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Human immunodeficiency virus type 1 coat protein neurotoxicity mediated by nitric oxide in primary cortical cultures

(gp120/AIDS/glutamate/*N*-methyl-D-aspartate/microglia)

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ABSTRACT **The human immunodeficiency virus type 1 coat protein, gp120, kills neurons in primary cortical cultures at low picomolar concentrations. The toxicity requires external glutamate and calcium and is blocked by glutamate receptor antagonists. Nitric oxide (NO) contributes to gp120 toxicity, since nitroarginine, an inhibitor of NO synthase, prevents toxicity as does deletion of arginine from the incubation medium and hemoglobin, which binds NO. Superoxide dismutase also attenuates toxicity, implying a role for superoxide anions.**

AIDS is often associated with multiple neurological abnormalities including deficits in cognitive and motor functions (1–3). While the human immunodeficiency virus type 1 (HIV-1) can enter the central nervous system, the pathogenesis of the HIV-1-associated cognitive motor complex, the AIDS dementia complex, has remained elusive (4), as HIV-1 infection has been detected in macrophages and microglia but not in neurons (5–8). Despite HIV-1 not directly infecting neurons, there is profound neuronal loss in the cortex and retina (9, 10). Neuronal cell death might involve the HIV-1 coat protein, gp120, which is shed by the virus and which can elicit neurotoxicity in very low concentrations in primary hippocampal (11, 12) and retinal ganglion (11, 13, 14) cultures. Death of gp120-treated retinal ganglion cells is preceded by a marked increase in intracellular calcium, which is blocked by L-type calcium channel antagonists (11, 13). *N*-methyl-D-aspartate (NMDA) receptor antagonists also attenuate gp120-induced neurotoxicity (14). We showed that glutamate neurotoxicity in primary neuronal cultures induced by stimulation of NMDA receptors is mediated, in part, by nitric oxide (NO) (15, 16). In the present study, we demonstrate that gp120 neurotoxicity in primary cortical neurons involves NO and superoxide anions.

MATERIALS AND METHODS

Cell Cultures. Primary cell cultures were prepared from fetal rats on gestation day 14 (17). The cortex was dissected under a microscope, incubated for 20 min in 0.027% trypsin/saline (5% phosphate-buffered saline/40 mM sucrose/30 mM glucose/10 mM Hepes, pH 7.4), and transferred to modified Eagle's medium (MEM)/10% horse serum/10% fetal bovine serum/2 mM glutamine. Cells were dissociated by trituration, counted, and plated in 15-mm multiwell (Nunc) plates coated with polyornithine at a density of 3–4 × 10^5 cells per well. Four days after plating, the cells were treated with 5-fluoro-2'-deoxyuridine (10 μg/ml) for 3 days to inhibit proliferation of nonneuronal cells. Cells were maintained in MEM/5% horse serum/2 mM glutamine in humidified 8% CO_2/92% air at 37°C. The medium was changed twice a week with freshly prepared medium in which glutamine was added at the time of feeding. Mature neurons (>21 days in culture) were used in all experiments.

Cytotoxicity. Cells were exposed to test solutions as described (15). The cells were washed three times with a Tris-buffered control salt solution (CSS) (120 mM NaCl/5.4 mM KCl/1.8 mM $CaCl_2$/25 mM Tris·HCl/15 mM glucose, pH 7.4), exposed to test solutions for 5 min, and then washed with CSS followed by MEM containing 21 mM glucose. The cells were then returned to the incubator. At 20–24 hr after exposure to test solutions, the cells were exposed to 0.4% trypan blue in CSS to stain the residue of nonviable cells. Two to four photoprints at ×10 to ×20 were made of each well. Viable and nonviable cells were counted with at least 500–1500 cells counted per well. At least two experiments were performed using four separate wells so that a minimum of 4000–12,000 neurons were counted for each data point. Ten percent of the photomicrographs were counted by an additional observer blinded to the arrangement of photomicrographs, study design, and treatment protocol. An interrater reliability of >90% was consistently observed for the cell counting.

cGMP Assay. The formation of cGMP in primary cortical cultures was determined by a RIA. Neuronal cultures were washed three times with CSS. This was followed by application of the test solutions for 5 min in the presence of 100 μM isobutylmethylxanthine to inhibit phosphodiesterases. The reaction was stopped with 15% trichloroacetic acid followed by ether extraction. cGMP levels were measured according to the manufacturer's instructions (Amersham).

