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Cytokine-Stimulated, But Not HIV-Infected, Human Monocyte-Derived Macrophages Produce Neurotoxic Levels of L-Cysteine

Michael W. Yeh,* Marcus Kaul,** Jialin Zheng,† Hans S. L. M. Nottet,§ Michael Thylin,‡ Howard E. Gendelman,§ and Stuart A. Lipton*†

Approximately one-quarter of individuals with AIDS develop neuropathological symptoms that are attributable to infection of the brain with HIV. The cognitive manifestations have been termed HIV-associated dementia. The mechanisms underlying HIV-associated neuronal injury are incompletely understood, but various studies have confirmed the release of neurotoxins by macrophages/microglia infected with HIV-1 or stimulated by viral proteins, including the envelope glycoprotein gp120. In the present study, we investigated the possibility that L-cysteine, a neurotoxin acting at the N-methyl-D-aspartate subtype of glutamate receptor, could contribute to HIV-associated neuronal injury. Picomolar concentrations of gp120 were found to stimulate cysteine release from human monocyte-derived macrophages (hMDM) in amounts sufficient to injure cultured rat cerebrocortical neurons. A TNF-α antagonist partially blocked gp120-induced cysteine release, suggesting that these cytokines may mediate the actions of gp120. Interestingly, hMDM infected with HIV-1 produced significantly less cysteine than uninfected cells following stimulation with gp120. Our findings imply that cysteine may play a role in the pathogenesis of neuronal injury in HIV-associated dementia due to its release from immune-activated macrophages but not virus-infected macrophages. Such uninfected cells comprise the vast majority of mononuclear phagocytes (macrophages and microglia) found in HIV-encephalitic brains.

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Abbreviations used in this paper: HAD, HIV-associated dementia; hMDM, human monocyte-derived macrophages; NMDA, N-methyl-D-aspartate; IRA, IL-1β receptor antagonist; αTNF, TNF-neutralizing Ab.

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that mouse peritoneal macrophages stimulated with TNF-α release cysteine. Finally, cysteine was shown to be an endogenous neu-
rototox in that causes via excessive NMDA receptor activation (29, 30).
These studies led us to hypothesize that infection with HIV-1 or
stimulation with its envelope glycoprotein gp120 might cause hu-
mans macrophages to release cysteine in excessive quantities. We
had previously shown that low or chronic levels of excitoxins
acting at the NMDA receptor can cause neuronal apoptosis (31).
We have therefore investigated whether cysteine released from
macrophages could contribute to the neuronal damage and apo-
potosis observed in HIV infection. In the present study, we also consider
the corollary hypothesis that the cytokines TNF-α and IL-1β could be
mediators of gp120-induced cysteine release via immune activa-
tion of human macrophages.
Additionally, we explored potential intracellular signaling path-
ways involved in cysteine release. Both TNF-α and IL-1β have been
shown to activate a sphingolipid-derived messenger system in
several myeloid cell lines. The pathway is initiated by the ac-
tivity of a membrane-associated sphingomyelinase which hydro-
lyzes sphingomyelin to ceramide (reviewed in Ref. 32). Down-
stream targets of the sphingomyelinase pathway include phospholipase A₃ and mitogen-activated protein kinases, which
have been shown to be activated by ceramide in HL-60 human
leukemia cells (32, 33). Ceramide is also in the pathway to trans-
location of NF-κB, an important transcriptional regulator of many
immune and inflammatory response genes (reviewed in Ref. 34).
In several systems, including the U937 human monocyte cell line,
TNF-α and IL-1β have been shown to induce sphingomyelin hy-
drolysis. Furthermore, many of the actions of these two cytokines
could be mimicked by cell-permeable ceramide analogues, such as
acetyl ceramide (reviewed in Ref. 35). Therefore, we explored the
possibility that ceramide analogues could induce cysteine release
from human macrophages.
To test our hypotheses, we measured cysteine released by hu-
man monocyte-derived macrophages (hMDM) stimulated with pi-
comolar concentrations of gp120 or pathophysiologically relevant
concentrations of TNF-α, IL-1β, or acetyl ceramide. The possibil-
ity that these cytokines mediate the effects of gp120 was investi-
gated using a monoclonal TNF-α-neutralizing Ab and an IL-1βR
antagonist. Cysteine release by HIV-infected hMDM after immune
activation with TNF-α was also investigated. Finally, the neuro-
toxic potential of the cysteine released by hMDM was assessed on
cultured rat cerebrocortical neurons.

