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# Secretion of Neurotoxins by Mononuclear Phagocytes Infected with HIV-1

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**Mononuclear phagocytes (microglia, macrophages, and macrophage-like giant cells) are the principal cellular targets for human immunodeficiency virus-1 (HIV-1) in the central nervous system (CNS). Since HIV-1 does not directly infect neurons, the causes for CNS dysfunction in acquired immunodeficiency syndrome (AIDS) remain uncertain. HIV-1–infected human monocytoid cells, but not infected human lymphoid cells, released toxic agents that destroy chick and rat neurons in culture. These neurotoxins were small, heat-stable, protease-resistant molecules that act by way of *N*-methyl-D-aspartate receptors. Macrophages and microglia infected with HIV-1 may produce neurologic disease through chronic secretion of neurotoxic factors.**

THE ACQUIRED IMMUNODEFICIENCY syndrome (AIDS) has a devastating effect on the central nervous system (CNS). Most clinical studies indicate that more than 60% of all patients with AIDS eventually develop cognitive dysfunction (*1*), and at least 25% show motor impairment (*2*). These disabilities range in severity from mental slowing and mild weakness to global dementia and paralysis. At autopsy, the CNSs of patients with AIDS show atrophy, white matter pallor, and modest loss of neurons (*1, 2*). Cellular changes within the brain include an invasion of macrophages, clusters of microglial cells (microglial nodules), and giant cell formation. The autopsy findings can, however, be rather sparse despite the fact that patients have had profound cognitive impairment (*3*).

The first isolations of human immunodeficiency virus-1 (HIV-1) from the brains of AIDS patients were reported by Shaw *et al.* (*4*) and Ho *et al.* (*5*). A number of other groups (*6*) have now identified mononuclear phagocytes (microglia, macrophages, and multinucleated macrophage-like cells) as the principal cellular targets for HIV-1 in the CNS. Since HIV-1 infection of neurons occurs very rarely, if at all (*1, 7*), the mechanisms impairing neuron function remain uncertain. Proposed etiologies for AIDS dementia have included the coexistence of opportunistic fungal or cytomegaloviral infections, white matter destruction by cytokines, growth factor blockade, and direct neuron killing by gp120, the HIV-1 envelope glycoprotein (*3, 8, 9*). We considered the possibility that HIV-1–infected mononuclear cells released substances that would disrupt the function of neurons (*3*). We tested for the presence of neurotoxins by means of two different culture systems. In the first, dissociated neurons from ciliary ganglia of embryonic chicks were seeded at low density on glass coverslips coated with poly-L-lysine. Under such conditions, neurons (about 50% of the total cell population) were readily identified by phase microscopy or histochemical markers (*10*). We also examined toxic effects on neurofilament (NF)–containing neurons from embryonic rat spinal cord (*11*) growing atop dense feeder layers of glia. [In these cultures <5% of the total population are $NF^+$ cells.] Several different types of $CD4^+$ human cell lines (the lymphoid cell H9, the promonocytic cell U937, and the monocytic cell THP-1) constitutively infected with $HTLV_{IIIB}$ prototypic strain of HIV-1 were used as models for HIV-1–infected human mononuclear cells (*12*).

Infected cells were seeded into chambers with filtered bottoms (0.4-μm pores) and then placed into culture wells containing neurons grown on cover slips (*13*). Using this coculture system to prevent direct contact between $CD4^+$ cells and neurons, we found that the HIV-1–infected monocytoid cells, U937 and THP-1, but not infected H9 lymphoid cells, secreted substances that reduced neuron growth and survival (Fig. 1). Dead or dying neurons could be detected within 10 hours of coculture, often showing

