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Immune Activation, Viral Gene Product Expression and Neurotoxicity in the HIV-1 Transgenic Rat

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Abstract

The HIV-1 transgenic (TG) rat has been shown to be a useful model of nervous system disease that occurs in human HIV-1 infection. Studies were, therefore, performed to examine characteristics of the immune response in the periphery and brain of the animals and expression of factors in the nervous system that might be associated with neurotoxicity. Activated splenocytes from wild-type (WT) and TG rats were stimulated with either CD3/CD28 or with lipopolysaccharide (LPS) and examined for proliferative responses and for proinflammatory cytokine (IFN- γ , TNF- α and IL-1 β) secretion. Brain tissue lysates from the rats were also examined for proinflammatory cytokine levels and tissue sections were stained by immunofluorescence for class II MHC+, ED1+ or Iba1+ (for macrophages and microglial cells), and for GFAP+ (for astrocytes) cells and for co-labeling of these cells for TNF- α . Co-labeling was also performed to identify cells expressing HIV-1 gp160, tat, nef and vif. Finally, on Western blots brain tissue lysates were examined for phosphorylation of Erk1/2, p38, JNK-SAPK and Erk5. TG rat splenocyte proliferative responses were higher than for WT with CD3/CD28-stimulation but lower than WT with LPS stimulation. CD3/CD28-stimulated TG rat splenocytes also secreted higher levels of IFN- γ , TNF- α and IL-1 β whereas LPS-stimulated TG rat splenocytes secreted higher levels of only TNF- α than cultures from WT rats. Levels of all three cytokines were higher in brain lysates from TG rats than for WT rats. On immunofluorescence staining of corresponding sections of brain, TG rats contained increased numbers of class II MHC+ and ED1+ cells, and there was also increased co-labeling of these cells as well as astrocytes for TNF- α . Iba1+ cells showed positive staining for all of the HIV proteins whereas astrocytes showed significant positive staining for only nef and vif. Phosphorylation of Erk1/2, p38 and JNK/SAPK was detected for both TG and WT rat tissue with higher levels of phosphorylation forms of these proteins detected in the TG rat brain. Phosphorylation of Erk5, a marker that is associated with specifically neuronal repair, was detected only in TG rat brain. These studies suggest that activated nervous system

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mononuclear phagocytes and astrocytes expressing HIV-1 gene products in specific patterns are associated with neurodegeneration in the HIV-1 TG rat.

INTRODUCTION

HIV infection is associated with the development of neurological complications that, with the progression of infection, can occur at a high frequency. One of the most devastating consequences of HIV infection is HIV-associated neurocognitive impairment (NCI), which has been shown to occur in a significant number of individuals even after initiation with otherwise effective antiretroviral therapies (Boisse et al., 2008; Kraft-Terry et al., 2010). Indeed, the virus is able to develop resistance to such treatment, and it is the persistent replication and the direct and indirect effects of virus on cells that results in clinical neurological impairment. Pathological studies performed on brains from individuals with NCI have shown the presence of activated mononuclear phagocytes (macrophages and microglial cells) and astrocytes, a subset of which are productively infected with HIV and express proinflammatory cytokines and other neurotoxic molecules that can potentially induce damage to nearby tissue (Yadav and Collman, 2009). Such activation can be replicated in vitro by exposing neural cells to the HIV gene products gp120, tat and nef, which can also directly induce cell injury and death (Kaul and Lipton, 2004). Therefore, studies that lead to an increased understanding of the immune and virologic factors that are responsible for the detrimental effects of HIV infection on the nervous system are important to pursue.