Materials and Methods
Isolation and culture of hMDM
Monocytes were recovered from PMBC of HIV-1- and hepatitis B-negative donors after leukapheresis and purified by centrifugal elutriation, as we have described previously (36). The monocytes were cultured as adherent monolayers at a concentration of 10⁶ cells/ml in 1.0 ml DMEM (formula D5671; Sigma, St. Louis, MO) with 10% heat-inactivated human serum, 50 μg/ml gentamicin, and 1000 U/ml recombinant human M-CSF (a generous gift from Genetics Institute, Cambridge, MA). The cells were cultured for 1 wk before HIV-1 infection or exposure to gp120 and cytokines.

HIV-1 infection of monocytes
M-CSF-treated monocytes were exposed to the monocytotropic viral strain, HIV-1ADA, at a multiplicity of infection of 0.1 infectious virus particles/target cell. All viral stocks were tested and found to be free of Mycoplasma and endotoxin contamination (Gen-Probe II; Gen-Probe, San Diego, CA). Half of the culture medium was replaced every 2–3 days (on a Monday-
Wednesday-Friday schedule in every case). Reverse transcriptase activity was determined in replicate samples of culture supernatant, as described elsewhere (23).

Preparation and administration of agents
Recombinant gp120 (Genentech, South San Francisco, CA) was pro-
duced by transfection of a Chinese hamster ovary cell line, as previously
detailed (37). The glycosylated envelope protein was purified by immuno-
affinity chromatography to >99.9% purity. An alternative source of recom-
binant gp120 was obtained from the National Institutes of Health
AIDS Research and Reference Reagent Program, catalogue no. 386; this
glycosylated gp120α was also expressed in Chinese hamster ovary cells
and purified by a nonaffinity method in the absence of organic or denatur-
ing reagents to a purity of 94.8% by SDS-PAGE under reducing condi-
tions. Similar results were obtained in the experiments described here with
either preparation of the envelope protein. The gp120 was stored at milli-
gram per milliliter concentrations in citrate-buffered saline or PBS at
4°C. Aliquots were stored on ice, diluted in standard medium, and
used within 1 h of thawing. Recombinant human TNF-α, IL-1β, TNF-α-
neutralizing Ab (αTNF), and IL-1βR antagonist (IRA) were purchased
from R&D Systems (Minneapolis, MN). The cell-permeable ceramide an-
dogue acetyl ceramide (Molecular Probes, Eugene, OR) was dissolved in
100% ethanol and diluted 1:100 for use; controls consisted of the diluent
solution and manifest no effect by themselves.

Quantitation of cysteine levels
The assay for acid-soluble cysteine was a modification of that described by Gaitonde (38). An aliquot of the culture supernatant was mixed with 50% 5-sulfosalicylic acid (to a final concentration of 2.5% v/v) to precipitate proteins. The mixture was vortexed briefly and incubated at 4°C for 10 min. It was then centrifuged at 3000 rpm in an Eppendorf microfuge for 15 min. The supernatant (acid-soluble fraction) was mixed with an acid nin-
hydrin reagent (140 mM ninhydrin in a 3:2 mixture of acetic acid and
concentrated hydrochloric acid), which reacts specifically with cysteine at
acid pH to form a colored product that can be quantitated by spectropho-
tometry. After heating (100°C for 10 min) and cooling, the samples were
diluted 1:2 with 100% ethanol. The relationship between cysteine concen-
tration and absorbance at 560 nm was linear between 5 and 1000 μM.
Percentage values for cysteine were compared between experiments because
of the variability in absolute values among monocytes from different
 donors. Such variability among donors is to be expected (39–43). However,
the data were qualitatively similar among donors.

