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Short Communication

Chronic systemic administration of tumor necrosis factor alpha and HIV gp120: effects on adult rodent brain and blood-brain barrier

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Since tumor necrosis factor alpha (TNF-α) and HIV gp120 glycoprotein are both neurotoxic, the possibility that systemic sources of these two agents mediate AIDS-associated blood-brain barrier (BBB) breakdown and brain damage was tested in two murine models: (1) intramuscular implantation of a TNF-α-transfected tumor in nu/nu mice and (2) daily subcutaneous injections of HIV gp120 in BALB/c mice. The BBB remained intact; brain damage was not found, and apoptotic cell numbers did not increase. These results show that normal adult brain and BBB is unaffected by exposure to TNF-α or HIV gp120 and suggest that severity of brain disease is not directly affected by systemic levels of these compounds.

Keywords: AIDS; human immunodeficiency virus; central nervous system

Neurodegenerative changes are common in patients with HIV and with the acquired immunodeficiency syndrome (AIDS) and are closely related to human immunodeficiency virus (HIV) encephalitis. They include dendritic abnormalities, neuronal loss, and enhanced vascular permeability (Budka *et al*, 1991; Masliah *et al*, 1997; Petito and Cash, 1992; Power *et al*, 1993). Indirect mechanisms secondary to neurotoxins, rather than direct damage due to viral infection, are likely causes since productive brain infection is limited to microglia and monocytes (Koenig *et al*, 1986; Wiley *et al*, 1986). High on the list of potential neurotoxins are tumor necrosis factor alpha (TNF-α) and HIV gp120 glycoprotein (Epstein and Gendelman, 1993; Gendelman *et al*, 1994; Brenneman *et al*, 1994; Benveniste and Benos, 1995). Both damage or kill rodent and human neurons, glia and endothelium (Bagetta *et al*, 1995; Flitter *et al*, 1997) and both are increased in serum and brains of AIDS patients. Because there is evidence that TNF-α or gp120 in the systemic circulation could damage brain by bypassing, or altering, the normal blood-brain barrier (BBB) (Mintz *et al*, 1989; Hill *et al*, 1993), we administered these substances by implantation of a TNF-α-secreting tumor cell line and by acute intravenous or chronic subcutaneous injections of HIV gp120 to experimental animals. Brain and BBB damage were assessed by light microscopy, *in situ* end labeling (ISEL) for DNA fragmentation associated with apoptosis and immunohistochemistry to detect alterations in synaptophysin and to demonstrate leakage of serum proteins in brain parenchyma as a marker of enhanced vascular permeability.

TNF-α model

The gastrocnemius muscles of 16-week-old nude mice (nu/nu) were injected with 1×10^7 Chinese hamster ovarian (CHO) cells that had been genetically transfected with the human TNF-α gene. Tracey *et al* (1990) previously have shown that this produces serum TNF-α levels of 0.44 ± 0.17 ng/mL after 10 days and 0.48 ± 0.13 ng/mL after 50 days. Animals were sacrificed at 10 and 40 days after implantation. Controls were implanted with non-transfected CHO cells (CHO-neg). There were six animals in each group.

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At the time of sacrifice, animals were deeply anesthetized and brains perfused-fixed with 4% paraformaldehyde in 0.1 M phosphate buffer. The entire brain was sectioned, embedded in paraffin and examined with routine histological stains for cellular, myelin and axonal changes. Standard immunohistochemical methods identified synaptophysin, mouse serum, mouse albumin and mouse fibrinogen by sequential incubation with primary antibodies; secondary antibody; the avidin-biotin complex and diaminobenzidine and hydrogen peroxide: anti-synaptophysin: 1 : 1000, Dako Corp., Carpinteria, CA, USA; anti-albumin: 1 : 2000, Accurate Corp., Westbury, NY, USA; and anti-fibrinogen 1 : 1000, Nordic, Capistrano Beach, CA, USA. The ISEL technique identified fragmented nuclear DNA, according to manufacturer's instructions (ApopTag, Oncor Laboratory, Gaithersburg, MD, USA). The number of positive nuclei per 400 × microscopic field × 100 were quantitated in the coronal section at the level of the basal ganglia. Blood-brain barrier damage was assessed by leakage of serum proteins, identified by immunohistochemistry, into brain parenchyma (Petito and Cash, 1992). Since we obtained similar results with serum, albumin and fibrinogen, we elected to quantitate the data by determining the number of fibrinogen-positive foci per 200 × microscopic field × 100. All studies were evaluated without knowledge of the experimental protocol.