To examine the mechanisms that underlie these effects, a number of small animal models have been developed that mimic the human disease to varying degrees (Bruce-Keller et al., 2008; Dickie et al., 1991; Dou et al., 2006; Gorantla et al., 2007; Kim et al., 2003; Reid et al., 2001; Tyor et al., 1993). The HIV transgenic rat, which incorporates a non-infectious HIV genome, has been shown to develop neurological abnormalities that are similar to those observed with natural HIV-1 infection in humans (Basselin et al., 2011; June et al., 2009; Liu et al., 2009; Peng et al., 2010; Reid et al., 2001; Royal, III et al., 2007; Sultana et al., 2010; Webb et al., 2010). In particular, the animals develop cognitive, behavioral and motor abnormalities which may be linked to the presence of increased systemic immune activation and loss of specific neuronal populations in the brain (June et al., 2009; Mazzucchelli et al., 2004; Royal, III et al., 2007). It has also been previously demonstrated that monocyte/macrophages from TG rats can efficiently transcribe HIV genes and can specifically express env and tat transcripts (Mazzucchelli et al., 2004). In the studies described in this report, we examined activation responses and proinflammatory cytokine production by T cells and monocyte/macrophages in splenocyte cultures from TG and WT rats. Also examined were associations for these findings with the presence of immune activation and evidence of neuronal damage in the brains of the animals. The studies suggest that, as with the human infection, there occurs in the HIV TG rat an alteration in the systemic immune response in association with brain mononuclear phagocyte and astrocyte activation and HIV gene expression patterns which likely play key roles in the neurodegeneration that occurs in this model.

METHODS

Animals

The construction of the HIV TG rat has been previously described (Reid et al., 2001). For the described studies, TG and WT rats were anesthetized, exsanguinated with the blood placed in heparinized tubes and the spleens removed. The animals were then perfused with cold 0.1 M phosphate buffered saline, the brains removed and coronal blocks of one

hemisphere of brain was frozen in OCT and stored at -80°C and the other was hemisphere snap frozen in liquid nitrogen for later immunohistochemical and biochemical analysis, respectively. All procedures were performed under a protocol approved by the Institutional Animal Care and Use Committee of the University of Maryland, Baltimore.

Splenocyte Cultures and Proliferation Assays

Splenocytes from TG and WT rats ($n=2$ rats per group) were plated in triplicate in 96-well, round-bottom plates at 4×10^5 cells/well in $200\ \mu\text{l}$ of complete RPMI 1640 medium (Invitrogen Life Technologies) supplemented with $2\ \text{mM}$ L-glutamine, $25\ \text{mM}$ HEPES, $100\ \text{U/ml}$ penicillin, $100\ \mu\text{g/ml}$ streptomycin, $5.5 \times 10^{-5}\ \text{M}$ 2-mercaptoethanol, and 5% FCS (all supplements from Invitrogen Life Technologies) containing either medium alone or CD3/CD28 beads (Invitrogen) at cell:bead ratios of either 5:1 or 10:1 and incubated at 37°C in 5% CO_2 . In addition, separate splenocyte cultures were incubated with either $1\ \mu\text{g}$ or $5\ \mu\text{g}$ of lipopolysaccharide (LPS). All cultures were incubated for either 24, 48 or 72 hours. To 72 hours cultures was added $20\ \mu\text{l}$ of $5\ \text{mg/ml}$ MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) per well and the cells were subsequently incubated for 4 hours at 37°C . The plates were then be centrifuged at $1000\ \text{rpm}$ for 10 minutes, the supernatants were then discarded and then $150\ \mu\text{l}$ of DMSO was added to each well. The absorbance at $570\ \text{nm}$ with a reference of $630\ \text{nm}$ was then determined for each well using a spectrophotometric 96 well plate reader.

Enzyme-Linked Immunosorbent Assays

Brain tissue lysates and splenocyte culture supernatants ($100\ \mu\text{l}$ volumes) from each TG and WT rat were analyzed using commercial ELISA kits for levels of IFN- γ (BD Biosciences), TNF- α (BD Biosciences) and IL-1 β (R&D Systems) according to the manufacturers' directions. Lysates were prepared from $80\text{--}100\ \text{mg}$ of previously frozen tissue brain frontal cortex and subcortical white matter disrupted with a hand rotor and with a pestle. The protein was extracted from the tissue using $1\times$ RIPA buffer ($100\ \text{mM}$ Tris, $\text{pH}\ 7.5$, $100\ \text{mM}$ NaCl, $1\ \text{mM}$ EDTA, $1\ \text{mM}$ EGTA, 1% Triton X-1000, 10% glycerol 0.1% SDS) with $1\ \text{mM}$ PMSF and protease cocktail (Roche). The lysates were then centrifuged at $13200\ \text{rpm}$ at 4°C for $60\ \text{min}$. Supernatants were then collected and re-centrifuged for $30\ \text{min}$ and the resultant supernatants collected and the protein measured with Bradford reagent for equal protein loading in the ELISA wells. ELISA was performed using duplicate samples from $n=2$ rats per group analyzed in triplicate.

