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Short Communication

NFκB activation, TNF-α expression, and apoptosis in the AIDS-Dementia-Complex

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The role of NFκB activation and its relationship to inflammatory mediators and apoptosis in the HIV-infected brain have remained uncertain. The cellular and regional distribution of NFκB, TNF-α, and apoptosis was examined in the frontal cortex (FC), deep white matter (DWM) and the basal ganglia (BG) of 17 patients with ADC. Nuclear staining for NFκB was localized predominantly to perivascular microglia/macrophages in the BG and DWM and correlated with ADC severity. Correlations were further found with HLA-DR, iNOS, TNF-α, and gp41 expression in these regions. The number of TUNEL-positive cells, particularly in the BG, correlated with ADC stage. Logistic regression analysis further showed a significant relationship between the likelihood of TUNEL staining in the BG and worsening cognitive impairment. *Journal of NeuroVirology* (2000) **6,** 537–543.

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It is now widely believed that HIV-induced brain injury and AIDS-Dementia-Complex (ADC) are related to indirect mechanisms mediated either by HIV proteins including gp120, Tat, and gp41 and/or host factors such as TNF-α, iNOS and the alpha chemokine SDF-1α (Lipton and Gendelman, 1995; Magnusen *et al*, 1995; Talley *et al*, 1995; Adamson *et al*, 1996; Kaul and Lipton, 1999). These factors have been shown *in vitro* to induce or amplify cellular injury and apoptosis. Further, cytokines, particularly TNF-α, and chemokines may act to mediate or potentiate the neurotoxic effects of Tat and gp120 (Yeung *et al*, 1995; Shi *et al*, 1998; New *et al*, 1998). Additional support for the role of these factors has come from *in vitro* studies that have correlated immune activation as well as elevated levels of TNF-α and iNOS mRNA in the deep white matter with the degree of dementia (Wesselingh *et al*, 1993; Adamson *et al*, 1996).

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In concert with the *in vitro* findings, neuronal loss and apoptosis have been reported in brains of subjects with ADC as well as in asymptomatic individuals and are believed to comprise an important component of the pathogenetic process leading to this disorder (Wiley *et al*, 1991; Masliah *et al*, 1992; Everall *et al*, 1993; Petito and Roberts 1995; Shi *et al*, 1996; An *et al*, 1996). Nonetheless, the relationship of these findings to dementia and the underlying pathogenetic mechanisms have remained uncertain.

Nuclear factor-κB (NFκB)/Rel proteins comprise a family of host transcription factors that have become recognized as central mediators of rapid and coordinated induction of host and viral genes in response to various stimuli including cytokines, oxidative stress, viral products, glutamate and beta-amyloid (Baeuerle and Henkel, 1994; Grilli and Memo, 1999; Guerrini *et al*, 1995; Kaltschmidt *et al*, 1999; Rattner *et al*, 1993). NFκB activation has also been recently implicated in the pathogenesis of several neurodegenerative and inflammatory disorders, including Parkinson's Disease, Alzheimer's Disease and multiple sclerosis (Hunot *et al*, 1997; Kaltschmidt *et al*, 1997; Gveric *et al*, 1998).

To determine the cellular localization and activation status of NFκB in the HIV-infected brain and the role of apoptosis in relationship to ADC severity, we studied 17 patients with AIDS at various stages of cognitive impairment and without a clinical history of a focal CNS process. The neurological status was determined in 13 cases within 1–2 weeks of death and by retrospective chart review in the remaining four cases. The diagnosis for ADC was based on American Academy of Neurology criteria (AAN AIDS Task Force, 1991) and severity was scored using the Memorial Sloan Kettering scale as described by Price and Brew (Price and Brew, 1988). ADC staging was as follows: stage 0: four subjects; stage 1: five subjects, stage 2: three subjects; stage 3/4: five subjects. The severity of the HIV-encephalitis was graded based on a modified scale by Brew *et al* and scored independently of gp41 as previously described (Brew *et al*, 1995; Rostasy *et al*, 1999).

