 0.012, Mann-Whitney U test).

The effect of the numbers of cycle on the amplification by nested PCR is shown in Figure 2. Following 28 cycles in the first round, a linear relationship between the logarithm (log) of the amount of PCR product and the log of the initial amount of sample DNA (ranging from 1–10 to 1,000 copies) could be obtained when 15 to 20 cycles of amplification were performed in the second round. The results of PCR for 4 HIV-
Table 1. Results of Neuropathology and Load of HIV-1 DNA

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Neuropathology</th>
<th>Dementia</th>
<th>Copies/150,000 Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>HIV</td>
<td>T + yes</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>HIV</td>
<td>T + no</td>
<td>150</td>
</tr>
<tr>
<td>3</td>
<td>HIV, CMVE</td>
<td>T + yes</td>
<td>540</td>
</tr>
<tr>
<td>4</td>
<td>HIV</td>
<td>T + no</td>
<td>65</td>
</tr>
<tr>
<td>5</td>
<td>HIV</td>
<td>T + yes</td>
<td>5 - 10</td>
</tr>
<tr>
<td>6</td>
<td>HIV</td>
<td>T + no</td>
<td>540</td>
</tr>
<tr>
<td>7</td>
<td>HIV</td>
<td>T + no</td>
<td>150</td>
</tr>
<tr>
<td>8</td>
<td>HIV</td>
<td>T + yes</td>
<td>210</td>
</tr>
<tr>
<td>9</td>
<td>HIV leukoencephalopathy</td>
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<tr>
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<td>Focal pontine leukoencephalopathy</td>
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<tr>
<td>11</td>
<td>No abnormalities</td>
<td>T + yes</td>
<td>5 - 10</td>
</tr>
<tr>
<td>12</td>
<td>No abnormalities</td>
<td>T + no</td>
<td>180</td>
</tr>
<tr>
<td>13</td>
<td>No abnormalities</td>
<td>T + no</td>
<td>160</td>
</tr>
<tr>
<td>14</td>
<td>No abnormalities</td>
<td>T + no</td>
<td>105</td>
</tr>
<tr>
<td>15</td>
<td>No abnormalities</td>
<td>T + no</td>
<td>280</td>
</tr>
<tr>
<td>16</td>
<td>No abnormalities</td>
<td>T + no</td>
<td>90</td>
</tr>
<tr>
<td>17</td>
<td>Meningitis (Lym), microglia (+W), gliosis (+W)</td>
<td></td>
<td>120</td>
</tr>
<tr>
<td>18</td>
<td>Microglia (+W, ±G), gliosis (+/+/+W)</td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>19</td>
<td>Meningitis, microglia (+W), gliosis (+/+/W, ±G)</td>
<td></td>
<td>15</td>
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<tr>
<td>20</td>
<td>Microglia+/++, gliosis (+W)</td>
<td></td>
<td>215</td>
</tr>
<tr>
<td>21</td>
<td>Microglia ±, gliosis (+/+/W, ±G)</td>
<td></td>
<td>30</td>
</tr>
<tr>
<td>22</td>
<td>Microglia ±, gliosis (+/+/W, +G), hypoxic changes</td>
<td></td>
<td>5 - 10</td>
</tr>
<tr>
<td>23</td>
<td>Microglia (+W), gliosis (++W, +G), hypoxic changes</td>
<td></td>
<td>5 - 10</td>
</tr>
<tr>
<td>24</td>
<td>Meningitis, microglia (+W, +G), gliosis (+++, +G), hypoxic changes</td>
<td></td>
<td>60</td>
</tr>
<tr>
<td>25</td>
<td>Meningitis (focal), microglia ±, gliosis (+W), hypoxic changes</td>
<td></td>
<td>70</td>
</tr>
<tr>
<td>26</td>
<td>Meningitis (Mac), gliosis (+W, ±G), hypoxic changes</td>
<td></td>
<td>50</td>
</tr>
<tr>
<td>27</td>
<td>Microglia (+W, ±G), gliosis (+/+/W, +G)</td>
<td></td>
<td>70</td>
</tr>
<tr>
<td>28</td>
<td>Microglia (+W), gliosis (++W, +G), hypoxic changes</td>
<td></td>
<td>160</td>
</tr>
<tr>
<td>29</td>
<td>Microglia infiltrate+/++ (Mac)</td>
<td></td>
<td>5 - 10</td>
</tr>
<tr>
<td>30</td>
<td>Meningitis, gliosis (+)</td>
<td></td>
<td>5 - 10</td>
</tr>
<tr>
<td>31</td>
<td>Meningitis (Lym), gliosis (+W, ±G)</td>
<td></td>
<td>50</td>
</tr>
<tr>
<td>32</td>
<td>Meningitis, microglia +, gliosis (+W)</td>
<td></td>
<td>45</td>
</tr>
<tr>
<td>33</td>
<td>Microglia (++W, +/++G), hypoxic changes</td>
<td></td>
<td>27</td>
</tr>
</tbody>
</table>

HIVE = HIV encephalitis; CMVE = cytomegalovirus; Lym = lymphocyte; Mac = macrophage; W = white matter; G = gray matter; ± = positive pre-AIDS and 5 AIDS individuals are shown in Figure 3.

