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Anomalies in Nef expression within the central nervous system of HIV-1 positive individuals/AIDS patients with or without AIDS dementia complex

Dale A McPhee[1,3], Alison L Greenway[1], Gavan Holloway[1,3], Karen Smith[2,3], Nick Deacon[2,3], Louise Pemberton[4] and Bruce J Brew[4,5]

[1]AIDS Cellular Biology and [2]Molecular Biology Units, [3]National Centre in HIV Virology Research, Macfarlane Burnet Centre for Medical Research, Melbourne and [4]Centre for Immunology, St Vincents Hospital, Sydney, [5]National Centre in HIV Epidemiology and Clinical Research, University of New South Wales, Sydney, Australia

In determining levels of expression of HIV-1 Nef protein within the central nervous system (CNS) we assessed antibody responses to the protein both peripherally and in CNS. Antibodies to Nef were not detected within the CNS despite detection of antibodies to both gp41 and Nef in peripheral blood and representative virus isolates derived from CNS and peripheral blood (PB) samples containing full length *nef* sequence and virus-infected cells expressing Nef protein. We conclude from this that expression of Nef within the CNS is such that little or no antibody production occurs and that these differences indicate that Nef protein may not be directly contributing to the AIDS dementia complex. Expression of Nef protein in PHA-activated peripheral blood mononuclear cells from CNS derived isolates was different to that of coincidental PB derived isolates in that partial surface expression was observed for the latter. The results suggest that antigenic presentation of Nef within the CNS is anomalous and that Nef protein expression, at least for the limited number of *in vitro* derived isolates tested, has a different localization pattern.

Keywords: human immunodeficiency virus; central nervous system; Nef; antibodies

Introduction

Infection with HIV is complicated by the development of a subcortical dementia termed the AIDS dementia complex. This is usually a feature of advanced HIV infection and almost invariably occurs at the time of waning immunity. In contrast to systemic HIV infection the dominant infected cell population within the central nervous system (CNS) appears to be microglial cells. Migration of HIV-infected monocytes across the blood brain barrier may also be contributing to the infection (Brew *et al*, 1995; Persidsky *et al*, 1997). Mechanisms of viral pathogenesis resulting from CNS infection are unclear and appear more complicated than just direct viral effects. Patients may have clinically severe ADC yet little in the way of a productive viral infection of the brain. Factors involved may be viral products (Sabatier *et al*, 1991; Werner *et al*, 1991), cytokine induction or interaction of several factors.

Nef protein is expressed early and in abundance during HIV-1 replication (Ranki *et al*, 1994, 1995). Overexpression of this protein relative to other viral proteins has been observed in HIV-infected astroglial cells and in brain biopsies of infected individuals particularly children (Brack-Werner *et al*, 1992; Tornatore *et al*, 1994; Saito *et al*, 1994). The potential overabundance of Nef protein expression in an infected astroglial cell population and early levels of expression in microglial cells within the CNS led us to investigate a possible link between Nef expression and ADC. HIV-1 isolates within the CNS of infected individuals

Correspondence: D McPhee, AIDS Cellular Biology Unit, Macfarlane Burnet Centre for Medical Research, PO Box 254, Fairfield, Victoria 3078, Australia
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may constitute a distinct subgroup of viruses (O'Brien, 1994). Certainly, there appear to be a predominance of NSI or macrophage tropic isolates within the CNS (Brew *et al*, 1996). However, little is known about levels of Nef protein expression within CNS apart from immunohistochemical studies (Saito *et al*, 1994; Ranki *et al*, 1995, Wiley *et al*, 1996; Brack-Werner *et al*, 1997; Wiley and Aichim, 1997).

We assessed patients for an antibody response against HIV infection *in vivo* by screening for antibodies against a universally recognized epitope in envelope protein gp41 (Cumming *et al*, 1990; Wilson *et al*, 1991) and for antibodies against Nef protein. The results proved quite startling in that whilst there were antibodies against the HIV-1 Nef protein in peripheral blood (PB), there were none detected in coincident CSF samples. We were able to compare virus isolations made from five coincident pairs from PB/CSF samples and sequenced clones of *nef* derived from three of these. Although quite variable relative to a reference isolate all clones had the potential to encode a full length Nef protein. These data question how Nef is being presented antigenically within the CNS relative to that in peripheral blood.

Results

Clinical status of patients and virus isolation

Most patients studied were in advanced stages of virus infection/disease, as evidenced by CD4 cell counts, with ADC ranging from 0 through 3 in severity (Table 1). Virus was isolated from PB with most patients while the isolation rate was considerably lower (30% c/w 80%) from CSF. Additionally, virus was more readily isolated from patients with greater ADC severity, although not statistically significant. This left few paired isolates for direct comparison.