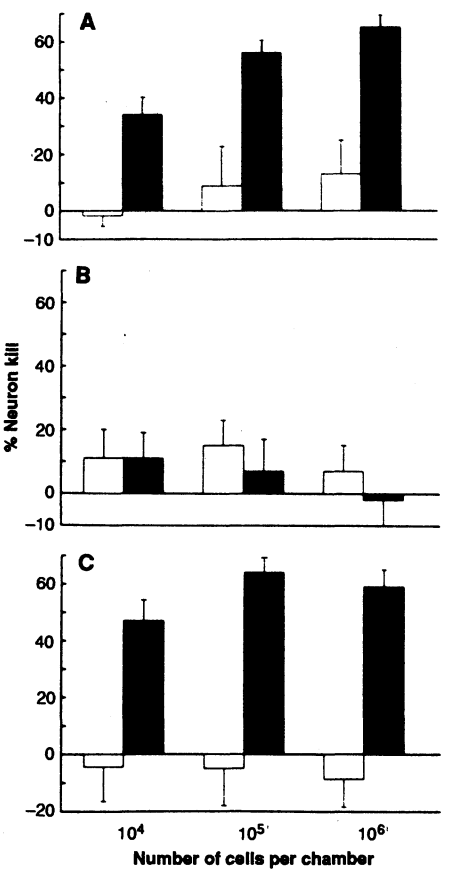


**Fig. 1.** HIV-1–infected monocytoid cells but not lymphoid cells release neuron-killing factors. The uninfected (white bars) or HIV-1–infected (black bars) cell lines were seeded at the indicated densities into Millicell-CM chambers which had been placed into wells (12-well plates; 3.8 $cm^2$ per well) with each well holding approximately 8000 chick ciliary neurons growing on a poly-L-lysine–coated glass cover slip in 1.0 ml of culture medium. The cocultures were incubated for 48 hours before fixation. (**A**) THP-1 monocytoid cells, (**B**) H9 lymphoid cells, (**C**) U937 promonocytoid cells. The percent neuron survival for each experiment was expressed as the ratio of the mean number of neurons per field for a treated culture to the mean number of neurons per field for untreated control cultures. Every value, expressed as mean percent neuron kill ± standard error (100% − % neuron survival), was obtained from at least three cultures. Negative percent kill indicates an increase in survival when compared to untreated control cultures. All comparisons between infected and uninfected THP-1 or U937 cells at each of the three cell concentrations showed $P < 0.05$ when analyzed by Student's *t* test.

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such early degenerative changes as cytoplasmic vacuoles and loss of nuclei as seen by phase microscopy. Within 48 hours of coculture, significant destruction of neurons had occurred in the presence of both the HIV-1–infected THP-1 and U937 cells (Fig. 1). The maximum level of killing for either cell type was about 60% when $10^5$ infected cells were seeded into the chambers. No significant destruction of neurons was noted when the uninfected monocytoid cells or the HIV-1–infected lymphoid cells were tested at various densities up to $10^6$ cells per chamber. Determination of p24 antigen levels (*14*) in culture media showed that the viral core protein diffused across the chamber filter in amounts ranging from 585 pg/ml (THP-1 infected with HIV-1) to 4500 pg/ml (H9 infected with HIV-1). The virus or viral gene products had no apparent direct effect on chick neurons, since normal survival was observed with neurons cultured in the presence of the HIV-1–infected H9 cell line, which secreted the highest titers of virus. The killing activity was not likely to be a result of excessive cell metabolism or depletion of essential nutrients since neuron loss was observed within 10 hours in the presence of relatively few monocytoid cells ($10^4$). Only conditioned medium from the HIV-1–infected THP-1 or U937 cells killed ciliary neurons, and killing was dose-dependent (Fig. 2). Similarly, secretion products from infected monocytoid cells, but not lymphoid cells, destroyed nearly 75% of $NF^+$ neurons found in cultures of rat spinal cord (Fig. 3).

It is well recognized that activated mononuclear phagocytes, including microglia, release cytotoxic substances (*15, 16*). However, our initial characterization of the neurotoxic activity secreted by HIV-1–infected monocytic lines showed properties distinct from those molecules previously identified as macrophage-secreted cell poisons (*16*). For example, the neuron-killing activity was stable when cells were stored at −80°C for at least 1 month or boiled for 10 min at pH 7.2, suggesting that short-lived substances such as free radicals (*16*) were not responsible for neuron destruction. Moreover, the neurotoxic activity was not inactivated with proteinase K (100 μg/ml for 60 min at 37°C) or with other proteases (trypsin and papain), ruling out protein cytokines and large peptides as the toxic agents. Finally, the neurotoxic activity had a molecular mass of <2 kD as suggested by ultrafiltration with Centriprep 10 (approximate cutoff 10 kD) and an Amicon YM-2 filter (approximate cutoff 2 kD). From these experiments, we conclude that the HIV-1–associated neuron-killing activity consists of heat-stable, protease-resistant molecules of <2 kD, which are distinct from previously identified cytotoxins secreted by mononuclear phagocytes as well as from gp120, the envelope glycoprotein of HIV-1 recently reported to be a neurotoxin (*9*).