Discussion

PCR has a critical role in assessing the load of HIV-1 DNA [24-28]. In our investigation a nested PCR was applied because of obvious advantages over the single-round PCR method. It eliminates nonspecific products that could interfere with quantitation and has an increased sensitivity, and therefore allows a lower threshold of detection [25-28]. The sensitivity of this technique was further improved by chemiluminescence, which has been applied to the Southern blot [25, 28] and PCR [29].

The sensitivity of detection of HIV-1 DNA by this technique is in the range of 1 to 10 copies. As shown in Figure 3, the results obtained with this technique are superior to those produced by traditional ethidium bromide staining. A comparative study by Ou and associates [27] confirmed that hybridization protection +H T assay by chemiluminescent-labeled oligonucleotide was as sensitive as the method that uses 3P-labeled DNA probes.

Quantiative PCR analysis of HIV-1 DNA in asymptomatic carriers has been performed on peripheral blood mononuclear cells (PBMCs) [28, 29], CD4 T lymphocytes [41-42], lymphoid organ [43], semen, and saliva [44]. Comparison between data in these individuals and in AIDS patients showed that the titer of HIV was significantly higher among the latter [93] and that viral load had an inverse correlation with the number of CD4 T cells [106]. On the other hand, when individuals (both asymptomatic and symptomatic) with a low CD4 T cell count (< 500/muI) were taken into consideration, no significant difference in viral load was found between the two groups [45]. As a
Fig 1. Distribution of levels of HIV-1 DNA in AIDS and pre-AIDS groups.

Fig 2. Effect of cycle numbers of amplification by nested polymerase chain reaction (PCR). A to E, F to J, and K to O illustrate the chemiluminescence results when 14, 16, and 20 cycles were amplified, following 28 cycles of amplification in the first round of PCR, respectively. A, F, and K represent negative controls with normal human DNA only; B, G, and L represent 1 copy of HIV-1 DNA; C, H, and M represent 10 copies; D, I, and N represent 100 copies; and E, J, and O represent 1,000 copies.

Fig 3. A semiquantitative detection of HIV-1 DNA in specimens from pre-AIDS (A–D) and AIDS (E–L) patients. J to L illustrate a standard HIV-1 DNA; J, 200 copies; K, 100 copies; and L, 10 copies. The top figure represents an agarose gel electrophoresis stained by ethidium bromide and the bottom one represents a chemiluminescence result.

strated that HIV-1 proviral load in seropositive patients, at all stages, was significantly higher in the CSF than in the blood (median: 25 copies vs 0.6 copy/1,000 CD4+ cells [p = 0.0001]); in addition, proviral load was greater in the blood and CSF of subjects with more advanced systemic disease and HIV-1 neurological disease. However, viral levels in the CSF may not closely correlate with those in the brain tissue. Indeed, although the brain and CSF represent two interconnected compartments, the concentration of various substances was shown to be not necessarily the same [145]; by analogy, we could assume that the viral levels too may differ in the two media.

Our results using brain tissue confirmed that levels of HIV-1 DNA in the brains of the AIDS group were higher than those found in asymptomatic individuals (median: 135 vs 45 copies/150,000 cells [p < 0.012]). The possibility that the low copy numbers (5–10) in pre-AIDS brains could be the result of contamination from circulating blood [26] at the time of death can be ruled out on the basis of the following calculation: Sources of possible contamination could be three components of blood: PBMCs, CD4+ cells, and plasma [58]. Regarding the third possibility, the chance that unintegrated DNA molecules could be released into the circulation has not yet been confirmed. With regard to PBMCs and CD4+ T cells, assuming the blood distribution to be even throughout the whole body at the time of death, that blood flow has a normal rate of 50 to 55 ml/100 gm of brain/min (50 × 14 [1,400 gm weight of the organ/100 gm] = 700 ml = 700,000
Table 2: List of Load of HIV-1 DNA in Peripheral Blood Mononuclear Cells (PBMC), CD4+ T Cells, and RNA in Plasma by Published Data

<table>
<thead>
<tr>
<th>Source</th>
<th>AIDS</th>
<th>Pre-AIDS</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBMC</td>
<td>1/4,606</td>
<td>1/10,714</td>
<td>4</td>
</tr>
<tr>
<td>PBMC</td>
<td>450–10,516/10^6 PBMC</td>
<td>50–2,570/10^6 PBMC</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>(mean: 2,403)</td>
<td>(mean: 779)</td>
<td></td>
</tr>
<tr>
<td>PBMC</td>
<td>1/700–3,300</td>
<td>1/6,000–80,000</td>
<td>13</td>
</tr>
<tr>
<td>PBMC</td>
<td>200–4,000/10^6 PBMC</td>
<td>2–1,000/10^6 PBMC</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>(mean: 1,245)</td>
<td>(mean: 213)</td>
<td></td>
</tr>
<tr>
<td>CD4*</td>
<td>1/10 cells</td>
<td>1/10,000 cells</td>
<td>27</td>
</tr>
<tr>
<td>CD4*</td>
<td>1/100–10,000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4*</td>
<td>18–2,857/10^4 CD4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4*</td>
<td>1/2,500–26,000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4*</td>
<td>10.7/1,000 cells</td>
<td></td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>0.09/1,000 cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma</td>
<td>100–22,000,000 copies/ml</td>
<td></td>
<td>46</td>
</tr>
<tr>
<td>Plasma</td>
<td>10^4–8 × 10^9/ml</td>
<td></td>
<td>51</td>
</tr>
</tbody>
</table>

The proviral load in patients, all stages, was significantly greater in cerebrospinal fluid than blood (median: 25 vs 0.6 copies/1,000 CD4+ cells [p = 0.0001]).

