Platelet-Activating Factor: a Candidate Human Immunodeficiency Virus Type 1-Induced Neurotoxin

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The pathogenesis of central nervous system disease during human immunodeficiency virus type 1 (HIV-1) infection revolves around productive viral infection of brain macrophages and microglia. Neuronal losses in the cortex and subcortical gray matter accompany macrophage infection. The question of how viral infection of brain macrophages ultimately leads to central nervous system (CNS) pathology remains unanswered. Our previous work demonstrated high-level production of tumor necrosis factor alpha, interleukin 1 β , arachidonic acid metabolites, and platelet-activating factor (PAF) from HIV-infected monocytes and astroglia (H. E. Gendelman, P. Genis, M. Jett, and H. S. L. M. Nottet, in E. Major, ed., *Technical Advances in AIDS Research in the Nervous System*, in press; P. Genis, M. Jett, E. W. Bernton, H. A. Gelbard, K. Dzenko, R. Keane, L. Resnick, D. J. Volsky, L. G. Epstein, and H. E. Gendelman, *J. Exp. Med.* 176:1703–1718, 1992). These factors, together, were neurotoxic. The relative role(s) of each of these candidate neurotoxins in HIV-1-related CNS dysfunction was not unraveled by these initial experiments. We now report that PAF is produced during HIV-1-infected monocyte-astroglia interactions. PAF was detected at high levels in CSF of HIV-1-infected patients with immunosuppression and signs of CNS dysfunction. The biologic significance of the results for neurological disease was determined by addition of PAF to cultures of primary human fetal cortical or rat postnatal retinal ganglion neurons. Here, PAF at concentrations of ≥ 300 pg/ml produced neuronal death. The *N*-methyl-D-aspartate receptor antagonist MK-801 or memantine partially blocked the neurotoxic effects of PAF. The identification of PAF as an HIV-1-induced neurotoxin provides new insights into how HIV-1 causes neurological impairment and how it may ultimately be ameliorated.

Human immunodeficiency virus type 1 (HIV-1) infection of the central nervous system (CNS) results in cognitive and motor abnormalities in a majority of infected individuals. HIV-1-associated dementia or AIDS dementia complex (ADC), a devastating complication of direct viral infection of the brain, arguably represents one of the most severe and significant clinical manifestations of HIV infection (6, 13, 16, 26, 43). The pathological hallmarks coincident with cognitive and motor dysfunctions include HIV-1 infection of brain macrophages, microglia, and multinucleated giant cells; astrocyte proliferation; and neuronal loss in discrete areas of the retina, neocortex, and subcortical brain (14, 18, 29, 40, 49, 50, 53, 59, 60). Interestingly, this disease complex is most prevalent in virus-infected children (6).

The molecular mechanisms governing neurologic dysfunction during HIV-1 infection of the brain remain an enigma. No laboratory has convincingly demonstrated HIV-1 mRNA or p24 antigens in neurons or oligodendrocytes within virus-infected brain tissue. How does HIV-1 cause neuronal loss

when neurons are not infected and perhaps only a small number of macrophages support productive viral replication (13, 17, 40)? Indeed, secretory products from HIV-1-infected macrophages are the likely source of neurotoxic activities. Neuronal death can occur soon after exposure to culture fluids from HIV-1-infected monocytes. Monocyte production of neurotoxic factors appears to require HIV-1 infection (21, 47), monocyte activation, and astroglia and/or neuronal monocyte interactions (2, 12, 17, 20, 52). HIV-1 gene products also affect neuronal viability. Picomolar amounts of HIV-1 gp120 added to mixed microglial-neuronal cells in the presence of sublethal concentrations of glutamate are toxic to rat retinal ganglion cells (10, 11, 33–35). This toxicity is associated with increased neuronal Ca²⁺ and is reversed by calcium channel or *N*-methyl-D-aspartate (NMDA) antagonists (33–35).

Our recent work has demonstrated that coculture of HIV-1-infected monocytes and human astrocytoma cell lines induces cytokines, arachidonic acid metabolites, and platelet-activating factor (PAF) from virus-infected monocytes (20). Astroglia cells serve to activate macrophages for their production of these neurotoxic factors (17). Our initial studies, however, did not provide evidence that any or all of the factors are neurotoxins or determine whether they play a biologically important role in neurological disease. The purpose of the

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present study was to identify PAF as a candidate HIV-1 neurotoxin and to provide a conceptual framework for how "putative" HIV-1 neurotoxins are identified. PAF is produced within cocultures of HIV-1-infected monocytes and astroglia and is present in the cerebrospinal fluid (CSF) of HIV-infected patients during neurologic dysfunction. PAF added to primary human or rat neurons at levels approximating those found in the CSF of HIV-1-infected patients produces dose-dependent neurotoxicity. NMDA receptor uncompetitive antagonists MK-801 and memantine (4, 22, 35, 39) block PAF-mediated neurotoxicity. These findings, taken together, provide insights into the pathobiology of ADC and new ideas for therapeutic interventions for neurological impairments associated with HIV-1 infection.