Untreated nu/nu mice, 10 day and 40 day CHO-neg mice and 10 day CHO-TNF-α mice had normal weight gain and behavior. Weight loss and listlessness developed between 30–40 days post-inoculation in CHO-TNF-α mice as described previously (Tracey *et al*, 1990). Microscopic examination of brain was normal save for a basilar meningitis in one 10 day CHO-neg animal. Nuclei displaying ISEL-positivity were rare or absent in untreated nude mice and in all experimental groups. There were no significant differences between the number of FIB+ foci following 10 day or 40 day exposure to CHO-neg implants and CHO-TNF-α implants (Table 1).

HIV gp120 BALB/c mouse model

HIV $gp120_{SF2}$ (courtesy of the AIDS Reagent and Research Program, Catalog #385 and of Chiron Corp.), 10 ng protein per g body weight in sterile phosphate buffered saline (PBS), was administered daily by subcutaneous injections between the shoulder blades for 20 or 40 days to 16-week-old BALB/c mice. The gp120 used was produced in CHO secretor cells and forms syncytium when the transfected cells are co-cultured with $CD4^+$ T cells (Weiss and White, 1993). In order to exclude a possible immune response to gp120 causing its accelerated clearance, a second series of BALB/c mice were thymectomized under general anesthesia on post-natal day one (Adkins and Du, 1998) and subcutaneous injections of gp120 for 20 days were administered when they were 16-weeks-old. Lastly, we administered HIV gp120 intravenously to BALB/c mice 4 h or 24 h prior to sacrifice. Animal sacrifice and brain preparation were identical to those used with the TNF-α animals. Controls were injected with PBS or with Epstein Barr virus (EBV) glycoprotein gp125, a non-HIV glycoprotein of similar molecular weight.

Weight gain, behavior and light microscopic brain examination was unremarkable in all groups. Nuclei displaying ISEL-positively averaged 1.3 ± 1.7 per field in untreated BALB/c mice. Their numbers increased after daily subcutaneous injections but the differences between PBS, EBV and HIV groups were not significant (Table 1). Although the number of FIB+ foci were higher than the untreated BALB/c mice, there were no significant differences between HIV gp120 administered for 20 days (data not shown) or 40 days (Table 1) when compared with their PBS or EBV gp125 treatment control groups. Similarly, intravenous administration of viral glycoproteins did not alter the BBB. Thymectomized-treated gp120 mice were similar to their PBS-treated controls, suggesting that the failure of injected HIV gp120 to alter the brain or BBB was not related to immune-mediated clearance of this antigen. Neither group contained ISEL-positive cells. The number of FIB+ foci averaged 1.7 ± 1.6

Table 1 *In situ* end labeled cells and fibrinogen extravasation following chronic exposure to TNF-α in nude mice and to HIV gp120 in BALB/c mice

	nu/nu	*nu/nu 10 days*		*nu/nu 40 days*		*BALB/c*	*BALB/c thymectomized 20 days*		*BALB/c mice 40 days injection*		
	untreated	*CHO−*	*CHO+*	*CHO−*	*CHO+*	*untreated*	*PBS*	*HIV*	*PBS*	*EBV*	*HIV*
No. of animals	3	6	6	5	4	3	4	4	3	3	3
ISEL+	0	0	0	0	0	1.3 ± 1.7	0	0	1.8 ± 2	4.4 ± 3.1	2.7 ± 2.4
FIB+foci	8.6 ± 1	10.9 ± 4.7	8.7 ± 5.7	1.4 ± 1.6	4.2 ± 1.4	1.7 ± 1.6	0.8 ± 1	1.2 ± 1.1	5 ± 2	6 ± 4	7 ± 2

Abbreviations: ISEL: number of *in situ* end label positive nuclei per 400× power field ×100. FIB+foci: number of foci with fibrinogen immunoreactivity in brain parenchyma per 200 × power field × 100; CHO−: non transfected Chinese hamster ovarian tumor cell; CHO+: human TNF-α-transfected Chinese hamster ovarian tumor cell, PBS: phosphate buffered saline; HIV: human immunodeficiency virus gp120; EBV: Epstein-Barr virus glycoprotein gp125, nu/nu mice received intramuscular implants of CHO cell lines. BALB/c mice received subcutaneous injections of viral glycoproteins or PBS.

in untreated mice and 0.8 ± 1 and 1.2 ± 1.1 PBS and HIV gp120-treated thymectomized BALB/c mice following 20 day injection.