Immunoprecipitation. Immunoprecipitation of gp120 was performed by incubating 0.96-ng samples of gp120 with 4.8 μg of a mouse monoclonal anti-gp120 (IgG) antibody (American Biotechnologies, Cambridge, MA) overnight at 4°C. Control experiments were performed by incubating 0.96 ng of gp120 with 4.8 μg of mouse IgG or subjecting 0.96 ng of gp120 to identical conditions except for the addition of antibody. After overnight incubation, the solutions described above were incubated with 100 μl of goat anti-mouse IgG agarose beads (Sigma) overnight at 4°C. After centrifugation, the supernatants were used for cytotoxicity assays.

Inositol Phospholipid Turnover. Inositol phospholipid turnover was assayed as described (18). Rat brain cortical slices (400 × 400 μm) were prepared, allowed to recover (19), and then incubated for 1 hr in Krebs/Hepes buffer containing 2-[^{3}H]cytidine at 0.4 μCi/ml (1 Ci = 37 GBq). Lithium chloride (final concentration, 10 mM) was then added fol-

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Abbreviations: DNQX, 6,7-dinitroquinoxaline-2,3-dione; DPI, diphenyleneiodonium; HIV-1, human immunodeficiency virus type 1; NMDA, *N*-methyl-D-aspartate; NOS, nitric oxide synthase; SOD, superoxide dismutase.

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lowed 10 min later by gp120 (100 pM). The reaction was terminated after 1 hr of gp120 exposure. [^{3}H]cytidine diphosphate diacylglycerol ([^{3}H]CDP-DAG) was extracted and radioactivity was counted. Simultaneous experiments were performed with carbachol (1 mM). Typical ratios of carbachol-stimulated to basal [^{3}H]CDP-DAG were 10:1.

Materials. HIV-1SF$_2$ gp120 was obtained from the National Institutes of Health AIDS Reagent Program through Monique Dubois-Dalcq (National Institute of Neurological and Communicative Disorders and Stroke/National Institutes of Health). Recombinant gp120 and mouse anti-gp120 antibody were obtained from American Biotechnologies. Diphenyleneiodonium (DPI) was purchased from Kodak and MK801 was purchased from Research Biochemicals (Natick, MA). cGMP RIA kits were purchased from Amersham. Tissue culture reagents were obtained from GIBCO/BRL. [^{3}H]Cytidine was obtained from DuPont/NEN. Unless otherwise noted, all other chemicals were purchased from Sigma. Reduced hemoglobin was prepared by the method of Martin and colleagues (20).

RESULTS

gp120 Neurotoxicity Is Dependent on Extracellular Glutamate. In our initial experiments, gp120-induced neurotoxicity in cortical cultures was variable with the potency of gp120 apparently related to the interval following the exchange of the growth medium (data not shown). When we exposed neuronal cultures to gp120, 12 hr after exchanging the standard growth medium, we observed minimal cell death. Lipton *et al.* (14) showed that gp120 toxicity in retinal ganglia cell cultures depends on extracellular glutamate. Accordingly, we exposed cells to 100 pM gp120 with various concentrations of glutamate. gp120 toxicity is absolutely dependent on extracellular glutamate (Fig. 1*A*). No toxicity is evident in the absence of glutamate while 25 μM glutamate plus 100 pM gp120 produces ≈75% of the maximal cell death obtained at 50–75 μM glutamate plus 100 pM gp120. The maximal percentage of cells killed by gp120 in the presence of glutamate is ≈60%. All subsequent experiments used 25 μM glutamate, as 50 μM glutamate itself elicits some toxicity.

gp120 is extremely potent in eliciting cell death with significant effects at 0.1 pM gp120, half-maximal influences at 10 pM gp120, and maximal toxicity at 0.1–1 nM gp120 with ≈50% of cells dying (Fig. 1*B*). Toxicity following 100 pM gp120 is attenuated 75% by immunoprecipitating with antiserum to gp120 but not by nonimmune mouse IgG (data not shown).

gp120 Toxicity Involves Glutamate Receptors and Calcium. In retinal ganglion cell cultures, NMDA antagonists block gp120 toxicity (14). In cortical cultures, the NMDA antagonist MK801 (10 μM) reduces gp120 (100 pM)-induced toxicity by 70% (Table 1). 6,7-Dinitroquinoxaline-2,3-dione (DNQX), which blocks non-NMDA glutamate receptors, reduces neurotoxicity to a similar extent as MK801. The combination of MK801 and DNQX results in even greater protection, reducing neurotoxicity by >90%.