MATERIALS AND METHODS

HIV-1 infection of monocytes. Monocytes were recovered from peripheral blood mononuclear cells of HIV- and hepatitis B-seronegative donors after leukapheresis and purified by countercurrent centrifugal elutriation. Cell suspensions were >98% monocytes by criteria of cell morphology on Wright-stained cytosmears, by granular peroxidase, and by nonspecific esterase (15, 19). Cells were cultured as adherent monolayers (10^6 /ml in 24-mm-diameter plastic culture wells) in Dulbecco modified Eagle medium (Sigma, St. Louis, Mo.) with recombinant human macrophage colony-stimulatory factor (Genetics Institute, Cambridge, Mass.). The human brain astroglia tumor-derived cell line U251 MG, a generous gift from D. Bigner, was utilized in cocultivation assays with HIV-1-infected monocytes as described previously (20). These cells were grown as adherent monolayers in Dulbecco modified Eagle medium (Sigma)-10% heat-inactivated fetal calf serum (Sigma)-50 μ g of gentamicin per ml.

Monocytes were exposed to HIV-1_{ADA} (accession number M60472) (42) at a multiplicity of infection of 0.01 infectious virions per target cell (19, 57). The viral inocula were free of mycoplasma contamination (Mycoplasma Detection Kit III; Geneprobe, San Diego, Calif.). Human macrophage colony-stimulating factor-treated monocytes were cultured as adherent monolayers 7 to 10 days prior to use as viral target cells. Under these conditions, 10 to 20% of the monocytes are productively infected 7 days after HIV-1 inoculation (27). All cultures were refed with fresh medium every 2 to 3 days. Reverse transcriptase activity was determined in culture fluids added to a reaction mixture of Nonidet P-40 (Sigma), poly(rA)-oligo(dT) (Pharmacia, Piscataway, N.J.), dithiothreitol (Pharmacia), MgCl_2 , and [α - 32 P]dTTP (400 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) for 24 h at 37°C. The mixture was applied to chromatography paper, air dried, and washed five times in 0.3 M NaCl-0.03 M sodium citrate (pH 7.4) and twice in 95% ethanol. The paper was dried and cut, and the radioactivity was counted by liquid scintillation spectroscopy (27). Five to 7 days after infection and during the peak of reverse transcriptase activity (10^7 cpm/ml) in HIV-1-infected monocytes, equal numbers of astroglial cells, U251 MG, were added and cell lysates were recovered for PAF determinations.

PAF assay. Cultured cells or CSF samples were frozen in dry ice-ethanol mixtures. CSF samples were frozen immediately after procurement from patients. Cell samples were extracted within 1 week of freezing to preserve the integrity of the lipids, which are generally unstable in aqueous solutions. The extraction procedure was performed with a C18 disposable cartridge (Waters, Milford, Mass.) as previously described (18). PAF was eluted as a separate fraction, with approximately 70%

TABLE 1. PAF levels in CSF of HIV-1-infected patients with or without neurological impairment

Patient no.	Age	ADC stage ^a	No. of CD4 ⁺ T cells	Mean PAF concn (pg/ml) $\pm$ SEM
1	7 mo	1 ^b	480	22
2	10 yr	1 ^b	182	32
3	7 yr	0 ^b	20	61
4	47 yr	1	530	124 $\pm$ 17
5	5 yr	3 ^b	256	152 $\pm$ 6
6	61 yr	0	355	157 $\pm$ 13
7	37 yr	0	116	192 $\pm$ 27
8	35 yr	0	446	195 $\pm$ 11
9	48 yr	2	4	197 $\pm$ 73
10	41 yr	2	12	198 $\pm$ 20
11	34 yr	1	374	232 $\pm$ 62
12	42 yr	4	4	241 $\pm$ 22
13	31 yr	2	258	245 $\pm$ 32
14	23 yr	0	320	260 $\pm$ 2
15	31 yr	1	157	287 $\pm$ 35
16	17 mo	3 ^b	85	299 $\pm$ 24
17	25 mo	3 ^b	354	322 $\pm$ 51
18	37 yr	3	6	335 $\pm$ 20
19	34 yr	3	20	343 $\pm$ 56
20	45 yr	2	58	353 $\pm$ 151
21	42 yr	3	12	398 $\pm$ 17
22	35 yr	3	45	624 $\pm$ 45
23	34 yr	1	50	853 $\pm$ 248

^a Neurologic dysfunction (ADC stage) (46a) rating was done in accordance with the guidelines of the American Academy of Neurology Task Force on AIDS (26).