Antibodies against gp41 and Nef in plasma and CSF

A panel of paired samples of plasma and CSF were assembled from 27 HIV-1 infected patients who had defined ADC with severity ranging from 0 to 3 (Table 1). With only one exception (patient 15), all patients had serum antibodies to gp41 peptide (1 : 3000 to $>$1 : 100 000) and Nef protein (1 : 3000 to $>$1 : 10 000) by EIA (Table 2). Patient 15 had low titre CSF antibodies (1:300) to the gp41 peptide but undetectable gp41 antibodies in plasma ($<$1 : 300). Although there were antibodies reactive to gp41 in CSF there was no detectable response to Nef for all 27 patients ($<$1 : 100; Table 2). Differences in antibody titrations to gp41 compared with Nef in plasma ($<$0.3–10-fold) were generally less than 1 (11/20), where end points available. In contrast these differences were $>$30-fold (24/27) with CSF samples (Table 2).

Table 1 Patient background

Patient no.	*CD4 cells/mm^3*	*%CD4*	*ADC stage (0–4)*	*plasma prot (mg/dl)*	*CSF prot (mg/dl)*
1	186	31	0	ND	ND
2	8	1	0.5	6000	53
3	408	34	0	8100	44
4	0	0	2	4800	47
5	34	12	1	6800	77
6	16	5	0.5	6200	ND
7	141	8	0.5	9300	44
8	78	6	0.5	7000	ND
9	0	0	3	5400	5
10	21	6	0	7700	47
11	ND	ND	0.5	8900	33
12	52	10	3	6700	38
13	6	2	0.5	6300	161
14	414	23	1	8500	78
15	6	3	0.5	6600	83
16	5	1	2	ND	48
17	42	6	2	8200	ND
18	ND	ND	1	5100	63
19	39	8	2	7900	89
20	72	9	0	6800	158
21	8	1	0	7400	25
22	16	2	1	8300	ND
23	ND	ND	1	6200	157
24	42	7	3	ND	ND
25	0	0	0.5	ND	ND
26	ND	14	3	ND	ND
27	5	2	2	ND	ND

The negative antibody response to Nef within the CSF was consistent across the spectrum of ADC (Table 2). In support of this the total protein concentrations revealed that all the CSF samples appeared preserved for possible breakdown in the blood-brain barrier or sample contamination (Table 1). The complete absence of antibodies to Nef prompted us to test for evidence of Nef protein expression in virus isolates derived from patient samples.

Virus isolation and replication

Paired blood and CSF HIV-1 isolates from five ADC patients (ranging from 0.5 to 3 in severity) were tested for replication in PHA-PBMCs at equivalent multiplicities of infection (MOI). All viruses isolated were replication competent but CSF isolates from patients 16 and 24 replicated very poorly compared to coincident blood isolates (Figure 1a and b, respectively). We assessed virus infections of PBMC where there were detectable levels of virus antigen expression by IF using a polyclonal anti-HIV serum (data not shown) to assess Nef protein expression.

Determination of nef *sequences*

Paired blood and CNS HIV-1 isolates which replicated to high titre in PHA-PBMCs were sequenced to assess the *nef* open reading frame.

Table 2 EIA results

	Plasma[a]			*CSF*[a]		
Patient no.	*gp41 EIA*	nef *EIA*	nef/*gp41*	*gp41 EIA*	nef *EIA*	nef/*gp41*
1	>100 000	>10 000	ND	10 000	<100	>100
2	10 000	>10 000	<1	3000	<100	>30
3	10 000	>10 000	<1	300	<100	>3
4	10 000	>10 000	<1	10 000	<100	>100
5	>100 000	>10 000	ND	3000	<100	>30
6	10 000	>10 000	<1	3000	<100	>30
7	>100 000	>10 000	ND	>30 000	<100	>300
8	100 000	>10 000	<10	3000	<100	>30
9	100 000	>10 000	<10	10 000	<100	>100
10	>100 000	>3000	>3	>30 000	<100	>300
11	>100 000	>10 000	ND	>30 000	<100	>300
12	100 000	>10 000	<10	1000	<100	>10
13	100 000	10 000	10	3000	<100	>30
14	>100 000	>10 000	ND	>30 000	<100	>300
15	<300	10 000	<0.33	300	<100	>3
16*[0]	100 000	>10 000	<10	3000	<100	>30
17	100 000	>10 000	<10	3000	<100	>30
18	>100 000	10 000	>10	>30 000	<100	>300
19	100 000	>10 000	<10	3000	<100	>30
20	3000	>10 000	<1	3000	<100	>30
21	>100 000	>10 000	ND	3000	<100	>30
22	3000	>10 000	<1	10 000	<100	>100
23	3000	>10 000	<1	3000	<100	>30
24*	3000	>10 000	<1	>30 000	<100	>300
25*[×]	10 000	>10 000	<1	3000	<100	>30
26*[×0]	>100 000	>10 000	ND	10 000	<100	>100
27*[×0]	10 000	>10 000	<1	3000	<100	>30

*Cultured virus in PBMC ex PB and CSF. [0]Detection of Nef protein on *in vitro* culture in PBMC. [×]Sequence data for Nef gene ex PBMC PB and CSF *in vitro* culture. [a]Reciprocal titration determined as highest dilution >3 times the negative control.

