Inflammatory Cytokines and HIV-1-Associated Neurodegeneration: Oncostatin-M Produced by Mononuclear Cells from HIV-1-Infected Individuals Induces Apoptosis of Primary Neurons

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Neurologic abnormalities are common in HIV-1-infected patients and often represent the dominant clinical manifestation of pediatric AIDS. The neurological dysfunction has been directly related to CNS invasion by HIV-1 that is principally, if not exclusively, supported by blood-derived monocytes/macrophages and lymphocytes. By using primary long term cultures of human fetal sensory neurons as well as sympathetic precursors-like neuronal cells, we determined that blood-derived mononuclear cells from HIV-1-infected individuals spontaneously release soluble mediators that can potently inhibit the growth and survival of developing neurons as well as the viability of postmitotic neuronal cells by inducing apoptotic cell death. Analysis of the cytokines produced by lymphomonocytic cells, HIV-1 infected or activated, indicated that oncostatin M (oncM) is a major mediator of these effects. Since low TGF-β1 concentrations were capable of enhancing oncM-mediated neuronal alterations, our data indicate that by acting in concert with other cytokines, oncM may induce neuronal demise in both the developing and the mature brain. Thus, this cytokine may contribute to the setting of the neuronal cell damage observed in HIV-1-infected individuals. The Journal of Immunology, 1999, 162: 6268 – 6277.

D egenerative neurological abnormalities are frequent in HIV-1-infected patients (1–3) and represent a common manifestation of pediatric AIDS, often accompanied by developmental delay or the loss of motor and intellectual milestones (4–7). In both adults and children the neuropathology is characterized by brain atrophy with sulcal widening and ventricular dilatation and histologically by the presence of microglial nodules with multinucleated giant cells, diffuse astrocytosis, perivascular mononuclear inflammation, rarefaction of the white matter with microvacuolation, and neuronal loss (8).

Although the neurological dysfunction has been directly related to CNS invasion by HIV-1 (9–15), the pathogenesis of neurologic disorders remains unclear. The conspicuous neuronal loss does not appear to depend upon direct cytopathic effects mediated by HIV-1 (1, 16–21). In fact, viral burden in the brain is not abundant and does not seem to correlate with the clinical severity of the disease (22–24). The limited extent of viral burden as well as the lack of evidence directly involving HIV-1 in neuronal demise suggested that the dementia in AIDS patients might be due to bioactive substances produced by resident and blood-derived mononuclear cells,

such as microglia, monocyte-macrophages, and, to a lesser extent, lymphocytes, which compelling evidence indicate to be major targets and the principal reservoir of the virus in the brain (8, 10, 20–21, 25–27). Indeed, the extent of macrophage/microglia recruitment in the CNS of patients with AIDS appears to better correlate to the dementia than to the viral burden (22–24). In addition to the production of viral structural and regulatory proteins (i.e., gp1201 and Tat), HIV-1-infected and/or functionally activated mononuclear cells can produce a number of soluble mediators, such as TNF-α, TGF-β, platelet-activating factor, arachidonic acid metabolites, free radicals, nitric oxide, and excitatory amino acids, that can alter neural cell function and survival and have been shown to possess potential neurotoxic properties in vitro and in animal models (28–39).

Consistent with this evidence, recent studies indicated that apoptotic mechanisms of neuronal injury may be involved in HIV-1 neuropathogenesis in the absence of direct HIV-1 infection of neuronal cells (40, 41), and that soluble mediators produced by PBMC from HIV-1-infected subjects can alter the survival of neuronal and glial cells in primary explants from fetal CNS (42). Thus, the mechanism of AIDS-associated neurodegeneration appears complex, probably involving more than one potentially neurotoxic mediator and possibly others, yet unidentified, factors that may act in concert in altering the function and survival of neuronal cells. In addition, the neurological impairment and neurodevelopment retardation observed in newborn and infants may depend upon mechanisms related to the specific requirements of actively developing and maturing cells, which may have peculiar vulnerabilities.

To investigate the role of immune-derived mediators responsible for the severity of CNS disease, particularly during nervous

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1 Dep. Allergy and Clinical Immunology, University of Rome “La Sapienza,” Rome, Italy; Department of Anatomy and Histology, University of Florence, Florence, Italy; and Dep. Cell and Molecular Biology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892

2 Address correspondence and reprint requests to Dr. Fabrizio Ensoli, Department of Allergy and Clinical Immunology, University of Rome “La Sapienza,” Viale dell’Università, 00185 Rome, Italy. E-mail address: ensolifior@axrma.uniroma1.it

3 Abbreviations used in this paper: gp120, glycoprotein 120; oncM, oncostatin M; CM, conditioned medium; NBD, normal blood donors; M/M, monocyte/macrophage; PI, propidium iodide; A-CM, lectin-activated PBMC CM.

