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Effects of human immunodeficiency virus encephalitis and drug abuse on the B lymphocyte population of the brain

Iain C Anthony,[1] Dorothy H Crawford,[2] and Jeanne E Bell[1]

[1]Neuropathology Unit, University of Edinburgh, Western General Hospital, Edinburgh, United Kingdom; [2]Basic and Clinical Virology Laboratory, University of Edinburgh, Royal (Dick) Veterinary School, Edinburgh, United Kingdom

We aimed to assess the effects of human immunodeficiency virus (HIV) encephalitis (HIVE) on the B-lymphocyte population of the brain. We also tested the effects of intravenous opiate drug abuse because this is a major risk factor for infection, with known immunosuppressive properties. Immunohistochemistry was used to identify B lymphocytes in the brains of clinically well-characterized HIV-negative drug abusers, individuals with HIVE, and, for comparison, HIV-negative individuals with encephalitis. Perivascular and parenchymal B lymphocytes were studied in 11 regions of each brain. We found that despite a small apparent rise, the abuse of opiate drugs had no significant effect on the B-lymphocyte population of the brain. Individuals with HIVE were found to have a greater number of B lymphocytes in brain tissue than individuals with acquired immunodeficiency syndrome (AIDS) who had no central nervous system (CNS) pathology. However, in comparison to nonimmunocompromised individuals with encephalitis, the B-lymphocyte population of HIVE brains was greatly reduced. We suggest that this latter finding may be linked to declining CD4 T-lymphocyte levels in end-stage AIDS, and that CD4 T lymphocytes may be required for efficient entry of B lymphocytes to the CNS. The brain B-lymphocyte population correlated well with CD4 T-lymphocyte level in the blood, in cases with viral encephalitis. These findings suggest that systemic immune competence is required to mount a full B-lymphocyte response to viral CNS infections. Furthermore, we suggest that CD4 T lymphocytes may play a key role in the humoral immune response to viral infection of the brain.
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Keywords: B lymphocytes; brain; drug abuse; HIVE

Introduction

Human immunodeficiency virus (HIV) enters the brain early in infection (Hughes *et al*, 1997; An *et al*, 1999) and can induce significant neuropathological changes, including HIV encephalitis (HIVE) in the late stages of infection. The introduction of highly active antiretroviral therapy (HAART) has resulted in dramatic improvements in the course and prognosis of HIV-related disorders, with infected individuals now living much longer. HIV/acquired immune deficiency syndrome (AIDS) has become a chronic disease and AIDS-defining illnesses are no longer the major cause of death in western countries; instead, patients are just as likely to die from non–HIV-related diseases or events, including hepatitis B– or hepatitis C–associated cirrhosis, suicide, drug overdose, cardiac events, and non–HIV-related malignancies (Bonnet *et al*, 2002). As a consequence of more effective treatment, the prevalence of HIV

Address correspondence to Professor Jeanne E. Bell, Department of Pathology (Neuropathology), University of Edinburgh, Alexander Donald Building, Western General Hospital, Edinburgh, EH4 2XU, United Kingdom. E-mail: Jeanne.Bell@ed.ac.uk