Cerebrocortical cell cultures
Cortical cultures, containing neurons and glia in similar proportions to that
found in the brain, were derived from the cerebral hemispheres of embry-
doing Sprague Dawley rats on fetal day 15 or 16, as we have described
previously (44). Briefly, following dissociation in 0.027% trypsin, cere-
brocortical cells were plated at a density of 4.5 × 10⁵/35-mm dish contain-
ing poly-L-lysine-coated glass coverslips in DMEM with Ham’s F12 and
heat-inactivated, iron-supplemented calf serum (HyClone, Logan, UT)
in a ratio of 8:1:1. After 15 days in culture (when the astrocyte layer had
become confluent), the cultures were treated with cytosine arabinoside for
72 h. The culture medium was replenished three times weekly. Cultures
were incubated at 36°C in a 5% CO₂/95% air-humidified atmosphere.
The cultures were used for experiments ~3 wk after plating. Neurons could be
reliably identified by morphological criteria under phase-contrast optics
and immunostaining with microtubule-associated protein-2 or NeuN, as
later confirmed by patch-clamp recording (44). For neurotoxicity experi-
ments, the medium was switched to one containing cysteine in an amount
sufficient to produce the same effect as the gp120- or cytokine-stimulated macro-
phages. Sibling cultures were also incubated with the NMDA antagonists
MK-801 (Research Biochemicals, Natick, MA) or memantine (Dr. G.
Quack, Merz, Frankfurt, Germany or Dr. J. Larrick, Panorama, Palo Alto,
CA). After a 6-day incubation, cell survival was determined by directly
counting viable neurons, as described previously (31, 44).

Results
HIV-1 envelope glycoprotein gp120 stimulates hMDM to release
cysteine
In these experiments, cultured hMDM stimulated with 200 pM
gp120 released cysteine in a time-dependent manner, with peak
levels achieved at 96 h after application (Fig. 1). Compared with
control cultures, cysteine levels rose to 130 ± 3.3% (mean ±
SEM, n = 27).
TNF-α, IL-1β, and ceramide stimulate cysteine release from hMDM

We found that pathophysiological amounts of TNF-α and IL-1β cause cysteine release in a dose-dependent manner. At 500 U/ml, either cytokine induced ~80% of the maximal response, and this concentration of the cytokines was used in all additional experiments. Peak cysteine levels, observed 24 h after application, were 183 ± 68% of control for TNF-α and 186 ± 14% of control for IL-1β (mean ± SEM, n = 12, Fig. 2). Acetyl ceramide (C2-Cer) also induced cysteine release with a peak response at 24 h after application. A concentration of 1 μM C2-Cer generated cysteine levels 235 ± 14% of control, an effect similar to that of 500 U/ml of TNF-α or IL-1β (Fig. 3). Cysteine levels produced by macrophages exposed to the ethanol vehicle alone (final concentration of 1%) did not differ from untreated macrophages.

gp120-induced cysteine release is partially inhibited by TNF-neutralizing Ab and IL-1R antagonist

The finding that peak cysteine levels occurred 96 h after gp120 application but only 24 h after cytokine application was consistent with the hypothesis that gp120 must first cause the elaboration of cytokines, a known event (26), to induce cysteine release. To test this hypothesis, we pretreated macrophages with TNF-neutralizing Ab (αTNF), IL-1 receptor antagonist (IRA), or both. A concentration of αTNF (6 μg/ml) sufficient to neutralize 500 U/ml of TNF-α was added to the culture media 1 h before application of gp120. IRA was utilized in an analogous fashion. αTNF reduced gp120-induced cysteine release by 65% (Fig. 4). IRA reduced cysteine levels by 65% (Fig. 4).