^b Pediatric patients were assigned "best-approximation" ADC stages to conform with the adult data. All of the subjects were male.

recovery, based on inclusion of internal standards of [^3H]PAF during extraction. The material was dissolved in 1.2 ml of methanol and aliquoted into 0.1-, 0.4-, and 0.6-ml fractions. These were dried by vacuum centrifugation (Savant Instruments, Inc., Farmingdale, N.Y.), and PAF was quantitated by radioimmunoassay (E. I. Du Pont de Nemours & Co., Boston, Mass.). The detectable levels of PAF in these assays were 30 to 1,200 pg/ml (20).

Patient material. Forty-four patients were clinically evaluated at the University of Nebraska, the University of Medicine and Dentistry of New Jersey, the Wadsworth VA Medical Center (University of California Los Angeles), and the Massachusetts General Hospital. Patients were retrospectively evaluated. Nine HIV-1-seropositive patients had complete postmortem examinations. The HIV-positive patients ranged in age from 7 months to 61 years (Table 1). Twenty-one control patients were between 13 and 78 years old (Table 2). The HIV-seropositive patients were classified as to severity of neurologic dysfunction in accordance with ADC guidelines (46a) and those established by the American Academy of Neurology Task Force on AIDS (26).

Human neuronal cell cultures. Human fetal brain tissue was obtained from elective therapeutic abortions under the strict ethical guidelines of the National Institutes of Health and the University of Rochester Medical Center. Neurons were obtained from the telencephalon with both cortical and ventricular surfaces of second-trimester (13 to 16 weeks of gestation) human fetal brain tissue by a modification of the procedure of Banker and Cowan (1). Briefly, brain tissue was collected and washed in 30 ml of cold Hanks balanced salt solution (containing Ca^{2+} , Mg^{2+} , HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid], and 50 μ g of gentamicin per ml). Brain tissue was separated from adherent meninges and blood and

TABLE 2. PAF levels in CSF of non-HIV-1 disease and control subjects

Patient no.	Age (yr)/sex ^a	Systemic disease	Mean PAF concn (pg/ml) $\pm$ SEM
1	38/F	None ^b	30 $\pm$ 4
2	14/F	None	32 $\pm$ 7
3	78/F	None	37 $\pm$ 12
4	61/F	MS ^c (minimal deficits)	64 $\pm$ 13
5	49/F	Localized melanoma	68 $\pm$ 7
6	56/M	None	97 $\pm$ 4
7	71/M	None	97 $\pm$ 13
8	72/M	Myocardial infarction	111 $\pm$ 10
9	30/M	None	121 $\pm$ 13
10	13/M	Leukemia (CNS involvement)	125 $\pm$ 8
11	36/M	TIA ^d	144 $\pm$ 21
12	54/M	TIA	160
13	32/F	MS (minimal deficits)	168 $\pm$ 26
14	79/M	Pneumonia	172 $\pm$ 24
15	93/M	Prostatic Ca ^e	190 $\pm$ 16
16	61/M	Prostatic Ca	213 $\pm$ 40
17	44/M	Hepatic failure	235 $\pm$ 79
18	4/M	Leukemia (CNS)	366 $\pm$ 55
19	57/M	Nasopharyngeal Ca	417 $\pm$ 53
20	22/M	Diffuse histiocytic lymphoma	709 $\pm$ 201
21	58/M	Metastatic melanoma	825 $\pm$ 446

^a F, female; M, male.^b For subjects without systemic disease, lumbar punctures were performed to exclude meningitis. Patients presented with headache, fever, and/or weakness. CSF examinations showed normal protein and glucose levels and cell counts.^c MS, multiple sclerosis.^d TIA, transient ischemic attack.^e Ca, carcinoma.