The predicted Nef amino acid sequences were highly conserved for the three PB/CSF pairs tested with minor but consistent differences observed between the PB and coincident CSF isolate (Figure 2a). This indicated a different predominant genotype from a different location in an infected individual with additional differences between individuals. In the alignment of consensus Nef protein sequences for each pair with the reference strain NL4-3 (data not shown) the known functional motifs were highly conserved (myristylation motif, acidic charged motif, SH3 binding proline motif, polypurine tract, second proline motif (see Shugars *et al*, 1993); but there were some anomalies. For example all of the patient 25, 26 and 27 *nef* clones had extra cysteine residues at positions 8, 169 and 138, respectively. A study of the AIDS database (Myers *et al*, 1995) showed these cysteines to be quite rare, 4/65, 1/65 and 3/65, respectively. Sequences separated into three clear groups, corresponding to the three patient sources. Within each patient group, there was a consistent and significant clustering of CSF-derived sequences away from PBMC-derived sequences (Figure 2b). These results had bootstrap values that were consistently high using the algorithms maximum parsimony, neighbour joining and maximum likelihood. Thus, for the virus pairs assessed they were not only replication competent but encoded a predicted Nef protein with each series of clones being distinct, more so between patients.

Differences observed between the PB and CSF Nef protein sequences were not consistent between patients, indicating no obvious general selection for a particular *nef* sequence within PB or CSF. There were some changes in amino acid makeup from uncharged polar to basic (Q to R), uncharged polar to non-polar (Y to F), acidic to non-polar (D to A), and/or acidic to basic (E to K). The PB/CSF isolates from patient 27 showed the greatest differences among the paired isolates with eight changes in *nef* (Figure 2a). Additional to the predominant changes in amino acid makeup from PB to CSF there were single amino acid changes in single clones that may represent natural variability within an isolate and/or a reading error from the Taq polymerase in PCR amplification. The changes observed in all clones from one source clearly indicate different genotypes being present in PB and CSF at the time of virus isolation.

Expression of Nef protein in infected cells

Having identified competent virus that contained a full *nef* open reading frame we then assessed the ability of selected isolates (16, 26 and 27; Table 2) to express Nef protein in infected PHA-PBMCs. All