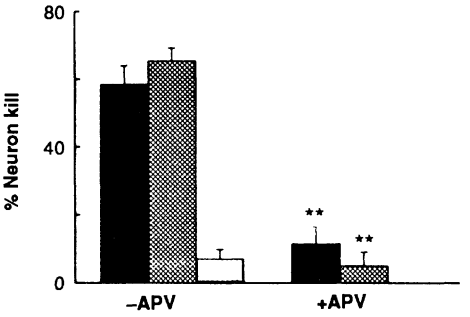


**Fig. 3.** Destruction of rat spinal cord neurons by toxins released from HIV-1–infected human monocytoid cell lines. Conditioned medium (20%) from HIV-1–infected THP-1 monocytoid cells (black bars), U937 promonocytoid cells (shaded bars), or H9 lymphoid cells (white bar) were incubated for 72 hours with spinal cord cells from embryonic rats. Only media from HIV-1–infected monocytoid cell lines exhibited significant neurotoxic activity. The use of 2-amino-5-phosphonovalerate (APV), an NMDA receptor antagonist, at a concentration of 100 μM protected against this cytotoxic action. Counts of $NF^+$ neurons were obtained for each group from at least 20 randomly selected fields (at ×200 magnification) from each of three cultures from five experiments scored by two observers. Data are expressed as mean percent neuron kill ± standard error. Kill effects associated with HIV-1–infected THP-1 and U937 cells were significantly reduced with APV when analyzed by Student's *t* test (**$P < 0.01$).

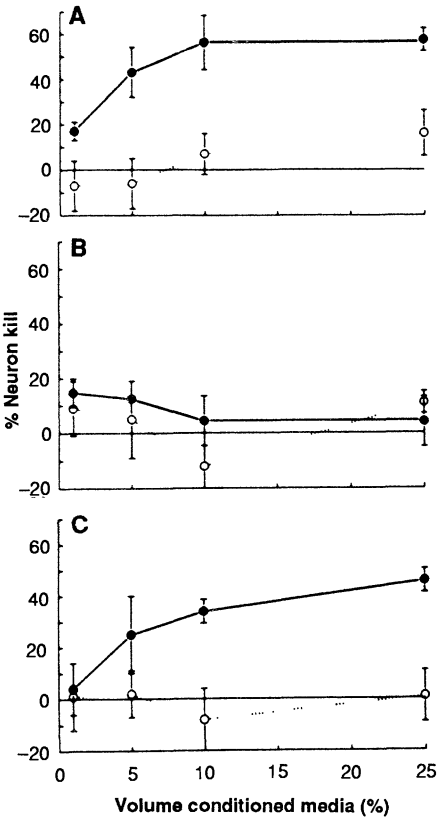


**Fig. 2.** Dose-response curves showing neuron-killing activity released from uninfected (○) and HIV-1–infected (●) human cell lines. Conditioned media produced by $5 \times 10^5$ cells/ml were recovered after 48 hours (*14*). Chick ciliary neuron counts for each group were obtained from at least 20 randomly selected fields (at ×200 magnification) from each of three cultures from five different experiments with three different preparations of conditioned media. Data are expressed as mean percent neuron kill ± standard error. (**A**) THP-1 monocytoid cells. (**B**) H9 lymphoid cells. (**C**) U937 promonocytoid cells. Despite a range of media concentrations, HIV-1–infected monocytoid cell lines release significant toxic activity in contrast to the infected lymphoid cells and all uninfected cells. Negative percent kill indicates an increase in survival when compared to untreated control cultures. All values for HIV-1–infected cell lines U937 and THP-1 at >5% conditioned media show significant killing ($P < 0.01$) when analyzed by Student's *t* test.

