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Human immunodeficiency virus coat protein gp120 inhibits the β-adrenergic regulation of astroglial and microglial functions

(acquired immunodeficiency syndrome/cyclic AMP/protein phosphorylation/tumor necrosis factor)

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ABSTRACT **The goal of our study was to assess whether the human immunodeficiency virus (HIV) coat protein gp120 induces functional alterations in astrocytes and microglia, known for their reactivity and involvement in most types of brain pathology. We hypothesized that gp120-induced anomalies in glial functions, if present, might be mediated by changes in the levels of intracellular messengers important for signal transduction, such as cAMP. Acute (10 min) exposure of cultured rat cortical astrocytes or microglia to 100 pM gp120 caused only a modest (50–60%), though statistically significant, elevation in cAMP levels, which was antagonized by the β-adrenergic receptor antagonist propranolol. More importantly, the protein substantially depressed [by 30% (astrocytes) and 50% (microglia)] the large increase in cAMP induced by the β-adrenergic agonist isoproterenol (10 nM), without affecting that induced by direct adenylate cyclase stimulation by forskolin. Qualitatively similar results were obtained using a glial fibrillary acidic protein (GFAP)-positive human glioma cell line. The depression of the β-adrenergic response had functional consequences in both astrocytes and microglia. In astrocytes we studied the phosphorylation of the two major cytoskeletal proteins, vimentin and GFAP, which is normally stimulated by isoproterenol, and found that gp120 partially (40–50%) prevented such stimulation. In microglial cells, which are the major producers of inflammatory cytokines within the brain, gp120 partially antagonized the negative β-adrenergic modulation of lipopolysaccharide (10 ng/ml)-induced production of tumor necrosis factor α. Our results suggest that, by interfering with the β-adrenergic regulation of astrocytes and microglia, gp120 may alter astroglial "reactivity" and upset the delicate cytokine network responsible for the defense against viral and opportunistic infections.**

The pathogenesis of AIDS encephalopathy, a complication present in 50–80% of AIDS patients, is still largely unknown (1–4). Besides direct infection of brain cells by human immunodeficiency virus (HIV), which seems to occur mainly in microglia (2–6), the intrinsic brain macrophages, other factors could contribute to the development of brain damage, such as neurotoxic substances produced by brain cells and/or by invading hematic cells, or proteins encoded by the viral genome. Among the latter, the envelope glycoprotein gp120 was reported to cause neuronal death in cell cultures from the rodent central nervous system (reviewed in ref. 7; see also ref. 8) and to induce interleukin 1 (9) and to cause learning impairment (10) in the rat brain *in vivo*. In the present study we evaluated the possibility that the viral protein may cause functional alterations in glial cell types (astrocytes and microglia) known for their reactivity and involvement in most neurological diseases. We found that acute exposure to picomolar gp120 depressed the β-adrenergic agonist-induced formation of cAMP and altered important cAMP-regulated functions in both cell types. We propose that by such a mechanism gp120 may have a critical role in the establishment and consolidation of brain damage in AIDS.

MATERIALS AND METHODS

Cell Cultures. Astroglial and microglial secondary cultures were prepared from mixed primary glial cultures obtained from cerebral cortex of 1-day-old rats. For astrocytes the procedure described in ref. 11 was followed. The subcultures consisted of >96% GFAP (glial fibrillar acidic protein)-positive type 1 astrocytes and <4% oligodendrocyte precursors, type 2 astrocytes, fibroblasts, and microglia, as assessed by specific cell markers. They were used at confluency, after 3–4 days of subculture. Microglial cells were obtained from 12- to 15-day primary glial cultures by mild shaking. The cells were plated on plastic at a density of 2×10^4 cells per cm^2, allowed to adhere for 20 min, washed with fresh medium to remove nonadhering cells, and cultured for 3–4 days. The cultures consisted of >99% microglia/macrophages (positive for the macrophage marker ED1) and contained <1% astrocytes and oligodendrocyte precursors, as assessed by specific cell markers. All cultures were grown in basal Eagle's medium supplemented with 10% fetal bovine serum, 100 μg of gentamicin per ml, and 2 mM glutamine. In the case of microglial preparations, all media were lipopolysaccharide (LPS)-free as determined by *Limulus* amebocyte assay by the manufacturer (GIBCO).