cut into 2-mm³ pieces. Dissociated tissue was transferred to and then forced through a 230- μ m Nitex bag. The dissociated tissue was gently triturated and then centrifuged at 100 $\times$ g for 5 min at 4°C. The pellet was resuspended in 5 to 10 ml of MEM-hipp (2 mM D-glucose, 10 mM HEPES, 1 mM Na pyruvate, 20 mM KCl) containing N1 components (insulin at 5 mg/liter, transferrin at 5 mg/liter, selenite at 5 μ g/liter, progesterone at 20 nM, and putrescine at 100 μ M), as well as 10% fetal calf serum, PSN antibiotic mix (penicillin at 50 mg/liter, streptomycin at 50 mg/liter, and neomycin at 100 mg/liter), and amphotericin B (Fungizone; 2.5 mg/liter). The cell count and viability were determined by diluting Hanks balanced salt solution with 0.4% trypan blue (1:1, vol/vol) and counting with a hemocytometer. Cells were gently triturated five times with a 10-ml pipet, plated at a density of 10⁵/12-mm-diameter glass coverslip precoated with poly-L-lysine (70K-150K MW; Sigma), and placed in 24-well culture dishes. Cells were cultured for 10 to 28 days at 37°C in a humidified atmosphere of 5% CO₂-95% air, and the medium was changed every 3 days (7).

Under these conditions, neuronal cultures were >70% homogeneous as determined by anti-human PGP 9.5 staining (Ultraclone, Ltd., Wellow, Isle of Wight, England) (28) and anti-MAP-2 staining. Glial fibrillary astrocyte protein staining-positive astrocytes made up 25% of the total cell population. Microglia-macrophages made up less than 5% of the population as determined by RCA-1 lectin and CD68 staining.

Neuronal cultures maintained for >28 days under the conditions described above expressed NMDA receptors. Neurons exposed to 300 μ M NMDA for 10 min were reduced in number to 20% of that of untreated neurons. This neurotoxicity was blocked by coincubation with 10 μ M MK-801 during exposure to NMDA (data not shown). Evaluation of PAF-

induced human neurotoxicity was performed with 1-O-hexadecyl-2-O-acetyl-sn-glycero-3-phosphorylcholine PAF (Biomol Research Laboratories, Inc., Plymouth Meeting, Pa.).

Coverslips with human neurons were fixed with 4% paraformaldehyde at 48 h after exposure to culture fluids from HIV-1_{ADA}-infected monocytes and/or astroglial cells. Cells were incubated with Neurotag Red (Boehringer Mannheim, Indianapolis, Ind.) to stain somas and neuritic processes. Cell morphology of neurons was examined by fluorescence microscopy. In replicate experiments, neurons were evaluated as described above at 48 h after exposure to PAF with or without MK-801. Cells were immunocytochemically stained with PGP 9.5 antiserum. To assay neurotoxicity, examiners (K.A.D., H.A.G., and L.G.E.) were blinded to the neuronal treatments. All assays were performed in triplicate determinations. Digitized images of PGP 9.5-stained neurons in ≥ 15 microscopic fields were analyzed for numbers of intact neuronal somas per 50 $\times$ field with a densitometer (Imaging Research Inc., St. Catharines, Ontario, Canada). Data were expressed as mean neuronal cell counts $\pm$ the standard errors of the means (SEM). Tests of statistical significance of the differences between control and experimental treatments were determined by paired *t* tests.

Rat neuronal cell cultures. Rat retinal ganglion cells from 7- to 10-day-old postnatal Long-Evans rats were labeled in situ by retrograde transport of the fluorescent dye granular blue. Since ganglion cells are the only cells to project from the retina to deeper brain structures, this labeling was accomplished by injection of granular blue (as a 1% solution in saline) into the superior colliculus; maximum labeling of retinal ganglion cells occurred within 2 days of the injections, so the retinas were harvested at this time after the rats were sacrificed by cervical dislocation (36). With the retrograde labeling technique, the ganglion cell neurons could be specifically identified by the presence of the blue fluorescent label, even following dissociation from the retina and placement in culture, as previously described (31, 36). The resulting retinal cultures contained mixed neuronal and glial elements. These cells were plated onto glass coverslips coated with poly-L-lysine and cultured in Eagle's minimum essential medium supplemented with 0.7% (wt/vol) methylcellulose, 2 mM glutamine, 1 mg of gentamicin per ml, 16 mM glucose, and 5% (vol/vol) rat serum. This culture medium contained 1.8 mM CaCl₂ but no MgCl₂, since at negative membrane potentials magnesium is known to block NMDA receptor-operated channels and therefore might have partially masked NMDA receptor-operated channels and NMDA receptor-mediated neurotoxicity (4, 22, 39, 44). In parallel experiments, however, qualitatively similar results were obtained with a physiologic concentration of Mg²⁺ (0.8 mM). Replicate cultures received PAF with or without the NMDA antagonist MK-801 (dizocilpine) or memantine. Retinal cultures treated in this manner were incubated overnight at 37°C in a humidified atmosphere of 5% CO₂-95% air, and then viability was assessed as described below. For these experiments, carbamyl-PAF (c-PAF; Biomol Research Laboratories, Inc.) was used to avoid rapid metabolism of PAF by tissue hydrolases (45). The existence of PAF degradation products might have obfuscated the results of the neuronal assays. In this respect, we would not have known if PAF itself or a metabolite was a neurotoxic factor in this system. c-PAF has a pharmacologic profile similar to that of PAF but is 5- to 10-fold less potent in binding and physiological studies of membrane surface receptors for PAF (45). Previous studies using high-pressure liquid chromatographic analysis showed that these retinal cultures contain approximately 25 μ M glutamate (an NMDA receptor agonist) and 15 μ M glycine (an

