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*J Immunol* 1999; 162:6268-6277; 
http://www.jimmunol.org/content/162/10/6268
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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 lymphomononuclear 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.

Received for publication July 27, 1998. Accepted for publication March 1, 1999.

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This work was supported by Istituto Superiore di Sanità AIDS Projects 96 (Grant 9403-63) and 97 (Grants 40 A.0.02 and 30 A.0.33), by Istituto Pasteur, Fondazione Cenci Bolognetti (to F.E.); and by a grant from ANLAIDS, 1997 (to V.F.).

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.
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 sympathetic 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 cell 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 1:20 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 G1 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 SH-SY5Y neuroblastoma cells, was used in some experiments. This cell line is composed of a homogeneous neuronal population that has been previously established and extensively characterized both phenotypically and biochemically. The biochemical, phenotypical, and morphological data suggest a close resemblance with their normal sympathetic counterpart of the developing nervous system (46–48). In addition, SH-SY5Y cells can be used in experiments involving sympathetic neurons and neurotransmitters). Differentiation in vitro by appropriate stimuli (i.e., retinoic acid) (49). This process involves virtually all the cells present in these cultures, indicating that they are composed of a relatively homogeneous population of neuroblasts consistent with sympathetic precursors.

The FNC-B4 primary neuronal cells and the SH-SY5Y cell line show different biochemical and functional neuronal phenotypes that mimic some of the neural functional heterogeneity characteristic of both the developing and the mature nervous system. Both cell types were cultured in Coons’s modified F-12 medium supplemented with 10% FBS and antibiotics in a 5% CO2 atmosphere at 37°C as previously described (43).

PBMC and conditioned medium (CM) preparation from HIV-1 infected individuals and normal blood donors (NBD)

Twenty-eight HIV-1-infected individuals at different stages of disease progression and 10 healthy individuals (NBD) were enrolled in the study. In addition to immunological and virological assessment, all patients had neuropsychiatric evaluation and diagnostic neuroimaging. Those presenting severe cognitive decline as their initial clinical manifestation of AIDS (n = 6) were considered to have pure dementia. They had no history of CNS opportunistic infections. Eight of the remaining patients had subjective CNS involvement, documented by neuropsychiatric assessment and neuroimaging studies that showed signs of brain alterations (cortical atrophy). The remaining patients (n = 14) had normal mental status as assessed by psychiatric evaluation and no signs of CNS alterations by computed tomographic scan analysis.

Preliminary experiments were performed to assess the efficiency of monocytic/macrophagic (M/M) isolation through plastic adherence in HIV-1-infected patients and NBD. Briefly, freshly collected PBMC were allowed to adhere in 75-cm2 plastic tissue culture flasks at a concentration of 4 × 106 cell/ml in RPMI 1640 supplemented with 10% FBS and 20% normal human serum. After 24–72 h of incubation nonadherent cells were harvested, and pre- and postadherence cells were analyzed by flow cytometry to test the expression of monocytic/macrophagic markers (CD14). The results were consistent with those of previous studies (42) and indicated a high variability in the recovery of M/M cells from the peripheral blood of HIV-1-infected patients. Therefore, to assess the production of soluble mediators from unselected M/M, subsequent experiments with HIV-1-infected individuals were performed on PBMC not subjected to plastic adherence.

PBMC and CM were isolated from whole blood by Ficoll-Hypaque gradient centrifugation, counted, and cultured at 2 × 106 cells/ml in RPMI 1640 medium supplemented with 10% FBS in a 5% CO2 atmosphere at 37°C as previously described (50, 51). CM were prepared from either unstimulated PBMC from HIV-1-infected individuals and NBD or activated PBMC or M/M preparations from NBD as previously described (50, 51). Briefly, 2 × 106 cells were cultured in the absence of stimuli to verify the spontaneous production of putative neurotoxic mediators. Alternatively, PBMC or M/M preparations from NBD were activated by incubation with PHA (0.1, 0.5, and 2 µg/ml), Con A (0.1, 1, and 5 µg/ml), or LPS (0.001, 0.1, and 1 µg/ml), respectively. CM from PBMC were collected after 72 h, while CM from M/M were collected at different time points (24 h, 72 h, and 6 days). CM were then centrifuged at 3000 rpm at 4°C for 30 min and tested for cytokine content and biological activity on neuronal cells. To avoid the loss of cytokines, all samples were handled in plasticware precoated with PBS/0.1% BSA.

Cytokines

Recombinant purified human TNF-α, TNF-β, IL-6, IL-1α, IL-1β, IFN-γ, and IL-2 were purchased from Boehringer Mannheim (Indianapolis, IN). OncM was obtained from R&D Systems (Minneapolis, MN). TGF-β1 was purchased from Genzyme (Cambridge, MA).