Some amino acids and their metabolites, including glutamate, cysteine, cysteic acid, and quinolinic acid, are heat-stable, protease-resistant molecules of low molecular mass that act as neurotoxins by specifically activating the *N*-methyl-D-aspartate (NMDA)–sensitive receptor, a subclass of glutamate-binding membrane proteins (*17*). A growing body of evidence has implicated NMDA receptor agonists in a variety of neuropathic conditions (*18*), including stroke, brain trauma, degenerative neurological diseases, and AIDS dementia (*19*). We observed that some NMDA agonists had potent neurotoxic effects in our cultures of chick ciliary ganglia or rat spinal cord. For these reasons, we explored the possibility that the neuron-killing factors secreted by HIV-1–infected cells also acted on the NMDA receptor. We found that kynurenic acid, an antagonist for glutamate receptors (*17*), blocked >90% of the ciliary ganglion cell killing by either HIV-1–infected THP-1 or U937 cells (Fig. 4). Furthermore, the selective NMDA antagonist 2-amino-5-phosphonovaleric acid (APV) (*17*) protected against the HIV-1–induced neurotoxicity, whereas the antagonists to non-NMDA–type excitatory amino acid receptors [6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (*17*) and γ-D-glutamylaminomethylsulfonic acid (GAMS) (*17*)] had no protective effect (Fig. 4). Similar results were obtained with rat spinal cord neurons in which APV again prevented neuron killing (Fig. 3). Ion channel blockers were used to confirm the involvement of NMDA receptor–ionophore complex (which increases permeability to $Na^+$, $K^+$, and $Ca^{2+}$) (*17*). Whereas MK-801, which blocks the NMDA-mediated

channels, gave good protection against neuron killing (Fig. 4), the calcium L-channel blockers nifedipine and verapamil did not prevent cell death (*17, 18*). It should be noted that the neurotoxic activity ascribed to gp120 could be blocked by calcium L-channel antagonists (*9*).

Since the neuron-killing factor or factors released by HIV-1–infected monocytoid cells were not common inflammatory cell cytotoxins (free radicals, proteases, or cytokines), we next examined neurotoxic amino acids. Our standard culture medium contained 50 μM each of glutamate and aspartate. When added at concentrations up to 1 mM, these amino acids did not significantly affect ciliary neuron survival. Moreover, the concentrations of glutamate, aspartate, or cysteine did not increase significantly in medium conditioned by HIV-1–infected cells (*20*). We did find that the NMDA agonist quinolinic acid was a potent neurotoxin for chick ciliary neurons and rat spinal cord neurons with 40 to 60% killing at 100 nM; this effect was blocked by such NMDA antagonists as APV, MK-801, and kynurenic acid but not by such non–NMDA receptor antagonists as CNQX and GAMS. However, the amount of quinolinic acid as measured by gas chromatography–mass spectrometry (GC-MS) in media conditioned by HIV-infected THP-1, U937, and H9 cells was found to be too low (≤0.2 ng/ml) to account for the neurotoxicity (*21*). To date, we have not determined the composition of the HIV-1–elicited neurotoxic activity. It may be that an unusual cytotoxin or a collection of cytotoxins secreted by HIV-1–infected cells are responsible for destruction of neurons.