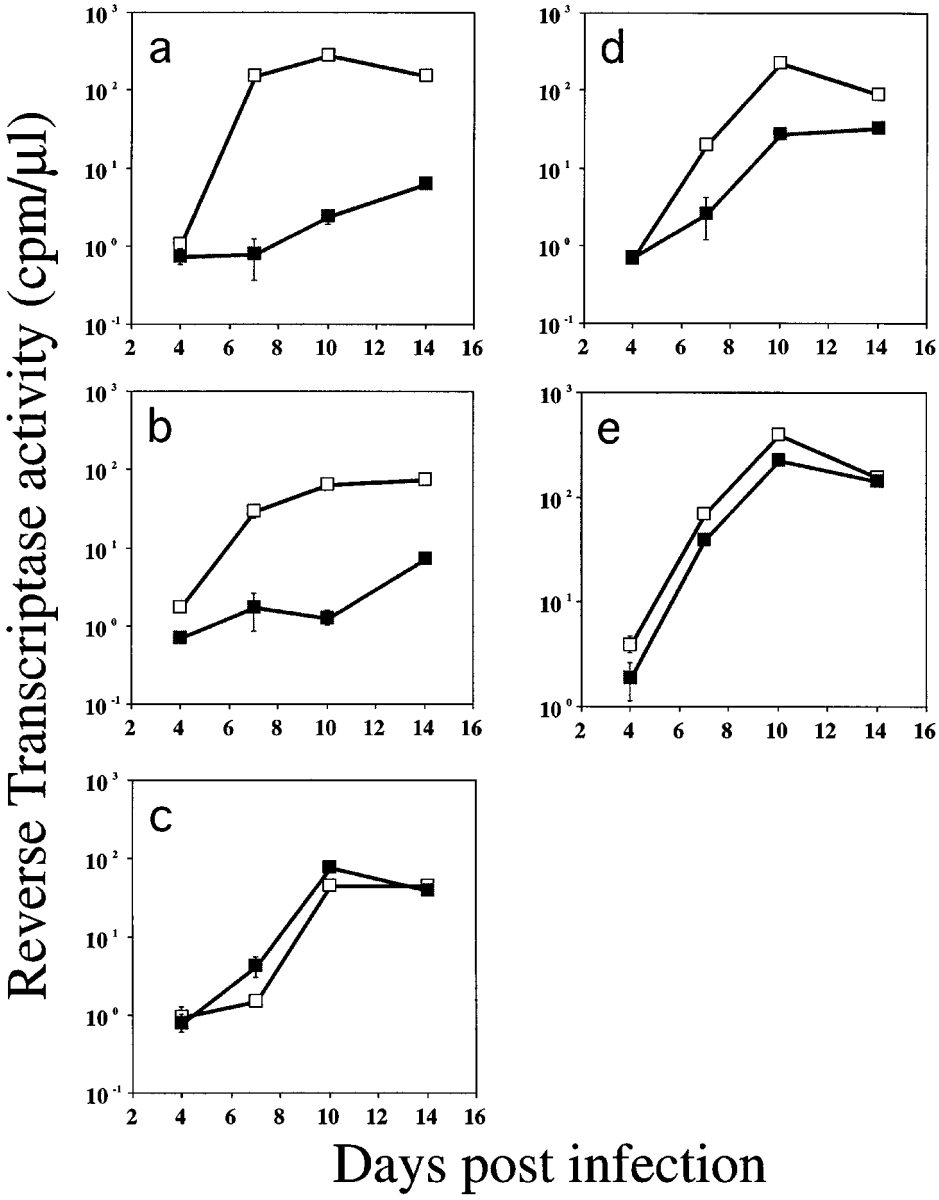

Figure 1 Replication of coincident PB and CSF isolates. PHA-PBMC were infected with five paired isolates at an moi of 0.001 $TCID_{50}$/cell. The production of virus from PB (open squares) or CSF (closed squares) isolates was assessed by cell-free reverse transcriptase assay for patients 16(a), 24(b), 25(c), 26(d) and 27 (e). Standard error bars are shown for all time points but are often obscured due to the point size.

three pairs of virus isolates were shown to express Nef protein using an N-terminal and C-terminal antibody with permeabilized virus-infected cells (Table 3; Figure 3). In contrast when non-permeabilized cells were examined the C-terminal antibody was not reactive at all and the N-terminal antibody was only positive for the PB isolate from each pair (Table 3; Figure 3). These data indicate that the localisation of Nef differed depending on the source of the primary isolate. At least part of the Nef protein was apparently exposed on the surface of infected PHA-PBMCs infected with the PB isolates but not on cells infected with the CNS isolates. When compared to reference T-cell and macrophagetropic isolates HIV-1_{NL43} and HIV-1_{BaL}, respectively, we found the PB isolates corresponded to NL43 and the CSF isolates BaL (Table 3; Figure 3). This correlation extended to other reference T-cell and macrophagetropic isolates (unpublished data). Additionally, we assessed Nef expression with reference isolate BaL in macrophages and the results were the same as seen in

PBMC where Nef was only detected in permeabilized cells using the N-terminal antibody (Figure 3e and f compared with m and n).

Conclusions and Discussion

There were no Nef antibodies detected within the CSF, in contrast to a consistent antibody response in PB, despite the presence of antibodies against gp41 in both CSF and PB and a competent *nef* open reading frame being present and expression of Nef protein *in vitro* with both PB and CSF strains from all selected coincident virus isolations.

In PB the dominant productively infected cell type is the CD4+ lymphocyte in contrast to CNS where it is the microglial cell (Glass *et al*, 1995; Johnson *et al*, 1996; Bagasra *et al*, 1996; Brew *et al*,

Table 3 Detection of Nef protein expression by immunofluorescence in permeabilized and non-permeabilized virus-infected PHA-activated PBMC

Virus infection	*Detection in permeabilized cells*		*Detection in non-permeabilized cells*	
	N-terminal Ab[a]	*C-terminal Ab*[b]	*N-terminal Ab*[a]	*C-terminal Ab*[b]
PB 16	+	+	+	−
CSF 16	+	+	−	−
PB 26	+	+	+	−
CSF 26	+	+	−	−
PB 27	+	+	+	−
CSF 27	+	+	−	−
HIV-1_{NL43}	+	+	+	−
HIV-1_{BaL}	+	+	−	−

[a]Detection of Nef protein using an N-terminal antibody against aa 15–27 (Greenway *et al*, 1994). [b]Detection of Nef protein using a C-terminal antibody AE6 (Greenway *et al*. 1994).