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system development and maturation (fetal and early postnatal life), we examined the effects exerted by lymphomonocytes on the survival and maturation of primary fetal sensory neurons (43–45). These cell cultures maintain some proliferative potential in vitro and are representative of developing neurons. In addition, a sym pathetic neuronal cell line (46) was included in the study to represent some of the neuronal heterogeneity characteristic of the nervous system. Under appropriate experimental conditions, both cell cultures cease to grow and increase the expression of genes that correlate with a more mature phenotype (43, 44, 47, 48). Thus, they provide a useful tool for the investigation of pathological alterations of the development and survival of embryologically and biochemically distinct neuroblasts as well as mitotically quiescent neurons such as those present in the mature brain.

Here we show that soluble mediators spontaneously produced by immune cells from HIV-1-infected individuals or released by normal lymphomonocytes upon functional activation can potentially inhibit the proliferation and survival of primary sensory neurons as well as the viability of mitotically quiescent cells by inducing apoptotic cell death. We find that oncostatin M (oncM) is a major mediator of these effects, and it may act in concert with other cytokines in inducing neuronal damage in both the developing and the mature brain.

Materials and Methods

Primary neuronal long term cultures and neuroblastoma cell line

The primary human neuronal long term culture FNC-B4 has been established, cloned, and propagated in vitro from the human fetal olfactory system and cryogenically preserved (43). These cells, which have a limited life span (15–20 passages in culture), express a normal human karyotype which is conserved after cryogenic preservation (43). FNC-B4 cells have been phenotypically, biochemically, and functionally characterized (43–45). The expression of both neuronal proteins and olfactory genes as well as their capability to generate action potentials indicate that they derive from the olfactory stem cell compartment that gives rise to mature olfactory neurons throughout life (43–45). In fact, in addition to its chemosensory function, this system represents a model of neurogenesis that, simplifying the cellular heterogeneity of the developing nervous system, may help in investigating in vitro pathologic perturbations of the activation, self renewal, differentiation, and survival of primary sensory neuronal precursors. Cryogenically preserved, early passages of FNC-B4 cells have been used in the present study. In some experiments, proliferating neuroblasts were growth arrested and allowed to spontaneously differentiate in vitro. The latter experimental setting was obtained by growing FNC-B4 cells to a confluent monolayer. This supports close cell-to-cell contacts and induces primary olfactory neuronal precursors to arrest the growth, accumulate in the G0 phase of the cell cycle, and increase the expression of tissue-specific genes such as the olfactory marker protein that correlate with a more mature phenotype (43, 44) (data not shown).

The neuroblastoma-derived SH-SY5Y cell line, a clonal derivative from primary human embryonic adrenal medulla (42), was purchased from Genzyme (Cambridge, MA) at 0.5–1 × 10^6 and 5 × 10^6 cells/well, respectively, and 1 day later the corresponding CM, the recombinant cytokines, or the medium in duplicates was added to each well. The cells were then reincubated and the counts per minute of incorporated thymidine were determined with a beta counter (1250 β Plate, LKB/Pharmacia, Piscataway, NJ). The results were expressed as the percentage of growth inhibition (1–cm of treated cells/cpm of untreated controls × 100). For the cell-counting method, PBMC or conditioned medium (CM) preparation from HIV-1-infected individuals and normal blood donors (NBD) were seeded in 96-well plates (Costar, Cambridge, MA) at 0.5–1 × 10^6 and 5 × 10^6 cells/well, respectively, and 1 day later the corresponding CM, recombinant cytokines, or the medium in which the cytokines were resuspended (PBS/0.1% BSA) were added to each well. Cells were then incubated for an additional 6 days. During the last 18 h of incubation, 1 μCi of [3H]thymidine (6.7 Ci/mmol; New England Nuclear Research Products, Boston, MA) was added to each well (four replicates per sample) in medium containing 10% FBS (Life Technologies, Grand Island, NY). Cells were then trypsinized and harvested, and the counts per minute of incorporated thymidine were determined with a beta counter (1250 β Plate, LKB/Pharmacia, Piscataway, NJ). The results were expressed as the percentage of growth inhibition (1–cm of treated cells/cpm of untreated controls × 100). For the cell-counting method, PBMC or CM preparation from SH-SY5Y cells were added in triplicate containing 10% FBS (Life Technologies), 10^3 and 2.5 × 10^4 cells/well, respectively (in duplicate), and cell number was determined 6 days after addition of CM or cytokines by trypan blue dye exclusion as previously described (51).