FIGURE 1. Cysteine release by gp120-stimulated hMDM (10⁶/ml). Cysteine levels in the culture supernatant were measured at various time points up to 144 h after application of 200 pM gp120. The cysteine concentration 96 h after gp120 stimulation was 167 ± 7 μM (mean ± SEM, n = 27 experiments, each performed in triplicate). Cysteine levels are expressed as percentage of control since the absolute value of cysteine released by control macrophages within any one experiment was quite consistent but varied from experiment to experiment. This probably occurred because each experiment was performed with hMDM from a different donor, each manifesting a different degree of endogenous activation. For this reason all figures are shown with data expressed as percent of control for that experiment. *, p < 0.005 compared with control by paired one-tailed t test. **, p < 0.005.

FIGURE 2. Cysteine release by cytokine-stimulated human macrophages. Five hundred U/ml of either TNF-α (solid line) or IL-1β (dashed line) were used. Some error bars in this and subsequent figures are not visible because the SEM for that value was smaller than the size of the symbol. *, p < 0.005 compared with control by paired one-tailed t test (n = 12).

FIGURE 3. Cysteine release by macrophages stimulated with a cell-permeable ceramide analogue. Cysteine levels were measured 24 h after application of 1, 5, 10, or 20 μM acetyl ceramide (Cer 1, Cer 5, Cer 10, and Cer 20, respectively). Cysteine level released by macrophages stimulated with 500 U/ml TNF-α is shown for comparison. **, p < 0.0001 compared with control by an ANOVA followed by a Scheffé multiple comparison of means (n = 6).

FIGURE 4. Effects of αTNF and IRA on gp120-induced cysteine release. Macrophages were pretreated with 6 μg/ml αTNF, 100 ng/ml IRA, or both 1 h before application of 200 pM gp120. Cysteine levels were measured 96 h after gp120 exposure. In these experiments, control cysteine concentration was 118 ± 8.4 μM (mean ± SEM, n = 6). **, p < 0.0001 compared with control by ANOVA.
teine levels below that of controls (representing a 1.3-fold reduction). The combination of αTNF and IRA did not have any additional significant effect compared with either agent alone. As a control, when administered in the absence of cytokine, αTNF- and IRA-treated hMDM did not differ from control macrophages with respect to cysteine production (data not shown).

**HIV-infected hMDM display attenuated cysteine release**

When compared with cytokine-stimulated/HIV-infected macrophages, cytokine-stimulated/uninfected macrophages released a 5–20-fold greater amount of cysteine. Compared with control, HIV-1-infected macrophages failed to respond to stimulation with either 100 or 1000 U/ml of TNF-α with increased cysteine production (Fig. 5). Neither was IL-1β effective in stimulating increased cysteine production by HIV-1-infected macrophages (data not shown).

**Concentrations of cysteine released by cytokine- or gp120-stimulated hMDM induce NMDA receptor-mediated neurotoxicity**

To investigate the neurotoxic potential of the cysteine released by human macrophages, we exposed mixed neuronal/glial cerebrocortical cultures to medium containing cysteine at a level equal to that typically released by hMDM that had been stimulated by either cytokines or gp120, as determined in the aforementioned experiments. Accordingly, the concentration of cysteine used (210 μM) was the mean level measured in cultures of 10⁶ hMDM/ml that had been stimulated by cytokines or by gp120, and this level also exceeded the concentration of cysteine encountered in any of the controls. We incubated the cultures for 6 days in this low level of cysteine to simulate chronic exposure in a relatively slowly progressing neurodegenerative condition such as HAD and also because Brenneman et al. (20) had reported that gp120 toxicity in rodent hippocampal cultures was manifest maximally after several days of exposure; additionally, similar findings concerning the length of exposure were recently reported for human neurons (20).

Cysteine-exposed cultures displayed a 58 ± 10% decrease in neuronal viability (mean ± SEM) compared with controls (Fig. 6). We also found that the NMDA receptor antagonists, MK-801 and memantine, each protected from cysteine-induced neurotoxicity.