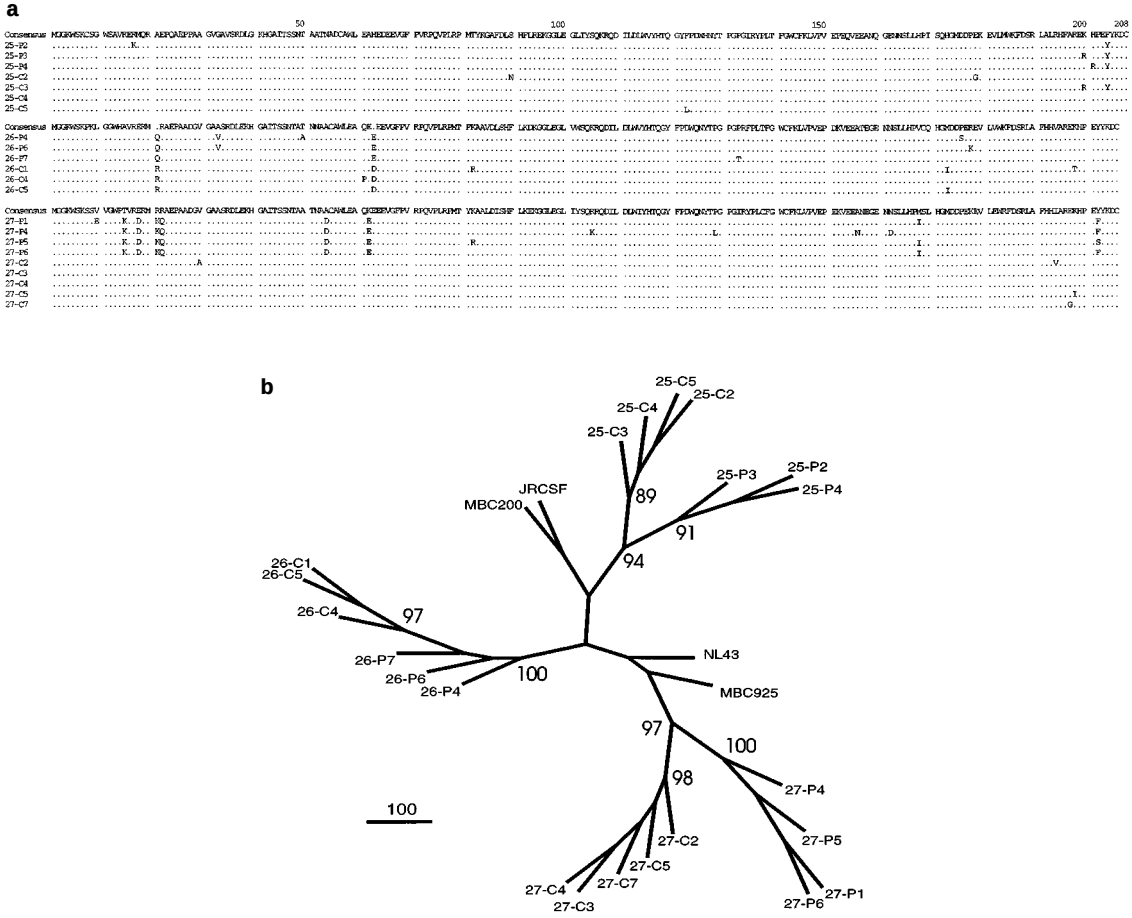

Figure 2 (**a**) Alignment of PB and CNS *nef* sequences from patients 25, 26 and 27. Alignment was using the Geneworks program (Version 2.5) with the predominant sequence shown as the consensus and the various PB (P) and CNS (C) clones aligned with (•) denoting consensus and amino acid changes with the one letter code. (**b**) Unrooted maximum parsimony tree depicting the phylogenetic relationships between the DNA sequences of the 22 *nef* genes isolated. Significant bootstrap values (as a percentage of 100 resamplings) are shown beside nodes. Labels for the clones are as in (**a**) with *nef* gene sequences from four reference HIV-1 isolates included; two CSF-derived isolates (JRCSF and MBC925[x]) and two lymphotropic isolates (NL4-3 and MBC200[x]). [x]Oelrichs and MePhee, unpublished data.

1995; Takahashi *et al*, 1996). Different localization of an expressed Nef protein in CNS infection due to virus infection of different cell types, microglial *versus* CD4+ lymphocytes, allied with virus strains of different tropisms may be responsible for the undetectable Nef antibody response.