Measurement of neuronal cell growth and viability

FNC-B4 and SH-SY5Y cell growth was evaluated by both the [3H]thymidine incorporation assay and the cell counting method. For the [3H]thymidine incorporation assay, cells were seeded in 96-well plates (Costar, Cambridge, MA) at 0.5–1 × 105 and 5 × 105 cells/well, respectively, and 1 day later the corresponding CM, the 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 fast 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 – cpm of treated cells/cpm of untreated controls × 100). For the cell-counting method, 1 × 104 and 1 × 105 SH-SY5Y cells were added in medium containing 10% FBS (Life Technologies) 104 and 2.5 × 104 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

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streptomyacin, 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 iodide 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 x 10^4 and 5 x 10^4 cells/well, respectively) and were cultured in Coon's/F-12 medium with 100 U/ml penicillin, 100 mg/ml streptomyacin, 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 streptomyacin, and 10% FBS (Life Technologies, Gaithersburg, MD). Twenty-four hours later 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 cells. 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 Ab. 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 plastic ware 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 unstimulated PBMC 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 inhibits neuroblast growth (HIV-CM, left panel). Unstimulated PBMC from healthy subjects do not show any neurotoxic activity (HIV-CM, center panel), while cell growth inhibition is observed in the presence of CM from lectin-activated PBMC from healthy individuals (A-CM, right panel). The results are expressed as the percentage of growth inhibition (1 – number of CM-treated FNC-B4 cells/number of untreated FNC-B4 cells x 100; see also Materials and Methods). B and C. Upon functional activation, lymphomonocytes and enriched M/M preparations from NBD produce a soluble mediator(s) responsible for inhibitory effects on neuroblast growth. Primary human neuroblasts were cultured in the presence of CM prepared from activated PBMC (left panel) or M/M preparations (right panel) from NBD (see also Materials and Methods). The results are expressed as the percentage of growth inhibition (1 – number of CM-treated FNC-B4 cells/number of untreated FNC-B4 cells x 100)

**FIGURE 1.** Soluble neurotoxic mediators are spontaneously produced by PBMC from HIV-infected patients and can be induced in normal PBMC or M/M preparations by functional activation. A, PBMC from HIV-1-infected subjects release a factor(s) that inhibits neuroblast growth (HIV-CM, left panel). Unstimulated PBMC from healthy subjects do not show any neurotoxic activity (HIV-CM, center panel), while cell growth inhibition is observed in the presence of CM from lectin-activated PBMC from healthy individuals (A-CM, right panel). The results are expressed as the percentage of growth inhibition (1 – number of CM-treated FNC-B4 cells/number of untreated FNC-B4 cells x 100; see also Materials and Methods). B and C. Upon functional activation, lymphomonocytes and enriched M/M preparations from NBD produce a soluble mediator(s) responsible for inhibitory effects on neuroblast growth. Primary human neuroblasts were cultured in the presence of CM prepared from activated PBMC (left panel) or M/M preparations (right panel) from NBD (see also Materials and Methods). The results are expressed as the percentage of growth inhibition (1 – number of CM-treated FNC-B4 cells/number of untreated FNC-B4 cells x 100).
4.9-fold increase of PI staining; 1.5–1.9 enrichment factor; 2–4 enrichment factor.

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.

DNA fragmentation: +, 1.5–1.9 enrichment factor; +++, 2–4 enrichment factor.

Cell growth inhibition/cell loss: +, 10–29%; +++, 30–49%; +++++, ≥50%.

The inhibitory effects exerted by HIV-1 infected or functionally 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 presence 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.

Results were calculated as enrichment factor of mono- and oligonucleosomes released into the cytoplasm fraction of the cells and by altered permeability of the cell membrane to cation-charged dyes such as PI (Table II). These data indicate a reduced cell survival and suggest that the induction of apoptosis or apoptosis-related mechanisms may be responsible of these events (53). To confirm this hypothesis we investigated the temporal kinetic of chromatin alterations induced by oncM in primary sensory neurons.

**Time-course analysis of oncM-induced neuronal cell death**

Extensive DNA degradation is a characteristic event occurring in the early stages of apoptosis. Such DNA cleavage may yield double-stranded DNA fragments of low m.w. (mono- and oligonucleosomes, detectable by ELISA or gel electrophoresis) as well as extensive single-strand breaks of high m.w. DNA. The latter can be demonstrated in situ, in cell nuclei, by enzymatic end labeling of the free 3'-OH termini with nucleotides modified to include a target molecule (i.e., fluorescein-dUTP). Such a method, termed TUNEL, appears one of the most sensitive techniques to detect apoptosis, even in early stages of the process (53); thus, we performed a time-course TUNEL analysis to determine the extent and temporal kinetics of DNA strand breaks triggered by oncM in primary sensory neurons. The appearance of high m.w. DNA strand breaks was determined at serial time points (24, 48, and 72 h) following incubation of the cells with a single concentration (10 ng/ml) of oncM, and the relationship to alterations of cellular integrity (exclusion of cationic-charged fluorochromes) and the kinetics of cell proliferation were examined (Table III). The results of these experiments strongly suggest the involvement of apoptosis or apoptosis-related mechanisms in the oncM-mediated demise of primary neurons. Specifically, the first evidence of extensive DNA strand breaks occurred within the first 24–48 h of incubation of the cells with oncM (Table III). At these times the cells maintained 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 loosing their membrane integrity, as indicated by the increased permeability to PI. Peak levels of cell growth inhibition were maintained through later time points (6 days) and were accompanied by extensive DNA cleavage, as low m.w. DNA fragments (mono- and oligonucleosomes) were released from the nuclei and were found in the cytoplasmic cell fractions (Table II). These results indicated that oncM may 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.