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dementia and HIVE declined initially, although these disorders have not disappeared. However, in recent years several reports have suggested that the prevalence of HIVE is again increasing (Gray *et al*, 2003; Masliah *et al*, 2000). In addition to the recent resurgence in the numbers of cases demonstrating HIVE at autopsy, new forms of HIVE are now beginning to be described, including severe leukoencephalopathy with intense perivascular macrophage and lymphocyte infiltration, perhaps due to an exaggerated response from the newly reconstituted immune system, as well as chronic "burnt out" forms of HIVE similar to varicella-zoster virus encephalitis, in long-term survivors (Gray *et al*, 2003). The factors that lead to the emergence of HIVE are not clearly understood, although declining T-lymphocyte responses probably play an important role. T lymphocytes have been well studied in the central nervous system (CNS) during HIV infection with several studies showing increased numbers of T lymphocytes in patients with presymptomatic HIV infection (Bell *et al*, 1993; Gray *et al*, 1996; Tomlinson *et al*, 1999; Anthony *et al*, 2003). In contrast to T lymphocytes, few studies have focused on the prevalence of B lymphocytes in the brain during HIV infection, despite the central role of systemic B lymphocytes in anti-HIV antibody production, and consistent reports both of HIV and of antiviral antibodies in the cerebrospinal fluid (CSF) throughout the infection (Mergener *et al*, 1987; Kaiser *et al*, 1989). Several animal models of viral encephalitis have demonstrated the importance of B lymphocytes in the clearance of other viruses from the CNS (Hatalski *et al*, 1998a, 1998b; Tyor *et al*, 1989). Furthermore, Knopf *et al* (1998) have demonstrated in rats that intrathecal antibody production to previously unseen antigen can occur within the brain, despite the presence of an intact blood-brain barrier (BBB). If HIV is present in the CNS from an early time point in infection and anti-HIV antibodies are present within both the serum and CSF of infected individuals throughout the course of infection, then the question arises as to whether B lymphocytes play a role in the development of immune reaction to HIVE. There are at present no data in the literature to answer this question.

In a study designed to provide data on B-lymphocyte prevalence in the brains of HIV-infected individuals, we selected relevant cases from the Edinburgh HIV Brain Bank. In Edinburgh, a high proportion of HIV-infected individuals acquired infection through shared equipment used for the intravenous injection of drugs (Peutherer *et al*, 1985). Before March 1997, 1176 HIV infections had been reported in Edinburgh and the surrounding area, of which 52% were linked to injecting drug use (Davies *et al*, 1985). In this cohort at least, HIVE has proved significantly more common in drug users compared with homosexual men (59% *versus* 15%, prior to effective treatment) (Bell *et al*, 1996), suggesting perhaps that the combination of drug abuse and HIV infection may have additive consequences for the CNS. It was therefore considered important to also establish the effects of drug abuse, independent of HIV infection, on the B-lymphocyte population of the brain. Drug abuse is not associated with an increase in T lymphocytes in the brain (Tomlinson *et al*, 1999).

We have shown recently that activated (CD23 positive) B lymphocytes are present in the parenchyma of normal brain in small numbers (Anthony *et al*, 2003). During presymptomatic HIV infection, the number of B lymphocytes was found to increase significantly in the perivascular compartment but not in the brain parenchyma. Our previous study was designed to assess if increased B-lymphocyte prevalence in the brain of HIV-infected individuals was a predisposing factor in the development of AIDS-associated primary CNS lymphoma. We established that the brains of AIDS subjects with PCNSL contained few or no B lymphocytes outside the area of malignancy, closely similar to AIDS brains with no overt CNS pathology, which also proved to be almost devoid of B lymphocytes. These findings suggested that Primary Central Nervous System Lymphoma (PCNSL) does not develop in a setting of HIV-associated increased B-lymphocyte trafficking into the CNS.

However, there have been no previous studies, to our knowledge, directed to B-lymphocyte responses in the brains of subjects with HIVE; nor have the effects of drug abuse on the CNS B lymphocyte population been investigated. Our hypothesis was that the number of B lymphocytes might be increased in the former group, but not in the latter.

Results

Figure 1 shows the prevalence of HIVE in the Edinburgh cohort from 1989 to 2002. In recent years, there has been decline in the number of AIDS autopsy cases displaying evidence of HIVE in Edinburgh. However, in common with other reports, we are seeing a re-emergence of HIVE within the last 2 years.

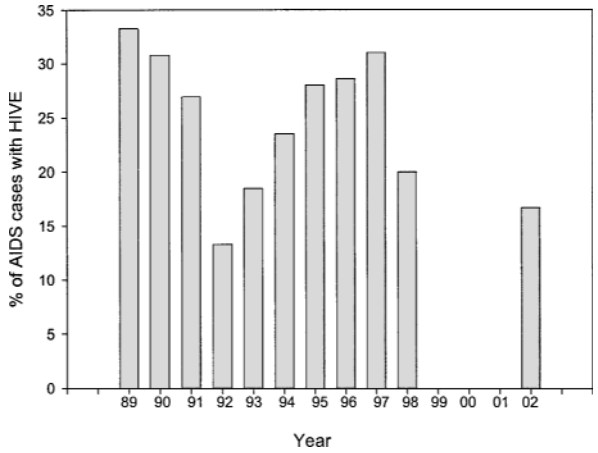

Figure 1 Prevalence of HIVE in the Edinburgh cohort.