NMDA receptor coagonist), but by themselves these levels of the amino acids are not neurotoxic in this system (35).

The ability of retinal ganglion cells to take up and cleave fluorescein diacetate to fluorescein was used as an index of their viability and lack of injury, as described previously (17, 31). Under a combination of phase-contrast microscopy and UV epifluorescence, retinal ganglion cells are the only cells that appear blue with a standard 4',6-diamidino-2-phenylindole filter set because of the presence of retrogradely transported dye; viable cells, visualized with a fluorescein filter set, appear yellow-green. Thus, viable retinal ganglion cell neurons appear blue with one fluorescence filter set and yellow-green with a second, facilitating their rapid scoring. This test appears to be a more sensitive indicator of subtle forms of cell injury than dye exclusion studies, which have been used to monitor cell death in many studies. Results were replicated in triplicate dishes on at least four separate occasions. Viable rat retinal ganglion cells were scored in each culture dish over an area of 75 μm^2 .

RESULTS

PAF detected in HIV-infected monocyte-astroglial mixtures.

Our previous work demonstrated that tumor necrosis factor alpha (TNF- α), interleukin 1 β , arachidonic acid metabolites, and PAF are produced during interactions between HIV-infected monocytes and astroglia tumor cell lines. These factors predict neurotoxicity (20). The contribution that each of these factors made to neurotoxicity was not demonstrated. Therefore, we performed a series of experiments to determine whether PAF is a neurotoxin associated with HIV-1 infection and neurological disease.

PAF levels were examined by radioimmunoassay in HIV-1-infected cell cultures. Monocytes were infected with HIV-1_{ADA} at a multiplicity of infection of 0.01 for 7 days then added in a 1:1 cell-to-astroglia ratio (U251 MG cells). Methanol extracts of cells were prepared during monocyte-astroglial coculture at 1, 3, 6, 12, 20, and 30 min. As shown in Fig. 1, high levels of PAF were selectively observed (3 to 20 min) in cocultures of HIV-infected monocytes and astroglia. PAF concentrations were significantly lower (i) in HIV-1-infected monocytes or (ii) in uninfected monocytes in the presence or absence of astroglia. PAF could not be detected reproducibly at longer time intervals (hours to days) (data not shown). This may have been due to the temporal regulation of PAF and its instability in culture solutions for longer time intervals (3, 8, 9, 20). Alternatively, PAF may be synthesized only after the initial cell-to-cell contact between HIV-1-infected monocytes and astroglia.

Measurement of PAF in CSF of HIV-1-infected control subjects. CSF samples from 23 patients with documented HIV-1 infection and 21 controls were collected for measurement of PAF levels (Tables 1 and 2). Neurologic dysfunction, defined by the clinical parameters of the ADC, was scored on a scale of 0 to 4. Immunosuppression was measured by determining numbers of CD4⁺ T cells in blood. Six of the 23 patients were children, with ages ranging from 7 months to 10 years. In the pediatric group, three of six children had elevated PAF levels (152 to 322 pg/ml), correlating with neurologic dysfunction and immunosuppression (Fig. 2 and Table 1). In adult patients, however, elevated PAF levels (124 to 853 pg/ml) correlated in 17 of 23 patients with the onset but not progression of neurologic dysfunction (ADC stages 1 to 4). PAF levels in CSF from this group of patients were significantly different from those in CSF from control patients ($P < 0.0002$). Five of 23 patients with no neurologic dysfunction (ADC stage 0) had

