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Regulation of glutathione and cell toxicity following exposure to neurotropic substances and human immunodeficiency virus-1 *in vitro*

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The aim of the present study was to assess the toxic potential of drugs of abuse and other neuropharmacological agents in the pathogenesis of AIDS dementia complex (ADC), the neurological complication of AIDS. Neuroblastoma and glioblastoma cell lines expressing the dopamine transporter, as well as primary macrophages exposed to human immunodeficiency virus-1 (HIV-1), were used to investigate the possibility of any synergistic effect between the mode of toxicity of such substances and virus exposure. The drugs of abuse used in our experiments were cocaine and morphine, which exert their action, among others, on the dopaminergic system. Effects were compared to treatment with dopamine itself and a typical dopaminergic drug used pharmaceutically, selegiline. In macrophage cultures, glutathione (GSH) was upregulated strongly after treatment with dopamine, morphine or selegiline, and this effect was enhanced when cells were pre-exposed to virus. This upregulation is discussed as a compensatory reaction to an oxidative signal. When hydrogen peroxide plus iron sulfate was used as a strong oxidant in macrophages, GSH concentrations decreased as a result of cell injury. Cell numbers remained constant in all treatment groups. In contrast, in both neuroblastoma and glioblastoma cell lines, the modulation of GSH concentrations by neurotropic substances was accompanied by significant cell loss, which was exacerbated by HIV-1 pretreatment. Selegiline did not change cell numbers when incubated alone. However, when incubated following treatment with HIV-1 cell death was highly significant. Ascorbic acid (AA), included as antioxidant, totally restored cell loss in cultures treated with dopamine. However, no effect was observed in combined treatment of AA and morphine or selegiline. The results demonstrate a synergistic role in cellular toxicity due to neurotropic substances and HIV-1, and suggest that neuropharmacological agents may contribute to the pathogenesis of ADC.

Keywords: oxidation-reduction; HIV-1; selegiline; dopamine; abuse

Introduction

A chronic encephalopathy termed AIDS dementia complex (ADC) is manifested clinically in patients suffering from human immunodeficiency virus-1 (HIV-1) type infection (Spencer and Price, 1992). Macrophages and resident microglia are the principal cell types primarily infected with HIV-1 in the CNS (Watkins *et al*, 1990). Moreover, immunohistochemical studies demonstrated that the distribution of HIV-positive cells preferentially involve basal ganglia (Brew *et al*, 1995), suggesting a special role of the dopaminergic system for the infection. Interestingly, neurons, astrocytes and oligodendrocytes are not infected with this virus *in vivo*. However, the pathological findings include marked neuronal loss, reactive astrogliosis and myelin damage (Spencer and Price, 1992). The causes of such neuronal damage remain obscure, although several hypotheses for the neuronal deficits being the indirect result of infection have been proposed (Price *et al*, 1988). Several lines of evidence suggest that oxidative stress is involved in the pathogenesis of AIDS. HIV-infected individuals have abnormally low levels of antioxidants, such as cysteine and glutathion (GSH), the latter being the major

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intracellular defence against the production of reactive oxygen species (ROS) (Eck *et al*, 1989; Buhl *et al*, 1989). Additionally the reduction in GSH levels is accompanied by increased levels of malondialdehyde, an indicator of oxidative damage (Sonnerborg *et al*, 1988). Furthermore, ROS such as hydrogen peroxide induce HIV gene expression and virus replication in human T cells, an effect which can be counteracted by the addition of antioxidants (Legrand-Poels *et al*, 1990; Schreck *et al*, 1991).

Epidemiologically, it seems that the frequency of CNS pathology in HIV-infected drug abusers is much higher in comparison to other risk groups (Martinez *et al*, 1995), and drugs of abuse have been postulated as a cofactor in the pathogenesis of HIV-1 infection (Donahoe and Falek, 1988). It is unclear whether this apparent enhanced incidence of HIV encephalopathy among drug abusers is due to immunomodulation caused by addictive substances. Drugs of abuse have been found to promote HIV-1 replication and it has been shown that this amplifying effect is blocked by the respective receptor antagonists (Peterson *et al*, 1990, 1991). Furthermore, previous studies suggested that addictive substances are implicated in oxidative stress processes (Singhal *et al*, 1994; Vaz *et al*, 1993). These data suggest that addictive drugs and probably other neuropharmacological agents may exert an influence on the development of ADC. Thus, we used cells which express the dopamine transporter (Koutsilieri *et al*, 1996a) to investigate whether these substances are able to initiate or to potentiate the toxicity of HIV-1 via a synergistic role in oxidative stress processes.