Analysis of neuronal cell death: membrane permeabilization assay

FNC-B4 cells were seeded at 40% confluence in T75 flasks (Costar) and cultured in Coon’s/F-12 medium with 100 U/ml penicillin, 100 mg/ml...
streptomycin, and 10% FBS (Life Technologies, Grand Island, NY). Twenty-four hours later the cells were incubated with the corresponding PBMC-CM, recombinant cytokines, or the buffer in which they were resuspended. Cells were harvested at serial time points, stained with propidium isodide solution (PI; 1 mg/ml) for 30 min, and analyzed by FACS to determine the frequency of cells with altered membrane permeability as previously described (52).

**Immunological determination of internucleosomal DNA fragmentation**

FNC-B4 cells were seeded in six-well plates (Costar) at subconfluent or confluent densities (2 × 10^5 and 5 × 10^5 cells/well, respectively) and were cultured in Coon’s/F-12 medium with 100 U/ml penicillin, 100 mg/ml streptomycin, and 10% FBS (Life Technologies, Gaithersburg, MD). Twenty-four hours later both subconfluent and confluent cultures were incubated with the corresponding PBMC-CM, recombinant cytokines, or the buffer in which they were resuspended. Cells were collected at 6 days, and nuclear and cytoplasmic cellular fractions were prepared and analyzed following the manufacturer’s instructions (Boehringer Mannheim. Cell Death Detection ELISA Plus). The assay, which is based on the immunological demonstration of DNA fragmentation, a hallmark of apoptosis, in dying cells, may help distinguish late apoptotic events from necrosis (53). Specifically, the test provides an immunological quantification of internucleosomal DNA fragmentation, expressed as the enrichment of histone-associated DNA fragments (mono- and oligonucleosomes) in the cytoplasmic fraction of the cells, which corresponds to the appearance of a DNA ladder by gel electrophoresis analysis.

**Time-course analysis of early chromatin alterations by in situ end labeling of DNA strand breaks (TUNEL assay)**

A time-course TUNEL analysis was performed to determine the extent and the temporal kinetics of DNA strand breaks triggered by oncM treatment in primary sensory neurons. Briefly, FNC-B4 cells were seeded in T75 tissue culture flasks (Costar) at 40% confluence and cultured in Coon’s/F-12 medium with 100 U/ml penicillin, 100 mg/ml streptomycin, and 10% FBS (Life Technologies, Gaithersburg, MD). Twenty-four hours later subconfluent cultures were incubated with oncM (10 ng/ml) or the buffer in which the cytokine was resuspended. Cells were collected at serial time points, membrane permeability was assessed by PI staining, and the extent of high m.w. DNA strand breaks was determined by in situ labeling of the free 3’-OH termini by terminal-transferase, followed by flow cytometric visualization of the incorporated fluorescein-dUTP according to the manufacturer’s instructions (In Situ Cell Death Detection Kit, Boehringer Mannheim).

**Bioactivity of released oncM and blocking assays**

To determine whether neuronal cell growth inhibition induced by CM was due to oncM, blocking assays were performed by preincubating the PBMC-derived soluble fractions at 4°C for 8 h with 5–20 μg/ml of affinity-purified anti-oncM neutralizing polyclonal Abs (R&D Systems) or with control preimmune antiserum (control Abs; Endogen, Boston, MA) and by adding these fractions to primary neuronal cultures. Alternatively, PBMC-CM were preincubated at 37°C for 12–16 h on petri plastic dishes coated for 2 h at room temperature with anti-oncM neutralizing Abs (50 μg/ml) to deplete them of the cytokine. Control dishes were coated with the control Abs. OncM depletion was confirmed by ELISA. Subsequently these fractions were added to neuronal cells. Recombinant oncM (R&D Systems) was used as a positive control.

**Measurements of oncM in PBMC and M/M supernatants**

PBMC or M/M supernatants from HIV-1-infected individuals or NBD were collected as described above. CM were then tested for oncM content by ELISA according to the instructions provided by the manufacturer (R&D Systems). To avoid loss of oncM, all samples were handled in plasticware precoated with PBS/0.1% BSA.

**Results**

**Soluble neurotoxic mediators are spontaneously produced by PBMC from HIV-infected patients and can be induced in normal PBMC by functional activation**