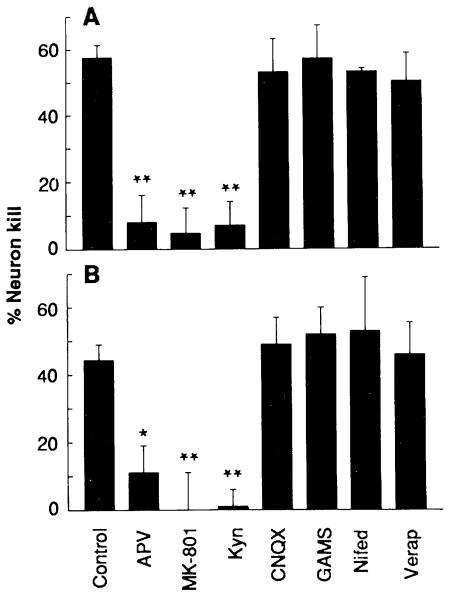


**Fig. 4.** Effects of glutamate receptor antagonists and calcium channel blockers on neurotoxic activity secreted by HIV-1–infected cells. Conditioned medium prepared from HIV-1–infected THP-1 (**A**) or U937 (**B**) cells at $5 \times 10^5$ cells/ml was added to chick ciliary neuron cultures at a concentration of 25% (v/v) in the presence of the indicated drugs and incubated for 48 hours. The NMDA receptor antagonists APV (10 μM) and MK-801 (10 μM) blocked the killing activity, as did a mixed-type glutamate antagonist, kynurenic acid (Kyn; 10 μM). Antagonists of kainate- and quisqualate-type glutamate receptors, CNQX (100 μM) and GAMS (100 μM), were ineffective. Similarly, the calcium channel blockers nifedipine (Nifed; 10 μM) and verapamil (Verap; 10 μM) were without protective effect. Comparisons with control groups were analyzed by the Student's *t* test with a confidence level for seven comparisons estimated at 0.0071 by the Bonferroni method (*$P < 0.01$; **$P < 0.001$).

Our observations suggest a series of cellular events that may account for neurologic complications associated with AIDS. Perhaps $CD4^+$-reactive microglia and invading macrophages infected with HIV-1 persist within the CNS as active secretory cells releasing toxic substances that are capable of disrupting nervous system function. A few infected cells could release toxins for prolonged periods, thereby affecting neurons not in direct contact with macrophages or microglia (*3*). Active viral replication or high extracellular levels of viral proteins may not be necessary to perpetuate nervous system disease. Since the neurotoxic activity involves the NMDA receptor, which is common to vertebrate neural systems, we believe animal models and cell culture systems will prove useful in the study of AIDS encephalopathy. However, further investigation in vivo (perhaps with subhuman primates) will be needed to confirm our observations from cultures of rat and chick neurons. Once the neurotoxic molecules secreted by the HIV-1–infected U937 and THP-1 cell lines have been identified, more direct assays can be performed to uncover the basic mechanisms of neurologic disease and to determine the toxin-producing capacities of $CD4^+$ cells found in the CNSs of patients with AIDS.

The clinical data on AIDS encephalopathy clearly indicate that marked dysfunction of the nervous system occurs despite subtle or minimal neuropathic changes (*2*). We believe macrophages and microglia infected with HIV-1 release poisons that disrupt and, if in sufficient amounts, destroy neurons. The degree of neuronal loss may be determined in part by the chronicity of toxin secretion and by the proximity of HIV-1–infected cells to neurons. We do not suggest that this is the only mechanism by which HIV-1 causes diffuse CNS injury, nor do we suggest that HIV-1 is the only signal to elicit neurotoxin secretion from mononuclear phagocytes. On the contrary, it is quite likely that activated mononuclear phagocytes release neuron-killing factors in a variety of neuropathic states and that this activation results from a variety of signals (*15*). We anticipate that the underlying mechanisms of AIDS encephalopathy are similar to events responsible for delayed loss of neurons seen after stroke and trauma (*15*). What may distinguish AIDS from other neurologic disorders is the presence of chronically infected (and therefore activated) inflammatory cells that continue to disrupt neurologic function until the death of the patient. NMDA agonists disturb the nervous system in many ways, as indicated by the appearance of seizures and degeneration of neurons (*18, 22*). The clinical presentation of AIDS encephalopathy is consistent with a diffuse and persistent presence of neurotoxic agents. Based on the data reported here, strategies to reduce the neurologic complications of AIDS would include suppression of mononuclear phagocyte activation, blockade of neurotoxin biosynthesis, or protection of neurons by competition for NMDA receptors.

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10. Ciliary ganglia from day 9 chick embryos were dissociated by trituration after a 9-min incubation at 37°C with 0.08% trypsin (ICN) in $Ca^{2+}$- and $Mg^{2+}$-free medium [K. Vaca, S. Stewart, S. Appel, *J. Neurosci. Res.* **23**, 55 (1989)]. The cells were collected by centrifugation at 600*g*, resuspended in a chemically defined neuron culture medium [J. E. Bottenstein and G. H. Sato, *Proc. Natl. Acad. Sci. U.S.A.* 76, 514 (1979)] supplemented with 10% fetal bovine serum (FBS; Gibco), and plated on poly-L-lysine–coated glass cover slips at a density of about 2000 cells/$cm^2$. After 2 days in culture, neurons were fixed for 2 hours with 3% formaldehyde in phosphate-buffered saline (PBS; pH 7.2) and viewed at ×200 by phase-contrast microscopy. For each cell count determination, we defined a healthy,