In a previous study in which we compared copy numbers of HIV-1 DNA obtained from frozen and fixed brain tissues of AIDS patients determined by semiquantitative PCR, we noted that levels from the latter were on the whole less reliable than those from the former [23]. With single-round semiquantitative PCR, the exponential phase was obtained when 20 to 2,000 copies were amplified. By using double rounds of PCR (the phase is now between 1 to 1,000 copies), the technique has become more reliable for the quantitation of samples with low copy of HIV-1 DNA.

By using nested semiquantitative PCR we showed that copy numbers were higher in the group of patients with more advanced disease than in asymptomatic individuals. Our results received indirect confirmation by observations [4, 12, 13, 28] and CD4+ T cells [10] showing that HIV-1 DNA levels were higher in patients with AIDS than in those with pre-AIDS. However, when the results of single individuals were considered, some degree of overlapping could be noted: large numbers of copies were present in some asymptomatic whereas some AIDS patients showed low copy numbers. Regarding the relatively high level of HIV-1 DNA found in the brains of asymptomatic patients (Cases 17, 20, and 28), we cannot rule out the possibility that since all members of this group were "normal" and died of causes unrelated to AIDS (suicide, road traffic accident, etc) without CD4 count available, they could have reached a stage of the infection just preceding AIDS. On the other hand, no copy of HIV-1 DNA per infected cell, or no more than 1.54 (0.5 + 0.66 + 0.83) copies while we assume 2 copies per infected cell. In the above formula 1/1,000 PBMCs, 1/100 CD4+ T cells, and 10^4 copies/ml reflect values higher than average levels, as shown in Table 2.

Usually, 1 to 2 µg of DNA is obtained from 10 mg of brain tissue. This figure refers to the number of PBMCs infected in an asymptomatic individual in [4].

This figure refers to the number of CD4+ T cells infected in an AIDS patient in [27].

This figure refers to the copy number of HIV-1 RNA in plasma in an AIDS patient in [5].
plausible reason could be produced for the low copy number found in some AIDS brains. On the basis of these results we conclude that whereas the use of HIV-1 DNA load in CNS is a valuable indicator of progression of the disease, its application should be restricted to investigation of large series and not to single patients.

Within the AIDS group, we were able to find any correlation between levels of viral DNA and presence or absence of neuropathological changes; this would suggest that the relationship between HIV and encephalitis is much more complex than previously thought, as shown recently by Brew and coauthors, who could find only limited correlation between dementia and the amount of HIV detectable by immunohistochemistry. Further studies to examine whether there are specific sequence differences in brain-derived HIV-1 clones between patients with and those without HIV encephalopathy, as recently demonstrated by Power and colleagues in patients with and those without HIV-associated dementia, are necessary to better understand the pathogenesis of this disease.

It is still unclear whether HIV-1 existing in the CNS during the early pre-AIDS stage is only latent as proviral DNA, or whether the infection of HIV-1 is reactivated during this stage. Indeed Seshamma and associates observed that during the period of viral latency, regulatory transcripts rather than full-length HIV-1 RNA are predominant in PBMCs. If this did apply also to the brain, it would explain why p24 and p41 (which represent viral structural proteins) could not be detected immunohistochemically in pre-AIDS brains. Therefore, a study of the expression and/or replication of HIV-1 and its localization would be of great interest for the understanding of HIV-1 in the CNS in the early pre-AIDS stage following infection.

This work was supported by grants from the Medical Research Council (MRC) (to S. F. A. and F. E.) and the Brain Research Trust (to F. S. V. D.); Giometto received a grant from the Istituto Superiore di Sanità of Italy.

We wish to thank Prof S. Lucas and Mrs M. Kasevitsky for providing the brain tissue, and Mr A. Becker for the detection of the HIV core antigen p24, RCAs10, and glial fibrillary acidic protein. We are grateful to the MRC AIDS directed programme for providing the PCR reference kit and β-globin primers.

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manifest by nuclear and cytoplasmic viral inclusion bodies and positive immunohistochemistry. The remaining cases with systemic disease showed a patchy uveal inflammation and occasional cytoid bodies which may indicate that uveitic involvement predates and is a necessary step in the evolution of retinal disease and that retinitis is a secondary, rather than primary phenomenon.