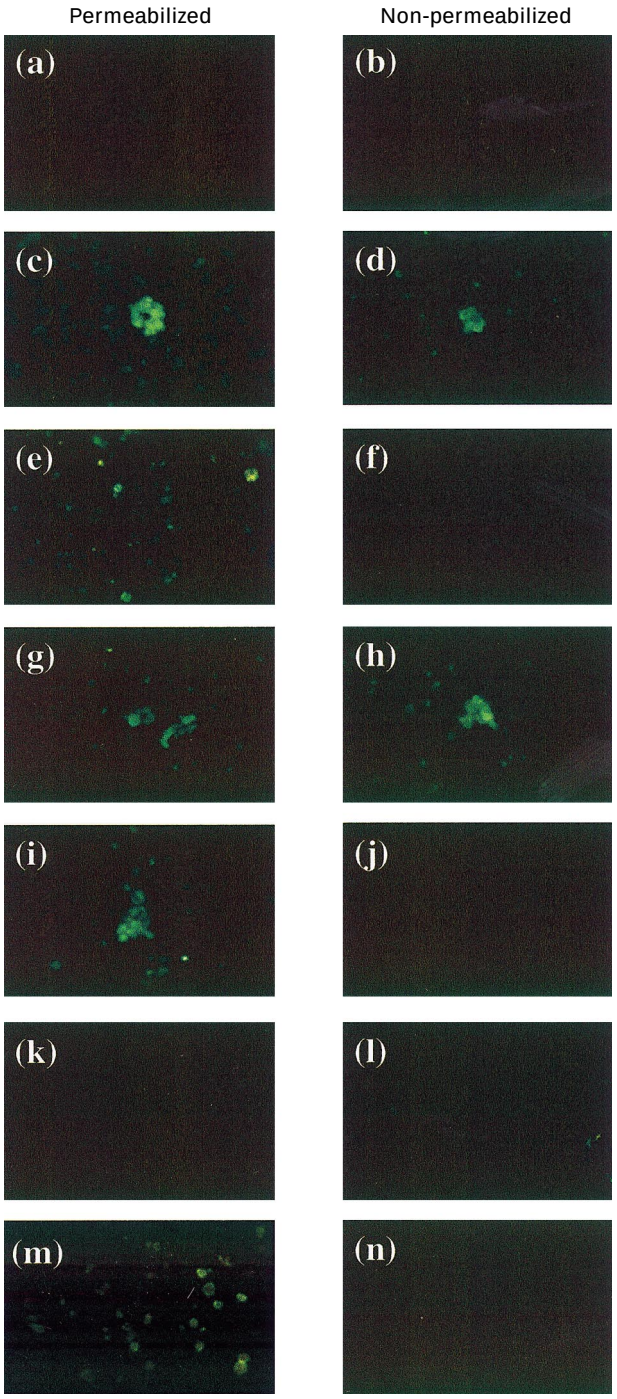

Figure 3 Immunofluorescence in HIV-infected cells stained with an N-terminal antibody to Nef before and after permeabilization. PHA-activated PBMC (**a–j**) or adherent macrophages (**k–n**) mock-infected (**a,b,k,l**) or infected with HIV-1_{NL43} (**c,d**), HIV-1_{BaL} (**e,f,m,n**), PB 27 (g,h), or CSF 27 (i,j) were reacted with polyclonal anti-$Nef_{(15-27)}$ 7 to 11 days post-infection before (**b,d,f,h,j,l,n**) and after (**a,c,e,g,i,k,m**) permeabilization with 0.05% NP-40. (Final magnification ×100).

Despite antibodies being assessed against antigen from NL4-3 Nef protein there are many conserved epitopes among primary HIV-1 isolates. Certainly all but one of the 27 patients tested were positive and we have data with a further 37 patients where all PB samples were Nef antibody positive using the same assay (Greenway *et al*, in press). Additionally, antibodies were clearly present against gp41 within the CNS indicating virus replication *in situ*, and antibodies against other HIV protein antigens have been observed consistently within the CNS of AIDS patients (V3, Lucey *et al*, 1993; gp120, Trujillo *et al*, 1996). In fact with HIV-1 infected patients there is an increased level of intrathecal IgG synthesis, particularly in patients with neurological disease (Mathiesen *et al*, 1990; Singer *et al*, 1994). This suggests that antibodies to Nef protein would be present in the CNS at a detectable level according to the presence of gp41 antibodies with our assay using a human anti-Ig that detects all subclasses.

The sequence data and phylogenetic analyses indicate separate sequences were derived from coincident CSF and PB isolates, although, there were no consistent differences observed that suggest a unique genotype in PB or CNS. Certainly, *nef* sequences derived from patient cells and from *in vitro* replication can vary but in general there is overall conservation in sequence with little evidence of sequence evolution (Blumberg *et al*, 1992; Michael *et al*, 1995a,b; Huang *et al*, 1995). Virus isolation of macrophagetropic/non-syncytium inducing isolates from PHA-activated PBMCs has proved most successful for CSF samples (Brew *et al*, 1996). Although there may be some selection process for strains that replicate best in PBMCs this cell type has certainly been shown to be the most permissive for all virus types thus far. This allied with little or no change in sequence data *in vivo* and *in vitro* would argue that the isolations made relate to the virus present *in vivo*. However, we cannot exclude subtle changes without sequence analysis directly from patient material (experiments in progress). Recent *in vitro* analysis of Nef protein function from multiple primary isolate sequences revealed that the most subtle amino acid changes could affect function (Mariani *et al*, 1996), hence nothing can be concluded with respect to function from the sequence data.

The observed expression of Nef protein, at least in part, on the surface of infected cells has been made by at least one other group (Fujii *et al*, 1993; Otake *et al*; 1994). The defined region or regions of Nef involved remain to be determined. The reasons for these differences in surface expression with T-cell tropic compared to macrophagetropic virus strains is currently being assessed.

