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HIV-1 gp120 PRODUCES DNA FRAGMENTATION IN THE CEREBRAL CORTEX OF RAT

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SUMMARY In the present experiments we have used morphological techniques to study the neuropathological profile of the brain of rats after intracerebroventricular (i.c.v.) injection of recombinant HIV-1 gp 120. Using brain cryostat sections (10 μm) from rats treated with a single, daily dose of gp120 (100 ng/rat) given for 7 and 14 consecutive days, *in situ* DNA fragmentation was revealed in the neocortex but not in the hippocampus by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end-labelling (TUNEL). In these rats, dark degenerating neurones were observed in the neocortex but not in the hippocampus. Treatment with bovine serum albumin (300 ng/rat, i.c.v.) for up to 14 days did not produce DNA fragmentation nor did it yield neuropathological lesions of the neocortex or hippocampus. In conclusion, the present data demonstrate that gp 120 given i.c.v. produced DNA fragmentation in the neocortex, thus suggesting that apoptosis is the mechanism through which neurones of the neocortex are killed.

The acquired immunodeficiency syndrome (AIDS) is often accompanied by signs of neurological deficits such as loss of memory and progressive dementia (see 9, 14); loss of cortical neurones has also been reported at *post-mortem* in the brain of AIDS patients (6). It has been suggested that human immunodeficiency virus type-1 (HIV-1) glycoprotein gp120 may contribute to the observed brain neuronal loss because it causes death of several types of neurones maintained in culture (see 10).

In vitro exposure to gp120, in fact, produces death of rodent cortical and hippocampal neurones, retinal ganglion cells (4, 10) and cerebellar granule cells (15). The mechanism of gp120 cytotoxicity involves excessive Ca^{2+} entry into neurones via N-methyl-D-aspartate (NMDA) receptor associated cation channel and through voltage operated Ca^{2+} channels

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since NMDA antagonists and Ca^{2+} channel blockers prevent neuronal death (see 10). *In vivo* experiments have shown that systemic administration of purified gp120 in neonatal rats produces dystrophic changes of cortical pyramidal neurones accompanied by abnormalities of developmental behaviours (9). In addition, neuronal damage has been recently reported in the brain of transgenic mice expressing gp120 mRNA in astrocytes (17). Here we now report evidence demonstrating that subchronic intracerebroventricular (i.c.v.) microinfusion of gp120 produces DNA fragmentation in the neocortex suggesting that the neuronal death reported in this region of the rat brain might be of the apoptotic type.

MATERIALS AND METHODS

Adult male Wistar rats (250-280 g) were housed in a temperature (22 °C)- and humidity (65%)-controlled colony room. Anaesthetised (chloral hydrate; 400 mg/kg i.p.) rats were implanted with a guide cannula (25 gauge) into one lateral cerebral ventricle (i.c.v.) under stereotaxic guidance (13). Microinfusion of recombinant HIV-1 gp120 IIIB (from baculovirus expression system; >90% pure) or bovine serum albumin (BSA) was carried out via a Hamilton syringe (5 µl) connected through a teflon tube to an injector (1 µl volume of injection; 1 µl/min rate). Twentyfour hours after 1, 7 and 14 days treatment the animals were perfused through the left ventricle of the heart with 150 ml 4% paraformaldehyde in phosphate buffered (pH 7.4) saline (PBS) preceded by 60 ml of heparinized PBS. Two hours after perfusion, the brain was removed from the skull, post-fixed in 4% paraformaldehyde overnight, cryoprotected in sucrose (30%), immersed in nitrogen and stored at -80 °C until use. Cryostat brain coronal sections (10 µm) were mounted on polylysinized slides and processed for routine morphological analysis and neuronal cell counting (see 2). For *in situ* analysis of DNA fragmentation brain coronal sections from rats treated with gp120 or bovine serum albumin (BSA) were processed according to the TUNEL method (8). Briefly, tissue sections were incubated with proteinase K (20 µg/ml) for 15 min at room temperature (RT). The sections were rinsed with bidistilled water, washed once with TdT buffer (0.2 M potassium cacodylate, 25 mM Tris-HCl, 0.025% bovine serum albumine, 2.5 mM $CoCl_2$) and incubated with TdT (0.5 U/µl; Boehringer Mannheim) and biotinylated dUTP (0.025 nmol/µl; Boehringer Mannheim) in TdT buffer in a humid atmosphere at 37 °C for 60 min. The reaction was terminated by transferring the sections to citrate buffer (300 mM sodium chloride; 30 mM sodium citrate) for 15 min at RT. After incubation with 2% BSA for 10 min at RT, the sections were covered with alkaline phosphatase-conjugated streptavidin (BioGenex) for 20 min at RT, then developed with alkaline phosphatase substrate Fast red (BioGenex) and slightly counterstained with Mayer's haematoxylin.