gp120 toxicity in retinal ganglion cells is diminished by the L-voltage-dependent calcium channel antagonists nifedipine and nimodipine as well as by calcium-free medium (11, 14). We observe a 50% reduction in gp120 (100 pM) toxicity in cortical cultures with 100 μM nifedipine and complete protection when calcium is omitted from the CSS medium. Release of calcium from intracellular calcium pools may also contribute to neurotoxicity. In cultured cortical neurons, dantrolene inhibits glutamate neurotoxicity as well as increases in intracellular calcium that are both dependent and independent of external calcium (21, 43). Dantrolene (30 μM) reduces gp120 (100 pM) neurotoxicity nearly to control values (Table 1). Since inositol phospholipid turnover increases intracellular Ca^{2+}, we monitored effects of gp120 on inositol phospholipid turnover in cortical slices (18) but observed no changes (data not shown).

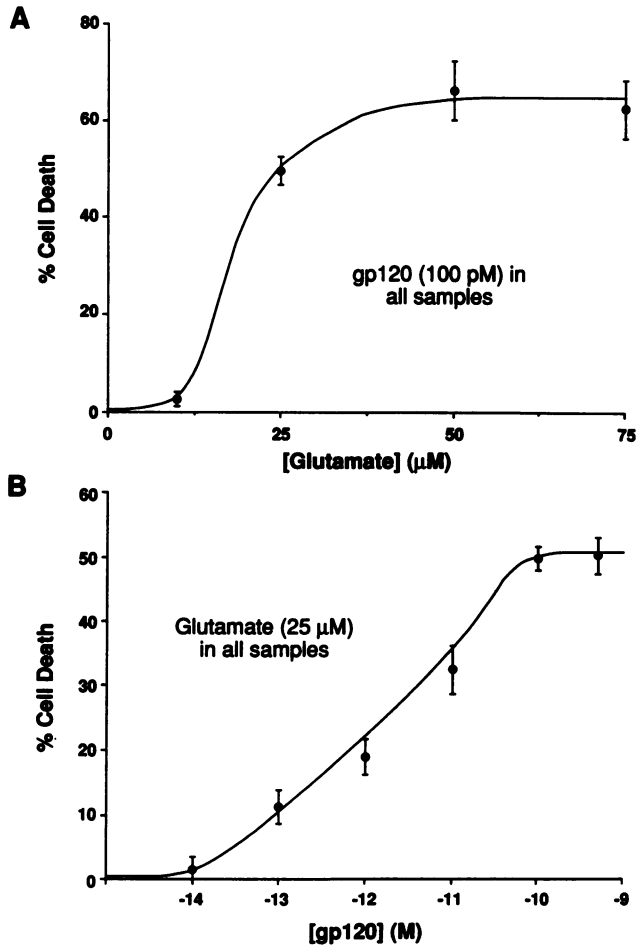

FIG. 1. gp120 neurotoxicity is dependent on extracellular glutamate. (*A*) Effect of various glutamate concentrations in the presence of 100 pM gp120. (*B*) Effect of various gp120 concentrations in the presence of 25 μM glutamate. All data points represent means ± SEM of at least two individual experiments with a total of $n \geq 8$.

gp120 Neurotoxicity Involves NO. Cortical cultures were depleted of endogenous arginine by incubating them 20–24 hr before addition of gp120 in MEM with arginine deleted and glutamine added to block arginine synthesis (22). In arginine-free medium, gp120 toxicity is reduced by nearly 70% (Table 2). Nitroarginine (100 μM), a potent inhibitor of NO synthase (NOS), reduces cell death by 70%. Protection by nitroarginine is reversed by L-arginine (1 mM). NOS contains tightly bound flavin groups (23). Flavin-containing enzymes are

Table 1. Inhibition of gp120 neurotoxicity by glutamate receptor antagonists and calcium channel blockers

	Cell death, %
100 pM gp120 + 25 μM glutamate	51.0 ± 4.2
+ 10 μM MK801	16.2 ± 4.7*
+ 100 μM DNQX	21.7 ± 6.7*
+ 10 μM MK801 + 100 μM DNQX	5.5 ± 5.7*
+ 100 μM nifedipine	30.8 ± 6.2*
+ Ca^{2+} free medium	8.1 ± 5.3*
+ 30 μM dantrolene	6.0 ± 3.9*

Data are means ± SEM (n = 8–20). Cell death was determined by 0.4% trypan blue exclusion by viable cells (see text). Significance was determined by Student's t test for independent means.
*$P < 0.001$.