Paraffin blocks of the frontal lobe with adjacent deep white matter and basal ganglia including the globus pallidus were obtained from 15 patients and from frontal region only in two patients. The samples were provided by the following institutions: the New England Medical Center (9), Boston City Hospital (4) and the Western General Hospital/Edinburgh (Scotland) (4). All cases were coded as previously described (Rostasy *et al*, 1999). Pathological examination did not reveal focal opportunistic infections in any of these cases. Moderate to severe HIVE with perivascular and less often parenchymal multinucleated giant cells (MNGC) in the white matter and/or basal ganglia was found in seven of 17 of the ADC subjects, six of whom were ADC stage 2–4. The remaining cases showed occasional perivascular MNGC collections (n=3) or no brain pathology (n=7).

Serial 7-μm sections were cut from paraffin-embedded blocks, were floated onto warm water without gelatin and mounted onto precleaned Superfrost/Plus slides (Fisher Scientific). Hidden antigen activity was reactivated as previously described (Hedreen and Mucci, 1995; Rostasy *et al*, 1999). The following commercially available antibodies were used: TNF-α (1 : 1000, Sigma), NFκB p65 (1 : 500, Santa Cruz Biotechnology). Immunohistochemical staining was carried out using the ABC method (Rostasy *et al*, 1999). Negative controls included use of sections from two HIV seronegative individuals without evidence of brain pathology. The primary antibody was also omitted on corresponding sections from the HIV cases. One case showed positive staining for all the above mentioned antigens tested and served as a positive control.

Apoptosis was determined by the TdT-mediated *in situ* DNA nick end labeling method (TUNEL) (Migheli *et al*, 1994). Serial sections of two HIV seronegative individuals without evidence of brain pathology were used as negative controls. Four cases with Alzheimer's disease were used as positive controls. In addition, serial and adjacent sections from 10 of 17 cases were incubated with DNase I and used as an internal control for TUNEL positive cells.

The amount of staining was quantified by two independent observers (KR and BN), who were blinded to the ADC or HIVE scores. The number of TNF-α, NFκB and TUNEL positive stained cells was determined by analyzing two serial sections from the frontal lobe and basal ganglia in 15 and the frontal cortex in 17 cases. The entire section was scanned for positive staining cells. The degree of staining was scored as follows: 0=no or only nonspecific staining; 1 (rare)=1–5 positive cells; 2 (mild)=5–10; 3=10–20 (moderate); 4 (severe)= greater than 20 or too numerous to count (Rostasy *et al*, 1999).

The severity of ADC and HIVE was graded in a four-step scale and the measure of association between the two determined by the Spearman correlation coefficient. The abundance of immunohistochemical staining was compared with ADC and HIVE scores (univariate analysis) using nonparametric methods (Spearman rank order correlation and Wilcoxon rank sum test). The abundance of staining was also compared between the basal ganglia and frontal lobe, adjusting for ADC stage (multivariate analysis) by logistic regression analysis.

In the HIV-infected subjects, intranuclear staining for NFκB using the p65/rel antibody was predominantly found in perivascular microglia/macrophages in the basal ganglia and frontal white matter as opposed to the cortex, particularly in subjects with severe dementia (Figure 1). As previously described, TNF-α staining was localized to the cytoplasmic area of perivascular microglia/macrophages in a similar distribution (Wesselingh

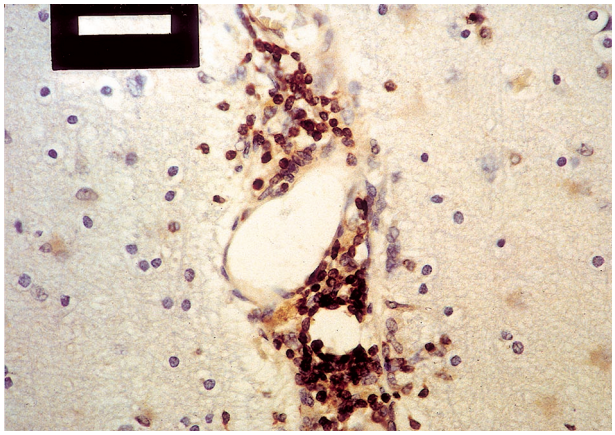

Figure 1 Intranuclear NFκBp65 staining of microglia/macrophages in the basal ganglia in a patient with severe ADC (×400).

et al, 1997). There was no difference in the abundance of NFκB or TNF-α staining between the frontal white matter and the basal ganglia. Further, NFκB activation generally correlated with the abundance of TNF-α staining in the basal ganglia and the deep white matter (P=0.012 and 0.028 respectively). In seven out of 17 cases (ADC stage 0–2 cases; stage 2–2, stage 3/4–3) and one negative control brain, nuclear and cytoplasmic staining of NFκB was detected in neurons and astrocytes in both the frontal cortex and the basal ganglia (data not shown). The number of these positively stained cells was independent of the degree of pathology or dementia score, consistent with previous reports of constitutive NFκB expression in neuronal and glial cells (Kaltschmidt *et al*, 1994; Dollard *et al*, 1995).