Immunofluorescence Staining

For immunofluorescence staining the sections were fixed in 100% methanol (Fisher) for 10 minutes and then the slides were washed three times for five minutes in PBS and then blocked with blocking solution (5% BSA in 0.1% Triton X-100) for 30 minutes at room temperature. Immunofluorescence staining for HIV proteins was performed using primary antibodies to HIV-1 gp160 ($1:400$ dilution), tat ($1:1000$ dilution), vif ($1:1000$ dilution) and nef ($1:1000$ dilution) (all antibodies against HIV proteins were obtained from the NIH AIDS Reagent Program). Sections were also stained with rat anti-ED1 ($1:20$ dilution; Serotec), rat anti-Iba1 ($1:50$ dilution; Serotec), rat anti-GFAP ($1:50$ dilution; BD Biosciences), and rat anti-class II MHC ($1:50$ dilution; Pierce). After incubations at 37°C for 1 hour or 4°C overnight with appropriate primary antibody, the sections were washed 3 times in PBS for 5 minutes and then treated with, as appropriate, either fluorescein isothiocyanate (FITC)-conjugated donkey anti-goat-IgG ($1:200$ dilution; Santa Cruz Biotechnology), either Alexa Floor 488- or Alexa Floor 568-conjugated donkey anti-rabbit-IgG ($1:400$ dilution; Invitrogen), or Alexa Floor 568-conjugated donkey anti-mouse IgG ($1:200$ dilution; Invitrogen) for 1 hour at room temperature, The slides were then washed 3 times in PBS for 5 minutes and the nuclei in the sections stained with Vectashield and coverslipped in the

dark. The specificity of the procedures was verified by negative control sections incubated without primary antibody. Fluorescence imaging was performed using a Zeiss Axiovert 40 microscope equipped with a AxioCam mrc fluorescence camera (Zeiss). The percent area of staining and mean fluorescence for two randomly selected 20x microscopic fields on two sections from two TG and two WT rats was determined using ImageJ software (National Institutes of Health).

Western blots

Western blots was performed for total and phosphorylated (phospho-) Erk1/2, p38, JNK/SAPK and Erk5 using 50 µg of protein from brain lysates prepared as described above. The separated protein was then transferred onto Millipore membranes using a semidry blot transfer apparatus (Bio- Rad). The membranes were then blocked with 3% BSA in Tris buffered saline/Tween-20 (TBST) for 1 hour at room temperature and then incubated with primary antibodies against phospho- Erk1/2, phospho-p38, phospho-JNK/SAPK and phospho-Erk5 (all antibodies were purchased from Cell Signaling Technologies) overnight in TBST plus 3% BSA at 4 °C with shaking on the platform of a rotating shaker. After three 15-minute washes in TBST, the filters were incubated with the corresponding anti-species secondary antibody (all antibodies were BIO-RAD Laboratories) for 1 hour at room temperature. After three 15 minute washes, the western blots were developed using chemiluminescence detection reagent and the signal was detected on X-ray film. At that point the membranes were stripped and then incubated with the corresponding rabbit antibodies against total Erk1/2, p38, JNK/SAPK and Erk5 (antibodies purchased from Cell Signaling Technologies). The filters were then washed, incubated again with secondary antibody and then developed and the signal detected again on X-ray film as described above. After each membrane was stained with antibodies to detect phosphorylated and total Erk1/2, p38, JNK/SAPK and Erk5, the membranes were stripped a second time and incubated with antibodies against β-actin, as an internal control, then washed, incubated with secondary antibody, developed, and the signal detected. The film was then digitally scanned and the band intensities on the scanned images were measured using ImageJ software (National Institutes of Health).

Statistical Analyses

Comparison of mean proliferative responses, cytokine levels and the percent area and intensities for the immunofluorescence measurements for the TG and WT rat groups were performed unpaired t-tests (GraphPad Software). Statistical significance was indicated by a p-value < 0.05.