Lack of recognition of HIV-1 Nef protein by the immune system within the CNS may be due to one of several reasons: the protein may not be expressed at all *in vivo*, protein may be expressed but not available to the immune system or the protein may be seen as self within the CNS and hence excluded from the immune system. Expression of Nef is most probably occurring due to the observation of a complete open reading frame from multiple clones from those isolates tested and *in vitro* expression results. Due to the different cell types infected in CNS and the different strains present compared to PB and differences in Nef protein expression or its interaction with other viral or host cell proteins that preclude it from being surface expressed or released from the infected cell, expression may not be amenable to a significant immune response. Preliminary studies of Nef protein expression during virus-infection of primary macrophages, a cell type analogous to that productively infected within the CNS, have shown altered localisation of Nef relative to that of T-cell tropic strains in CD4+ lymphocytes and indeed macrophagetropic virus infection of PBMC (Figure 3; Newton and Greenway, manuscript in preparation).

The lack of antibodies to Nef in the CNS of HIV-1 infected individuals parallels the lack of antibodies to virion matrix protein in the CNS of measles infected individuals despite detectable antibodies in PB (Hall and Choppin, 1981). Results indicate that this may be due to lack of expression or expression of a defective or altered form of matrix in measles virus encephalitis (Ballart *et al*, 1991; Cattaneo *et al*, 1992; Baczko *et al*, 1993; Suryanarayana *et al*, 1994; Hirano *et al*, 1992). Expression of Nef could be altered in HIV infection of the CNS. Our data indicate there is altered expression, due to the lack of local antibodies in all patients tested and the altered expression of Nef in selected patients. Due to the varied Nef expression in PB and CNS this could be related to the cell type infected and/or the dominant viral strain present. Due to the importance of *nef* in disease pathogenesis (Deacon *et al*, 1995) altered expression in the CNS eludes to a different role for Nef protein at different sites in an infected individual. We are currently assessing the function of Nef protein in CD4+ lymphocytes, macrophages and astrocytes.

Materials and methods

Patient selection and ADC assessment
Paired cerebral spinal fluid (CSF) and plasma specimens were collected from 27 HIV-1 infected patients. None of the CSF samples had detectable red cells thereby excluding for practical purposes viral or antibody contamination from blood. Patients were diagnosed as having ADC if abnormal neurologic symptoms and signs and characteristic neuroimaging findings were present. Severity was rated on a functionally based scale ranging from 0 to 4 (Price and Brew, 1988): stage 0 (normal), stage 0.5 (equivocally abnormal symptoms or signs), stage 1 (mild), stage 2 (moderate), stage 3 (severe), and stage 4 (very severe).

Peptide and protein synthesis
A peptide corresponding to amino acid residues 549 to 569 of HIV-1_{NL43} *env* was synthesized using standard t-Boc chemistry and purified by high pressure liquid chromatography as described elsewhere (Fecondo *et al*, 1993). The method used for large scale expression of the 27 kDa form of HIV-1_{NL43} Nef in *E. coli* and subsequent purification have been described elsewhere (Azad *et al*, 1994).

Screening of plasma and CSF for reactivity against gp41 peptide and Nef protein by direct ELISA
For detection of antibodies that recognize gp41(549-569) and the Nef protein highly purified peptide or full length HIV-1_{NL43} Nef protein, respectively, were diluted in phosphate buffered saline (PBS) and coated onto the wells of 96-well polystyrene microtitre plates at 5.0 gM or 75 nM, respectively. Peptide or protein were allowed to bind for 2 h at 37°C. After this incubation period wells were washed three times with PBS containing 0.05% Tween 20 (PBS-Tween) and any remaining available binding sites on the wells blocked by incubation of 150 μl of gelatine (1% w/v) in PBS for 1 h at 37°C. Following washing with PBS-Tween as described above, 50 μl of CSF (1 : 30) or plasma (1 : 300) diluted in PBS/BSA (1% w/vol) was added to the wells, titrated from 1 : 30 to 1 : 10 000 or 1 : 300 to 1 : 100 000, respectively, and then incubated for 1.5 h at 37°C. The wells were again washed with PBS/Tween and subsequently incubated with 50 μl of biotinylated sheep anti-human Ig (diluted 1 : 1000 in 1% BSA/PBS; Amersham, Buckinghamshire, UK) for 1 h at 37°C. Following further washing, 50 μl of streptavidin-horseradish peroxidase (diluted 1 : 1000 in PBS containing 1% bovine serum albumin; Dakopatts, CA, USA) was added to the wells and the plate incubated at 37°C for 30 min. Substrate (*o*-phenylenediamine, Sigma, 100 μl/well) was added after a final wash and the plate allowed to incubate at room temperature for 15 min. The reaction was stopped by the addition of 1N H_2SO_4 and the absorbance measured at 450/630 nm using a Titertek® (Towing, UK) plate reader. A signal was considered to be positive if it was more than twice the mean value obtained with plasma from uninfected individuals.

Total protein levels
These tests were performed by the Victorian Infectious Diseases Reference Laboratory, Fairfield Hospital. The CSF total protein method was a turbidometric method using benzethonium chlor-

ide reagent (Trace Scientific) performed on a Beckman CX5 or CX7 analyser according to the method of Watson and Jenkins (1987).