Table 1 Quantitation of brain parenchymal and perivascular B lymphocytes

Group	*Age (years)/Gender*	*Intravenous drug abusers*	*Average number of CNS parenchymal B cells/cm²*	*Average number of CNS perivascular B cells/cm²*	*CD4 blood count (cells/mm³)*
HIV negative drug users	31M	Yes	0.96	0	NA
	34M	Yes	0.293	0	NA
	20F	Yes	0.05	0	NA
	20M	Yes	0.1	0	NA
	32M	Yes	0.174	0.12	NA
AIDS patients with HIVE	59M	No	0.249	0.829	NA
	28M	No	0.029	0.058	60
	35F	Yes	0	0.060	23
	28M	Yes	0.054	0.543	90
	41M	Yes	0.064	0.043	10
	49F	Yes	0.055	0.300	87
Non-HIV viral encephalitis	57M	No	3.183	12.561	NA
	24M	No	1.378	24.671	NA
	68F	No	14.851	155.778	NA
Case A: (Non-HIV viral encephalitis in pre-AIDS individual)	33F	Yes	1.930	39.109	240
*Normal subjects	N = 7 Average age 24 (5M, 2F)	No	0.112 (group average)	0 (group average)	NA
*Pre-symptomatic HIV infected drug users	N = 6 Average age 32 (6M)	Yes	0.417 (group average)	0.904 (group average)	259 (group average)
*AIDS patients with no significant CNS pathology					
	N = 4 Average age 39 (3M, 1F)	No	0.010 (group average)	0.006 (group average)	27 (group average)
	34M	Yes	0	0	4

*Details for individual cases in these groups can be viewed in Anthony *et al*, 2003.

Table 1 summarizes the results for anti-CD20 from each patient group. Results using the anti-CD79α antibody were not significantly different from those obtained using anti-CD20, and therefore the data for anti-CD79α are not presented here ($P = .6$). Data from normal control cases, presymptomatic HIV-positive drug users, and AIDS cases with no CNS pathology have been published previously, but are included here for reference and comparison with the new data presented above (Anthony *et al*, 2003).

Examination of the brains of five HIV-negative drug abusers revealed a small rise in the number of parenchymal B lymphocytes compared to normal controls, although this increase was not statistically significant ($P = .181$) (see Figure 2a, b and Table 1 for data). Perivascular B lymphocytes did not differ between the groups ($P = .255$). As no significant difference was detected between these two groups, we conclude that drug use *per se* has no influence on the trafficking of B lymphocytes into the CNS, and therefore it was legitimate to include both drug abusers and non–drug abusers in the 'AIDS with HIVE' group.

Examination of six AIDS brains with HIVE showed no significant differences between the number of B lymphocytes found in the brain parenchyma compared to either normal controls or to AIDS patients with no CNS pathology (see Figure 2a and Table 1). Conversely a significant increase was noted in the perivascular brain compartment of HIVE AIDS cases when compared to normal controls (Figure 2b and Table 1) ($P = .03$). Although an increase in

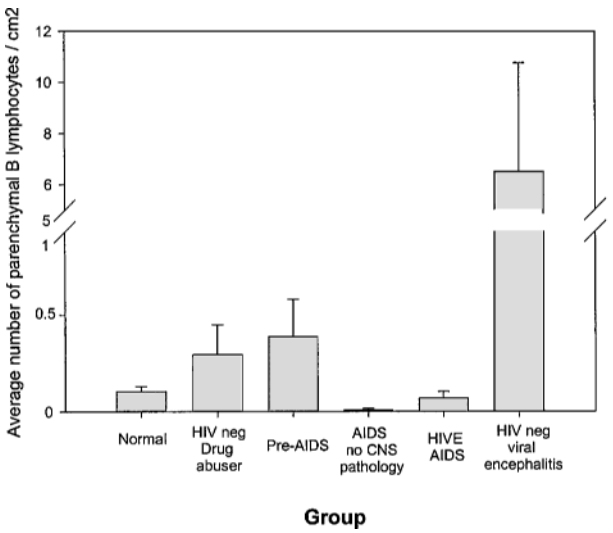

Figure 2a Quantitation of Parenchymal CD20 +ve B lymphocytes.

