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Immediate and Neurotoxic Effects of HIV Protein gp120 Act through CXCR4 Receptor

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Primary rat hippocampal neurones show pronounced elevations of intracellular calcium within minutes of exposure to the HIV coat protein gp120. Culture of hippocampal neurones with gp120 causes significant neurotoxicity. We find that the peptide VSLSYRCPCRFF, a competitive inhibitor of the CXCR4 chemokine receptor, markedly inhibits toxicity and eliminates the acute calcium elevation. CXCR4 receptors are thought to signal to the Gi/Go family of trimeric GTP binding proteins. Pretreatment of hippocampal neurones with pertussis toxin to inactivate Gi/Go proteins markedly reduced gp120 neurotoxicity. These results indicate that both short and long term effects of gp120 are the result of activation of the CXCR4 receptor.

Key Words: **rat; AIDS; HIV-associated dementia; HAD; CXCR4; chemokine; hippocampal culture; cortical culture.**

Human immunodeficiency virus type 1-associated dementia (HAD) is a devastating consequence of infection with HIV-1 characterized by behavioral, motor, and memory disturbances (1, 2). Although glial cells are infected by the virus, neurons are not, yet considerable neuronal death occurs (3). One major cause is the HIV coat protein gp120, which at picomolar concentrations causes neuronal death in isolated cell culture and *in situ* (4, 5).

Initially, attention focused on a proposed effect of gp120 on the NMDA subtype of ionotropic glutamate receptors. Early work by Lipton's group and others showed that antagonists of NMDA receptors rescued neurons from gp120 neurotoxicity, and that agents that rescued neurons from other excitotoxic insults, such as intracellular calcium buffer or low extracellular calcium, also protected against gp120 (4, 6). More recently, gp120 has been shown to bind directly to the glycine site of the NMDA receptor to increase its open probability (7–9). Consistent with a direct, glutamate-like action of gp120, early work reported that gp120 caused large calcium elevations in cultured neurons and that these calcium elevations could be blocked by glutamate antagonists (4, 6). Two lines of research have changed this simple picture. First, a number of studies have shown that chemokine receptors, initially thought to be restricted to leukocytes, are expressed by both glia and neurons (10, 11). The chemokine receptors CCR5 and CXCR4 have long been recognized as ligands for gp120 that, together with CD4, allow binding of HIV to lymphocytes and subsequent internalization. Neurons do not express CD4 and are therefore not infected, but this work raised the possibility that gp120 exerted its effects not by an action on the NMDA receptor but through an action on chemokine receptors (12). Secondly, the idea that gp120 (and whole virus) could directly damage neurons was called into question by a number of influential reviews by Lipton that drew attention to microglia, the resident macrophages of the brain, and to experiments indicating that they released neurotoxic agents upon stimulation (13–15).

HIV can infect HeLa cells that have been transfected so as to express both the CD4 and CXCR4 receptor. In these cells SDF-1α, the natural agonist at CXCR4, caused a rapid transient rise of intracellular calcium concentration. VSLSYRCPCRFF, a peptide derived from SDF-1α, protected HeLa cells against infection and blocked the calcium response to SDF-1α without triggering a calcium response itself (16). In order to test the hypothesis that gp120 binding to CXCR4 underlies both the rapid calcium response of neurones to gp120 and the subsequent slow neurotoxicity toxicity we tested the ability of VSLSYRCPCRFF to block these effects.

MATERIALS AND METHODS

Peptides and recombinant proteins. HIV-1 envelope glycoprotein from the SF2 strain was obtained from MRC (UK) AIDS Research and Reagent programme. Peptide VSLSYRCPCRFF was purchased from Alta Bioscience, Birmingham, U.K.

Cell culture. Growth medium comprised Neurobasal medium containing 292 mg/ml L-glutamine supplemented with 2% B27 (GIBCO). 5% horse serum was included in the growth medium for

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some cultures. One day old rat pups were killed by cervical dislocation and decapitation in accordance with the Scientific Procedures Act 1986. Cerebral cortices were dissected out into growth medium, triturated through a flame narrowed Pasteur pipette and plated onto autoclaved 25 × 40 mm cover glasses (thickness #1) that had previously been coated with poly-D-lysine (0.1 mg/ml) and washed with sterile water (density 2.6×10^5 cells per cover glass). The neurons were grown in a humidified atmosphere of 5% CO_2 and 95% air at 37°C and fed every 4–5 days with growth medium. Where horse serum was present, 10 μM (micromolar) cytosine arabinoside was added to the cultures within 48 h of plating to reduce glial growth and removed with the next medium change. Cells employed for these experiments were grown in culture for a minimum of 18 days. Hippocampal cultures were similar except that dissected hippocampi were first incubated in HEPES buffered solution (HBS: 20 mM HEPES; 120 mM NaCl; 1.8 mM $CaCl_2$; 1 mM $MgCl_2$; 5.5 mM KCl; 2.5 mM glucose; pH 7.2) containing 0.1% trypsin for half an hour at 37°C then centrifuged at 1000 RPM for 3 min. The pellet was washed once in HBS, twice in growth medium then dissociated mechanically by trituration through a 5 ml serological pipette before trituration with a flame-narrowed pasteur pipette and plating as for cortical cultures. All numerical data result from at least three independent platings.