Table 2. Modulation of gp120 neurotoxicity by NOS inhibitors or SOD

	Cell death, %
100 pM gp120 + 25 μM glutamate	51.0 ± 4.2
+ L-Arg-free MEM	16.8 ± 5.6*
+ 100 μM N-Arg	16.5 ± 6.7*
+ 100 μM N-Arg + 1 mM L-Arg	50.2 ± 6.5
+ 500 nM DPI	4.7 ± 4.3*
+ 500 μM Hb	12.6 ± 6.5*
+ 100 units of SOD	9.3 ± 8.0*
+ 100 μM N-Arg + 100 units of SOD	5.3 ± 4.4*
+ 20 μM Quis pretreatment	24.1 ± 6.0*

Data are means ± SEM (n = 8–20). Significance was determined by Student's t test for independent means. N-Arg, nitroarginine; Quis, quisqualate; SOD, superoxide dismutase.
*$P < 0.001$.

potently inhibited by DPI. DPI (500 nM) blocks gp120 toxicity by 90%. NO that passes between cells can be captured by hemoglobin, as NO binds with high affinity to iron in heme. Hemoglobin (0.5 mM) reduces gp120 toxicity ≈90%.

NO toxicity may result from the combination of NO with superoxide to form peroxynitrite, which degenerates to hydroxyl and nitrogen dioxide (NO_2) free radicals, which are highly reactive (24–26). To remove superoxide anions, we treated cultures with SOD (Table 2). SOD blocks toxicity to a slightly greater extent than nitroarginine. Coapplication of SOD and nitroarginine produces a further reduction in toxicity.

Microglia, astrocytes, and macrophages can produce NO (27–30). To differentiate between NO formed by neurons versus nonneuronal cells, we previously took advantage of the differential sensitivity of NOS-containing neurons to various glutamate derivatives. Although markedly resistant to NMDA toxicity, NOS neurons are uniquely sensitive to the toxic affects of quisqualate (16, 31). In cortical cultures, quisqualate (20 μM) kills >90% of NOS neurons but only 15–20% of the total neuronal population (16). Accordingly, we treated our cultures with 20 μM quisqualate for 5 min and 24 hr later exposed them to 100 pM gp120 (Table 2). Quisqualate pretreatment reduces gp120 toxicity by 65%, implying that NOS neurons are the primary source of NO in cortical cultures that mediates gp120 toxicity.

gp120 Influences cGMP Levels via NMDA Receptors and NO. NO enhances cGMP levels in cortical cultures and numerous other tissues by stimulating guanylyl cyclase activity (32–34). If gp120 were to stimulate NOS activity, one would anticipate elevation of cGMP levels. In cortical cultures gp120 increases cGMP levels 3- to 4-fold (Table 3). The increase is completely abolished by nitroarginine and nearly abolished by *N*-methylarginine, another selective inhibitor of NOS. Hemoglobin also blocks the increase in cGMP levels. MK801 prevents gp120 enhancement of cGMP levels. Whereas SOD blocks gp120-induced neurotoxicity, it augments gp120 stimulation of cGMP formation so that coapplication of gp120 and SOD elevates cGMP levels 9-fold (Table 3). SOD removes superoxide anion that combines with NO so that more NO is available to stimulate guanylyl cyclase. In previous studies, SOD increased NMDA stimulation of cGMP levels only 30–50% in cortical cultures (15). The greater stimulation of cGMP levels by gp120 implies that gp120 may increase superoxide formation more than NMDA.

Table 3. cGMP formation after stimulation by gp120 in the absence or presence of glutamate receptor antagonist, NOS inhibitors, and SOD

	cGMP, % basal
100 pM gp120	345 ± 146
+ 100 μM N-Arg	16.9 ± 1.5
+ 500 μM NMA	133 ± 27.2
+ 10 μM MK801	98.5 ± 6.3
+ 500 μM Hb	13.5 ± 0.5
+ 100 units of SOD	900 ± 262

Data are means ± SEM (n = 6–8). N-Arg, nitroarginine; NMA, *N*-methylarginine. Basal = 100%.