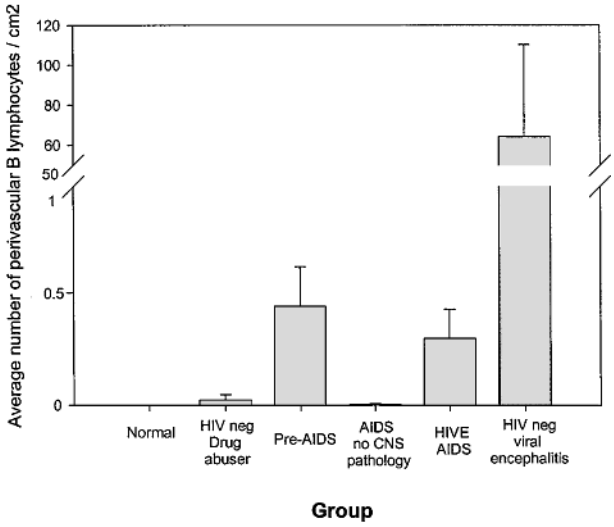

Figure 2b Quantitation of Perivascular CD20 +ve B lymphocytes.

perivascular B lymphocytes was also noted between HIVE cases and AIDS cases with no CNS pathology, this difference did not reach statistical significance ($P = .07$).

In comparison to non-HIV encephalitis, Figure 2a, b shows that HIVE induces only a fraction of the B-lymphocyte response seen in non-HIV encephalitis in terms of both parenchymal and perivascular B lymphocytes. Brains from immunocompetent patients with non-HIV encephalitis showed an average of 6.47 B lymphocytes/cm^2 in the brain parenchyma in comparison to 0.075 B lymphocytes/cm^2 in HIVE AIDS brains (Table 1). In the perivascular compartment, immunocompetent patients with encephalitis showed an average of 64.32 cells/cm^2 compared to 0.306 B lymphocytes/cm^2 in HIVE AIDS brains. No statistics were performed on these data as only three HIV-negative viral encephalitis cases were available for study.

Figure 3a, b show the average CD4 count for AIDS cases with HIVE, and non-HIV encephalitis cases compared to the average number of B lymphocytes

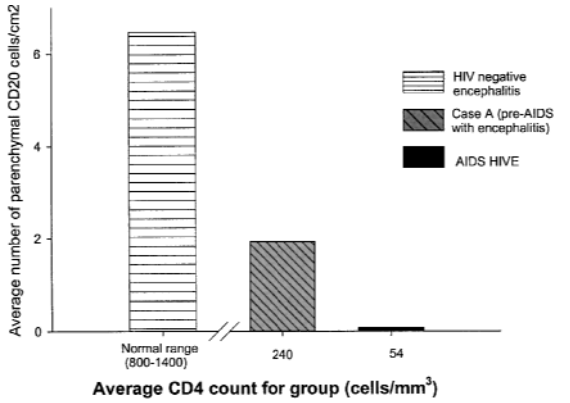

Figure 3a CD4 count versus brain parenchymal B lymphocytes in cases with viral encephalitis.