RESULTS

In situ labelling of fragmented DNA was observed in the neocortex of brain coronal sections of rats (n=6 per group) receiving daily injection of gp120 (100 ng/rat/day) for 7 and 14 days but not for 1 day. A typical example of neocortical cells positive to the TUNEL staining is

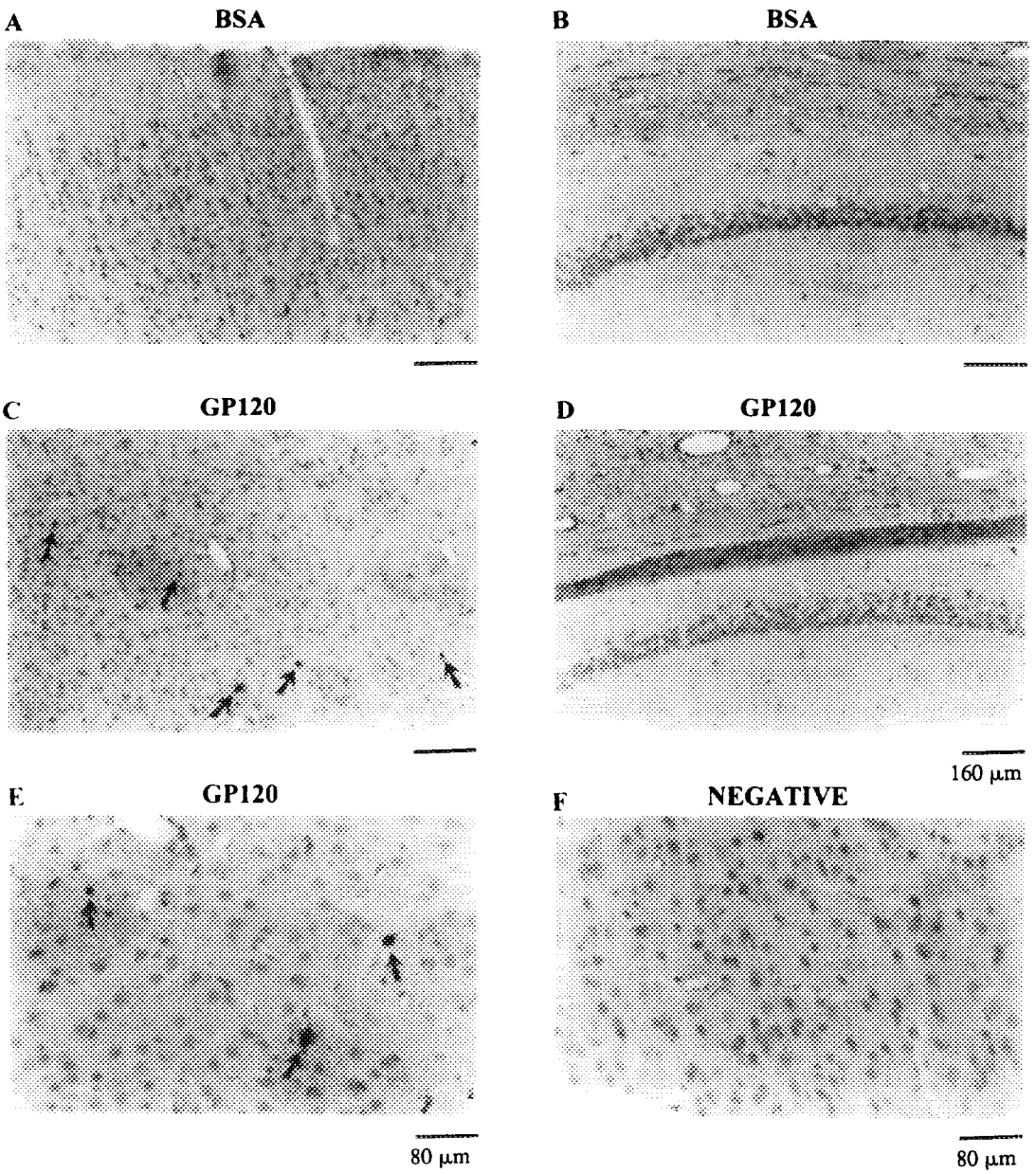

Fig. 1. (C, E) *In situ* DNA nick-end labelling of brain tissue emi-sections (10 μm) of a rat treated with a single, daily dose (100 ng/rat, i.v.c.) of gp120 given for 14 days. Note in (C) the presence of TUNEL positive cells (arrows) in the brain cortex but not in the hippocampus (panel D) ipsilateral to the side of i.c.v. injection of gp120. A similar view of positive nuclear labelling in the cortex is shown at greater magnification in (E). Lack of nuclear labelling in the cortex (A) and in the hippocampus (B) is evident in a brain tissue emi-section from a rat treated with bovine serum albumin (300 ng/rat, i.c.v.) for 14 days. (F) shows a rat brain tissue emi-section adjacent to the emi-section in (C-E) stained in the absence of TdT for negative control (see Methods for details); note the lack of nuclear labelling.