FIG. 1. Monocytes cultured for 7 days as adherent monolayers were exposed to HIV-1_{ADA} at a multiplicity of infection of 0.1. At 7 days after infection, HIV-infected or control uninfected monocytes were cocultured 1:1 (monocytes-astroglial cells). At various times (in minutes) after coculture, HIV-infected monocytes, uninfected monocytes, or monocyte cocultures were frozen on dry ice in ethanol and then cell lysates were combined with methanol washes of broken cells and extracted. PAF was eluted with approximately 70% recovery and quantitated by radioimmunoassay. The detectable levels of PAF in these assays were 30 to 1,200 pg/ml. Levels of PAF are shown as closed diamonds (HIV-1-infected monocytes and astroglial cells), closed circles (uninfected monocytes and astroglial cells), open boxes (HIV-1-infected monocytes), and open circles (uninfected monocytes). The error bars represent standard deviations.

low levels of PAF in CSF (61 to 195 pg/ml) (Fig. 3 and Table 1). Elevation of PAF in CSF was not specific for HIV-1-infected individuals. PAF was detected in patients with a variety of other medical conditions (multiple sclerosis, cerebrovascular disease, leukemia, disseminated cancer, and hepatic failure) in whom CNS symptoms (changes in cognitive and/or motor functions) were present. Patients with paraneoplastic syndromes that included CNS dysfunction (Table 2, patients 18 to 21), interestingly, had the highest PAF levels (366 to 825 pg/ml) of the non-HIV patients. This includes a single leukemic patient with CNS involvement. PAF levels in the CSF of this group of patients were also significantly different from those in the CSF of control patients ($P < 0.006$; Fig. 3).

Neurotoxic activity of PAF. Could the observed elevation of PAF in CSF mean that it plays an important role(s) in CNS dysfunction? To test this theory, we exposed primary neuronal fetal cultures to PAF at concentrations equal to and greater than those present in coculture fluids. PAF (50 to 6,000 pg/ml) applied to primary fetal cultures was toxic to human neurons in a dose-dependent manner within 48 h (Fig. 4). At PAF concentrations of 300 and 1,250 pg/ml, neuronal cell counts were reduced to 54 and 28%, respectively, of the control (untreated neurons) value. The PAF-induced neurotoxicity

FIG. 2. Box plots of PAF levels in CSF obtained from control (HIV-1-seronegative) patients without systemic disease ($n = 6$), HIV-1-seropositive patients without evidence of neurologic dysfunction (ADC stage 0; $n = 5$), and HIV-1-seropositive patients with neurologic dysfunction (ADC stages 1 to 4; $n = 18$). Comparisons by t test: control versus ADC stage 0 group, $P < 0.025$; control versus ADC stage 1 to 4 group, $P < 0.0002$; ADC stage 0 group versus ADC stage 1 to 4 group, $P < 0.05$. Because three comparisons were made, the level of significance was set at $\alpha = 0.05/3 = 0.017$. Therefore, only the difference between control patients and the ADC stage 1 to 4 group was significant.

was partially blocked by coincubation with 10 μ M MK-801 (Fig. 5A). The numbers of neurons treated with both PAF and MK-801 were 80% of the numbers of untreated control neurons.

Similar results were obtained with rat postnatal retinal cultures. Retinal ganglion cells were specifically depleted from cultures containing c-PAF. Assay of rat retinal ganglion cell viability was based upon the ability of healthy neurons to take up and cleave fluorescein diacetate to fluorescein. Retinal ganglion cells were specifically labeled by retrograde transport of a fluorescent dye injected into the superior colliculus. Viable retinal ganglion cells were dual labeled with granular blue and fluorescein yellow. A 35% reduction of neuronal viability was

FIG. 3. Box plot of PAF levels in CSF of control (HIV-1-seronegative) patients without systemic disease ($n = 6$) and patients (HIV-1 seronegative) with a variety of systemic diseases (metabolic, cancer, and others; $n = 15$). Comparison by t test of control patients versus patients with systemic disease, $P < 0.006$.