The human glioma cell line on which results are presented was derived by E. Vigneti (Institute of Cell Biology, National Research Council, Rome) from a surgical sample of a brain tumor classified as multiform glioblastoma. The cells were used at the 10th–15th passage and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% non-heat-inactivated fetal bovine serum, 50 units of penicillin per ml, 50 μg of streptomycin per ml, and 44 mM glucose. The cultures consisted of 95% GFAP-positive cells, which did not bind the monoclonal antibodies A2B5, anti-GD3 and anti-galactocerebroside (see refs. 11 and 12 for more information about these cell markers).

Determination of cAMP. Drugs were added to the culture medium for 10 min (for other details, see legend for Fig. 1), after which the cultures were washed with buffered Krebs–Ringer medium and processed for cAMP determination by a radiometric assay (Amersham kit) based on specific, high-affinity binding of cAMP to a purified and stabilized binding protein from bovine muscle. In some experiments, sister cultures were first washed with Krebs–Ringer medium and

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Abbreviations: HIV, human immunodeficiency virus; GFAP, glial fibrillary acidic protein; TNF-α, tumor necrosis factor α; LPS, lipopolysaccharide.

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then exposed to drugs in this medium. The results obtained were similar.

Protein Phosphorylation. Confluent astroglial secondary cultures (see Table 1) in 35-mm culture dishes were incubated for 150 min at 37°C in low-phosphate Krebs–Ringer medium (145 mM NaCl/3 mM KCl/1.8 mM $CaCl_2$/0.8 mM $MgSO_4$/ 0.1 mM Na_2HPO_4/10 mM glucose/20 mM Hepes at pH 7.4) containing [^{32}P]orthophosphate (125 μCi/ml; 1 μCi = 37 kBq). Isoproterenol (10 nM) and gp120 (100 pM) were added to the medium during the final 30 min of incubation. The cells were then washed and incubated for 30 min in 1 ml of ice-cold extraction buffer (20 mM Tris·HCl, pH 7.4/4 mM $MgCl_2$/1 mM EGTA/1 mM DL-dithiothreitol/0.5% Triton X-100/1 mM phenylmethylsulfonyl fluoride/10 mM NaF/50 μM sodium orthovanadate with 10 μg of leupeptin per ml). The Triton-insoluble material was collected with 100 μl of 50 mM Tris·HCl, pH 6.8/1% 2-mercaptoethanol/8 M urea/0.5% sodium dodecyl sulfate (SDS) and an aliquot was analyzed by two-dimensional gel electrophoresis (isoelectric focusing followed by SDS/10% PAGE). The radioactive peptides were visualized by autoradiography using XR-5 x-ray film (Kodak). The regions of the two-dimensional gel corresponding to vimentin and GFAP were excised, solubilized, and measured for radioactivity. The identity of the vimentin and GFAP spots was verified by immunoblotting (data not shown).

Determination of Tumor Necrosis Factor α (TNF-α). TNF-α production was measured over a period of 4 hr in the supernatant of microglial cell cultures grown in 96-well plates, by a biological assay based on the cytotoxicity of TNF-α for WEHI 164 clone 13 mouse fibrosarcoma cells, according to Mosmann (13). Typically, the cytokine was measurable in a concentration range between 0.0005 and 0.1 unit/ml.

Materials. Culture media and fetal bovine serum were purchased from GIBCO, and L-glutamine solution from Whittaker Bioproducts. Gentamicin, LPS (*Escherichia coli* 026:B6), isoproterenol, forskolin, and clonidine were obtained from Sigma. Recombinant glycosylated gp120 was donated by Genentech or purchased from Repligen (Cambridge, MA). A polyclonal antibody to gp120 (rabbit anti-gp120 serum, lot no. R2B16-38) was purchased from Microgenesis (West Aven, CT). Monoclonal antibody to rat ED1 was purchased from Serotec, and recombinant mouse TNF-α (4×10^7 units/mg) was from Genzyme. The cAMP radiometric assay kit was obtained from Amersham International and [^{32}P]orthophosphate (specific activity > 8.5 Ci/mmol) was provided by DuPont.

The gp120 from Genentech was used in the initial part of this study (part of the experiments presented in Table 2, Fig. 1, and Fig. 2). Before the stock was exhausted, gp120 from Genentech and from Repligen were compared in terms of effects on basal and isoproterenol-induced levels of cAMP and of protein phosphorylation. The results obtained were comparable, and subsequent results obtained with the Repligen gp120 were averaged with those obtained with the Genentech product. The results presented in Fig. 3 and those on human glioma were obtained with gp120 from Repligen.