Results

Effect of neuropharmacological substances and HIV-1 on GSH levels

In macrophage cultures, a strong upregulation of GSH was evident after treatment with dopamine, morphine or selegiline (by 99, 87 and 150% above control levels, respectively). When cells were pre-exposed to HIV-1, GSH levels were further increased (Figure 1). Cocaine treatment had no effect on GSH levels in HIV-1-free macrophages; however, when pretreated with virus, GSH levels were significantly increased. Although drug free HIV treatment in cultures induced an increase in GSH levels, this upregulation could not be attributed solely to HIV-1 exposure, as the net difference was greater when cells were incubated with both cocaine and HIV-1.

In cultures treated with the potent oxidants hydrogen peroxide/iron sulfate ($H_2O_2/FeSO_4$), GSH levels were reduced. When HIV-exposed cells were treated with these oxidants, GSH increased by 50% compared to untreated cells. This increase, however, was smaller than the increase seen in cells treated only with HIV-1 (Figure 1). This is possibly because the injury produced by $H_2O_2/FeSO_4$ is likely to be irreversible (Halleck *et al*, 1992).

In both cell lines only dopamine and morphine modulated GSH levels in cells, and this modulation was altered by HIV exposure. GSH levels were slightly increased in HIV-1-treated cells compared to cells not exposed to virus. However, this difference was not statistically significant. $H_2O_2/$

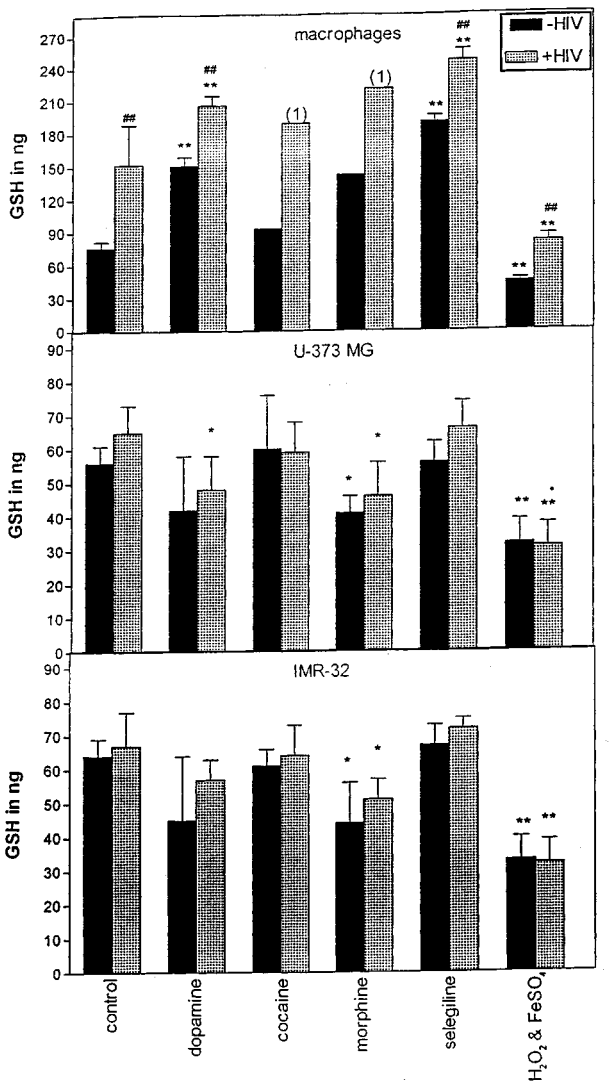

Figure 1 Effect of various neuropharmacological substances and HIV-1 on GSH levels in cell cultures. Substances used were: dopamine (100 μM), cocaine (10 μM), morphine (10 μM), selegiline (100 μM), H_2O_2 (500 μM)/$FeSO_4$ (50 μM) and HIV-1 (10^4/ml). Top panel: human primary macrophages. The number in parenthesis shows that results were obtained from one experiment. Middle panel: human U373 MG glioblastoma cells. Bottom panel: human IMR-32 neuroblastoma cells. Data represent mean values $\pm$ s.d. from three independent experiments. $^{*}P<0.05$, $^{**}P<0.01$, significantly different from controls, $^{\#\#}P<0.01$ from corresponding samples without HIV-1 exposure (Student's unpaired *t*-test).