FIG. 4. Dose response of human fetal neuronal cell cultures to PAF. Cultures were grown on coverslips as described in Materials and Methods. Human cortical neuronal cell cultures were incubated for 48 h with the vehicle alone or with increasing doses of PAF (50, 300, and 1,250 pg/ml [97 pM, 557 pM, and 2.3 nM, respectively]). The cultures were washed, fixed in paraformaldehyde, and subjected to PGP 9.5 immunocytochemical labeling. Neuronal cell counting was performed as described in Materials and Methods. Observers were blinded to the treatment groups. Each neuronal cell count shown represents the mean of ≥ 15 microscopic fields selected from four replicates $\pm$ SEM. Symbols: *, $P < 0.0001$, control versus PAF at 300 pg/ml; +, $P < 0.0001$, control versus PAF at 1,250 pg/ml.

observed following addition of c-PAF and was largely prevented by the specific NMDA antagonist MK-801 or memantine (each at 6 μ M) (Fig. 5B). Thus, PAF-induced neurotoxicity was seen in both human and rat neuronal cell culture systems.

DISCUSSION

Productive HIV infection of the brain occurs in brain macrophages and microglia. Cognitive and motor dysfunction occurs, as well as neuronal loss in infected brain tissue. HIV-1-infected brain macrophages and microglia may lead to HIV-1-associated dementia, but neurologic deficits may not directly correlate with productive viral infection. A discordance exists between the level of histopathologic changes and the small numbers of detectable HIV-infected cells. This supports the notion that diffusible virus-induced neurotoxins are responsible, in part, for the neurological manifestations of HIV-1 infection. Moreover, this hypothesis remains likely even if far more microglial cells and astrocytes (48) are infected with HIV-1 and low-level or restricted infection results in local neurotoxic activities. Here we demonstrate that PAF is one of possibly many HIV-induced neurotoxins. This was shown by several assay systems. First, we detected PAF in an experimental model system for HIV CNS disease. The levels of PAF in our laboratory system were high but not sustained, supporting the notion of its cyclical production. Second, we confirmed the biological relevance of our in vitro observations by assay of PAF in the CSF of HIV-infected patients. Although PAF elevations were not specific for HIV infection and CNS disease, they did correlate with the onset of neurological impairment. Finally, we inoculated PAF, in concentrations similar to those found in CSF, into primary neurons. Here PAF was a potent neurotoxin. These findings, taken together, strongly suggest that PAF plays a role in ADC. Moreover, the data provide the identity of a soluble neurotoxin, distinct from a viral gene product, produced by HIV-infected cells.

PAF is a lipid mediator that initiates a large repertoire of

FIG. 5. Neurotoxic effects of PAF on human fetal neuronal cell cultures (A) and rat postnatal retinal ganglion cells in culture (B) with partial blockade of neurotoxicity by NMDA antagonists. Cultures were grown on coverslips as described in Materials and Methods. (A) Human cortical neuronal cultures were incubated with PAF for 48 h. The cultures were then washed, fixed in paraformaldehyde, and subjected to PGP 9.5 immunocytochemical labeling. Neuronal cell counts were determined as described in Materials and Methods. Observers were blinded to the treatment groups. Each neuronal cell count shown represents the mean of ≥ 15 microscopic fields selected from four replicates $\pm$ SEM. PAF was used at 6,000 pg/ml (10.9 nM); MK-801 was used at 10 μ M. Symbols: *, $P < 0.0001$, control versus PAF; +, $P < 0.0001$, PAF versus PAF-MK-801. (B) Rat retinal cell cultures were incubated in c-PAF overnight. Retinal ganglion cell neurons were specifically identified with the fluorescent dye granular blue and then scored for viability in a masked fashion on the basis of the ability to take up and cleave fluorescein diacetate to fluorescein. Thus, viable retinal ganglion cells were dual labeled with granular blue and fluorescein yellow. c-PAF (54 ng/ml or 100 nM) injured 32% of the retinal ganglion cells (68% remained viable). MK-801 or memantine (each at 6 μ M) largely prevented the c-PAF-induced neuronal injury. In the experiments whose results are shown, all of the cultures contained 25 μ M glutamate. Control dishes contained approximately 150 viable retinal ganglion cell neurons and were used to normalize the data obtained from each of four separate experiments.

pathophysiologic responses, including bronchoconstriction, hypotension, neutropenia, thrombocytopenia, and increased vascular permeability leading to cardiac and renal impairment, as well as pulmonary edema, anaphylaxis, and death (8, 9). PAF has diverse effects at the cellular level, including phagocytosis, exocytosis, aggregation, chemotaxis, calcium mobilization, eicosanoid production, and superoxide production (24, 25). These responses are mediated by G-protein-coupled receptors (24, 25). PAF has been shown to induce transient increases in intracellular calcium in cultured neuronal cells (3, 30). PAF can facilitate excitatory neurotransmission and glutamate release in mammalian brains (3, 5, 37, 51, 58). Thus, experimen-

tal evidence suggests that PAF plays important roles in neuronal function. In contrast to its beneficial effects, it remains possible that PAF contributes to brain injury. This could occur by different mechanisms, including superoxide production, increased calcium mobilization, or by glutamate elevation. These events would collectively injure susceptible neurons.