Abnormalities of the intraparenchymal blood vessels of the brain in AIDS patients are common. They include a calcific vasculopathy in children, endothelial hypertrophy and hyperplasia in adults and a breakdown of the BBB that can develop in the apparent absence of focal brain lesions, including those due to HIV encephalitis (see Petito, 1997 for review). While the mechanism of the BBB breakdown is not certain, the elevated serum levels of TNF-α in AIDS patients is commonly hypothesized to be responsible, especially since TNF-α causes brain edema following intrathecal or brief intravenous administration (Saukkonen *et al*, 1990; Quagliarello *et al*, 1991). Tumor necrosis factor has limited, if any, passage across an intact BBB and any effects on brain would have to be mediated via alterations in endothelial function. HIV $gp120_{SF2}$, despite its high molecular weight of 120 000, crosses the BBB of adult mice with an influx constant of 4.62 (10^{-5} ml/g/min; adsorptive en docytosis is the hypothesized mechanism (Banks *et al*, 1997). Endothelial infection by HIV develops with lymphotropic variants (Poland *et al*, 1995) so the use of the SF2-derived HIV gp120 may be most appropriate to detect endothelial-related changes.

We found that cerebrovascular permeability is unaffected by chronic exposure to TNF in the systemic circulation. In addition, we found no evidence that prolonged exposure to systemic TNF caused any brain damage. Indeed, most studies of TNF-α-induced brain edema, including the original report in the CHO model used in this present study, require intrathecal administration of TNF-α in order for BBB breakdown and brain edema to develop. An analogous situation exists with cerebral malaria in which mechanisms of brain damage and cerebral edema, long considered to be due to elevated TNF-α in the serum, are more likely due to intrathecal production of this neurotoxic cytokine (Medana *et al*, 1997).

Although the model we used exposed murine brain to human TNF-α, it is unlikely that the species difference accounts for the BBB integrity since human TNF-α effects on mice include severe cachexia (Oliff *et al*, 1987; Tracey *et al*, 1990) and impaired collagen synthesis and wound healing (Buck *et al*, 1996) after systemic CHO-TNF-α implantation; and brain edema and inflammation after intracerebral CHO-TNF-α implantation (Tracey *et al*, 1990). Human TNF-α also causes intracellular Ca^{2+} accumulation in rat astrocytes (Koller *et al*, 1996), p53-dependent apoptosis in rat glioma cells (Yin *et al*, 1995), increased intracellular adhesion molecule-1 in rat lung (Mulligan *et al*, 1993), and increased vascular permeability in bovine endothelium (Royal *et al*, 1989) and piglet brain (Megyeri *et al*, 1992).

The present study also suggests that exposure to systemic gp120 produces neither a breakdown in the BBB nor brain damage in adult animals. While the evidence for this is not as strong as for TNF-α, since gp120 exposure was intermittent rather than chronic, the integrity of the BBB following intravenous administration supports the above interpretation. While gp120 clearly is neurotoxic *in vitro* and *in vivo*, it is important to recognize that these all required direct exposure of brain, either by intrathecal administration *in vivo* or direct application in culture, for the neurotoxicity to occur.

We cannot necessarily extrapolate our results to newborn animals since, in this age group, the BBB is not fully mature and is more permeable to macromolecules than the adult BBB (Tonra and Mendell, 1997). The factor may be responsible for the mild brain damage and impaired maze learning detected in newborn rat pups following similar chronic subcutaneous injections of HIV gp120 (Hill *et al*, 1993).

The results of the present study suggest that the levels of TNF-α and of gp120 in the systemic compartment alone will not affect the severity of disease in the CNS compartment. Thus, toxicity is likely to be related to intrathecal production. In those cases with HIV encephalitis, both could be operative, with TNF-α derived from activated inflammatory cells and reactive astrocytes and viral proteins from the HIV-infected monocytes. In those cases without HIV encephalitis, including preAIDS cases with minor or no productive HIV infection of brain, TNF-α toxicity could arise from the activated microglia and reactive astrocytes that have been described even in the preAIDS brains (Kibayashi *et al*, 1996) and thus could account for the enhanced apoptosis that can occur in those cases without HIV encephalitis (An *et al*, 1996; Gray *et al*, 1996). An alternative source of gp120 in both AIDS and preAIDS cases could be the infected cells within the stroma of the choroid plexus which are encountered in 43% of AIDS brains (Falangola *et al*, 1995) and 25% of preAIDS cases (personal observations).

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