**Discussion**

Our results show that picomolar concentrations of the HIV-1 envelope glycoprotein gp120 induce the release of cysteine from hMDM. Although the increase might at first appear to be a small one (~30%, Fig. 1), it is not only highly reproducible and statistically significant, but also pathophysiologically relevant because this level of cysteine is neurotoxic (Fig. 6). Our data further suggest that the process of gp120-induced cysteine release from human macrophages is at least partially mediated by the cytokines TNF-α and IL-1β. Direct stimulation with TNF-α or IL-1β resulted in more rapid cysteine release than gp120 and to even greater levels (~2-fold induction, Fig. 2). This is relevant to HAD because cytokines (especially TNF-α) have been found to be elevated in the brains and cerebrospinal fluid of patients with cognitive dysfunction and may herald the neurodegenerative process (47, 48). Several previous studies have demonstrated the release of putative neurotoxins by both gp120-stimulated and HIV-infected monocyteic cells (7–9, 11, 12, 19, 24, 25, 45, 46). Our new results lead us to propose that the amino acid L-cysteine, a molecule previously identified as a neurotoxin capable of acting at the NMDA receptor, should be added to the list of potential neurotoxins in AIDS brains. In the present study, we demonstrate that the concentrations of cysteine released by gp120- or cytokine-stimulated human macrophages are sufficient to result in neuronal damage in cerebrocortical cultures, and that this injury can be prevented by administration of specific NMDA receptor antagonists. In a prior investigation, Olney et al. (29) had shown that the excitotoxic threshold of cysteine in the chick retina is dependent on both pH and bicarbonate content. Under physiological conditions (pH 7.4, 24 mM bicarbonate), the threshold for neurotoxicity in the chick retina after a 30-min exposure was 500 μM cysteine. Our group
previously reported that cysteine concentrations of \( \geq 800 \, \mu M \) were necessary to cause toxicity in our cultures of rat cerebrocortical neurons after an 18-h exposure (30). Our new data show that cysteine can cause excitotoxic damage to rat cerebrocortical neurons at lower concentrations (~200 \( \mu M \)) if the cultures are exposed to cysteine for a prolonged period of time (i.e., at least 6 days). Neurodegenerative diseases in general, and HAD in particular, follow a progressive course. The natural history is consistent with a prolonged neurological insult of mild to moderate severity rather than a single, acute fulminating event. Therefore, our model may approach a more accurate representation of the actual clinical phenomenon.

The observation that TNF-\( \alpha \) and IL-1\( \beta \) are mediators of gp120-induced cysteine release by human macrophages suggests that immune activation can lead to neurotoxic production in AIDS brains. Either TNF-\( \alpha \) or IL-1\( \beta \) produced release of significant amounts of cysteine when administered alone. Furthermore, peak cysteine levels occurred earlier after cytokine stimulation than with gp120 stimulation, consistent with the notion that gp120 may act by first inducing cytokine secretion. In fact, previous studies have shown that gp120 stimulates the secretion of both TNF-\( \alpha \) and IL-1\( \beta \) by human monocytic cells (26, 27). As alluded to previously, TNF-\( \alpha \) and IL-1\( \beta \) are elevated in the brain, spinal cord, and cerebrospinal fluid of AIDS patients (47, 48). Importantly, the amount of TNF-\( \alpha \) present in brain parenchyma at postmortem appears to correlate with the degree of dementia determined praeagonally (47). Our findings suggest therefore that at least one pathway for the neuronal injury observed in HAD may be cytokine-induced release of macrophage toxins such as cysteine. TNF-\( \alpha \) and IL-1\( \beta \) have also been reported to be elevated in a variety of other CNS inflammatory, infectious, and degenerative conditions and are not unique to AIDS brains. However, it might be expected that the juxtaposition of either HIV-infected or immune-stimulated macrophages and NMDA receptor-bearing neurons might be unique in each of these conditions depending on the extent, predilection, and location of the insult. Thus, despite common cytokine abnormalities, unique patterns of neuropathology may evolve in disparate disorders.