To further assess the relationship between NFκB activation and ADC, the degree of staining was scored in both the frontal white matter and basal ganglia and compared to the ADC stage. For both NFκB and TNF-α, the degree of staining in the frontal deep white matter and the basal ganglia correlated with the stage of ADC (TNF-α: P=0.0001 and P=0.0017 respectively; NFκB: P=0.011 and P=0.014, respectively). The abundance of NFκB activation in the frontal white matter showed a statistical trend with the severity of HIVE (P=0.08).

As NFκB exerts a central role in regulating the expression of a number of proinflammatory and viral genes, we tested the hypothesis that its activation would correlate with the abundance of HIV infection and inflammatory mediators previously associated with ADC. We compared the extent of cellular expression of NFκB activation with that of gp41, iNOS and microglial activation as measured by HLA-DR staining (Rostasy *et al*, 1999). In both the basal ganglia and the frontal deep white matter, NFκB activation correlated strongly with the abundance of HLA-DR staining (Table 1). Additionally, NFκB activation correlated with the degree of iNOS expression in both brain regions and gp41 in the basal ganglia while a trend was noted in the white matter (Table 1). The expression of TNF-α also significantly correlated with the abundance of gp41 in the basal ganglia ($P<0.01$), and a trend was noted in the frontal white matter ($P<0.02$) (data not shown). Similar associations were observed between TNF-α and iNOS in these regions ($P<0.04$ for the frontal white matter and P=0.07 in the basal ganglia), further supporting the pathogenetic relationships that have been described *in vitro* between these factors.

To further understand the relationship of apoptosis in the HIV-infected brain to ADC, we compared the degree of TUNEL positive cells in both the basal ganglia and the frontal lobe to ADC stage and examined its relationship to the abundance of staining for the proinflammatory mediators described above. Apoptotic cells, including neurons, were found in five of eight cases with ADC stages 2–4, predominantly in the basal ganglia (see Figure 2) compared to two of nine subjects with ADC stages 0–1. Apoptosis in the basal ganglia correlated significantly with the ADC stage (P=0.021) and nearly reached statistical significance in the frontal cortex (P=0.052). This relationship was further supported by logistic regression analysis which showed a significant relationship between the likelihood of TUNEL staining in the basal ganglia and worsening ADC (Figure 3). Probability of staining increased from nearly 10% in subjects in ADC stage 0 and 0.5 to greater than 80% in those with advanced dementia (ADC stage 3/4) while a less pronounced effect was observed in the frontal lobe.

No correlations were found between any of the factors examined and apoptosis. Further, two cases with ADC stage 1 and minimal pathology showed numerous apoptotic cells in the basal ganglia but were found to have minimal staining for TNF-α and NFκB. Four brains with Alzheimer's Disease were also studied, showing various degrees of TUNEL positive cells in the hippocampal/parietal region. NFκB staining was found in neurons and astrocytes

Table 1 Spearman correlation coefficients (P values) of NFκB with various factors associated with brain injury.

	Factor			
Brain region	*gp41*	*HLA-DR*	*TNF-α*	*iNOS*
Basal ganglia	0.73 (0.0021)	0.84 (0.0001)	0.67 (0.0127)	0.64 (0.0110)
Frontal white matter	0.53 (0.0300)	0.79 (0.0002)	0.56 (0.0285)	0.60 (0.0106)

Given the number of comparisons within each brain region (four), P-values less than 0.0125 are considered statistically significant to an (overall) significance level of 5%. P-values between 0.0125 and 0.05 are considered 'statistical trends'.