The pathophysiologic relevance of PAF in HIV-1 infection of the CNS was first demonstrated by analyses of HIV-1-infected monocyte-astroglia mixtures. Cytokines, most notably TNF, are detected in brain tissues of patients with advanced ADC (54). Reports demonstrate that TNF- α and interleukin 1 β are regulated by PAF and arachidonic acid metabolites (23). Indeed, inhibitors of arachidonic acid metabolites, such as dexamethasone and nordihydroguaiaretic acid, decrease TNF- α production in HIV-1-infected cultures (20) and diminish neurotoxic activities (14a). Furthermore, PAF and arachidonic acid metabolites are potent stimuli for TNF- α production. The rise in cytokines, PAF, and arachidonic acid metabolites in these HIV-1-infected cell systems correlates with neurotoxicity, suggesting that all of these factors play important roles in progressive HIV-1 CNS disease.

PAF production in HIV-1-infected monocytes was ephemeral. This was reported previously for arachidonic acid metabolites induced in gp120-stimulated monocytes (56). Indeed, detection of PAF was difficult at later time points (hours and/or days). There are several explanations for this result, including the following: PAF biosynthesis may occur only during initial cell-to-cell contact between HIV-1-infected monocytes and astroglial cells; uninfected or infected monocytes may secrete a factor that inhibits astroglial cell-mediated biosynthesis of PAF; and enzymes important in the generation of PAF may be expressed at low levels in HIV-1-infected monocytes and astroglia but may undergo cell-to-cell transfer so that PAF biosynthesis is increased transiently (38).

Despite the evanescent nature of PAF metabolism in these experimental conditions, the marked increase in PAF biosynthesis in the CSF of HIV-1-infected patients and the ability of PAF to stimulate TNF production suggest that PAF is biologically relevant for ADC. One of the advantages of the CSF assays is that specimens were obtained with minimal risk to the patients and likely reflected ongoing brain pathology. The level of PAF in the CSF represents the average of different rates of production and degradation of PAF in all brain areas. Thus, levels of PAF in the CSF may be different from those observed in specific neocortical areas involved in ADC. Notwithstanding this limitation, several potentially important observations were made. First, in pediatric patients, elevated PAF levels in CSF correlated with severe encephalopathy. Second, in adult patients, elevated PAF levels in CSF correlated with both immunosuppression and onset of neurological impairment (ADC stages 1 to 4). PAF was not specific for neurological manifestations of HIV infection. PAF in CSF was elevated in a variety of non-HIV diseases with CNS manifestations, demonstrating that it is not a specific marker for HIV-1-associated CNS dysfunction.

PAF produced dose-dependent neurotoxicity (Fig. 4). Neurotoxicity associated with PAF was blocked, in part, by co-incubation with the NMDA antagonist MK-801 or memantine, demonstrating that PAF can directly activate NMDA receptors with subsequent excitotoxic neuronal damage. PAF can also increase neuronal Ca^{2+} and lead to enhanced excitatory neurotransmission through increased glutamate release (3, 5, 37, 51, 58). In addition, recent reports have demonstrated that arachidonic acid can inhibit high-affinity glutamate uptake in synaptosomes and astrocytes (55) and potentiate NMDA receptor currents by increasing open-channel probability (41).

These mechanisms might be responsible for an increase in glutamate in the synaptic cleft and subsequent NMDA receptor-mediated excitotoxic damage to neurons in HIV-infected brains. These findings, in toto, support a role of PAF in HIV-1-mediated neurotoxicity. This does not mean that PAF is an exclusive HIV neurotoxin. It is generally believed that many cellular and viral neurotoxins contribute to CNS disease. Indeed, arachidonic acid metabolites may play equally important roles in HIV-1-induced brain disorders.

The ability of NMDA antagonists to block neurotoxicity has been a central feature in other models of HIV-1-mediated neuronal damage (21, 35). The consequences of productive HIV-1 infection, including viral gene products, e.g., gp120, may stimulate arachidonic acid metabolite and cytokine production in macrophages (56). These alterations in macrophage secretory function may, in turn, result in neurotoxicity and support, for the first time, commonality among mechanisms for HIV-1 neuropathogenesis.

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