To verify whether mononuclear phagocytes and lymphocytes may potentially contribute to HIV-1 neuropathogenesis through the production of soluble neurotoxic mediators, we first determined whether blood-derived lymphomonocytes from HIV-1-infected patients spontaneously produce soluble mediators capable of altering the growth and survival of primary neurons (Fig. 1). This set of experiments was performed by culturing the primary human neuronal cell culture FNC-B4 in the presence of CM prepared from either HIV-1-infected patients (HIV−/CM) or NBD (HIV−/CM) and lectin-activated PBMC (A-CM) or unstimulated and LPS-activated M/M preparations from NBD (M/M-CM; Fig. 1, A–C). The results indicated that PBMC from HIV-1-infected subjects spontaneously produce and release a factor(s) that potently inhibits the growth of primary neuronal precursors (Fig. 1A, left panel), while unstimulated PBMC from healthy individuals did not show any neurotoxic activity (Fig. 1A, center panel). However, upon activation induced by lectins or LPS, respectively, uninfected PBMC or M/M produced a soluble mediator(s) with strong inhibitory effects on neuronal cell growth...
4.9-fold increase of PI staining; 111

harvested, stained with PI solution (1 mg/ml) for 30 min, and analyzed by FACS. In

release of histone-associated DNA fragments into the cytoplasm fraction and by al-

tion as assessed by both [3 H]thymidine uptake and cell number

stimulated neuronal cell proliferation (Fig. 2

not alter cell viability and morphology, although they variably

Effects of soluble mediators produced by functionally activated

Table I.

<table>
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<tr>
<th>Membrane permeabilityb</th>
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<tr>
<td>DNA fragmentationc</td>
<td>+ +</td>
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<td>Cell growth inhibition/cell lossd</td>
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* The inhibitory effects exerted by HIV-1 infected or functional activated
lymphomonocytes on primary neurons cell growth are accompanied by increased release of histone-associated DNA fragments into the cytoplasm fraction and by altered membrane permeability to PI staining. FNC-B4 cells were cultured in the pres-
ence of A-CM as described in Materials and Methods. After 6 days, cells were
harvested, stained with PI solution (1 mg/ml) for 30 min, and analyzed by FACS. In
addition, nuclear and cytoplasmic cellular fractions from FNC-B4 cells seeded at two
different densities were prepared and analyzed to detect low m.w. DNA fragments.
Results were calculated as enrichment factor of mono- and oligonucleosomes released
into the cytoplasm. ND, not done.

b Membrane permeability: +, 1.5- to 2.9-fold increase of PI staining; + +, 3- to
4.9-fold increase of PI staining; + + +, 5- to 8-fold increase of PI staining.

c DNA fragmentation: +, 1.5- to 1.9 enrichment factor; + +, 2- to 4 enrichment factor.

d Cell growth inhibition: +, 10-29%; + +, 30-49%; + + +, 50-66%.

(Fig. 1A, right panel; B and C). The slight growth inhibition ob-

erved with unstimulated M/M-CM (Fig. 1C) was probably due to an

increased baseline activation induced by the M/M separation

procedure, which is based on adherence to plastic surfaces.

To verify whether the alterations induced by HIV-1-infected or

functionally activated lymphomonocytes or M/M preparations

were limited to proliferating precursors, which are present during

nervous system development or may involve mitotically quiescent

neurons, FNC-B4 cells were growth arrested and allowed to sponta-

neously differentiate in vitro. Mitotically quiescent neurons were

then cultured in the presence of CM from HIV-1-infected or func-

tionally activated PBMC. The results of these experiments indi-

cated that HIV-1-infected or functionally activated PBMC can al-
ter the survival of neuronal cells independently from their mitotical

activity (Table I). With both proliferating and quiescent neurons, the

progressive neural cell loss induced by CM treatment became

morphologically evident 48–72 h or 4–6 days, respectively, after

incubation with the CM (data not shown) and was accompanied by

accumulation of histone-associated DNA fragments (mono- and

oligonucleosomes) into the cytoplasmic fraction of the cells and by

altered membrane permeability to PI staining (Table I). These re-

sults, consistent with previous observations (40–42), indicate that a

soluble factor(s) produced by lymphomonocytic cells upon either

HIV-1 infection or functional activation is capable of altering the

growth potential and survival of both immature and postmitotic

neurons and suggest that the decreased neuronal cell viability

may depend upon the induction of apoptosis or apoptotic-related

mechanisms.

Analysis of neuroblast growth and survival in the presence of recombinant inflammatory cytokines: effects of oncM

To verify whether inflammatory cytokines, which can be chroni-
cally induced by HIV-1 infection or stimulated by functional ac-
tivation, may play a part in these events, experiments were per-
formed to assess the effects of a panel of recombinant cytokines on

both primary sensory and sympathetic neuronal cell cultures. The

concentrations used were similar to those detected in the CM (50, 51) (Fig. 2A). The results indicated that inflammatory cytokines

such as TNF-α, TNF-β, IL-6, IL-1α, IL-1β, IFN-γ, or TGF-β1 did not

alter cell viability and morphology, although they variably

stimulated neuronal cell proliferation (Fig. 2A). However, incuba-
tion with oncM caused a pronounced inhibition of cell prolifer-
ation as assessed by both [3 H]thymidine uptake and cell number

(24 h 17%), 48 h (47%), and 72 h (51%; Table III). At these times the cells started losing their membrane integrity, as they appear capable of excluding PI.