An S.*, Ciardi A.* & Scaravilli F. Institute of Neurology, London WC1N 3BG
PCR detection of HIV proviral DNA in the brain of AIDS patients: comparison between results using fresh-frozen and paraffin-embedded specimens

The polymerase chain reaction (PCR), which detects low copy numbers of nucleic acid, has been used to detect HIV DNA in brain tissue of AIDS patients and HIV-positive individuals. Previous work (Sinclair & Scaravilli. AIDS. 6: 925. 1992; Sinclair et al., J Neurol. 239: 469, 1992) has used fresh frozen samples of brain tissue from post-mortem specimens. The technique has now been modified for the study of formalin-fixed, paraffin-embedded material and here we present the findings of a pilot study of 20 AIDS cases for which both frozen and fixed, paraffin material was available. Results showed that of the 16 cases that resulted positive on frozen samples, 15 were also positive when paraffin-embedded material was used. None of the paraffin samples which were positive gave negative results when examined on frozen material. These dates show that as far as the identification of HIV proviral DNA, reliable results can be obtained using formalin fixed, paraffin-embedded material.

Bell J., Keeling J.*, Lowrie S., Donaldson Y., Simmonds P. & Peutherer J.* University Department of Pathology, Edinburgh
Human immunodeficiency virus infection and the developing nervous system

Between 10% and 15% of infants born to HIV-positive mothers in Europe are infected vertically and the nervous system may be severely damaged in paediatric AIDS. The timing of vertical transmission is still uncertain—possibly in utero, or at birth, or during breast feeding. Examination of pregnancies terminated for maternal seropositivity provides an opportunity to investigate this problem. Full autopsies have been performed in 30 such fetuses. Apart from one case of spina bifida and one of facial dysmorphism, all were normal. No significant neuropathological abnormalities were discovered and quantitative polymerase chain reaction (PCR) for HIV gave negative results in all the tissues examined, including placenta, brain, spleen and liver. Negative controls included fetuses born to HIV-negative drug abusing mothers. these findings contrast with adult AIDS cases in which we have frequently found significant viral load in brain tissue, but may relate to adult pre-AIDS cases, many of which we have shown to be PCR negative for HIV in the central nervous system.

McQuaid S.*, Allen I. V., McMahon J.* & Kirk J. Neuropathology, Belfast BT12 6BL
Association of measles virus with neurofibrillary tangles in subacute sclerosing panencephalitis

Neurofibrillary tangle formation, a characteristic of Alzheimer’s disease, is also a feature of other neurodegenerative disorders, including subacute sclerosing panencephalitis. In this study the association of measles virus (MV) genome with neurofibrillary tangle formation has been studied in 5 cases of SSPE. using in situ hybridization (measles genome) and immunocytochemistry (tau, ubiquitin and B/A4 amyloid). In two cases with duration of disease less than one year, neurofibrillary tangle formation was not observed. However, in cases where the disease was of several years duration, numerous tau- and ubiquitin-positive neurofibrillary tangles were demonstrated. In the two cases of longest duration, double-labelling demonstrated the frequent association of neurofibrillary tangle formation with neuronal MV genome positivity. Immunocytochemistry for B/A4 amyloid failed to demonstrate amyloid in any of the cases. These findings support the hypothesis that neurofibrillary tangle formation can occur independently of amyloid formation in neurodegenerative disorders.

Hilton D. A.*, Love S., Pringle J. H.* & Fletcher A.* Department of Neuropathology, Frenchay Hospital, Bristol BS16 1LE
Absence of Epstein-Barr virus RNA in multiple sclerosis as assessed by in situ hybridization

Several recent epidemiological and serological studies have suggested a role for Epstein-Barr virus (EBV) infection in the aetiology of multiple sclerosis (MS). We have used oligonucleotide probes to EBV RNAs that are highly expressed in both productive and latent infection, to look
Quantitative PCR for detecting HIV-1 proviral DNA from formalin-fixed, paraffin-embedded brain tissue
AN S-F, DAVISON F, CIARDI A, GRAY F*, SCARAVILLI F.

Dept. of Neuropathology, Institute of Neurology, Queen Square, London WC1N 3BG.
*Dept. of Neuropathology, Henri Mondor Hospital, France.

Background: It is normal for post mortem brain material to be formalin fixed for very long periods before study. This may cause problems in DNA extraction and detection. However, we were able to detect successfully HIV-1 DNA by PCR from formalin-fixed, paraffin-embedded brain tissue. The ability to detect HIV DNA from this source of material correlated well with HIV DNA detection in fresh frozen brain (15/20 cases in paraffin embedded tissue, compared with 16/20 in frozen tissue, J Clin Path, in press).

Objectives: To extend this work by amplifying HIV DNA using a semi-quantitative PCR in 6 proven HIV + cases (4 were p24 antigen positive, 2 negative).

Methods: Total DNA was extracted from frontal lobe tissue of post mortem material. HIV DNA was amplified using pol gene digoxygenin-labelled primers and detected by chemiluminescence and densitometry. Cloned HIV DNA standards were amplified in parallel and used to construct a standard curve for quantification.

Results: The levels of HIV DNA from p24 antigen positive specimens were notably different from those in p24 negative cases. These results were compared with those obtained from frozen material of the same patients.