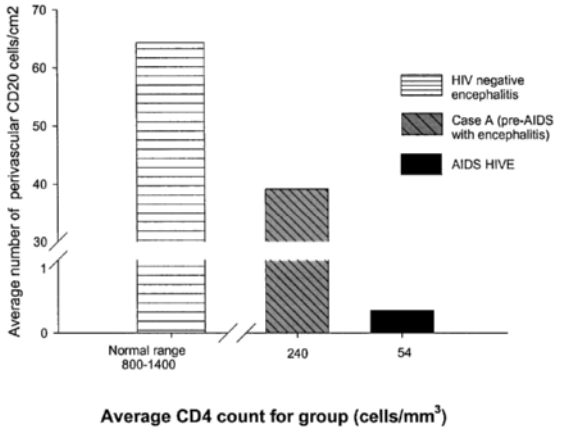

Figure 3b CD4 count versus brain perivascular B lymphocytes in cases with viral encephalitis.

in the brain parenchyma (Figure 3a) and perivascular region (Figure 3b). Figure 3a, b also show data from case A, a presymptomatic drug abuser, excluded from analysis with the presymptomatic group because there was pathological evidence of non-HIV viral encephalitis. This graph suggests that in cases with encephalitis, there is a correlation between systemic CD4 counts and the number of B lymphocytes in both the brain parenchyma and perivascular spaces. Figure 4 shows a comparison of perivascular B lymphocyte infiltrations in (a) pre-AIDS brains, (b) pre-AIDS brain with viral encephalitis, (c) HIVE, and (d) nonimmunocompromised viral encephalitis.

B lymphocytes were located in all 11 regions of the brain studied in all cases within each group. No preferential homing to any region of the brain was noted, so that with the following exceptions, B-lymphocyte counts showed very little variation around the brain. The exceptions to this are case A and the nonimmunocompromised group with encephalitis. In these individuals, there were localized areas within most sections studied that contained more concentrated infiltrations of B lymphocytes (Figure 4b, d). However, these focal collections were found in most regions of the brain, and within a background of generally increased numbers of CNS B lymphocytes. In addition to the infiltrating B lymphocytes, significant numbers of T lymphocytes were also present and were predominantly of CD8 phenotype.

It is not possible to comment on any positive gender differences in these results because the majority of our subjects were male.

Discussion

We have demonstrated previously that, contrary to widely held perceptions, B lymphocytes are present in the parenchyma of the normal human brain in very low numbers (Anthony *et al*, 2003). In HIV-positive presymptomatic drug users, a small increase was noted in the parenchyma, but there was a significant