However, a progressive cell growth inhibition was observed at 24 h (17%), 48 h (47%), and 72 h (51%; Table III). At these times the cells started losing their membrane integrity, as indicated by the increased permeability to PI. Peak levels of cell growth inhibi-
tion were maintained through later time points (6 days) and were accompanied by extensive DNA cleavage, as low m.w. DNA frag-
ments (mono- and oligonucleosomes) were released from the nu-
cleii and were found in the cytoplasmic cell fractions (Table II).
These results indicated that oncM can alter the survival of neuronal cells by inducing apoptosis or apoptosis-like mechanisms of cell
death and suggested that a chronic, dysregulated production of oncM may play a part in the pathogenesis of AIDS-associated neurological disorders.

Anti-oncM neutralizing Abs counteract the neurotoxic activity of

PBMC supernatants as well as the effects of recombinant oncM

To determine whether native oncM may contribute to the neuro-
toxic effects induced by HIV-1-infected or lectin-activated PBMC
on primary neurons, blocking experiments were performed by culturing primary neuronal cells with PBMC-CM or recombinant oncM in the presence of different concentrations of anti-oncM neutralizing Abs. Alternatively, PBMC supernatants were preincubated in anti-oncM Abs-coated culture plates to deplete them of the cytokine, and neuronal cells were then cultured with the oncM-depleted CM. The results of these experiments are summarized in Fig. 3, A–C. The addition of anti-oncM neutralizing Abs caused a dose-dependent neutralization of the inhibitory effects of A-CM or recombinant protein as a source of oncM. In contrast, control preimmune antiserum had no effect (Fig. 3A). Similarly, oncM depletion of PBMC supernatants consistently reduced, and in some cases abolished, the inhibitory effect of A-CM on primary neuron growth and survival. Results were consistently obtained using either the CM or the purified recombinant protein as a source of oncM. In contrast, control preimmune antiserum had no effect (Fig. 3A). Similarly, oncM depletion of PBMC supernatants consistently reduced, and in some cases abolished, the inhibitory effect of A-CM on primary neuron growth and survival. Results were consistently obtained using either the CM or the purified recombinant protein as a source of oncM.

OncM is spontaneously produced by PBMC from HIV-1-infected patients and can be induced in normal PBMC upon functional activation

The next set of studies was performed to determine the levels of oncM production by HIV-1-infected or functionally activated PBMC or M/M preparations and compare the results with those for other inflammatory cytokines such as TNF-α, whose production has been documented with both HIV-1-infected and lectin-activated PBMC and M/M (Table IV). The results of these experiments indicated that oncM is spontaneously produced by PBMC from most HIV-1-infected subjects (25 of 28; 89%); however, the cytokine was usually below the limit of detection of the assay with CM from unstimulated PBMC from NBD, while slight oncM production was detected with unstimulated M/M preparations from...
Table II. Effect of recombinant oncM on the viability of mitotically active and quiescent primary neurons

<table>
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<th>Mitotically Active FNC-B4</th>
<th>Quiescent FNC-B4</th>
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<tr>
<td>Membrane permeability&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>DNA fragmentation&lt;sup&gt;c&lt;/sup&gt;</td>
<td>+ +</td>
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<td>Cell growth inhibition/cell loss&lt;sup&gt;d&lt;/sup&gt;</td>
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<sup>a</sup> The inhibitory effects exerted by oncM on primary neurons cell growth are accompanied by accumulation of histone-associated DNA fragments into the cytoplasm fraction and by altered membrane permeability to PI staining. FNC-B4 cells were cultured in the presence of oncM (10 ng/ml) as described in Materials and Methods. After 6 days, cells were harvested, stained with PI solution (1 mg/ml) for 30 min, and analyzed by FACS. In addition, nuclear and cytoplasmic cellular fractions from FNC-B4 cells seeded at two different densities were prepared and analyzed to detect low m.w. DNA fragments. Results were calculated as enrichment factor of mono- and oligonucleosomes released into the cytoplasm. ND, not done.

<sup>b</sup> Membrane permeability: +, 1.5- to 2.9-fold increase of PI staining; + +, 3- to 4.9-fold increase of PI staining; + + +, 5- to 8-fold increase of PI staining.

<sup>c</sup> DNA fragmentation: +, 1.5-1.9 enrichment factor; + +, 2-4 enrichment factor.

<sup>d</sup> Cell growth inhibition: +, 10-29%; + +, 30-49%; + + +, 50%.