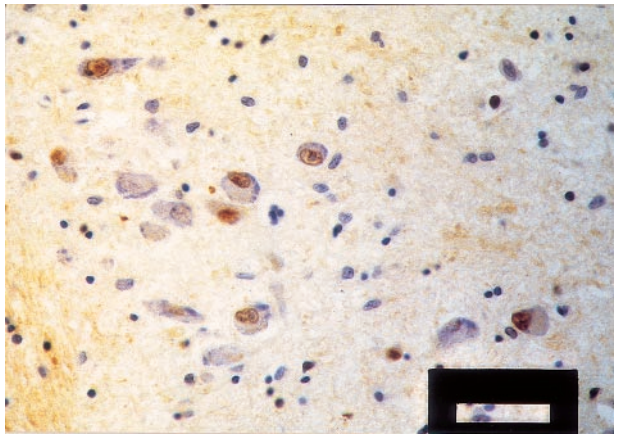

Figure 2 TUNEL positive cells including neurons in the basal ganglia in the same patient as above (×400).

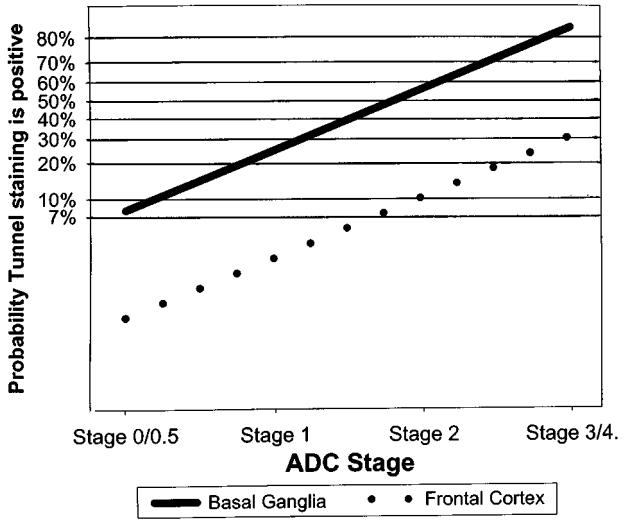

Figure 3 Probability of TUNEL staining in the basal ganglia (solid line) and the frontal cortex (dotted line), adjusted for the severity of acquired immunodeficiency syndrome dementia complex (ADC). TUNEL staining was more abundant in the basal ganglia and increased linearly with the severity of ADC stage (TUNEL Cortex/ADC=0.0452 and TUNEL Basal Ganglia/ADC P=0.021 respectively). The results shown in this figure were derived by means of a multivariate logistic regression analysis of the probability of TUNEL staining in the basal ganglia versus the frontal cortex.

in the cortical region of two subjects, as previously described (Kaltschmidt *et al*, 1997) but interestingly no TNF-α staining was detected in any of the four subjects.

The results show that NFκB activation is predominantly localized to perivascular macrophages/microglia in both the frontal white matter and basal ganglia and the abundance of staining in these regions correlates with the severity of dementia. Correlations were also found with microglial activation as well as iNOS, TNF-α and gp41 expression, consistent with the notion that the status of NFκB activation may be critical in modulating the activity of these factors and perhaps the course of injury in the HIV infected brain. In addition, the cellular and regional expression of TNF-α coincided with that found for NFκB and was highly correlated with the severity of ADC, consistent with previous reports (Wesselingh *et al*, 1993). As might be predicted, associations were also found with iNOS and gp41 staining in the basal ganglia and/or the frontal white matter. To our knowledge this is the first *in vivo* study to examine the regional expression between NFκB activation and TNF-α in relationship to ADC severity and the expression of other host and viral factors. Together, these results lend further support to the pathogenetic role of TNF-α and identifies NFκB as another central factor in the disease process.

The correlations found between TNF-α, NFκB and gp41 are in concert with the *in vitro* observations that TNF-α, regulates HIV replication via activation of NFκB which binds to regulatory sequences in the long terminal repeat (LTR; Duh *et al*, 1989; Atwood *et al*, 1994). NFκB also regulates the expression of TNF-α, in addition to other proinflammatory molecules while TNF-α has been suggested to regulate iNOS expression in the brain (Philippon *et al*, 1994; Bukrinsky *et al*, 1995). Further studies have shown that NFκB activation in response to TNF-α is mediated by reactive oxygen intermediates. Tat, which induces TNF-α activity, has been shown to downregulate the expression of Mn superoxide dismutase, thereby shifting the cell toward oxidative conditions that favor NFκB activation (Westendorp *et al*, 1995). Thus TNF-α, NFκB and HIV form part of a complex autoregulatory system that is significantly linked to the neuropathogenesis of HIV induced brain injury and the progression of cognitive impairment.