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

To verify whether inflammatory cytokines, which may be chronically induced by HIV-1 infection or stimulated by functional activation, may play a part in these events, experiments were performed 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, incubation with oncM caused a pronounced inhibition of cell proliferation as assessed by both [3H]thymidine uptake and cell number (Fig. 2, A–C). Culture of both primary sensory neurons and sympathetic neuronal cells performed in the presence of increasing concentrations of oncM indicated that the inhibitory effects of the cytokine are dose dependent and evident at concentrations (picograms per milliliter) that may be physiologically relevant (Fig. 2, B–D). OncM exerted inhibitory effects on both mitotically active and postmitotic neurons (Table II). Morphological changes induced by oncM in exponentially growing cells became evident after 48–72 h of incubation, while alterations in growth-arrested neurons were evident 4–6 days after incubation with the cytokine (data not shown). At these times the cultures were characterized by a progressive loss of cells associated with extensive DNA cleavage with the accumulation of histone-associated DNA fragments (mono- and oligonucleosomes) in the cytoplasmic fraction and altered permeability of the cell membrane to cation-charged dyes such as PI (Table II). These data indicate a reduced cell survival and suggest that the induction of apoptosis or apoptosis-related mechanisms may be responsible of these events (53). To confirm this hypothesis we investigated the temporal kinetic of chromatin alterations induced by oncM in primary sensory neurons.

**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 neurotoxic effects induced by HIV-1-infected or lectin-activated PBMC

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**Table I. Effects of soluble mediators produced by functionally activated lymphomonocytes on the viability of mitotically active and quiescent primary neurons**

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*a The inhibitory effects exerted by HIV-1 infected or functionally activated lymphomonocytes on primary neurons cell growth are accompanied by increased release of histone-associated DNA fragments into the cyttoplasm fraction and by altered membrane permeability to PI staining. FNC-B4 cells were cultured in the presence 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.

**Cell growth inhibition/cell loss**

- +: 10–29%; +++, 30–49%; +++++, ≥50%.

**Membrane permeability**

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

**DNA fragmentation**

- +: 1.5–1.9 enrichment factor;
- +++, 2–4 enrichment factor.

**Cell growth inhibition/cell loss**

- +: 10–29%; +++, 30–49%; +++++, ≥50%.
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 oncM 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 (Fig. 3B) or HIV+–CM (Fig. 3C) on the growth and survival of primary neurons. In addition, TNF-α blocking experiments performed with A-CM to verify the potential for a direct contribution to the neurotoxicity of the native protein compared with the effects of the recombinant protein indicated that neither blocking nor depletion of native TNF-α influences the inhibitory effects exerted by CM on our primary neuronal cells (data not shown). These results demonstrate that native oncM is biologically active and that it is specifically responsible for the biochemical alterations of primary neurons that can be prevented by anti-oncM Abs.

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

FIGURE 2. Analysis of neuronal cell growth and survival in the presence of recombinant inflammatory cytokines: effects of oncM. A, FNC-B4 cells were cultured in the presence of a panel of recombinant inflammatory cytokines, which are chronically induced by HIV-1 infection or stimulated by lectin activation. Dose-response analysis was performed on primary sensory neuronal cells with all the different factors tested to generate concentration-effect profiles, and the concentrations indicated in the figure were consistent with those detected in the CM (50, 51) (data not shown). The results indicate that inflammatory cytokines such as TNF-α (10 ng/ml), TNF-β (10 ng/ml), IL-6 (10 U/ml), IL-1α (10 U/ml), IL-1β (10 U/ml), IFN-γ (10 U/ml), IL-2 (20 U/ml), and TGF-β1 (0.05 ng/ml), do not alter neuroblast viability and morphology, although they can variably exert a positive regulation of neuroblast proliferation. On the other hand, in the presence of oncM (10 ng/ml), neuroblasts undergo a pronounced inhibition of cell proliferation as assessed by [³H]thymidine uptake. B–D. Increasing oncM concentrations induce a dose-dependent inhibition of [³H]thymidine uptake accompanied by a corresponding reduction of the cell number with both FNC-B4 (B and C, respectively) and SH-SY5Y cells (D).
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 subjects, 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 HTLVIIIIB 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
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 embryonic precursors as well as of tumor-derived 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,
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 of 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/permisive 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 such as TNF-α, TGF-β, OncM, and IL-6, which have partially overlapping effects and the potential to exert both direct and indirect CNS injuries, can play a major role in the pathogenesis of neurologic AIDS-associated disorders by acting through different mechanisms on different target cells (56, 67, 71). In addition, they can potentially cooperate by either additive or synergistic modality, thus amplifying their effects on responsive cell types.

Our findings contribute to identifying novel pathways of immunologically mediated neuronal injury and indicate that different 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.

Acknowledgments

We thank B. Ensoli (Laboratory of Virology, Istituto Superiore di Sanita) for the helpful comments and critical review of the manuscript.

References