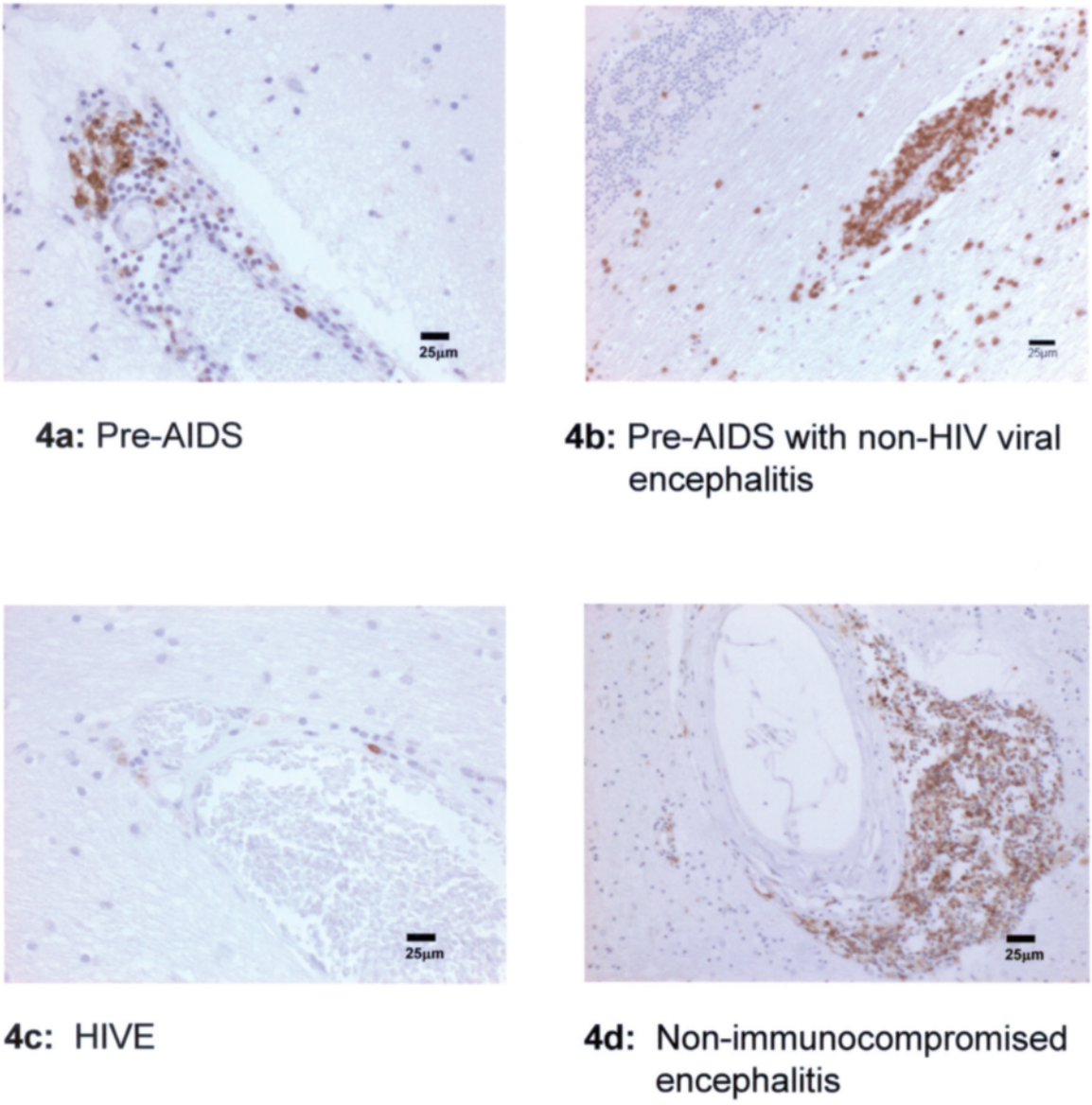

Figure 4 Comparison of brain perivascular (CD20-positive) B-lymphocyte infiltration in different patient groups. (**a**) pre-AIDS; (**b**) pre-AIDS with non-HIV viral encephalitis; (**c**) HIVE; (**d**) nonimmunocompromised encephalitis.

increase in perivascular B-lymphocytes in comparison to normal controls. We show in the present study that the abuse of intravenous drugs, in particular opiates, also leads to a small but not statistically significant increase in the B-lymphocyte population of the brain. Thus the increase noted in the pre-symptomatic HIV group is unlikely to be due to the effects of drug abuse alone, although a synergistic effect between drug use and HIV infection cannot be ruled out. Gurwell *et al* (2001) have demonstrated the synergistic ability of drugs and HIV to cause neuronal damage. They showed that acute opioid exposure *in vitro* exacerbated the neurodegenerative effects of the HIV protein Tat in striatal neurones. However, it seems more likely that the B lymphocytes may be localizing in the perivascular compartment of the brain as a result of early entry of HIV across the BBB (Gray *et al*, 1996). It is possible that both B and T lymphocytes help to control viral replication in the CNS during the presymptomatic phase of infection. In addition, perivascular B lymphocytes may potentially contribute to the level of antibody in the CSF.

Having previously shown that the brain B-lymphocyte population increases in pre-AIDS and then decreases below normal levels as patients progress towards AIDS, in the absence of HIVE, we sought to determine the effects of productive HIV infection on the B-lymphocyte population of the CNS. We have postulated that a B-lymphocyte response would be present in the CNS of HIVE patients for three reasons. Firstly, systemic B-lymphocyte responses are preserved until the very late stages

of AIDS, unlike T-lymphocyte responses, and are capable of producing high levels of anti-HIV antibodies (Amadori *et al*, 1998). Secondly, B lymphocytes have been shown to enter the CNS in response to antigen and can be found in large numbers in non-HIV viral encephalitis, as well as in animal models of encephalitis (Hatalski *et al*, 1998a, 1998b; Tyor *et al*, 1989), and this expectation was confirmed by our results. Finally, the parenchyma of HIVE brains contains HIV antigens that B lymphocytes are capable of recognizing and that might be expected to be a potent attractant for B-lymphocyte migration into the brain (Budka *et al*, 1987; Chiodi *et al*, 1988; Weber *et al*, 1989). This study showed that HIVE cases contained on average 10-fold more parenchymal B lymphocytes than AIDS cases with no CNS pathology and 60-fold more perivascular B lymphocytes. Although neither of these reached statistical significance, the perivascular results were close to achieving significance ($P = .130$ and $P = .07$, respectively). HIVE cases within this study represented both pre- and post-HAART samples and we found no discernable difference between HIVE occurring in these two treatment settings.