$FeSO_4$ treatment caused a reduction in GSH levels, which was not altered when the cells were exposed to virus.

Effect of neuropharmacological substances and HIV-1 on cell survival

To determine whether changes in GSH levels were accompanied by obvious toxicity, we counted the cell number during each treatment. In macrophage cultures, the number of cells remained the same under all conditions. When IMR-32 and U-373 MG cells were incubated with neuropharmacological substances only, cell survival was reduced followed by treatment with dopamine, morphine or H_2O_2/$FeSO_4$. When cells were pretreated with virus and subsequently treated with drugs, cell number was further reduced, suggesting a potentiating toxic effect (Figure 2). It is noteworthy that treatment with HIV-1 alone had no effect on cell number. A cell reduction was also observed in the case of selegiline in HIV-1 pretreated cells. In contrast, selegiline alone caused no apparent decrease in cell number (Figure 2), suggesting further that the combination of drugs with HIV-1 exposure may be very important in the pathogenesis of neurodisorders.

In order to determine whether such cell death was associated with oxidative stress, 200 μM ascorbic acid (AA) was included with all neuropharmacological substances which had caused cell death. In the absence of drugs, the number of cells was similar to control values when treated with AA (Tables 1 and 2). A greater cell number was observed in cultures treated with AA plus dopamine compared to cultures treated with dopamine alone, as well as in AA- plus H_2O_2/$FeSO_4$ treated cultures, compared with cultures treated with H_2O_2/$FeSO_4$ alone. In morphine- and selegiline-treated cultures, no effect of AA was noted (Tables 1 and 2). This suggests that the effect of the first two substances is mediated by oxidative processes, whereas that of the latter substances is more indirect.

A distinct darkening of the medium of the cell lines treated with dopamine indicated autoxidation of dopamine and melanization. The cells preserved their morphology, in contrast to macrophages, in

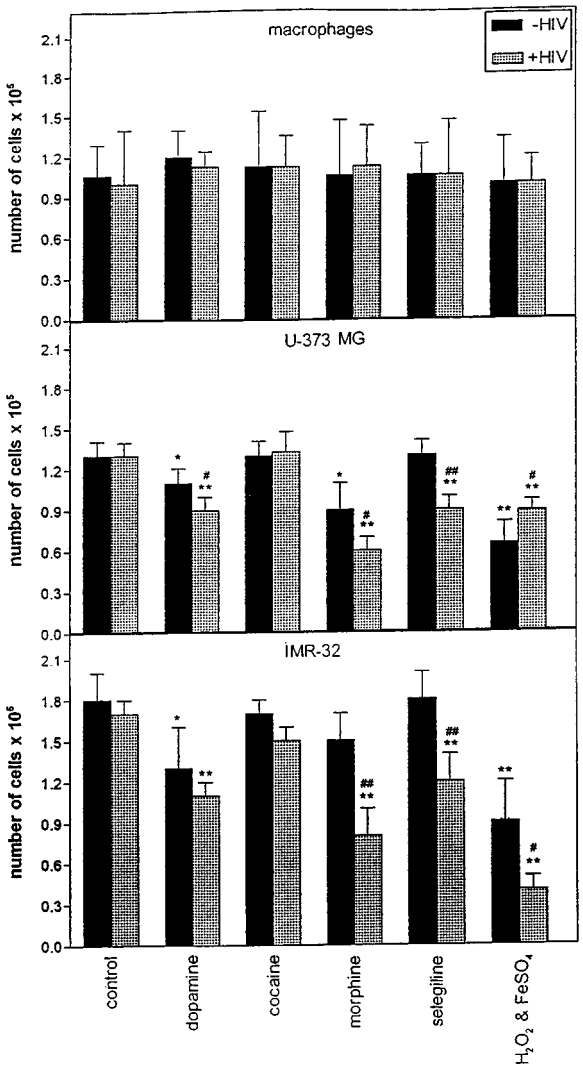

Figure 2 Effect of various neuropharmacological substances and HIV-1 on cell survival in cell cultures. Substances used were: dopamine (100 μM), cocaine (10 μM), morphine (10 μM), selegiline (100 μM), H_2O_2 (500 μM)/$FeSO_4$ (50 μM) and HIV-1 (10^4/ml). Top panel: human primary macrophages. Middle panel: human U373 MG glioblastoma cells. Bottom panel: human IMR-32 neuroblastoma cells. Data represent mean values $\pm$ s.d. from three independent experiments. $^*P<0.05$, $^{**}P<0.01$, significantly different from controls, $^{\#}P<0.05$, $^{\#\#}P<0.01$, from corresponding samples without HIV-1 exposure (Student's unpaired *t*-test).