Table III. Time course analysis of oncM-induced neuronal cell death<sup>a</sup>

<table>
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<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
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<tr>
<td>Cell growth inhibition&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>TUNEL assay&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>Membrane permeability&lt;sup&gt;d&lt;/sup&gt;</td>
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<sup>a</sup> The extent and the temporal kinetic of high m.w. DNA strand breaks triggered by a single concentration (10 ng/ml) of oncM in primary sensory neurons has been determined by in situ labeling of the free 3′-OH termini by TdT at serial time points (24 h, 48 h, 72 h, and 6 days), followed by flow cytomteric visualization of the incorporated fluorescein-dUTP (TUNEL assay). The appearance of high m.w. DNA strand breaks, the relationship with alterations of cellular integrity (exclusion of cat-ionic-charged dyes), and the kinetics of cell proliferation were examined. ND, not done.

<sup>b</sup> Cell growth inhibition: +, 10-29%; + +, 30-49%; + + +, 50%.

<sup>c</sup> Membrane permeability: +, 1.5- to 2.9-fold increase of PI staining; + +, 3- to 4.9-fold increase of PI staining; + + +, 5- to 8-fold increase of PI staining.

<sup>d</sup> TUNEL assay: +, 1.5- to 1.9-fold increase of % of positive cells; + +, 2- to 4-fold increase of % of positive cells.

healthy subjects, probably due to adherence-induced activation of M/M (Table IV). In addition, oncM detection was directly associated with evidence of neurotoxic effects of the corresponding CM (data not shown). Upon functional activation, the cytokine was potently induced with either normal PBMC or M/M at levels consistent with the higher concentrations used in the present study (Table IV). In contrast, spontaneous TNF-α production was detected at lower frequency, compared with oncM, in PBMC from HIV-1-infected individuals (8 of 17; 47%), and low, but measurable, concentrations of TNF-α were also detected with NBD (6 of 12; 50%). TNF-α production increased dramatically upon functional activation with both PBMC and M/M preparations (Table IV) at levels consistent with those used in the present study. Interestingly, these studies also suggested that native oncM may have a more pronounced biological activity than the recombinant protein. This may simply reflect a different efficiency of protein synthesis in vivo and in vitro. Alternatively, the higher activity of native oncM might depend upon the contribution of additional soluble mediators present in PBMC- or M/M-CM.

**Multiple inflammatory cytokines may act in concert in inducing neuronal demise: cooperative neurotoxic effects of TGF-β1 and oncM**

To verify whether other inflammatory mediators may act in concert with oncM in mediating neuronal demise, primary sensory neurons were cultured in the presence of low concentrations of oncM, consistent with those detected in the PBMC cultures from HIV-1-infected patients, and different inflammatory cytokines such as TNF-α, IL-6, IFN-γ, TGF-β1, or virus components such as gp120. The results indicated that TGF-β1, which alone had no inhibitory effect on primary neurons (Fig. 2A), can act in concert with oncM to increase the effects of this cytokine on neuronal cell growth and survival (Table V). Specifically, low concentrations of TGF-β1 potentiated the effects exerted by oncM on the cell growth and survival of primary neuronal cells compared with those observed using suboptimal concentrations of oncM alone (Table V). In contrast, none of the other inflammatory cytokines or gp120 had any consistent effect in either the presence or the absence of oncM (10–100 ng/ml concentrations of recombinant gp120 from the HTLVIIIB strain (obtained from Intracel, Cambridge, MA) were used, following the same experimental setting as that described in Materials and Methods for cytokine neurotoxicity testing; data not shown). These data indicate that TGF-β1 and oncM can act in concert in inducing neuronal demise and suggest that the combined actions of these cytokines may contribute to the pathophysiology of AIDS-associated neurological disorders.

**Discussion**

To identify a potentially neurotoxic mediator(s) produced by mononuclear cells as a consequence of HIV-1 infection, we developed cell culture conditions that allowed us to monitor the proliferation and differentiation of primary neuronal precursors. These cultures differ from more commonly used primary dissociated neuronal cell cultures in two important respects. Firstly, they represent homogeneous populations derived from single-cell progenitors that spontaneously proliferate in vitro. For this reason the effects of exogenous factors on cell growth and viability can be clearly evaluated. Secondly, under appropriate experimental conditions these cells cease to grow and increase the expression of tissue-specific genes that are consistent with a more differentiated phenotype, thus enabling an evaluation of potentially neurotoxic signals in quiescent (postmitotic) neurons such as those characteristic of the mature brain.