Conclusions: This study showed: 1. It was possible to estimate HIV-1 DNA levels in formalin fixed, paraffin embedded brain tissue by semi-quantitative PCR. 2. Digoxygenin incorporation followed by chemiluminescence and densitometry gave reliable results when used for this purpose. 3. p24 antigen positive and negative patients formed two well separated groups with clear differences in HIV DNA level in cortex.
Expression of TNF-α and its pathogenetic role in Vacuolar Myelopathy
A. Ciardi*, S.V. Tan†, S.F. Art#, R.J. Guiloff and F. Scaravilli*  
Department of Neuropathology, Institute of Neurology, Queen Square, & Department of Neurology, Charing Cross and Chelsea  
and Westminster Hospitals, London, UK

In the spinal cords of 18 AIDS patients we evaluated the presence of vacuolar myelopathy (VM) and the amount of microglial/macrophages, of cells expressing MHC Class II antigens and tumour necrosis factor (TNF-α). HIV encephalitis/leucoencephalopathy (as shown by the presence of MGCs and/or p24 antigen positive cells) was present in 56% of the cases. Other brain pathology were CMV encephalitis (16%), lymphoma (6%) and PML (6%); 16% of the patients had no specific pathology. HIV myelitis was seen in 1 out of 18 cords, which were devoid of opportunistic infections. Depending on the area involved and severity of vacuolar changes and inflammation VM was classified as mild in 7 cases, moderate in 2 and severe in 6. No VM was seen in 3 cases. p24 antigen was detected in the cord in 1/18 cases. In all 18 cases the number of inflammatory cells the expression of MHC II and the amount of TNF-α was higher than in controls; moreover there was correlation between the severity of VM and the amount of microglia/macrophages. On the other hand, whereas cords without VM showed low levels of TNF-α and microglial/macrophages, cases with VM showed amount of microglial/macrophages proportional to the severity of VM, whilst increased expression of TNF-α seemed to be correlate more with the presence of VM than with its severity.

HIV Infection of Post-Mortem Human Choroid Plexus
M.F. Falangola, B.G. Castro-Filho, C.K. Pedito. Departments of Pathology, University of Miami School of Medicine, Miami, FL and Centro Penicilina, Goncalo Moniz Foundation, Bahia, Brazil

The choroid plexus (CPx) may be an important site of entry of HIV into the nervous system, since fibroblast-like cells isolated from this structure are capable of latent HIV infection (Harras et al, Ana Neuroi, 1989), since its capillaries lack a blood-brain barrier, and since it has been implicated as a site of entry for other infectious agents. Consequently, we collected post-mortem choroid plexus from 23 AIDS patients and 13 nonAIDS controls employing routine H&E examination as well as immunohistochemistry to identify HIV gp41, T and B lymphocytes and monocytes. T lymphocytes infiltrated the CPx of all controls and 67% of AIDS cases whereas monocytes occurred in 22% of controls and 50% of AIDS cases. Eleven AIDS cases contained HIV-immunoreactive cells which were located mainly in the stroma but occasionally assumed an endothelial location. The distribution of the HIV+ cells and the inflammatory subtypes and double label immunohistochemistry suggest that infected cells, including those in capillary endothelium, were T lymphocytes as well as monocytes. Infection or lymphoma of the choroid plexus or brain did not correlate with the incidence of CPx HIV+ cells. These results demonstrate migration of T lymphocytes and monocytes in normal and diseased conditions. Support recent studies implicating this structure as a component of the neuroimmune system. Additionally, the study shows, for the first time direct HIV infection of the choroid plexus. This implies a hematogenous dissemination of infected T lymphocytes or monocytes into the choroid plexus from whence they could enter the CSF and periventricular brain. Thus, migration of HIV infected cells through the choroid plexus may be an important or alternative mechanism of HIV infection of the CNS. Supported by grants from the CNPq (MFP), and the NIH RO1NS27416, (CKP).

Zidovudine therapy and HIV encephalitis: a 10-year neuropathological survey
F. Gray, L. Bélec, P. De Trousch, B. Clair, A. Sobel, M. Durigon, Hôpital Raymond Poincaré, F-92380 Garches, France

Zidovudine has been shown to penetrate the CSF, to inhibit HIV replication in monocytes/macrophages and to improve neurological function in AIDS patients. In an attempt to assess the effect of zidovudine on productive HIV infection of the brain, we compared the incidence of HIV-specific pathology in the brains of patients treated or not treated with zidovudine and in patients whose treatment had been stopped.

We examined 192 AIDS cases neuropathologically; 87 had never been treated with zidovudine, 72 had received zidovudine for over 3 months and until death, 23 had their zidovudine treatment stopped more than 1 month before death.

The incidence of HIV encephalitis/HIV leuencephalopathy (HIVE/HIVL) and of multinucleated giant cells (MGCs) was significantly lower in patients who had been treated with zidovudine than in those who had never had zidovudine. The yearly incidence of HIVE/HIVL increased between 1982 and 1987 probably due to improved survival. It decreased between 1987 and 1990 while the percentage of patients treated with zidovudine increased concomitantly. In patients whose treatment was stopped, in 1989 and 1990 most had MGCs and HIV/HIVL. In 1991 and 1992 this incidence decreased markedly, co-inciding with the introduction of didoxyoxime therapy.