RESULTS

Proliferation Assays

Proliferative responses by splenocytes from TG and WT rats to T cell stimulation induced by incubating the cells either for 24 hours, 48 hours or 72 hours with either medium alone or with anti- CD3/anti-CD28 conjugated beads (at cell:bead ratios of either 5 or 10). These studies showed that responses were overall greater for cells from TG rats splenocytes than for the corresponding cells obtained from the WT rats (figure 1). This increase was noted specifically when the cultures were incubated in medium alone for 24 hours, when the cells were incubated with CD3/CD28 beads at cell:bead ratios of 5 and 10 for either 24 hours or 48 hours, and for cells incubated with the beads at a cell:bead ratio of 5 for 72 hours.

Proliferation in 24-hour, 48-hour and 72-hour splenocyte cultures was also examined after incubation in medium alone and following stimulation with 5 µg of LPS, which is an activator of monocytes and macrophages. These studies showed that, compared to WT rats,

there was a lower level of proliferation at the 24- and 48-hour time points for TG rat cultures incubated in medium alone. Similarly, at all three time points LPS stimulation induced proliferative responses that were also lower for the TG than for the WT rat cells.

Proinflammatory Cytokine Expression with Activation of T Cells and Monocyte/Macrophages in Splenocyte Cultures

Secretion of the proinflammatory cytokines IFN- γ , TNF- α and IL-1 β were measured in CD3/CD28- and LPS-stimulated splenocyte culture supernatants using antigen capture ELISA (figure 2a). The supernatants were obtained after the cultures were incubated with the activators for 48 hours and 72 hours. For the CD3-CD28-activated cultures, secreted levels of all three cytokines were higher for TG rat cells than for cells obtained from WT rats. This increase was noted at both cell:bead ratios with incubating the cultures for 48 hours. At the 72-hour time point, the increased cytokine secretion was present at only the lower cell: bead ratio.

Levels of IFN- γ , TNF- α and IL-1 β secreted following stimulation with either 1 μ g or 5 μ g of LPS were also measured (figure 2b). In contrast to what was observed for CD3/CD28 stimulation, for cells incubated with 5 μ g LPS for 48 hours and with 1 μ g LPS for 72 hours WT rat cells secreted significantly higher levels of IFN- γ than cells from TG rats. However, at both time points and at both LPS concentrations, higher levels of TNF- α was secreted by cells from TG rats than for WT cells. With respect to IL1- β measurements, in no instance was there was observed to be a significant difference in levels of IL1- β secreted by cells from the two rat groups.

Proinflammatory Cytokine Expression in Brain from the TG and WT Rats

Brain tissue lysates from the rats were examined by ELISA for expression of IFN- γ , TNF- α and IL-1 β . These studies showed there to be higher levels of all three cytokines in brain tissue lysates from the TG rats than for the WT rats (figure 3).

Immunofluorescence Staining for Class II MHC, ED1 and GFAP

Single color immunofluorescence staining of frozen sections of brain from the TG and WT rats were performed using antibodies against class II MHC, ED1 and GFAP (figure 4a). Quantitative analysis of the staining showed there to be more extensive staining, as determined by the mean area of the staining, with class II MHC and ED1 in the TG rat brain sections than for the WT sections. In addition, the intensity of staining for ED1 and class II MHC was also higher for the TG rat group. In contrast, for GFAP, there was no difference in the mean area of staining or in mean fluorescence intensity for the two animals groups.

To examine the subset of class II MHC-, ED1- and GFAP-positive cells also expressing proinflammatory cytokine, double-immunofluorescence staining was performed using these antibodies as well as antibody against TNF- α (figure 4b). Analysis of the mean double-stained area of the sections showed there to be more extensive co-labeling with for TNF- α in the TG rat brains sections than for the WT tissue. Also, the intensity of the co-labeled areas was greater in the TG rat sections.

Detection of HIV-1 Vif, Nef, Tat and gp160

Brain sections from TG and WT rats were also examined by immunofluorescence staining for expression of vif, nef, tat and gp160 (figure 5). No significant staining was noted in the tissue from the WT rats. In contrast, abundant staining was found for all of the HIV proteins in TG rat brain. To determine the localization of the staining, the TG brain sections were double-stained for Iba1, which labels activated macrophages and microglial cells, and for GFAP. These studies revealed that, for vif and nef, staining was present in cells that co-

labeled for macrophage/microglial marker and for cells that stained positive for GFAP. In contrast there was minimal specific staining for tat and gp160 in cells that were GFAP+. However, there was extensive co-labeling of Iba1+ cells for gp160 and tat.