RESULTS AND DISCUSSION

Our study started from the working hypothesis that gp120-induced anomalies in astroglial or microglial functions, if present, might be mediated by changes in the levels of intracellular second messengers important for signal transduction. cAMP is one of the most widespread of such messengers and is known to regulate important functions in glial cells (14–17). Its level is low under basal conditions but can be raised by the activation of several neurotransmitter receptors, among which β-adrenergic receptors are among the most studied and the most effective (14, 15).

Table 1. Effect of isoproterenol and forskolin on cAMP accumulation in astrocyte and microglia cultures

	cAMP pmol/100 μg of protein	
Addition(s)	Astrocytes	Microglia
None (control)	10.2 ± 1.1 (21)	7.9 ± 0.8 (13)
Isoproterenol		
1 nM	47.6 ± 16 (8)***	—
10 nM	461 ± 39 (20)*	89.2 ± 17 (11)*
100 nM	867 ± 41 (4)*	100 ± 25 (4)***
1 μM	887 ± 58 (3)*	114 ± 40 (5)***
Isoproterenol (10 nM)		
+ propranolol (1 μM)	16.3 ± 2.3 (5)	13.5 ± 3.4 (3)
Forskolin		
30 μM	67.8 ± 9 (4)**	—
100 μM	401 (2)	33.5 ± 2.6 (4)*

Purified rat astrocyte and microglia cultures were exposed for 10 min to the substances indicated in the first column. The level of cAMP was then measured by a radiometric assay (see *Materials and Methods*). Means ± SEM of *n* (in parentheses) experiments are presented. Each experiment was on a different cell preparation and was run in duplicate or triplicate. Statistical significance of the increase of cAMP concentration observed in the various conditions was evaluated by paired Student's *t* test. *, $P < 0.001$; **, $P < 0.01$; ***, $P \leq 0.05$.

Table 1 shows the massive (up to 90-fold), dose-dependent elevation in cAMP levels induced by a 10-min exposure to the β-adrenergic agonist isoproterenol (1 nM–1 μM) or to the adenylate cyclase activator forskolin (30 and 100 μM) in cultured rat astrocytes. A lower (up to 14-fold elevation) but still substantial cAMP response was observed in microglia. When the cultures were exposed to 100 pM gp120, a modest (40–60%) but statistically significant elevation in cAMP concentration was observed in both cell types (Table 2). Raising the gp120 concentration to 0.1 μM caused only a small further increase in cAMP (Table 2 and unpublished data). Interestingly, the β-adrenergic antagonist propranolol prevented not only the increase in cAMP induced by isoproterenol but also that evoked by gp120, suggesting that the viral protein might interact with β-adrenergic receptors.

When both isoproterenol and gp120 were added to the cultures, the cAMP increase was significantly inhibited (about 30% in astrocytes and 50% in microglia) (Fig. 1). Such inhibition was largely prevented by an anti-gp120 serum (1:500 dilution). Further, this suppression did not appear to be mediated by α_2-adrenergic receptors (18), since the α_2-adrenergic agonist clonidine caused only a minimal inhibition (in astrocytes) or no inhibition (in microglia) in the cAMP

Table 2. Effect of gp120 on the accumulation of cAMP in astrocyte and microglia cultures

	cAMP, percent of control	
Addition(s)	Astrocytes	Microglia
gp120 (100 pM)	158 ± 11 (15)*	144 ± 9 (8)**
gp120 (10 nM)	187 ± 38 (5)***	—
gp120 (100 pM)		
+ propranolol (1 μM)	102 ± 2 (5)	106 ± 4 (5)

Experimental conditions were as in the legend for Table 1. Recombinant gp120 was added for 10 min to the culture medium. The cAMP values are expressed as a percentage of control values (no additions). Means ± SEM are presented and the number of experiments (each run in duplicate or triplicate on a different preparation) is given in parentheses. Statistical significance of the increase of cAMP concentration elicited by gp120 was evaluated by paired Student's *t* test, using the absolute cAMP values in treated cultures and the corresponding controls. *, $P < 0.001$; **, $P < 0.01$; ***, $P < 0.05$.