We conclude that zidovudine treatment significantly reduces the occurrence of productive HIV infection of the brain in AIDS. Discontinuing zidovudine may favour the occurrence of HIV encephalitis. Substitution therapy with didoxyoxime also appears to protect against HIV-specific brain pathology.
The finding of normal visual ERPs with progressive P300 changes suggests that the earliest subcortical involvement is of the hippocampal circuitry rather than being diffuse.
HIV and the brain: contrasting patterns and virus load in homosexuals and drug abusers

Autopsies have been conducted in a cohort of human immunodeficiency virus (HIV1) positive individuals in Edinburgh who died with full-blown AIDS (Group A. drug misusers, n=39; Group B, homosexuals, n=27). Neuropathology findings were correlated with quantitative viral load, estimated by competitive polymerase chain reaction (PCR), in tissue retained from all areas of the central nervous system (CNS), and in blood. Drug misusers were found to have a high incidence of HIV encephalitis (60%), associated with high viral load, whereas homosexuals had a much lower incidence of HIV encephalitis (15%), and often with a smaller virus load. Both grey and white matter were PCR positive, correlating well with p24 immunocytochemical positivity. Primary cerebral lymphoma was more common in homosexuals (26%) than in drug abusers (3%). Opportunistic infection and CNS lymphoma were frequently present in the brain without co-existing HIV infection. HIV encephalitis correlated well with dementia. Zidovudine was prescribed, and taken, by both groups.

Quantitative PCR for detecting HIV-1 DNA in frozen and formalin fixed brain tissue

We were previously able to detect HIV-1 DNA by PCR from formalin-fixed and frozen brain tissue. We have extended this work by amplifying HIV DNA using semi-quantitative PCR in 13 AIDS cases (9 antigen p24+ve and 4— ). DNA extracted from frontal lobe tissue was amplified using HIV-1 pol gene digoxigenin-labelled primers and detected by chemiluminescence and densitometry. Cloned standards were amplified in parallel for quantification. HIV DNA levels detected in frozen tissue showed a good correlation with p24 antigen positivity and severity of histological diagnosis. However, in the formalin fixed material this correlation was less clear. This study showed: (i) HIV-1 DNA levels can be estimated in frozen and formalin fixed brain by semi-quantitative PCR; (ii) digoxigenin incorporation, chemiluminescence and densitometry gave reliable results when used for this purpose; (iii) p24+ve and —ve patients formed two well separated groups regarding HIV DNA levels in cortex.

In situ hybridization (ISH) study of changes in AMPA receptors in the cerebellum associated with HIV

There is in vitro evidence that glutamate is involved in HIV associated neuronal loss. We have investigated whether cerebellar glutamate AMPA receptor mRNA expression was altered in patients who died of AIDS. Fresh-frozen cerebellar sections from seven controls and 13 AIDS patients were examined by ISH using 35S labelled oligonucleotide antisense and sense probes to the mRNA flip and flop isoforms of the AMPA receptor GluR-A (Sommer et al., Science 1990, 249, 1580–5). There were no differences in the granular cell layer for flip (controls. 610 ± 377 nCi/g, and HIV. 560 ± 288 nCi/g); or flop isoforms (375 ± 273 nCi/g and 238 ± 129 nCi/g respectively). Purkinje cell density was unchanged, 10.7 ± 1.1 cells/mm, for controls, and 9.5 ± 1.6 for the HIV group. However, the density of flop mRNA expressing Purkinje cells was 7.6 ± 2.5 cells/mm for controls and 3.3 ± 1.6 in the HIV group. A reduction of over 60% (P=0.002. Student's t-test). Flip was not localized to Purkinje cells. This study demonstrates over a 60% decrease in the expression of mRNA AMPA flop isoform in cerebellar Purkinje cells, which is not accompanied by neuronal loss. Alteration of these isoforms may disrupt neuronal function.

In situ hybridization (ISH) study of changes in AMPA receptors in the cerebellum associated with HIV
Neuropathological variations in familial prion disease

**Introduction:** The spongiform encephalopathies (prion diseases) may exhibit remarkable histological heterogeneity. We report the atypical neuropathological findings in two brothers who were members of a large family with a previously characterized autosomal dominant prion disease involving a 144 base pair gene insertion.

**Methods:** A range of immunohistochemical stains including GFAP and prion protein were applied to the brain sections in these two cases. This was in addition to routine stains.

**Results:** The brains of other members of this family have shown typical histological features including spongiosis, neuronal loss, and astrocytosis. These features were, however, not all prominent in the brains of these brothers. Instead, the only significant histological findings were irregular deposits of prion protein in the molecular layer of the cerebellum.

**Conclusions:** This family illustrates the phenotypic heterogeneity of prion diseases and draws attention to the fact that for definitive diagnosis it may be necessary to employ immunohistochemical stains for prion protein.