Toxicity assays. After 18 days in culture, the medium bathing cortical or hippocampal cultures was changed to one containing 250 pM gp120. Control cultures had the medium changed at the same time. For rescue experiments the medium bathing cortical or hippocampal cultures was changed to 2 ml of medium containing either 50 μM CXCR4 blocking peptide VSLSYRCPCRFF or 500 ng/ml pertussis toxin (IAP component, Sigma); two hours later 1 ml of the same medium but supplemented with 750 pM gp120 was added. Cultures were then incubated for a further 48 h, then cover slips were rinsed with HBS and incubated at room temperature with 10 μg/ml Hoechst 33342 and 60 μM propidium iodide in HBS for 30 minutes. Using this method the nuclei of dead cells with compromised cell membranes fluoresce bright red, while apoptotic cells with condensed nuclei show intense Hoechst fluorescence. By this criterion, all neuronal death in this study was necrotic. Live cells were visualized by their relatively dim Hoechst fluorescence. On each cover slip an average of 250 cells were counted to give a value of % death; values for individual cover slips were then averaged.

Calcium measurement. Cells were loaded with the calcium indicator Fura-2 by incubating for 30–40 min at room temperature in 5 μM of the AM ester (Molecular Probes) in HBS supplemented with 63 mg/litre Pluronic F-127 (Molecular Probes). Coverslips were rinsed three times in fresh HBS then mounted in a rapid perfusion bath (RC26G, Warner, CT) and placed on the stage of a Zeiss IM inverted microscope fitted with an illumination system (Sutter, CA) and intensified CCD camera (Extended Isis, Photonic Science, UK). 350 nm/380 nm fluorescence image pairs were acquired at a repetition rate of 4 per minute under control of Kinetic Imaging software (Liverpool, UK). Fluorescence values were corrected for background light, estimated from an area of the field without cells, then converted to calcium values by applying $Ca^{++} = Kd.S.(R - R_{min})/(R_{max} - R)$ where $R = I_{350}/I_{380}$, R_{min}, R_{max} and S were measured from *in vitro* solutions as 0.352, 12.412 and 8.54 respectively, and the Kd was taken to be 236 nM (17). Neurons were identified by their phase-bright cell bodies and bright Fura-2 fluorescence. Cultures were frequently challenged with NMDA, all cells previously identified as neurons generated large calcium responses to this agonist.

RESULTS

We observed considerable variability across dissections in the ability of 250 pM gp120 to induce a calcium response. Results were consistent within cultures derived from one dissection, that is, when neurones on one cover glass responded to gp120 with a calcium rise, all neurones derived from that dissection responded. In order to investigate the role of the CXCR4 receptor, hippocampal cultures identified as giving calcium responses to gp120 were pretreated with 50 μM VSLSYRCPCRFF for 15 minutes then challenged with 250 pM gp120 in the continued presence of VSLSYRCPCRFF. In 82 neurones from 3 independent dissections, no calcium response to gp120 was observed, while a calcium response to gp120 was seen in 75 of 76 sister neurones not treated with VSLSYRCPCRFF (Fig. 1).

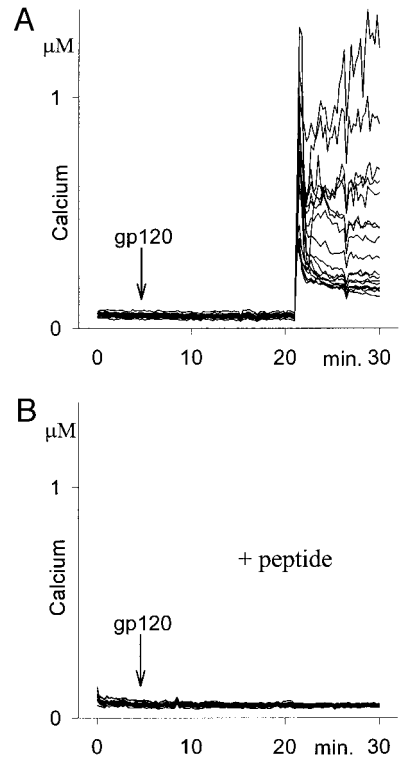

FIG. 1. The chemokine receptor CXCR4 mediates the calcium response to gp120. (A) All neurons in one field of view on a cover glass responded to gp120 with a massive, delayed rise of intracellular calcium. Overall 75 of 76 cells in 6 fields from 3 independent dissections responded to gp120. (B) On a second cover glass from the same dissection incubated with CXCR4 antagonist peptide VSLSYRCPCRFF no cells in the field of view responded to gp120. Overall 82 cells in 6 fields from the same 3 dissections were tested, none responded to gp120. Cultures were maintained in Neurobasal plus 2% B27 plus 5% horse serum.