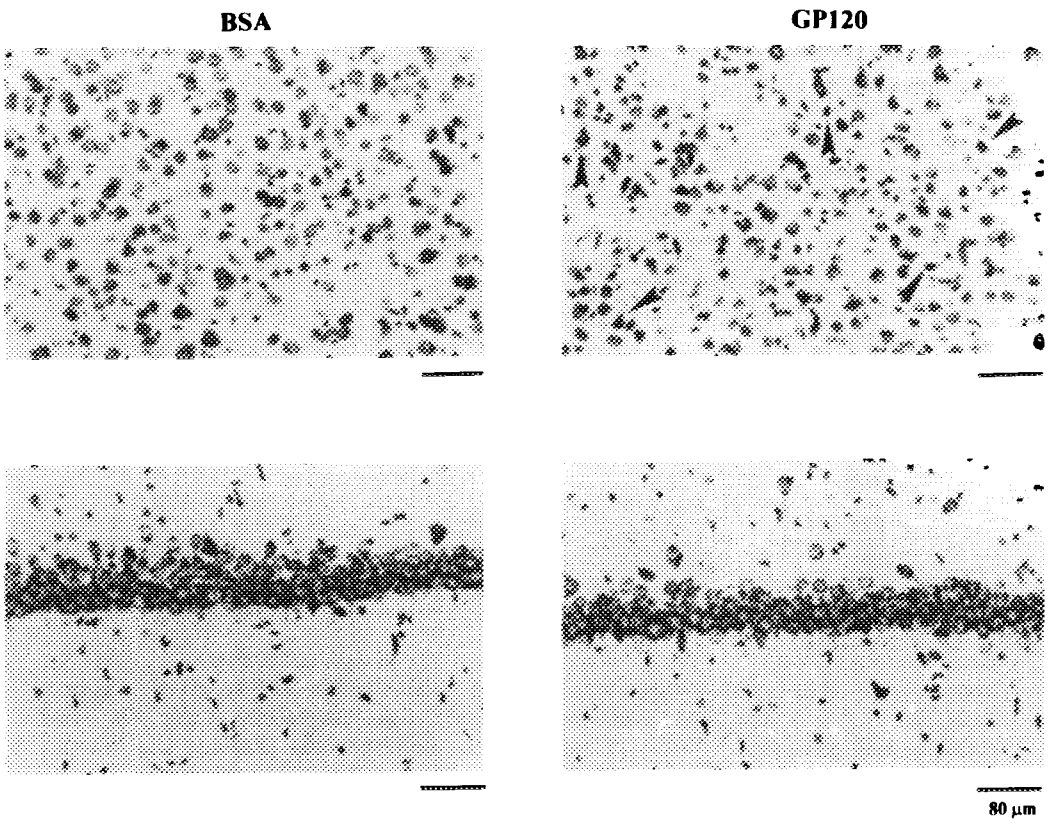

Fig. 2. Light photomicrograph of a brain tissue coronal emi-section (10 μm) depicting (panel GP120, top) a typical pattern of dark degenerating (triangular shaped) neurones (arrowheads) in the cortex ipsilateral to the side of injection of gp120 (100ng i.c.v., given daily for 14 consecutive days) in a rat. Note the lack of degeneration in the hippocampal CA1 pyramidal cell layer (panel GP120, bottom) from the same section as above. No evidence of degenerating neurones is observed in the cortex (panel BSA, top) and in the CA1 hippocampal area (panel BSA, bottom) ipsilateral to the side of injection of bovine serum albumin (BSA; 300ng i.c.v., given daily for 14 consecutive days) in a different rat.

shown in Fig. 1. The effects of gp120 were more evident in the neocortex of the treated side and concerned approximately 10% of the cortical cells counted per microscopical field (Table 1). Conventional histological examination of rat brain sections stained with cresyl fast violet revealed a similar proportion of dark degenerating neurones in the cortex (Fig. 2). In no instance was *in situ* DNA fragmentation (Fig. 1) or neuronal cell damage (Fig. 2) observed in the hippocampal formation of rats receiving daily injection of gp120 for 14 consecutive days. Lack of *in situ* DNA fragmentation (Fig. 1) or neuronal cell damage (Fig. 2) was observed in the neocortex and hippocampus of brain sections obtained from BSA (300 ng/rat i.c.v. given daily for 14 consecutive days)-treated rats (Table 1).