Differential neuropathology in cynomolgus macaques infected with HIV-2 and four SIVs

**Introduction:** The pathogenesis of neuronal damage in HIV infection remains uncertain but certain subtypes of virus are associated with CNS disease. The aim of this study was to investigate infection with a variety of viruses in a macaque model of AIDS. **Materials and methods:** The following viruses were used: HIV-2 (which does not cause significant immunosuppression in monkeys; \( n = 6 \)). SIVmac251 (a macrophage-tropic virus; \( n = 20 \)). SIVmac239 (a lymphocytotropic virus; \( n = 7 \)). SIVgs (a virulent molecular clone; \( n = 9 \)) and SIVcB (a ‘non-virulent’ molecular clone; \( n = 11 \)). Sixteen controls were examined. **Results:** SIVmac251, SIVgs and SIVcB induced neuronal atrophy and increased HLA-DR and GFAP expression. SIVmac239 infection led to no neuronal atrophy but gliosis was present. HIV-2 produced neuronal atrophy but little, if any, gliosis. **Conclusions:** These data demonstrate a dissociation between neuronal atrophy and immuno-suppression and that a lymphocytotropic virus causes gliosis but not neuronal atrophy. These observations will facilitate separation of different components of neuropathogenesis in AIDS.

MHC-II, cytokines in the brains of asymptomatic HIV-1 positive individuals

**Introduction:** Cytokines have found favour to explain the pathogenesis of HIV encephalitis. As in the brains of some HIV-positive pre-AIDS patients we found pathological abnormalities as well as presence of HIV-1 DNA, we undertook a study to show presence of a condition of immune reactivity with possible production of cytokines. **Materials and methods:** Thirty-six asymptomatic HIV-positive pre-AIDS individuals and controls were studied.
by morphological, immunohistochemistry (for MHC II and cytokines) and PCR methods. **Results:** Results showed PCR evidence of HIV proviral DNA in 17/36 brains from HIV positive pre-AIDS cases: MHC class II and cytokines could be detected predominantly within white matter even at this early stage. **Conclusion:** The data demonstrated that the state of immune activation described in AIDS is already present at the pre-AIDS stage and suggest that the presence of cytokines may already trigger the cascade of events leading to brain damage.

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**T-cell-rich post-transplant lymphoproliferative disorder (PTLD).** Radiological, histological and molecular characteristics

**Introduction:** Nearly one-quarter of PTLD involve the CNS. yet correlated clinical, pathological and radiological data remain sparse. The present case of a 12-year-old whose biopsy-proven PTLD has had 2 year remission on-withdrawal of immunosuppression is therefore an informative exception. **Methods:** Conventional histology. Immunocytochemistry (a-CD20. -CD3. -CD68. -CD45RO). PCR amplification: Immunoglobulin heavy-chain [IgH], T-cell antigen-receptor γ chain [TCRγ], EBV BamH1 W fragment from paraffin section: androgen-receptor trinucleotide-repeat segment from sections, donor and recipient lymphocytes. **Case report:** The patient presented 5 months post-transplant with diplopia. MRI showed multiple homogeneously contrast-enhancing lesions. Biopsy revealed numerous benign T-lymphocytes swarming around a small nidus of larger B-cells. PCR demonstrated clonal IgH bands and EBV genome, but no TCRγ clonality or cells of donor origin. **Conclusions:** (i) T-cell-rich PTLD may be a new member of this histopathological family, (ii) PCR is a powerful tool for characterizing PTLD.


Molecular pathology of familial amyotrophic lateral sclerosis (ALS) with a mutation of SOD 1

**Introduction:** We studied the molecular pathology of a patient with autosomal-dominant familial ALS (FALS) associated with a mutation of the SOD 1 gene at codon 48→His. **Methods:** A 54-year-old woman with FALS died 9 months after onset of progressive limb and ventilatory muscle weakness. Brain and spinal cord was processed for standard histopathology, immunocytochemistry and electron microscopy (EM). **Results:** There was extensive loss of lower motor neurons, but little evidence of corticospinal tract damage. Lower motor neurons showed hyaline inclusions and acromasia, and EM revealed bundles of intraneuronal filaments, but most of these were not labelled by antibodies against NFs at light microscope level. Rare and atypical ubiquitin-immunoreactive inclusions were present. SOD 1 and SOD 2 distribution was not different to controls. Vacuolation was present in one lumbar neuron. Some large pyramidal cells of the motor cortex showed accumulations of phosphorylated NFs. **Conclusions:** In comparison with sporadic ALS, the molecular pathology of this case with a SOD 1 gene mutation showed atypical features. Changes included prominent NF accumulations in upper and probably in lower motor neurons, and neuronal vacuolations. NF pathology may contribute to neuronal damage as a secondary phenomenon.
EXPRSSSION OF MAJOR HISTOCOMPATIBILITY COMPLEX (MHC) CLASS II AND CYTOKINES IN BRAINS OF ASYMPTOMATIC AND SYMPTOMATIC HIV-1 POSITIVE PATIENTS: CORRELATION WITH DETECTION OF HIV-1 DNA

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Among the mechanisms proposed to explain the pathogenesis of HIV encephalitis, a cytokine-mediated action has found most favour. Elevated expression of various cytokines, thought to be neurotoxic, has been found in AIDS patients. As a previous study had demonstrated the presence of HIV proviral DNA in brain of HIV positive non-AIDS patients, we undertook this investigation by morphological, immunohistochemical and PCR methods to detect in brains of the same group of individuals the expression of MHC II, the presence of HIV-1 proviral DNA and of the cytokines TNF-α, IL-1α, interleukins-1β, 6, 8, and monocyte chemoattractant protein-1 (MCP-1).