The present study determined that soluble mediators spontaneously produced by lymphomonocytes from HIV-1-infected individuals or released by normal PBMC or M/M upon functional activation can alter the growth and viability of immature neurons as well as the survival of mitotically quiescent neuronal cells. These effects are accompanied by both morphological and biochemical alterations of the cells consistent with the induction of apoptotic cell death. These observations are consistent with previous studies (33, 36, 40–42) and suggest that both HIV-1 infection and functional activation can induce lymphomononuclear cells to express neurotoxic potentials that rely upon the production of bioactive substances that, in turn, induce neuronal growth perturbations and apoptotic cell death. However, only a fraction (14 of 25) of the patients that showed immune-mediated neurotoxic activity (25 of 28) had clinical or subclinical evidence of CNS alterations at enrollment. This may simply reflect a dissociation between the presence of CNS histopathological alterations, undetected by neuroimaging studies, and clinical or subclinical evidence of CNS injury, which has been often described in HIV-1-infected individuals (1–3). Alternatively, these data suggest that to exert neurodamaging effects, the immune-mediated neurotoxic potential requires additional steps and/or components. The latter might be achieved by CNS influx of HIV-1-infected or activated lymphomononuclear cells that, in turn, depend upon the integrity
of the blood-brain barrier and the local production of different inflammatory cytokines and chemoattractants (54–56).

Studies performed with recombinant cytokines at concentrations similar to those detected in PBMC-CM (50, 51) (Table IV), identified oncM (57, 58) as one cytokine that caused a profound inhibition of neuronal proliferation and viability (Figs. 1 and 2). The decrease in cell viability, documented by the altered membrane permeability to PI staining, was preceded and accompanied by extensive DNA cleavage, which indicates that oncM induced apoptotic cell death (Table I–3). The effect of oncM was specific, as neutralizing anti-oncM Abs prevented the growth inhibition induced by either the CM or the recombinant protein (Fig. 3). These results indicate that native oncM can play a part in HIV-1-associated neurodegeneration; however, they also suggest that other factors are likely to be involved in the process. Additional experiments performed with primary sensory neurons indicated that TGF-β1, but not TNF-α, IFN-γ, IL-6, or virus components such as gp120 (data not shown), can act in concert with oncM, enhancing the inhibitory effects of the cytokine in our model system (Table V). Since low TGF-β1 concentrations increased the inhibitory effects induced by oncM, in the presence of both cytokines neuronal growth and survival alterations were elicited at very low oncM concentrations, consistent with those spontaneously released by PBMC from HIV-1-infected subjects in their supernatants (Table IV). Thus, the total neurotoxic activity potentially exerted by HIV-1-infected or functionally activated lymphomononuclear cells depends upon several parameters, including the class of cytokine production and its concentration, potency, and interaction(s) with other mediators.

OncM is a proinflammatory cytokine that belongs to a family of structurally and functionally related pleiotropic factors that use the gp130 or gp130-related receptor subunits on target cells (57–59). These cytokines exhibit differential effects on a variety of cell types, including cells of neural, hemopoietic, lymphopoietic, and vascular origins (59–62). Indeed, the same protein can act differentially on target cells depending upon the responsive cell population (59–61). In fact, oncM can exert a profound inhibition of the growth of primary embryonal precursors as well as of tumorderived cell lines of different origins (61, 62). Interestingly, expression of oncM in transgenic mice is detrimental to normal mouse development, and death is associated with expression in neurons (63), suggesting that oncM production in the brain may exert lethal effects on neuronal cells. The results of the present study provide support for previous observations and indicate that increased oncM production from HIV-1-infected or functionally activated lymphocytes and resident or blood-derived mononuclear phagocytes can directly alter neuronal development and survival by inducing apoptotic cell death. Thus, this cytokine may directly contribute to neuronal apoptosis in AIDS-associated neurodegeneration, which generally occurs in the absence of direct HIV-1 infection of these cells and appears to depend upon the induction of diffusible factors (40, 41). Other inflammatory mediators, however, are produced upon HIV-1 infection or functional activation of lymphomononuclear cells. Cytokines such as TNF-α, IL-6, and...
IL-1, IFN-γ, and TGF-β1, whose neurotoxic properties have been previously suggested or documented (13, 30, 54, 55, 64–66), did not directly alter neuronal viability or inhibit their growth in this model system (Fig. 2A). This evidence does not rule out their contribution to neuronal demise by both direct and indirect interactions with suitable target cells (67). For example, TNF-α can exert direct inhibitory or stimulatory effects depending upon the responsive neural cell type (68, 69). In fact, TNF-α can even protect primary neurons from different origins from metabolic-excitotoxic insults (70). This is not surprising, since cytokines such as TNF-α, TGF-β, OncM, and IL-6, key regulatory factors in the host response to injury or immunological challenge, can also act as instructive/permissive signals during nervous system development or in events that involve functional or structural tissue remodeling (71). In addition to directly interact with responsive neuronal cell targets, the contribution of these cytokines to neuronal demise involves multiple interactions with all the different nonneuronal cell types in the brain (67, 71). This idea further emphasizes the concept that the inappropriate, chronic production of different inflammatory cytokines can act in concert in directly inducing neuronal demise in both the immature and the adult brain. This evidence further suggests that a cascade of events triggered by HIV-1 infection and involving a chronic dysregulation of cytokine expression is implicated in the pathophysiology of AIDS-associated neurological disorders. This may have important implications for the understanding of neuropathogenesis of HIV-1 infection and provides a model for the interpretation of neurological disorders depending upon CNS inflammatory conditions from varied insults. These observations also suggest that in the setting of therapeutic strategies for AIDS-related neurologic disorders, efforts should be not only directed toward reducing the viral load in the brain, but also toward controlling the deleterious effects of cytokines such as OncM and TGF-β1.