Detection of total and phosphorylated ERK1/2, P38, JNK/SAPK and ERK5 on Western blots

Lysates of brain from the WT and TG rats were examined on Western blots for expression of total and phosphorylated Erk1/2, p38, JNK/SAPK and Erk5. The band intensity for total p38 and total JNK/SAPK Erk5 band was greater for the TG than for the WT rat brain lysate, whereas the intensity of the Erk1/2 and Erk5 bands were similar for the two animal groups. In contrast, the band intensity for the phosphorylated forms of all of the proteins was greater for the TG than for the WT samples. No phosphorylated Erk5 was detected in WT brain lysate.

DISCUSSION

Studies of infection with HIV in humans and SIV in primates have shown that virus enters the central nervous system early in infection and that the major route of entry is by transport in infected monocytes and macrophages (the “Trojan horse” theory) (Braathen, 1988). The infected monocytes and macrophages secrete inflammatory mediators and other products, such as TNF- α , IL-1 β , nitric oxide, quinolinate, platelet-derived growth factor and arachidonate (Meltzer et al., 1990). The secretion of these factors can be toxic to cells and contribute to the inflammation that is characteristic of HIV encephalitis and the neurodegeneration that can potentially lead to the development of cognitive and other neurological symptoms. In the TG rat model there is no requirement for infected cells to transport virus into the central nervous system since the cells that reside in the CNS already contain incorporated proviral DNA. Expression of HIV-specific RNA has been previously shown to be present in the brains of these animals (Peng et al., 2010; Sultana et al., 2010). In this report we demonstrate the presence of increased systemic and CNS inflammatory responses in the TG rats and also show for the first time the presence of significant expression of multiple HIV proteins in the TG rat brain. These two factors in combination are capable of inducing neurotoxic damage, which in the TG rat brain is implied by the demonstrated activation of markers cellular stress, indicated by expression of the Erk1/2, p38 and JNK/SAPK, and of neural cell repair, with expression of Erk5.

In previous studies it was demonstrated that mitogen-induced proliferation and proinflammatory cytokine secretion by peripheral blood mononuclear cells from the TG rats was similar to that which occurred with stimulation of cells from WT animals (Royal, III et al., 2007). This is in contrast to the results that are described here for splenocyte cultures, which showed increases in such responses with T cell stimulation for the TG rats as compared to cultures comprised of cells from WT animals. With stimulation of monocyte/macrophages in the cultures the results were variable and showed decreased proliferative responses for the TG rat and increased secretion of only TNF- α . The spleen is a site for primary sensitization of T cells to antigen presented by splenic macrophages (Debrick et al., 1991). The effector T cells that are produced as a result of this interaction are the likely source of the IFN- γ that is secreted in the cultures. IFN- γ is secreted by Th1 and cytotoxic T cells (proinflammatory T cells), which differentiate from naïve T cells under the influence of IL-12, which is produced by macrophages. Therefore, the observed decrease in IFN- γ production in the TG rat cultures relative to levels secreted by WT rat cells suggests that either monocyte/macrophages were present in lower numbers in the TG rat spleens or that these cells were deficient in their ability to stimulate proinflammatory T cell responses. Alternatively, it is possible that the macrophages present in the cultures are of a M2 phenotype, which, though capable of secreting TNF- α , are capable of inducing naïve T cells to differentiate into cells that possess an anti-inflammatory phenotype (Th2 cells or

regulatory T cells) (Cassol et al., 2010). In HIV infection M2 cells have been associated with increased immunosuppression and could promote more rapid HIV disease progression. Similar cytokine responses have been noted for activated monocytes obtained from HIV-infected humans (Molina et al., 1990; Roy et al., 1988). However, the identification of the specific factors and mechanisms that underlie the responses reported here will come through additional studies.