Whether NFκB activation contributes to neuronal injury or confers neuroprotection has remained a matter of debate. In a recent study, Kaltschmidt *et al* showed that increased NFκB activation in primary neuronal cell cultures induced with low doses of beta-amyloid and TNF-α protected against neuronal cell death, suggesting a neuroprotective role for this factor (Kaltschmidt *et al*, 1999). *In vitro* genetic studies through the use of dominant negative mutants that inhibit NFκB activation have also shown that NFκB may indeed provide a protective effect against cellular injury (Liu *et al*, 1996; Kneissl and Navia, unpublished observation). Further studies are needed to determine if NFκB may have a similar function in response to HIV-induced neuronal injury.

In contrast, NFκB activation in brains with multiple sclerosis has been localized to microglia and correlated with the course of clinical disease (Gveric *et al*, 1998), consistent with the reported observations here and those previously reported by Dollard *et al* who examined NFκB activation in the brains of children with HIV (Dollard *et al*, 1995). Similar to our findings, constitutive activation was also observed in a small percentage of neurons and astrocytes that did not differ from controls (Kaltschmidt *et al*, 1994; Dollard *et al*, 1995). Thus, NFκB activation in neurons is not a prominent feature in ADC and unlikely contributes directly to neuronal injury. These findings are also consistent with the *in vitro* observation that neuronal loss in response to Tat or TNF-α likely involves NFκB independent mechanisms (Talley *et al*, 1995; New *et al*, 1998), but are in contrast to a recent study by Tyler and colleagues who have shown that NFκB is a critical mediator of apoptosis in response to retroviral infection (Connolly *et al*, 2000). Together the results suggest that the role of NFκB is likely to be indirect, primarily functioning within microglia and macrophages to sustain the production of proinflammatory mediators and HIV replication within a

complex network of host and viral factors that can either contribute directly or amplify pathways leading to neuronal injury.

The relationship of apoptosis to the degree of dementia has largely remained an unresolved issue (Everall *et al*, 1993; Seilhean *et al*, 1993; Petito *et al*, 1995; An *et al*, 1996; Shi *et al*, 1996). Recently, Gray and colleagues found no global correlation between neuronal apoptosis, HIV dementia or the presence of encephalitis but noted a greater abundance of apoptotic neurons in the basal ganglia, particularly in subjects with HIV-encephalitis (Adle-Biassette *et al*, 1999). Our findings extend these observations, as apoptotic cells were predominantly found in the basal ganglia and to a lesser extent in the frontal cortex. Further, the number of apoptotic cells in the basal ganglia significantly correlated with ADC severity. This relationship was further supported by the results of a logistic regression model, which showed a significant relationship between the likelihood of TUNEL staining in the basal ganglia and worsening ADC. These findings are of interest as they further support the notion that the development and progression of cognitive impairment are related to pathogenic events in the subcortical regions, particularly the basal ganglia (Navia *et al*, 1986; Rostasy *et al*, 1999).

Although both microglial activation and the abundance of staining for iNOS and gp41 in the basal ganglia were significantly correlated with ADC, no such relationship between these factors and apoptosis was found. This result may reflect the small number of ADC cases with apoptosis compared to those that showed positive staining for these antigens or suggest that factors other than those examined here may be important in mediating apoptosis in the HIV-infected brain. The latter notion is further supported by the finding of significant apoptosis in the basal ganglia of two subjects with mild cognitive impairment and yet absent staining of TNF-α and NFκB. Recent studies have shown that strain differences in HIV may be partly responsible for variations in the degree of neuronal loss or apoptosis and that the envelope may be directly involved. In one study, T-tropic or CXCR4 using viruses caused greater neuronal apoptosis than M-tropic strains while in another study, blood-derived variants induced greater cytopathogenicity in primary brain cultures when compared to brain isolates (Ohagen *et al*, 1999; Zheng *et al*, 1999). These findings suggest the hypothesis that polymorphisms in the envelope region may contain determinants that may directly cause neuronal injury (Ohagen *et al*, 1999; Zheng *et al*, 1999).

Significant progress has been made in identifying potential agents of injury in the HIV-infected brain. Further studies are clearly needed to unravel the viral-host interactions and the signaling pathways that mediate their neuropathogenic effects. These efforts should help identify new targets for therapeutic intervention in what is rapidly becoming a chronic disease in response to dramatic changes in antiretroviral therapy (Ferrando *et al*, 1998; Palella *et al*, 1998).

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