Table 1 Effect of AA on cell viability after various treatments in IMR-32 cell cultures with and without exposure to HIV-1

	10^5 cells/ml	
Treatment	*Without HIV-1*	*With HIV-1*
Control	1.75 ± 0.19	1.70 ± 0.20
AA	1.80 ± 0.16	1.75 ± 0.10
Dopamine	1.30 ± 0.21^a	$0.95 \pm 0.10^{b,c}$
Morphine	1.30 ± 0.11^a	1.20 ± 0.28^a
Selegiline	1.70 ± 0.20	$1.15 \pm 0.47^{a,c}$
H_2O_2/$FeSO_4$	0.75 ± 0.19^b	$0.50 \pm 0.11^{b,c}$
AA and dopamine	1.75 ± 0.30^e	1.75 ± 0.19^f
AA and morphine	1.45 ± 0.19^a	1.30 ± 0.25^a
AA and selegiline	1.80 ± 0.23	$1.15 \pm 0.30^{b,d}$
AA and H_2O_2/$FeSO_4$	1.25 ± 0.25^e	1.35 ± 0.34^f

IMR-32 cells were incubated with the above substances for 24 h in presence or absence of AA either with or without pretreatment for 3 h with HIV-1. Data represent mean values $\pm$ s.d. from one experiment (four different wells per condition were analyzed). $^aP<0.05$, $^bP<0.01$ significantly different from controls, $^cP<0.05$, $^dP<0.01$ from the corresponding samples without HIV-1 exposure, $^eP<0.05$, $^fP<0.01$ from the corresponding samples without AA exposure (Student's unpaired *t*-test)

Table 2 Effect of AA on cell viability after various treatments in U-373 MG cell cultures with and without exposure to HIV-1

Treatment	*10^5 cells/ml*	
	Without HIV-1	*With HIV-1*
Control	1.33 ± 0.11	1.26 ± 0.11
AA	1.46 ± 0.11	1.33 ± 0.11
Dopamine	0.93 ± 0.23^{b}	0.80 ± 0.20^{b}
Morphine	0.93 ± 0.11^{b}	$0.73 \pm 0.11^{b,c}$
Selegiline	1.26 ± 0.23	$0.80 \pm 0.20^{b,c}$
$H_2O_2/FeSO_4$	0.53 ± 0.23^{b}	0.40 ± 0.20^{b}
AA and dopamine	1.40 ± 0.20^{e}	1.40 ± 0.20^{e}
AA and morphine	0.93 ± 0.11^{b}	0.73 ± 0.41^{a}
AA and selegiline	1.40 ± 0.20	$0.73 \pm 0.23^{b,d}$
AA and $H_2O_2/FeSO_4$	$1.06 \pm 0.11^{b,f}$	$1.00 \pm 0.20^{a,f}$

U-373 MG cells were incubated with the above substances for 24 h in presence or absence of AA either with or without pretreatment for 3 h with HIV-1. Data represent mean values $\pm$ s.d. from one experiment (four different wells per condition were analyzed). $^{a}P<0.05$, $^{b}P<0.01$ significantly different from controls, $^{c}P<0.05$, $^{d}P<0.01$ from the corresponding samples without HIV-1 exposure, $^{e}P<0.05$, $^{f}P<0.01$ from the corresponding samples without AA exposure (Student's unpaired *t*-test)

Table 3 IL-6 in macrophage cultures after treatment with neuropharmacological substances and HIV-1

	Dopamine	*Morphine*	*Selegiline*
Control	100 ± 6	100 ± 6	100 ± 6
Without HIV	158 ± 14**	115 ± 8*	114 ± 9
With HIV	171 ± 9**	124 ± 6**	123 ± 9**

Macrophages were incubated with the above substances for 24 h with or without pretreatment for 3 h with HIV-1. Data represent mean values in % $\pm$ s.d. from three cultures. $*P<0.05$, $**P<0.01$ significantly different from control (Student's unpaired *t*-test)

Table 4 Effect of dopamine on TNFα release in macrophage cultures

	TNFα (% of control)
Control	100 ± 14
LPS	857 ± 143**
Dopamine & LPS	1143 ± 143**

Macrophages were incubated with the above substances for 24 h without pretreatment with HIV-1. Data represent mean values in % $\pm$ s.d. from three cultures. $*P<0.05$, $**P<0.01$ significantly different from control (Student's unpaired *t*-test)

which deterioration was obvious. The latter cells were stained brown, suggesting that phagocytosis of melanin took place. In experiments in which the concentration of dopamine was increased to 500 μM, all cells were dead (data not shown).