In the brains of the TG rats it was also found that proinflammatory immune responses were higher in the TG compared to the WT rat. This was demonstrated by increased levels of IFN- γ , TNF- α , and IL-1 β in brain tissue lysates detected on ELISA. Also, in the TG rat brains there were increased numbers of monocyte/microglia cells and cells expressing class II MHC, which is expressed on the surface of mononuclear phagocytes, dendritic cells, B cells and astrocytes, and is involved in antigen presentation to T cells. A higher percentage of these cells co-labeled for TNF- α and stained more intensely for this cytokine, suggesting that these cells were in a higher state of activation than corresponding cells in the brains of the WT rats. These studies, therefore, provide evidence of enhanced systemic T cell and monocyte/macrophage responses in the TG animals that for both cell types result in increased proinflammatory cytokine, specifically TNF- α , production. Immunocytochemical staining for GFAP showed there to be similar levels of expression for this marker in TG and WT rat brains. This finding would suggest that there was no specific astroglial activation present in the TG rat brain tissue relative to the WT rat. However, as observed for ED1+ and class II MHC+ cells, astrocytes showed evidence increased activation, as demonstrated by increased co-labeling of GFAP+ cells for TNF- α and by the increased intensity of the staining that was observed for this cytokine. Astrocytes can be activated by IFN- γ , IL-1 and lipopolysaccharide to secrete TNF- α (Robbins et al., 1987), and TNF- α that is secreted by these cells and by mononuclear phagocytes can lead to further astrocyte production of this cytokine as well as to expression of MCHII (Wong et al., 1984; Zeinstra et al., 2000). Class II MHC is involved in the presentation of antigen to T cells to induce polarization and expansion of these naïve T cells and reactivation of effector memory T cells. Class II MHC staining has been previously demonstrated in brains from humans with HIV encephalitis (Tyor et al., 1992). We show here for the first time that such expression can be also demonstrated in the brain of the HIV TG rat. The expression of this protein on the surface of mononuclear phagocytes and astrocytes in the brains of these animals links these cells with an ability to present antigen and induce the production of antigen-specific immune responses.

In natural HIV infection, protective cellular immune responses occur in immunocompetent individuals that target intact virus and viral antigens to effect clearance of virus, free viral products and infected cells. In the TG rat the transgene is introduced into the fertilized egg and the viral proteins are expressed in all cells and not recognized as immunologically foreign. Although it has been shown that HIV infection can trigger autoimmune responses that target viral proteins antigens (Lindzen et al., 2008; Trujillo et al., 1993), it is likely that other mechanisms are responsible for the heightened proinflammatory responses that have been identified in the TG rat model. Indeed, HIV proteins can have other effects that can result in the generation of inflammation and neurotoxicity in the brains of these animals. For example, in vitro studies have shown that gp120 and tat can be toxic to neurons in culture and that gp41 and tat can induce toxicity in human neuronal and astroglial cultures in association with increased production of nitric oxide and inducible nitric oxide synthase (Adamson et al., 1996; Dawson and Dawson, 1994; Dreyer et al., 1990; Kruman et al., 1998). In our studies we examined the cells for evidence of expression of gp160, which is cleaved to form gp120 and gp41. Also, other HIV proteins, including nef, have been shown to have neurotoxic properties, and vif, by inhibiting proteosomal degradation of ubiquitinated proteins, could also promote the degeneration of cells in which this protein in

expressed (Kramer-Hammerle et al., 2005). In studies of brains from HIV infected humans, evidence of productive infection can be demonstrated frequently in association with pathological finding such as the presence of increased numbers of tissue macrophages and microglial cells and activated astrocytes (Tyor et al., 1992). Detailed analysis of HIV protein expression had not been previously performed in the HIV TG rat model. In our studies, immunostains of the brains from the TG rats for gp160, tat, nef and vif showed the presence of all of these proteins in mononuclear phagocytes that were co-labeled with Iba1, which labels activated macrophage/microglial cells. Iba1 staining was more extensive showed a greater intensity of fluorescence in the TG rats, suggesting that more phagocytes were present and that the cells were at a greater state of activation.