DISCUSSION

The present data demonstrate that microinfusion of recombinant gp120 into one lateral cerebral ventricle for 7 and 14 consecutive days produces bilateral neocortical cell death and this appears to be of the apoptotic type. This conclusion is based on direct *in situ* labelling

TABLE 1. Number of cells positive to the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end-labelling (TUNEL) staining technique in the cerebral cortex and hippocampus of rats receiving single, daily intracerebroventricular (i.c.v.) injection of recombinant HIV-1 gp120 for up to 14 days

Treatment	TUNEL Positive Cells per Microscopic Field (mean±s.e.m.)			
	Cortex		Hippocampus	
	C	T	C	T
Control (untreated)	0	0	0	0
BSA				
14 days	0	0	0	0
gp120				
1 day	0	0	0	0
7 days	2.2±0.2	2.8±0.3	0	0
14 days	2.6±0.3	6.9±0.4	0	0

Brain sections (10 μm) from rats (n=6 per treatment) treated with gp120 (100 ng, given i.c.v. once daily for up to 14 consecutive days) or bovine serum albumin (300 ng, given i.c.v. once daily for 14 consecutive days) were analysed under light microscopy (Leitz 40x). Cell counting was performed in areas of brain sections corresponding (Paxinos and Watson, 1982) to the fronto-parietal cortex and to the dorsal hippocampus. The number of TUNEL (see Methods for details) positive cells per microscopical field (only the cells in one field per area in each section were counted) was counted in the control (untreated side, C) and treated (T) side of the cortex and hippocampus in 6 sections per rat. The data from 6 rats per group were pooled and the results expressed as mean±s.e.m.

of DNA fragmentation (TUNEL). In agreement with previously reported data (7) we have failed to observe apoptotic neocortical cells in control (untreated) young adult rats by using the TUNEL staining technique. In addition, treatment with BSA for 14 days failed to produce DNA fragmentation in any of the brain regions studied suggesting that the injection procedure or other unspecific factors may not account for the effect observed in the neocortex of rats treated with gp120. Interestingly, lack of DNA fragmentation has been observed in the hippocampus of rats treated with gp120 for up to 14 days suggesting that a regional differential sensitivity to the detrimental effect of gp120 may exist in the rat brain. This is in agreement with the present and previous data demonstrating that i.c.v. injection of gp120 does not produce death or significant loss of neurones in the rat hippocampus (3). *In vitro* studies demonstrate that gp120 produces death of cortical, hippocampal, cerebellar and other types of rodent neuronal cells in primary culture (4, 10, 15). To date, however, apoptosis induced by gp120 has been reported only in primary cultures of cortical neurones by Muller et al. (12); in addition, these Authors failed to observe DNA fragmentation in

cultured human astrocytes (12). Whether this indicates a selective vulnerability of cortical neurones to the effect of gp120 remains to be established using other types of neuronal cells in culture. It is worth noting, however, that in the brain of AIDS patients neuronal loss has been often described in the neocortex but not in other regions (see 6). Fragmentation of genomic DNA into nucleosomal fragments is a hallmark of apoptosis probably initiated by an excessive raise in intracellular Ca^{2+} which in turn activates Ca^{2+}- Mg^{2+}-dependent endonucleases (see 1). *In vitro* studies have demonstrated that gp120 induces Ca^{2+} entry into neuronal cells (5). Furthermore, MK801 and memantine, two blockers of the Ca^{2+} channel associated to the NMDA receptor complex, prevented the DNA fragmentation caused by gp120 in primary cultures of rodent cortical neurones (12). Collectively, these data seem to suggest that the mechanism of apoptosis induced by gp120 in neuronal cells involves NMDA-gated Ca^{2+} entry. However, this conclusion has to be reconciled with the lack of observation of DNA fragmentation in the hippocampus (present data), an area of the brain endowed with a high density of NMDA receptors (11) and with the recent demonstration that gp120 *reduces* rather than *increases* NMDA-evoked Ca^{2+} currents and cytotoxicity in cultured cerebellar granule cells (16). In addition, we have recently failed to potentiate the excitotoxic effect of a subconvulsive dose of NMDA given stereotaxically into the dorsal hippocampus of rats pretreated i.c.v. with gp120 (3). In conclusion, the present study demonstrates that subchronic i.c.v. treatment with gp120 causes DNA fragmentation in the neocortex of rats suggesting that apoptosis may underlie neuronal cell death.

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