Effect of neurotropic substances and HIV-1 on cytokines

Dopamine is the major neurotransmitter involved in the mechanism of the neurotropic substances used. It was of interest to determine whether dopamine affected cytokines known to be released by HIV-infected macrophages, such as TNFα and IL-6. Lipopolysaccharide (LPS) known for its ability to stimulate macrophages, increased TNFα sevenfold compared to controls. Interestingly, the combined treatment of dopamine with LPS potentiated this effect by nearly threefold (Table 4).

IL-6 was measured in cultured macrophages following treatment with dopamine, morphine and selegiline after being exposed to HIV-1 or not. All three substances stimulated IL-6 release and the observed rise was further increased in HIV-pretreated cells (Table 3).

Discussion

It has been postulated that substances of abuse are a cofactor in the pathogenesis of HIV-induced encephalopathy (Donahoe and Falek, 1988). The common mode of action for most of the addictive substances is their activation of the dopaminergic system (DiChiara *et al*, 1988; Beitner-Johnson and Nestler, 1991). Besides altered transmission, this activation results in generation of ROS. Human primary macrophages and the IMR-32 neuroblastoma and U-373 MG glioblastoma cell lines, which express the dopamine transporter (Koutsilieri *et al*, 1996a), were thus used to evaluate the effects of drugs of abuse and other neuropharmacological substances alone or in combination with HIV-1 exposure on GSH levels and cell survival in cell culture.

In the current experiments, we used a general oxidant ($H_2O_2/FeSO_4$), dopamine itself, a neuropharmacological agent which inhibits dopamine metabolism via inhibition of MAO-B and consequent diminution of H_2O_2 (selegiline) (Gerlach *et al*, 1994) and two addictive substances (cocaine and morphine). Cocaine is a dopamine uptake inhibitor and stimulate macrophages to phagocyte (Vaz *et al*, 1993). Administration of morphine leads to an increased release of dopamine (DiChiara *et al*, 1988) and stimulates superoxide formation (Singhal *et al*, 1994). In the two cell lines, a reduction of GSH levels was apparent following dopamine or morphine treatment. GSH declined further when cells were treated with $H_2O_2/FeSO_4$; unfortunately, we could not measure the levels of glutathione disulfide (GSSG, one of the oxidation products of GSH), a rise in which would signify the presence of oxidative stress, because the values fell below the detection limit. In the same treatments, cell numbers declined significantly. This is in accordance with previous studies, in which toxicity of dopamine and other catecholamines was reported in cell culture (Rosenberg, 1988). Dopamine is susceptible to autoxidation, a process giving rise

to toxic products such as, H_2O_2, superoxide, quinones and semiquinones (Graham, 1978). Morphine has also been shown previously to stimulate the formation of ROS (Singhal *et al*, 1994). H_2O_2 at the concentrations used provides a model of cell injury through oxidative stress (Hinshaw *et al*, 1993). In combination with $FeSO_4$, H_2O_2 should be more potent in this stimulation as iron is the catalyst for the superoxide-driven Haber-Weiss reaction, which generates hydroxyl radicals (Haber and Weiss, 1934). When the cells were pretreated with HIV-1, cell numbers declined further. It is noteworthy that the cell lines used are susceptible to infection by HIV-1 although they do not possess the CD4 receptor (Harouse *et al*, 1989; Li *et al*, 1990). The toxic effect after the pretreatment with HIV-1 implies a synergism between the above substances and the virus. Although when selegiline was administered alone, it did not affect cell survival, when administered after exposing the cells to HIV-1, cell numbers decreased significantly. This suggests that exposure to the virus may trigger a situation in which selegiline becomes toxic. Selegiline is reported to be toxic at high concentrations in cultures of mesencephalic neurons (Koutsilieri *et al*, 1996b), contrary to its neuroprotective properties at very low concentrations (Tatton, 1993; Koutsilieri *et al*, 1994, 1996b).