In contrast to what was observed for brain phagocytes, astrocytes stained for nef and vif but did not show significant staining for gp160 and tat. It is possible that the mononuclear phagocyte staining resulted from non-specific uptake of HIV proteins by these cells as a part of their phagocytic function. However, astrocytes can also demonstrate phagocytic activity. Therefore, the positive staining of mononuclear phagocytes for all of the proteins likely reflects the well-known fact derived from human studies which show that mononuclear phagocytes are the only cells in the brain that frequently demonstrate productive HIV infection (Glass et al., 1995). As such, these cells can express both early viral genes, which include HIV regulatory proteins such as tat, nef and vif, and late proteins, such as the structural gag and envelope proteins (Brack-Werner, 1999). In the case of astrocytes, the presence of HIV proviral DNA can be demonstrated in a large percentage of these cells in brains from infected individuals. However, fewer astrocytes can be shown to express specific viral gene products. The HIV protein that is most frequently detected in astrocytes is nef, which may be produced in up to 40% of these cells. A similar percentage of astrocytes have been found by in situ RT-PCR to contain unspliced tat/rev mRNA; however, only rarely are the spliced products detected (Brack-Werner, 1999).

In response to the direct effects that result from HIV proteins, cytokines and other soluble factors binding and activating cell surface receptors, protective cellular mechanisms are triggered in association with activation of phosphorylation events mediated by mitogen activated protein kinases (Obara and Nakahata, 2010). In our studies we therefore examined activation of ERK1/2, ERK5, p38, JNK/SAPK. Erk1/2, p38 and JNK/SAPK are expressed in nearly all cell types in the body whereas ERK5 is expressed almost exclusively in the brain where, like Erk1/2, it promotes growth-enhancing effects in neurons that result from neurotrophin stimulation (Cargnello and Roux, 2011; Cavanaugh et al., 2001). These molecules are all primarily activated by extracellular stimuli such as cytokines, growth factors, G-protein-coupled receptors and stress as well as by intracellular derivatives of proto- oncogenes (Obara and Nakahata, 2010). Neuronal expression of both Erk1/2 and Erk5 can be induced by brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and NT-4. However, unlike Erk1/2, stimulation of Erk5 expression has been shown to occur exclusively as a result of the effects of these neurotrophins (Cavanaugh et al., 2001). In our studies, in only the TG rat brain was there found to be phosphorylation of Erk5, suggesting that neuroprotective pathways were stimulated by extracellular events that resulted in increased production of trophic factors. The specific factors and events that lead to Erk5 activation and the consequences of the activation require further study.

In summary, we demonstrate the presence of increased differing proinflammatory responses systemically and evidence of chronic immune activation in brains of TG rats as compared to the WT animals. Expression of all HIV proteins examined was detected in the brains of the TG rats with mononuclear phagocytes showing expression of all of the proteins whereas astrocytes did not show expression of tat and gp160. The selective replication of tat and gp160 in mononuclear phagocytes is consistent with the ability of these cells to produce

infectious virus and suggests that the TG rat should be useful for studies of viral neurotropism in HIV infection. Among the limitations of this report is that the TG rat fall short of what occurs in a true infection model, with all cells carrying at least one copy of a HIV genome. In addition, there is the remote possibility that the immune responses demonstrated in the TG rat are not specifically against the HIV gene products, per se, an issue that can be addressed in future studies. However, our findings reported here, and those from previous studies described in this report that describe clinical and virologic effects and responses in the model that are similar to those that occur with HIV infection in humans, suggest that opportunities remain for using the model in studies of the pathogenesis of nervous system disease that occurs with HIV infection.

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figure 1.

T cell proliferation and cytokine secretion in TG and WT splenocyte cultures were examined. The cultures were simulated with, respectively, anti-CD3/anti-CD28-conjugated beads and examined for (a) proliferative responses in MTT assays 24 hour, 48 hour and 72 hour time points and (b) by ELISA for secretion of IFN- γ , TNF- α and IL-1 β at 48 hour and 72 hour time points. (a) At the 24 hour time point, both non-activated and activated TG rat splenocytes showed greater proliferative responses than did WT cells. At the 48 hour and 72 hour time points no difference in proliferation was noted for cells incubated in medium alone; however proliferation was higher for TG splenocytes than WT cells with the increased noted at both time points at a cell: bead ratio of 5. (b) No increase in cytokine secretion was noted when the cells were incubated in medium alone. However, secretion of IFN- γ , TNF- α and IL-1 β were overall greater for splenocytes from the TG than for the WT rats, with the most consistent increase also noted at a cell:bead ratio of 5. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

figure 2.