nuclear membrane with characteristic nucleoli and a cytoplasm free of large vacuoles. To assess neuron survival in cultures exposed to putative neurotoxic factors for 48 hours, we determined the mean number of healthy cells by scoring the number of neurons in at least 20 randomly selected fields for each of three coverslips. Ciliary ganglion neurons also were identified by immunohistochemical staining for choline acetyltransferase [D. Giulian, K. Vaca, B. Johnson, *J. Neurosci. Res.* **21**, 487 (1988)] with a 1:500 dilution of mouse 1E6 monoclonal antibody from ascites fluid.

11. Spinal cords were removed from day 15 albino rat embryos (Holtzman, Madison, WI) and dissected free of attached meninges and ganglia. After incubation with 0.08% trypsin in $Ca^{+2}$- and $Mg^{+2}$-free PBS for 10 min at 37°C, the cords were transferred to a chemically defined neuron culture medium (*10*) supplemented with 10% heat-inactivated horse serum (Gibco), and dissociated by trituration with fire-polished Pasteur pipettes. Cells were plated at a density of 500,000 cells per well of a 24-well tissue culture plate (Falcon) with each well containing a poly-L-lysine–coated 12-mm diameter glass coverslip. Twenty-four hours after plating, control or conditioned medium was added and the cultures were incubated another 3 days before fixation for 45 min with 3% formaldehyde in PBS. Neuron identification in the mixed-cell population of rat spinal cord cells was performed by indirect immunofluorescence staining with a 1:50 dilution of mouse monoclonal antibody MO3B to the 160-kD neurofilament protein followed by a 1:100 dilution of rhodamine-conjugated rabbit antibody to mouse immunoglobulin G (Dakco). Under these conditions, control cultures contained <5% $NF^+$ neurons and >90% glia.
12. All of the cell lines were grown as suspension cultures in RPMI 1640 medium with L-glutamine supplemented with 10% FBS at 37°C in a humidified, 95% air and 5% $CO_2$ atmosphere. The cell lines were free of detectable mycoplasma contamination. Continuous human T cell lines, uninfected [H9, M. Popovic, M. C. Sarngadharan, E. Read, R. C. Gallo, *Science* **224**, 497 (1984); M. Popovic, E. Read-Cannole, R. C. Gallo, *Lancet* **ii**, 1472 (1984)] or persistently infected with the $HTLV_{IIIB}$ prototype strain of HIV-1 (H9/$HTLV_{IIIB}$ NIH 1983), were obtained through the AIDS Research and Reference Reagent Program, National Institute of Allergy and Infectious Diseases, NIH, from R. Gallo. Two human monocyte cell lines were obtained from the American Type Culture Collection: the promonocytic cell line U937 [ATCC CRL 1593, P. Ralph, A. S. Moore, K. Nilsson, *J. Exp. Med.* **143**, 1528 (1976)] and the monocytic leukemia line THP-1 [ATCC TIB 202, S. Tsuchiya *et al.*, *Int. J. Cancer* **26**, 171 (1980)]. Both the U937 and THP-1 cell lines were infected with the $HTLV_{IIIB}$ strain of HIV-1 at a multiplicity of infection of $5 \times 10^5$ tissue culture infectivity dose of 50% ($TCID_{50}$). The cells were passaged and monitored periodically for HIV antigens by indirect immunofluorescence with pooled polyclonal antiserum from HIV-1–infected patients. At 15 to 20 days postinfection, the cells were >80% HIV-antigen positive. These cell lines, which have been in culture for more than 1 year, grow well under standard culture conditions (without the addition of cytokines), produce virus that is cytopathic in the MT-4 cell line (approximately $10^4$ $TCID_{50}$ per milliliter of culture fluid), and show high titers of p24 antigen ($>1 \times 10^5$ pg/ml of culture fluid) as determined by EIA (Du Pont Biotechnology Systems). Electron microscopy of the infected THP-1 and H9 cells showed HIV-1–like retroviral particles.
13. To test the monocytoid or lymphoid cells for the production of neurotoxic factors, we plated human cells at concentrations of $10^4$, $10^5$, or $10^6$ in 1 ml of medium (RPMI 1640 with L-glutamine and 10% FBS) in Millicell-CM chambers (0.4-μm filter pore size; Millipore), which had been placed in 12-well plates (Falcon). Each well contained about 8000 ciliary ganglia neurons growing on an 18-mm round glass coverslip coated with poly-L-lysine in 1 ml of chemically defined neuron culture medium supplemented with 10% FBS. After 48 hours of coculture, neurons were fixed and scored as described in (*10*). Culture media were collected to measure p24 antigen concentrations.