If oxidative species are generated following treatment with the above substances, AA, as antioxidant, may be able to intercept them. In fact, AA restored cell numbers following treatment with dopamine and $H_2O_2/FeSO_4$; this accords with previous results in other cells (Mena *et al*, 1993). When these cell lines were treated with HIV and the above substances, AA also restored cell numbers, implicating the involvement of ROS following HIV-1 exposure. However, AA failed to restore cell numbers following treatment with morphine or selegiline, suggesting a more indirect mechanism of toxicity. Since AA penetrates cells (Kalir and Mytilineou, 1991), it may contribute to the intracellular removal of the oxidation products of dopamine. It is noteworthy that following AA treatment, the darkening of the medium, following dopamine treatment, disappeared. This darkening is due to the formation of quinones and melanin. Quinone formation has been accordingly detected after 24 h exposure of cell cultures to L-DOPA (Mena *et al*, 1993). The scavenging of superoxide radicals by ascorbate (Nishikimi, 1975) can suppress the superoxide-mediated catecholamine oxidation chain reaction (Misra and Fridovich, 1972), which leads to melanin formation.

In the macrophage cultures, a strong upregulation of GSH was evident after treatment with dopamine, morphine or selegiline. This is not surprising as in previous studies (Mytilineou *et al*, 1993) an increase in GSH content was observed in primary mesencephalic neurons after 48 h treatment with L-DOPA. The increase in GSH levels may be a compensatory reaction to an oxidative signal. However, the failure of AA to reduce the toxic effects of morphine and selegiline suggests another mechanism may be involved. The action of selegiline at high concentrations, such as that which was used may be mediated by one of its metabolites, which include methamphetamine and amphetamine (Reynolds *et al*, 1978). Furthermore, amphetamine was reported to cause expression of early genes (Ruskin and Marshall, 1994) which may result to an increase of GSH. Exposure to HIV-1 is apparently also a potent signal as pretreatment with HIV-1 potentiated the upregulation. The implication of oxidative stress in HIV-infected individuals is supported by reported changes in GSH levels (Eck *et al*, 1989). Additionally, previous studies have demonstrated that HIV-1 proteins, such as gp 120 and possibly *tat* and *nef*, can stimulate even uninfected cells to release neurotoxins (Brenneman *et al*, 1988). The neurotoxins include glutamate-like molecules, free radicals and cytokines (Lipton, 1994). We measured the changes in cytokines release, such as TNFa and IL-6 following our experimental scheme, since several reports have demonstrated previously that HIV-infected individuals exhibit elevated production and levels of these inflammatory cytokines (Gallo *et al*, 1989). In addition to triggering HIV replication in infected cells within the brain, these cytokines could also have indirect neuropathogenic effects by increasing the concentrations of neurotoxic viral components (Brenneman *et al*, 1988), causing an oxidative stress at both the systemic and cellular levels. Moroever, it has been postulated that the change in GSH levels in HIV-infected individuals may be due to chronic exposure to inflammatory cytokines (Roederer *et al*, 1992). Dopamine, morphine and selegiline treatment increased IL-6 levels in the supernatant of macrophage cultures, an effect of which became highly significant when cells were pretreated with HIV-1. TNFα was measured in LPS-stimulated macrophages with or without dopamine. The combined treatment increased the levels of TNFα by 286%. TNFα by itself can induce oxidant production, but it is far more potent in combination with other agents (synergistic stimulation) (Roederer *et al*, 1992). In *in vitro* experiments, it has been shown that the supernatants from LPS-stimulated microglial cell cultures contain TNFα and IL-6, and that they upregulated the expression of HIV-1 in a chronically infected human promonocyte clone U1 (Peterson *et al*, 1992).

The mechanism for cell damage in our experiments is not totally clear, nor is it certain that the stimuli for cell damage and for the rise in GSH levels are the same. Further experiments must be

undertaken with primary dopaminergic cells in order to resolve this question. However, it is evident that neurotropic substances can trigger a cascade of events which initiate or potentiate HIV-1-induced toxicity, an effect which must be taken into consideration in the current application of therapies used for neurodisorders.