The study included 36 asymptomatic HIV-1 positive patients and results were compared with those of AIDS patients either affected by HIV encephalitis (n=8) or exempt from neurological changes (n=10) and with normal controls (n=8). Results show that: HIV proviral DNA could be detected by PCR in 17/36 brains from HIV positive pre-AIDS cases; most (15/17) of PCR positive brains showed minimal to severe expression of MHC II; cytokines could be detected predominantly within white matter at this early stage. Results demonstrated that the state of immune activation is already present at the pre-AIDS stage and suggest that cytokines may already trigger the cascade of events leading to brain damage.

DETECTION OF EBV DNA IN CSF AND ITS CORRELATION WITH NERVOUS SYSTEM ENERGY PRODUCTION IN HIV-INFECTED INDIVIDUALS

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Cerebrospinal fluid (CSF) was examined for the presence of EBV DNA in 39 HIV-infected individuals undergoing diagnostic lumbar puncture (LP). A nested polymerase chain reaction was used with primers located in the internal repeats of the EBV genome. Results were correlated with clinical, radiological and histological investigations. Seventeen patients had a diagnosis of lymphocytic meningitis (CMN lymphoma), 2 CNS and systemic lymphomas, 4 systemic lymphomas. EBV DNA was detected in the CSF asymptomatic from 18 patients, including all 7 patients with CNS lymphoma, both patients with CNS and systemic lymphoma and 9 patients with no lymphoma at the time of LP. A further patient with systemic lymphoma had detectable EBV DNA in the CSF cellular pellet. Two patients with detectable EBV DNA in CSF but no lymphoma at the time of LP subsequently developed systemic and CNS lymphomas 15 and 19 weeks later. In summary, a diagnosis of CNS lymphoma was strongly associated with the presence of CSF EBV DNA. However, not all patients with detectable CSF EBV DNA had evidence of lymphoma emphasizing the need for caution when interpreting a positive result. This latter group of patients, however, are at risk of developing lymphoma.

PROGRAMMED CELL DEATH IN BRAINS OF HIV-1 POSITIVE AIDS AND PRE-AIDS INDIVIDUALS

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Neuropathological studies revealed that brains of HIV-1 infected AIDS patients show typical encephalitis and neuronal loss. More recently, this neuronal cell loss has been thought to take place via programmed cell death (apoptosis) which has been demonstrated by in situ end labelling (ISEL) technique.

In this study we investigated 54 brains of HIV-1 positive patients by ISEL technique. Our aim was to ascertain whether the presence of apoptosis was also present in brains at the asymptomatic stage. Of these, 10 were HIV encephalitis (HIVE); 8 were AIDS without neurological disorders and 36 belonged to HIV-1 positive pre-AIDS patients.

Apoptotic cells were detected in 6/10 HIVE, 1/8 AIDS without central nervous system (CNS) disease and 4/56 asymptomatic individuals. The difference between AIDS and pre-AIDS cases was that, in the latter, apoptotic cells were found in the white matter in all 4 cases whilst only 2/4 showed apoptotic neurons. The presence of apoptotic cells in a number, albeit small, of brains of HIV-1 positive pre-AIDS individuals, combined with abnormalities previously described in the same group of patients gives further support to the opinion that brain damage is already taking place during the early stages of HIV infection.

MUSCLE INVOLVEMENT IN HIV-INFECTED PATIENTS IS ASSOCIATED WITH MARKED SELENIUM DEFICIENCY

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Objectives. To evaluate the possible implication of selenium and vitamin E deficiencies in the occurrence of muscle involvement during HIV infection.

Background. Oxidative stress is implicated in tissue damage during HIV infection. Micronutrient deficiencies have long been recognized in HIV infected patients and involve vitamins and trace elements such as zinc, iron, and selenium. Selenium is a component of glutathione peroxidase, a major antioxidant agent; selenium deficiency, alone or in association with a deficiency in vitamin E, another antioxidant, is known to induce a skeletal muscle disorder manifesting by pain and proximal weakness (J Parent Ent Nutr 1985; 9:58-60; Am J Clin Nutr 1986; 43:549-54).

Methods. We studied serum levels of selenium and vitamin E (alpha-tocopherol) in 20 patients with muscular symptoms and 20 patients matched for CD4 count without muscular symptoms. Myopathic patients had widespread myopathy (8 patients); HIV polymyositis (6 patients), HIV-wasting syndrome (1 patient), and myopathies of unknown origin (5 patients).

Results. Selenium status (mean ± SE: 95.1 ± 0.04 µmol/L ± 0.05 vs. 95.6 ± 0.05 µmol/L ± 0.05). Student's t test: P = 0.005, but not vitamin E status (21.1 ± 0.6 µmol/L ± 2.0 vs. 21.6 ± 1.2, NS) was significantly impaired in patients with muscular symptoms. There was no correlation between selenium levels and the type of myopathy.

Conclusion. Since it is likely that selenium deficiency is not secondary to muscle damage, these results suggest that selenium deficiency might act as a co-factor of muscle involvement in HIV-infected patients, conceivably allowing oxidative stress in muscle tissue.