Incubation of both hippocampal and cortical cultures with 250 pM gp120 for 48 h gave significant neurotoxicity. We challenged cultures with gp120 in the presence of 50 μM of the CXCR4 inhibitor peptide VSLSYRCPCRFF. The peptide gave significant rescue of both hippocampal and cortical neurones (Figs. 2A and 2B). CXCR4 is thought to signal through the Gi/Go family of trimeric G proteins (18). Incubation of both

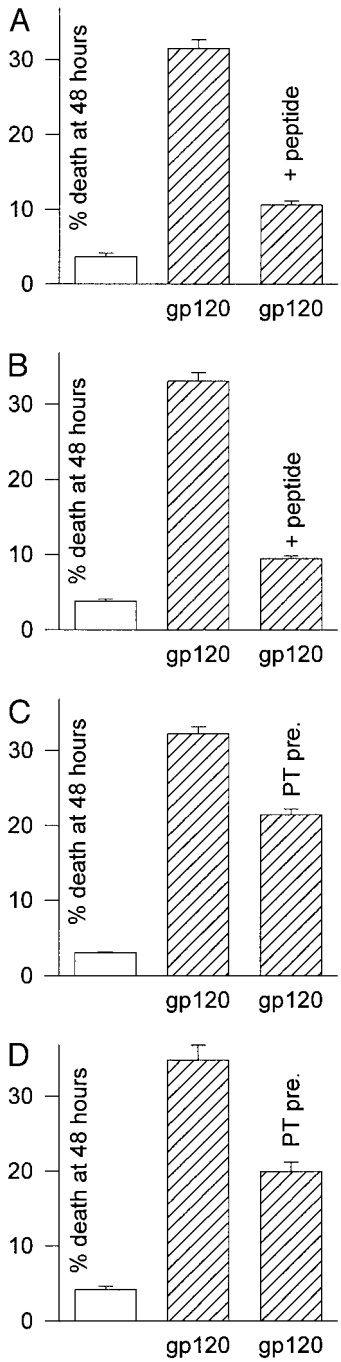

FIG. 2. A component of neurotoxicity is mediated by the CXCR4 chemokine receptor. Each histogram represents data from three independent platings, columns indicate % death in respectively control cultures, cultures with 250 pM gp120, and cultures with 250 pM gp120 and test agent. (A, B) CXCR4 antagonist peptide VSLSYRCPCRFF at 50 μM rescues in (A) hippocampal and (B) cortical cultures. (C, D) IAP component of pertussis toxin at 500 ng/ml rescues in (C) hippocampal and (D) cortical cultures. Test agents were present for two hours before, and during, gp120 application. In all 4 experiments, all values are significantly different ($P < 0.001$, Bonferroni corrected t test).

hippocampal and cortical cultures with 500 ng/ml pertussis toxin to inactivate Gi and Go significantly reduced gp120 neurotoxicity (Figs. 2C and 2D).

DISCUSSION

An inhibitory peptide derived from SDF-1α, the natural agonist of the CXCR4 chemokine receptor, blocks both the early calcium response of hippocampal neurones to gp120 and later toxicity. This indicates that the chemokine receptor plays an essential role in the generation of both short and long term responses of central neurones to gp120, and is therefore directly involved in the neuronal death that is responsible for the AIDS dementia complex. A number of potential downstream targets of chemokine receptors have been identified including trimeric GTP binding proteins of the Gi and Go subfamily, phosphoinositide 3-kinase and the protein tyrosine kinases Pyk2 and RAFTK (19–22). The finding that pertussis toxin treatment significantly reduces gp120 neurotoxicity indicates that a component of toxicity is mediated by the activation of Gi/Go family proteins.

Our results are in no way contradictory to the previous findings that glutamate receptor blockers inhibit calcium responses and toxicity, indeed in the same cultures we found that a cocktail of glutamate antagonists (20 μM MK-801, 20 μM CNQX, 500 μM MCPG) eliminated the calcium response to gp120 (data not shown). The simplest interpretation of our results is that gp120 acts on CXCR4 receptors, either on the nerve cells themselves or on other components of the culture such as astrocytes and microglia, and sets up conditions leading to further release of glutamate, intracellular calcium elevation, and exitotoxic cell death.

The peptide VSLSYRCPCRFF has been proposed as a lead molecule for the design of low molecular weight drugs for inhibiting HIV replication in lymphocytes (16). Our results indicate that such agents could also be extremely effective in preventing the development of the AIDS dementia complex.

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