DISCUSSION

In the present study, we demonstrate that the HIV-1 coat protein gp120 in extremely low concentrations elicits substantial neurotoxicity in cerebral cortical neuronal cultures, confirming the initial observations of Brenneman *et al.* (12). This toxicity requires extracellular glutamate, resembling results in retinal ganglion cells (14). The toxicity involves NMDA receptors, but non-NMDA receptors may also be required as the non-NMDA receptor antagonist DNQX prevents toxicity. However, DNQX does influence the glycine site on the NMDA receptor (35). External Ca^{2+} also is required for neurotoxicity in our cultures, as Ca^{2+} deletion prevents toxicity. Blockade of toxicity by nifedipine implicates L-type voltage-dependent calcium channels, although at the concentrations used nifedipine exerts numerous other actions (36). Block of toxicity by dantrolene suggests a role for dantrolene-sensitive intracellular calcium stores (21, 43).

The main finding of our study is that NO can play a role in gp120 toxicity. Thus, depletion of arginine from the incubation medium blocks toxicity, as does the NOS inhibitor nitroarginine. DPI, which inhibits NOS by binding to the flavin groups, also prevents toxicity. Hemoglobin, which binds extracellular NO, blocks neurotoxicity, implying that NO involved in mediating neurotoxicity passes between cells. The blockade of toxicity by SOD presumably involves the removal of superoxide anions, which can combine with NO to form peroxynitrite that degenerates into the toxic hydroxyl and NO_2 free radicals (24–26). Mediation of gp120 toxicity by NO implies that gp120 stimulates NO synthesis. The enhancement of cGMP levels by gp120 and its attenuation by NOS inhibitors further supports a role for NO in gp120 neurotoxicity.

What do these findings imply for the pathophysiology of AIDS dementia? gp120 does not directly activate NMDA receptor-associated channels (14) and does not stimulate cultures of monocytes/macrophages to increase their release

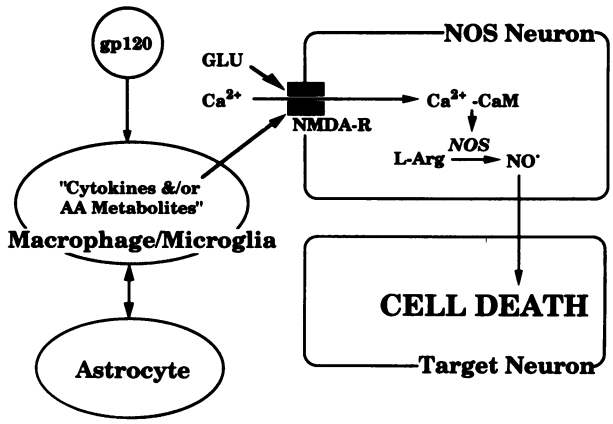

FIG. 2. Proposed mechanism of gp120-induced neurotoxicity. The HIV-1 coat protein, gp120, which is shed by the virus, may elicit neurotoxicity by interacting with macrophages/microglia and astrocytes to release cytokines and/or arachidonic acid metabolites (44, 45). These cytokines and/or arachidonic acid metabolites may act synergistically with glutamate to activate NMDA receptors, which increases intracellular calcium levels. NOS is subsequently activated and excessive formation of NO kills adjacent neurons.

of glutamate (S. A. Lipton, personal communication). However, gp120 neurotoxicity is dependent on the presence of glutamate (14) as well as macrophages and microglia in neuronal cultures (37). Arachidonic acid metabolites and cytokines are produced during cell-to-cell interactions between HIV-1-infected brain macrophages and astrocytes and mediate, in part, the neurotoxicity associated with HIV-1 infection (44). In addition, gp120 can directly induce arachidonic acid metabolites and cytokines (45). Arachidonic acid potentiates NMDA receptor currents by increasing the open channel probability (46). Thus, it is conceivable that gp120 interacts with macrophages and microglia in neuronal cultures to release arachidonic acid metabolites and cytokines, which act synergistically with endogenous glutamate to activate neuronal NMDA receptors. Ca^{2+} then enters NOS neurons (11, 14, 38) to stimulate the formation of NO, which is toxic to adjacent neurons (Fig. 2). Indeed, Giulian *et al.* (6) showed that HIV-1-infected mononuclear phagocytes release small, heat-stable, protease-resistant molecules that exert neurotoxicity through stimulation of NMDA receptors.

The discovery that NO mediates components of NMDA neurotoxicity (15, 16, 39–41) implied a role for NO in vascular stroke. In animal models of focal ischemia, nitroarginine administration after ligation of the middle cerebral artery markedly reduces neuronal damage (42). Conceivably, NOS inhibitors could exert corresponding therapeutic effects in treatment of AIDS dementia.

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