TNF-\( \alpha \) may also enhance HIV-1 replication. The mechanism of enhancement involves activation of the transcription factor NF-kB, which lies downstream in the signaling pathway of TNF-\( \alpha \) receptor type 1 (49). Proinflammatory cytokines, such as TNF-\( \alpha \) and IL-1\( \beta \), induce sphingomyelinases, which results in the production of ceramide (32, 50). Rivas et al. (51) demonstrated that both sphingomyelinase and C8-Cer (another membrane-permeable ceramide analogue) are capable of activating transcription of HIV-1 proviral DNA, presumably via activation of NF-kB. In the present study, we show that C2-Cer induces a robust release of cysteine from hMDM. C2-Cer (1 \( \mu M \)) elicited a response equivalent to that generated by a near-saturating dose of TNF-\( \alpha \). It is possible, therefore, that ceramide lies in the signaling pathway for cysteine production and release after cytokine stimulation.

Importantly, in the present study, we found that the elevated levels of cysteine produced by immune-stimulated hMDM (stimulated by gp120, TNF-\( \alpha \), or IL-1\( \beta \)) contrasted with the low levels of cysteine produced by HIV-infected macrophages. Neurotoxic concentrations of cysteine were released by immune-activated/uninfected macrophages but not by HIV-infected macrophages. This finding is not surprising in light of the known oxidative stress besieging HIV-infected cells. A major source of intracellular cysteine is glutathione (\( \gamma \)-glutamyl-cysteinyl-glycine). Glutathione is synthesized after uptake of extracellular cysteine by cells. A systemic decrease in both glutathione and cyst(e)ine has been noted in AIDS, presumably due to the oxidative stress of HIV-infected cells (52–54). Thus, HIV-infected macrophages may be rendered incapable of producing excessive cysteine because of oxidative stress and subsequent glutathione depletion. In fact, this cytokine-mediated induction of ceramide and glutathione depletion is redox sensitive, and therefore can be reversed with cysteine derivatives (55).

A major implication of our findings, therefore, is that neurotoxins associated with HAD may not only be produced by HIV-infected macrophages but also by immune-stimulated, uninfected macrophages. In fact, cysteine is produced in consequential amounts for neurons by HIV-infected macrophages, even if immune activated. Nonetheless, cysteine emanates in large excess from immune-activated, uninfected macrophages, and may thus represent a major contributor to neuronal damage. This finding represents a completely new concept in the pathogenesis of HAD, as heretofore HIV-infected macrophages had been primarily studied for their production of putative neurotoxins (7, 8, 20, 21, 34, 45). Importantly, since perhaps only 10–15\% of macrophages in AIDS brains are infected whereas the remainder may be immune activated (11), this new class of toxins from non-HIV-infected, immune-stimulated macrophages may possibly represent the predominant and more widespread mode of neuronal injury. Such damage would occur via localized release of toxins, such as t-cysteine, from brain macrophages onto nearby neurons. Clearly, the search for additional neurotoxins from immune-activated uninfected brain macrophages and microglia is indicated.

Our findings also raise the intriguing possibility that normal signaling molecules between macrophages and neurons may, if released in excess, contribute to neuronal injury. The idea of signaling between brain macrophages/microglia and neurons is a relatively new one. In our experiments, we found that “control” hMDM often released substantial amounts of cysteine (although when comparing any single human donor, immune-activated or gp120-stimulated macrophages always released more). Since cysteine is a known NMDA agonist and the NMDA subtype of glutamate receptor is important in many physiological functions such as long-term potentiation (LTP, a cellular correlate of learning and memory) (56, 57), this finding suggests that brain macrophages may communicate with neurons as part of a complex neuroimmune system. Our results are consistent with the notion that dysfunctional neuroimmune regulation can be effected by small molecules such as cysteine acting at neurotransmitter receptor sites. Moreover, this concept could prove important in a variety of neurologic disease states besides AIDS in which altered immune function can interrupt normal intercellular communication between neurons in the brain.

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References