In contrast to HIV-induced encephalitis, nonimmunocompromised brains showed a 86-fold increase in parenchymal B lymphocytes and a 210-fold increase in perivascular cells. A possible explanation for the decreased B lymphocyte response seen in HIVE is that CD4 T lymphocytes may be required to aid B lymphocytes' entry to the CNS. Tyor *et al* (1989) have suggested that, in mice, T lymphocytes are required for recruitment of B lymphocytes into the CNS in response to viral infection. Their study comparing B-lymphocyte responses to Sinbis virus encephalitis in BALB/c mice and athymic (nu/nu) mice confirmed the progressive failure of B-lymphocyte responses in the latter group. Our previous data (Anthony *et al*, 2003) demonstrates that B lymphocytes need to be activated in order to enter the CNS. The majority of B lymphocytes require signals from CD4 T lymphocytes for activation. Such activation occurs in the systemic compartment, indicating an "off-site" role for CD4 lymphocytes in B lymphocytes' entry to the brain. This function will fail in advancing HIV/AIDS. However, it should be noted that our data do not rule out a potential *in situ* role for T lymphocytes in addition to the proposed peripheral requirement, and the probability is that both functional roles of T lymphocytes are required for efficient B lymphocytes' entry to the CNS. Hyperactivation of B lymphocytes can occur during HIV/AIDS in the periphery; this is reported to be independent of T lymphocytes. The HIV protein TAT has been reported to activate B lymphocytes in culture (Huang *et al*, 1997) and gp120 is reputed to act as a B-cell superantigen for B lymphocytes (Muller and Kohler, 1997; Karray and Zouali, 1997). However, B-lymphocyte activation itself is probably not sufficient for B lymphocytes' entry to the CNS and other factors may be essential, for example chemokine expression and adhesion molecule expression on the BBB endothelium. In other words, immune competence is required for effective B-lymphocyte responses to acquired brain antigens. Collectively, these results suggest that despite the presence in the brain of antigens to which B lymphocytes are capable of responding, migration of these cells into the CNS is restricted by the immune deregulation seen in late-stage AIDS. This is supported by the data from the single presymptomatic case with non-HIV encephalitis (case A), which displayed results that fell between those of HIVE and nonimmunocompromised encephalitis.

The high levels of microglial activation seen in encephalitis (both HIV and non-HIV) may also play a role in providing necessary stimuli for B lymphocytes' migration into the brain. Microglial activation may lead to expression of chemoattractants, for example stromal cell derived factor-1 (SDF-1), which has the potential to attract B lymphocytes along with other immune cells into the brain (Ohtani *et al*, 1998). Drug use, in particular cocaine, may also have the potential to directly affect the expression of adhesion molecules at the BBB (Gan *et al*, 1999). Gan *et al* (1999) have shown that the use of cocaine stimulates expression of adhesion molecules, including intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1). However, it should be noted that the drug users in this study are not generally cocaine users and different drugs may have different effects on the BBB. Our study suggests that microglial factors alone are not sufficient to recruit significant numbers of B lymphocytes to the CNS in T lymphocyte–depleted individuals.

The data shown in Table 1 and Figure 3a, b also support a correlation between CD4 lymphocyte levels in the blood and the B-lymphocyte population of the brain within groups with encephalitis (Table 1), and between groups (Figure 3a, b). The data for the HIVE group show that perivascular or total B-lymphocyte numbers correlate better with the CD4 count than do parenchymal B lymphocytes.