Materials and methods

Tissue culture

Cell lines The derivations of IMR-32 (neuroblastoma) and U-373 MG (glioblastoma) cell lines have been described (Tumilowicz *et al*, 1970; Bigner *et al*, 1981, respectively). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Heymanns and Unsicker, 1987) supplemented with 10% inactivated fetal calf serum, 4 mM L-glutamine, and 10% HEPES. Cells were incubated for 7 days in 25 cm^2 plastic flasks (Falcon, Lincoln Park, NJ) at 37°C in a water-saturated atmosphere of 5% CO_2. The medium was changed three times a week. Monolayer cultures were harvested by trypsinization (0.125% trypsin/1% EDTA in phosphate-buffered saline (PBS) and subcultured in 24-well dishes (Primaria, Falcon, NJ) until the assays were performed. Cultures were routinely harvested and stored in liquid nitrogen until required. All chemicals were supplied by Boehringer Mannheim, Germany.

Monocyte-derived macrophage cultures Macrophages were derived from the peripheral blood of healthy human donors. Mononuclear cells were purified from heparinized blood (Sopper *et al*, 1996) by centrifugation through Ficoll-Hypaque (Pharmacia, Freiburg, Germany) gradients and plated in 24-well plates in an RPMI 1640 medium supplemented with 10% inactivated fetal calf serum. After 2 h of incubation at 37°C, nonadherent cells were removed by replacing the medium including floating cells with fresh medium. Cells were seeded at a concentration of 10^5 well and cultured at 37°C in a water-saturated atmosphere of 5% CO_2 for 4 days until treatment.

Cell number and viability for both cell lines and primary cells were determined by trypan blue dye exclusion in a Neubauer hemocytometer.

Reagents and solutions

The neurotropic substances used in our experimental scheme were: dopamine (100 μM), cocaine (10 μM), morphine (10 μM) and selegiline (100 μM). In some experiments H_2O_2 (500 μM) plus $FeSO_4$ (50 μM) or AA (200 μM) were also included. All stock solutions were prepared in physiological solution according to Merck Index indicatons and stored in light-resistant containers at -70°C.

Virus

For the viral exposure of cells, the virus strain HIV-1 IIIB, kindly provided by Dr C Jassoy was used. Stock virus was prepared in the human T-cell line C8166. Cultures were incubated with 300 μl virus for 3 h (TC ID_{50}; 10^4/ml). The supernatants of uninfected C8166 cells were checked for toxicity.

GSH/GSSG detection

GSH/GSSG was determined after modifying the previously described assay of Krien *et al*. (1992). Cells were washed twice with PBS and homogenized (W 250 Sonicator, Heinemann, Schwäbisch Gmünd, 20 KHz for 30 s) at 0°C in 300 μl of a solution containing 150 mM *o*-phosphoric acid and 500 μM bis(2-aminoethyl)-amine-N,N,N′, N″,N″-pentaacetic acid-Ca,3Na-salt (DTPA). For the separation of larger cell fragments, the cell suspension was centrifuged at 4°C for 20 min at 48 000 × g (Sorvall RC5C, Rotor SM 24, DuPont-Sorvall, Bad Homburg).

The supernatants were filtered through UltrafreeR MC-cartridges (exclusion limit 5 kDa; Millipore, Eschborn, Germany) and centrifuged at 4°C for 90 min at 10 000 × g in a Micro rapid-K centrifuge, Hettich, Tuttlingen. For determination of GSH and GSSG, the filtrates (10 – 20 μl) were injected into a high performance liquid chromatography (HPLC) system equipped with a coulometric detector (ESA Coulochem 5100A, Bedford, MA, USA) with a dual analytical cell (model 5011;+0.6 V GSH; 0.9 V GSSG). HPLC separation was carried out at room temperature on a 300 × 4.0 mm Nucleosil 120, 5 μm C18 column (Bischoff, Leonberg, Germany). The mobile phase consisted of 25 mM sodiumphosphate, pH 3.0. The flow rate was set at 1.0 ml/min. Identification of peaks was achieved by comparison with commercial standards (Sigma, Deisenhofen). Detection limit for both compounds was 5 ng.

Determination of cytokines

Concentrations of TNFα and IL-6 in the supernatant of cultured macrophages were measured using commercially available sandwich ELISAs (Endogen, Boston, MA, detection limit 5 pg/ml and R&D Systems, Minneapolis, MN, detection limit: 3.5 pg/ml, respectively). The assays used the procedures recommended by the manufacturers.

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