Total protein in the plasma was determined by the timed endpoint-Biuret method (Beckman Reagents) performed on a Beckman CX5 or CX7 analyser according to the method of Hiller *et al* (1948).

Virus isolation and replication

CSF was filtered (0.45 μM) and inoculated onto 3×10^6 PHA activated PBMC (PHA-PBMC) which had been pretreated with DEAE-dextran (25 μg/ml) at 3×10^6 cells/ml for 45 min. Supernatants were sampled and assayed for the appearance of p24 antigen every 3 days. The cultures were maintained for 30 days in RPMI-1640 supplemented with 10% foetal calf serum and 10% rIL-2 and received 1×10^6 fresh PHA-PBMC every 6 days. Viral isolates were also obtained by co-cultivation of PBMC from each subject with normal PBMC.

HIV negative donor PBMC were stimulated by culture in RPMI 1640 containing 10% (v/v) fcs, 15 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid, 0.1% (w/v) sodium bicarbonate with 100 IU/ml penicillin and 100 μg/ml streptomycin with the addition of 10 μg/ml PHA (Wellcome, Temple Hill, Dartford, England) for 72 h prior to co-culture. 40×10^6 cells were spun down and resuspended in 1 ml of virus cell-free inoculum to give an MOI of 0.001 $TCID_{50}$/cell. After incubation for 2 h the PHA-PBMC were resuspended at 1×10^6 cells/ml in RPMI 1640 containing 10% (vlv) fcs, 15 mM HEPES, 0.1% (w/v) sodium bicarbonate, 25 μg/ml glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 μg/ml polybrene (Sigma, St Louis, MO, USA), 20 U/ml interleukin 2 (Boehringer Mannheim, W. Germany) and 120 nU/ml anti-interferon (ICN Biochemicals, Costa Mesa, CA, USA). Cells were maintained by half medium changes every 3 to 4 days, with the addition of fresh PHA-PBMC on day 7. Virus production was assayed by cell-free reverse transcriptase activity (Willey *et al*, 1988) or p24 activity (Abbott Diagnostics assay).

DNA preparation and nested PCR amlification

5×10^6 PBMC's per culture were lysed in 0.5 ml lysis buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3, 2.5 mM $MgCl_2$, 0.1 mg/ml gelatin, 0.45% NP40, 0.45% Tween 20) containing 0.05 mg/ml Proteinase K and incubated at 55°C for 1 h. After deactivation of proteinase K by incubation at 95°C for 10 min cell lysates were centrifuged to pellet cellular debris. 1 μl of each cell lysate supernatant was then subjected to a first round PCR amplification using oligonucleotide primers SK-68 and Cl-6, from which 2 μl of product was added to a second round amplification using primers Nef-5′ and LTR-3′. Primer sequences and PCR reaction conditions have been previously reported (Deacon *et al*, 1995).

Cloning and sequencing

Nef PCR products were blunt end cloned into *Sma*I cut pGEM−7zf(+) (Promega) which were used to transform competent *E. coli* DH5α cells. Colonies, positive by blue/white selection, were picked and lysed in 50 μl H_20 for 3 min at 100°C. l μl of each lysate was then subjected to denaturation at 94°C for 120 s followed by 35 cycles of PCR amplification (94°C for 15 s, 50°C for 15 s, 72°C for 120 s) then 72°C for 7 min using M13 Universal and Reverse oligonucleotide primers. PCR products were ethanol precipitated and sequenced using Sequitherm™ Long Read™ Cycle Sequencing Kit-LC with fluorescent labeled M13 Forward and Reverse primers (Epicentre, WI, USA) and the LI-COR Model 4000L Automated Sequencer (Lincoln, Nebraska, USA).

Phylogenetic analysis

The *nef* sequences (nt 8787 to 9404 in NL,43) for 22 sequences from three patients were analysed. All computations were done through the Australian National Genomic Information Service (ANGIS), WAG interface. The alignment was performed using the CLUSTAL algorithm and the tree shown was constructed using the maximum parsimony algorithm DNAPARS, from the PHYLIP package. The bootstrap values shown (for 100 replicates) were generated using SEQBOOT and CONSENCE, also ported from the PHYLIP package. The Fitch-Margoliash method (FITCH), using a distance matrix generated by DNADIST (with Jukes-Cantor or Kimura nucleotide substitution methods) as well as NEIGHBOR (neighbour joining method) and the maximum likelihood algorithm DNAML, PHYLIP package, resulted in trees with no significant differences (data not shown).

Detection of Nef protein by indirect immunofluorescence

Virus-infected or mock-infected cells, day 7 post-infection, were washed twice with PBS and prepared for indirect immunofluorescence. The method used for staining permeabilized and non-permeabilized cells was as described previously (Greenway *et al*, 1994) using sheep anti-$nef_{(15-27)}$ antibody or mouse monoclonal antibody AE6 (reactive to the C-terminus; Dr J Hoxie, AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH).

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