In conclusion, we have shown that the abuse of intravenous drugs does not lead to a significant increase in the overall number of B lymphocytes found in the brain. In addition, we have shown that in AIDS patients with HIVE, the B-lymphocyte response is smaller than seen in immunocompetent individuals, and we suggest that this may be due to a lack of CD4 T-helper lymphocytes in the late stages of AIDS. CD4 T lymphocytes may be required for activation and subsequent efficient B lymphocytes' entry to the CNS. Thus presymptomatic HIV-positive individuals are still able to mount a B-lymphocyte response in the brain, focused on the perivascular spaces. This response may form a significant component of ongoing brain protection in HIV-positive patients treated with those drugs that are effective in maintaining T-cell numbers.

Material and methods

We selected a group of HIV-negative drug users ($n = 5$), a group of AIDS subjects with HIVE ($n = 6$), and three cases of viral encephalitis of non-HIV origin in subjects with normal immune function. Information on individual subjects, including history of drug abuse, age, sex, and HIV status is given in Table 1. The drug users within this study were all principally opiate users; heroin initially then methadone, and supplemented with dihydrocodeine, cannabis, diazepam, alcohol, and nicotine.

One further individual case was informative in the context of the present study. Case A, an HIV-positive drug user, displayed evidence at postmortem examination of p24-negative non-HIV viral encephalitis with viral inclusion bodies and typical heavy lymphocytic infiltrate (i.e., viral encephalitis occurring in an HIV-positive individual but which is not attributable to the presence of HIV within the brain), but had no evidence of AIDS-defining illness (female, age 32 years). This patient's CD4 count at death was 240/μl blood.

For essential comparative purposes, we reviewed previously published data on three further groups. These included normal control brains ($n = 7$), all of whom were age matched, had died as a result of accidents, and displayed no evidence of CNS disease; presymptomatic HIV-positive drug users ($n = 6$); and AIDS cases with no significant pathology ($n = 5$). These cases form part of the Edinburgh HIV Brain Bank and use of this resource in research is approved by the Lothian Ethics of Research Committee.

In each case, the brain was removed at autopsy and fixed intact in formalin for 3 to 12 weeks. Blocks were removed for histology from the frontal, parietal, and occipital lobes, central white matter, temporal hippocampus, basal ganglia, thalamus, mid brain, pons, medulla, and cerebellum. All samples were paraffin embedded and 5-μm sections were cut from each block. Sections were placed on superfrost slides (BDH) and stored at 37°C for 24 h. Sections were dewaxed in xylene and rinsed in alcohol in preparation for immunohistochemistry. The antibodies used to detect B lymphocytes were anti-CD20 and anti-CD79α (both from DAKO). In order to allow comparison of B lymphocytes with T lymphocytes, consecutive sections were stained with an anti-CD3 and anti-CD8 antibodies (both DAKO). Antigen retrieval pretreatments were used for all antibodies (microwave heating in 0.1 M citric acid, pH 6.0, for CD20; microwave heating in 0.1 M EDTA, pH 8.0, for CD79α, CD3, and CD8). Following pretreatments, sections were incubated in 3% hydrogen peroxide for 10 min, and then in normal rabbit serum for 10 min, followed by incubation with primary antibody for 30 min at room temperature (CD20 1/300, CD79α 1/75, CD3 1/100, CD8 1/20). The secondary antibody used for each primary was biotinylated rabbit anti-mouse (DAKO) diluted to 1/200 for 30 min. DAKO ABC (peroxidase) kit was used as a tertiary step, with diaminobenzidine (Vector) as the visualization agent. Sections were counterstained with hematoxylin, and pertex mounted.

Sections from the 11 named areas of the brain in each case were assessed with CD20 and CD79α antibodies. The numbers of parenchymal and perivascular B lymphocytes were quantified in each slide. Positive cells were sufficiently rare that subjective counting was deemed more accurate than automated image analysis. The number of cells per square centimetre in each 5-μm section was calculated from the total cell count in the entire section. Statistical analysis was applied to the results obtained for the parenchymal and perivascular B-lymphocyte counts from the different groups using the Student's *t* test.

All cases were examined and quantified blind to group status.

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