14. To prepare conditioned media, test cells were seeded at a density of $5 \times 10^5$ cells/ml and cultured for 48 hours under standard conditions (*12*). To monitor HIV-1 production, we evaluated 200-μl samples of culture media by an EIA specific for the p24 antigen of HIV. Media were centrifuged for 10 min at 300*g* and then subjected to ultracentrifugation for 60 min at 200,000*g* to remove viral particles. The media were then heat treated at 60°C for 60 min, filter-sterilized (0.2 μm; Acrodisc; Millipore), and stored at −80°C.
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21. Quantitative measurements of quinolinic acid levels in culture media were carried out by S.-N. Lin, Analytical Chemistry Center, University of Texas Health Science Center at Houston, with a Finnigan MAT Incos 50 for GC-MS. As described by Heyes *et al.* (*19*), samples containing quinolinic acid were washed with an equal volume of chloroform, and the aqueous phase was passed over a small ion-exchange column (0.2 cm by 1.0 cm) containing Dowex 1-X2 (formic form), washed with 10 volumes of distilled $H_2O$ and 10 volumes of 0.1 N formic acid, eluted with 5.0 ml of 6 N formic acid, and lyophilized with a Speed-Vac (Savant). Under these conditions, we found an ~15% loss of [$^3$H]quinolinic acid during the chloroform extraction and another 10 to 15% loss on the ion-exchange column. Lyophilized samples eluted from Dowex ion-exchange columns were derivatized with bis-trimethylsilyl trifluoroacetic acid and passed through a 60-m DB-1 fused-silica capillary column (GC oven temperature programmed from 190°C to 210°C at the rate of 1°C/min). Under these conditions, we showed good separation of quinolinic acid (2,3-pyridinedicarboxylic acid) from dipicolinic acid (2,6-pyridine-dicarboxylic acid). Selective ion monitoring by MS allowed detection of an internal standard 4-chlorobenzoic acid (mass-to-charge ratio *m/z* 213) and quinolinic acid (*m/z* 296) with detection sensitivity (after column chromatography) of <1 ng. Standards of quinolinic acid measured by GC-MS showed a linear range from 2 to 50 ng, thus providing sufficient sensitivity to detect toxic levels of quinolinic acid in conditioned media. Using large batches of culture media (10 ml), we found that the amounts of quinolinic acid secreted by various cell lines with or without HIV-1 infection did not significantly differ and were far below the toxic range for the cultured ciliary neurons.
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# Poliovirus Mutants Resistant to Neutralization with Soluble Cell Receptors

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**Poliovirus mutants resistant to neutralization with soluble cellular receptor were isolated. Replication of soluble receptor-resistant (*srr*) mutants was blocked by a monoclonal antibody directed against the HeLa cell receptor for poliovirus, indicating that the mutants use this receptor to enter cells. The *srr* mutants showed reduced binding to HeLa cells and cell membranes. However, the reduced binding phenotype did not have a major impact on viral replication, as judged by plaque size and one-step growth curves. These results suggest that the use of soluble receptors as antiviral agents could lead to the selection of neutralization-resistant mutants that are able to bind cell surface receptors, replicate, and cause disease.**

SOLUBLE CELL RECEPTORS HAVE BEEN shown to block infection by HIV-1 (*1–5*), Epstein-Barr virus (*6*), and rhinovirus (*7*). We have shown that infection of cells with poliovirus, a small, nonenveloped animal virus, is blocked by cytoplasmic extracts of insect cells expressing cell receptors for poliovirus (*8*). As a result of these studies, it has been proposed that soluble cell receptors might be effective antiviral therapeutics. It has been suggested that viral mutants resistant to the antiviral effects of

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