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**References**


Table IV. OncM and TNF-α production by PBMC or M/M from HIV-1 infected or healthy individuals

<table>
<thead>
<tr>
<th></th>
<th>OncM (pg/ml)</th>
<th>No. positive samples/ No. tested samples</th>
<th>TNF-α (pg/ml)</th>
<th>No. positive samples/ No. tested samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV-1-CM</td>
<td>67 ± 59</td>
<td>25/28</td>
<td>11 ± 15</td>
<td>6/12</td>
</tr>
<tr>
<td>HIV+1-CM</td>
<td>1212 ± 76</td>
<td>4/4</td>
<td>3162 ± 224</td>
<td>4/4</td>
</tr>
<tr>
<td>A-CM</td>
<td>39 ± 19</td>
<td>3/3</td>
<td>92 ± 2</td>
<td>3/3</td>
</tr>
<tr>
<td>UNS/M-M-CM</td>
<td>297 ± 36</td>
<td>2947 ± 159</td>
<td>3/3</td>
<td></td>
</tr>
<tr>
<td>LPS-M-M-CM</td>
<td>297 ± 36</td>
<td>2947 ± 159</td>
<td>3/3</td>
<td></td>
</tr>
</tbody>
</table>

* Cell supernatants were prepared from unstimulated PBMC from HIV-1-infected or healthy subjects and from activated PBMC or M/M preparations from NBD and assessed for OncM content by ELISA. OncM is spontaneously produced by PBMC from HIV-1-infected subjects (25 of 28; 89%) and can be strongly induced in normal PBMC or M/M by functional activation, in a dose-dependent manner. In contrast, the cytokine is consistently below the limit of detection with CM from unstimulated PBMC, whereas a slight OncM production was detected with unstimulated M/M preparations from healthy subjects. The latter was probably due to adherence-induced activation of M/M and was associated with inhibitory effects of the corresponding CM on primary neuroblast cell growth (see also Fig. 1C). HIV+1-CM; CM from unstimulated PBMC from NBD; A-CM; CM from activated PBMC from NBD; M/M-CM; CM from unstimulated or activated M/M preparations from NBD; HIV+1-CM; CM from unstimulated PBMC from HIV+1 individuals. Undetectable.

Table V. Multiple inflammatory cytokines may act in concert in inducing neuronal demise: cooperative neurotoxic effects of TGF-β1 and OncM

<table>
<thead>
<tr>
<th></th>
<th>Cell Growth Inhibition (%)</th>
<th>DNA Fragmentation (enrichment factor)</th>
</tr>
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<tbody>
<tr>
<td>OncM (0.1 ng/ml)</td>
<td>28.6</td>
<td>1.16</td>
</tr>
<tr>
<td>TGF-β1 (0.05 ng/ml)</td>
<td>0</td>
<td>1.10</td>
</tr>
<tr>
<td>OncM (0.1 ng/ml)</td>
<td>61.9</td>
<td>2.00</td>
</tr>
<tr>
<td>+ TGF-β1 (0.05 ng/ml)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Low TGF-β1 concentrations, which alone have no inhibitory effects on primary neuroblast cell growth, can cooperate with OncM and increase the effects of the cytokine on neuronal cell growth (0.1 ng/ml OncM; 0.05 ng/ml TGF-β1). In addition, the enhanced cell growth inhibition exerted by TGF-β1 and OncM is accompanied by an increased release of histone-associated DNA fragments into the cytoplasmic fraction, as compared with that observed with OncM alone (1 ng/ml OncM; 0.05 ng/ml TGF-β1; 5 days of assay). In contrast, other inflammatory cytokines such as IL-6, IFN-γ, TNF-α, or virus components such as gp120 had no measurable effect on OncM-mediated neuronal alterations (data not shown). Primary neuroblast cell growth was evaluated by [3H]thymidine incorporation assay, and DNA fragmentation was determined as described above (see also Materials and Methods). The results are expressed as percent of growth inhibition ([1–cpm of FNC-B4 treated cells]/[cpm of untreated FNC-B4 cells]) × 100), and as enrichment factor of mono- and oligonucleosomes released into the cytoplasmic fraction.