Effects from activating monocytes in splenocyte cultures were examined by incubating cultures with either medium alone or with 5 µg of LPS followed by analysis for proliferation in MTT assays at 24 hour, 48 hour and 72 hour time points (a). Cultures were also incubated with either 1 µg or 5 µg of LPS followed by assessment of cytokine secretion at the 48 hour and 72 hour time points by ELISA (b). (a) At the 24 hour and 48 hour time points proliferation in both medium alone and in the presence of 5 µgm LPS was higher for WT then for TG cells. At 72 hours, there was no difference in proliferation between the two groups in the absence of LPS; however, LPS-induced proliferation remained higher for cells from the WT than for the TG rats. (b) Measurement of cytokine secretion at both the 48 hour and 72 hour showed there to be no difference in levels secreted in the absence of LPS stimulation. Incubating the cells for 48 hours in 5 µg LPS and for 72 hours in 1 µg LPS induced higher levels of IFN-γ secretion by WT cells than for TG cells. In contrast, at both 48 and 72 hours both 1 µg and 5 µg LPS induced higher levels of TNF-α secretion by TG cells than WT splenocytes. No significant difference in IL-1β secretion was noted at either time point. * = p< 0.01; ** = p< 0.01; *** = p<0.001.

Figure 3. Proinflammatory cytokine levels in brain lysates from TG and WT rats. Brain lysates were prepared as described in the Materials and Methods section and then IFN- γ , TNF- α and IL- β levels in the samples were measured by ELISA. Levels in TG rat samples were higher for all three cytokines as compared to those for the WT animals.

figure 4.

Quantitation of mononuclear phagocytes and TNF- α expression by cells in TG and WT rat brains. Expression of class II MHC (i), ED1 (ii) and GFAP (iii) were examined by indirect immunofluorescence staining using Texas Red-conjugated (for ED1, class II MHC and GFAP detection) and FITC-conjugated (for TNF- α detection) secondary antibodies. The mean area and fluorescence intensity of the staining are shown on the right side of the figures (iv-vi). (a) Staining for class II MHC, ED1 and GFAP was detected in both WT and TG samples and an increase in the area and intensity of the staining noted for TG as compared to WT rats (iv and v), whereas that area and intensity of GFAP staining was similar for the two groups (vi). (b) Co-expression of class II MHC+ (i), ED1+ (ii) and GFAP + (iii) cells for TNF- α . The stained sections were analyzed as described above for the mean area and fluorescence intensity of the staining, which is to the right of the figure (iv-vi). Co-

labeling for TNF- α was observed for all of the stained samples, and both the mean area and the mean intensity of the co-staining with all three markers were greater in the TG than in the WT rat tissue.

figure 5.
Double immunofluorescence staining of WT and TG rat brain cortex using primary antibodies against GFAP and against the HIV proteins vif, nef, tat and gp160. For GFAP double- staining with vif, nef or tat the secondary antibodies were conjugated to FITC for GFAP and to Texas Red for vif, nef and tat. For the GFAP double-staining with gp160 the secondary antibodies were conjugated to Texas Red for GFAP and to FITC for gp160. There was double-staining of astrocytes for HIV vif and nef. However, there was little or no co-localization of gp160 or tat with GFAP staining.

figure 6.
Double immunofluorescence staining of WT and TG rat brain using primary antibodies against Iba1 and against HIV vif, nef, tat and gp160. The secondary antibodies were conjugated to Texas Red for Iba1 labeling and to FITC for labeling of the HIV proteins. Co-labeling was observed for all of the HIV proteins in Iba1+ cells.

Figure 7.

Activation of intracellular signaling pathways in TG and WT rat brains. Shown is a representative Western blot demonstrating levels of total and phosphorylated p38, JNK, Erk1/2 and Erk5 in brain from the TG and WT. Higher levels of the non-phosphorylated p38 and JNK were detected in the TG as compared to the WT brains whereas levels in these animals were similar for Erk1/2 in and Erk5. In contrast, levels of phosphorylated protein and phosphorylated to non- phosphorylated protein ratios were higher for the TG rat with no phosphorylated Erk5 detected in brain from the WT rat.