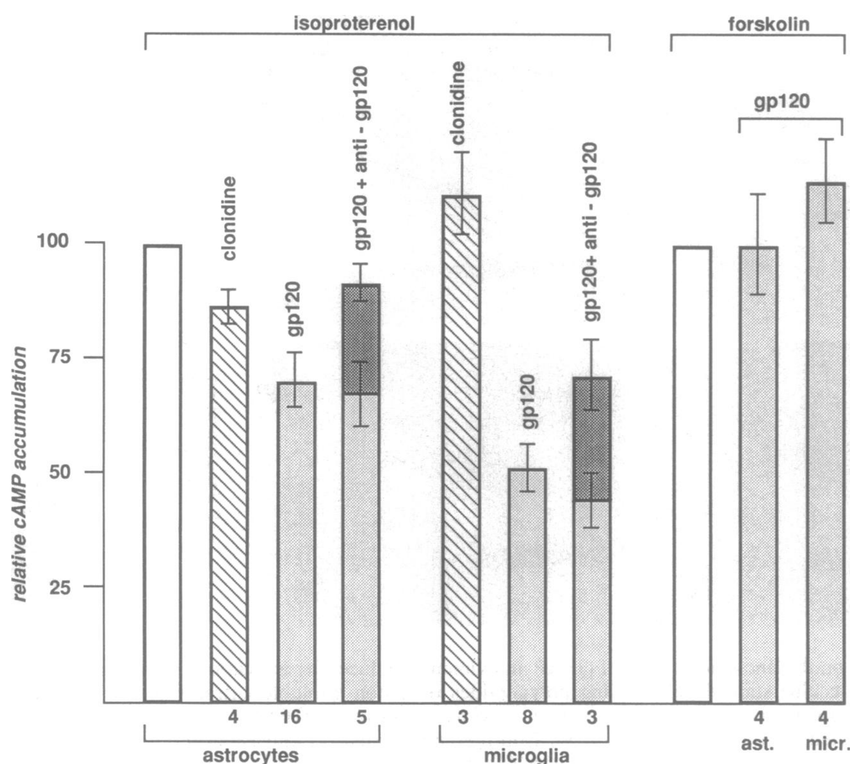

FIG. 1. HIV coat protein of gp120 inhibits the accumulation of cAMP induced by isoproterenol, but not that induced by forskolin, in rat astrocyte (ast.) and microglia (micr.) cultures. Experimental details are given in the legend for Table 1. When indicated, gp120 was added to the culture medium immediately before isoproterenol or forskolin. When the effect of anti-gp120 antibodies (which, by themselves, did not affect cAMP accumulation; data not shown) was tested, polyclonal antibodies (1:500 dilution) were preincubated for 30 min at room temperature with gp120, and the mixture was then added to the culture medium, immediately before isoproterenol. The accumulation (above controls) of cAMP induced by isoproterenol or forskolin in the absence of other substances was taken as 100. In the case of treatments with gp120 and anti-gp120 antibodies, the lower parts of the bars refer to the effect of gp120 alone, and the whole bars to the effect of gp120 plus antibodies. The substances indicated were used at the following concentrations: isoproterenol, 10 nM; gp120, 100 pM; clonidine, 1 μM; forskolin, 30 μM for astrocytes and 100 μM for microglia. In three experiments, no significant increase in the effect of gp120 was observed when the protein was used at 10 nM (data not shown). The number of experiments (run in duplicate or triplicate, each from a different preparation) is shown at the bottom of each bar. Means ± SEM are shown. Statistical significance was evaluated by paired Student's t test, using the absolute cAMP concentrations measured. The following differences were found to be statistically significant: gp120 plus isoproterenol versus isoproterenol; $P < 0.001$ (astrocytes, set of 16 experiments) and $P < 0.01$ (microglia, set of 8 experiments); gp120 plus isoproterenol versus isoproterenol, $P < 0.05$ (astrocytes, set of 5 experiments, and microglia, set of 3 experiments); gp120 plus isoproterenol versus gp120 plus anti-gp120 antibodies plus isoproterenol; $P < 0.02$ (astrocytes and microglia).

response to isoproterenol in the conditions tested (Fig. 1). Finally, the elevation of cAMP induced by forskolin was unaffected by gp120 (Fig. 1), suggesting that the viral protein did not influence the synthesis or the degradation of the cyclic nucleotide directly. Although other explanations should also be considered (for example, interaction with receptor-associated guanine nucleotide-binding proteins), the above results are compatible with the possibility that the viral protein acts as a partial agonist at astroglial β-adrenergic receptors.

In the attempt to confirm the data described above with human cells, astrocyte-enriched cultures prepared from embryonic human brain (12) and four astroglioma cell lines were tested for expression of functional β-adrenergic receptors by measuring the isoproterenol-induced cAMP formation. In six astrocyte preparations and in three astrogliomas, isoproterenol was ineffective (probably due to the absence of mature β-adrenergic receptors, since forskolin did raise cAMP levels), whereas in one glioma cell line consisting of >95% GFAP-positive cells (kindly donated by E. Vigneti), 1 μM isoproterenol caused a 3-fold increase in cAMP that was 50% antagonized by 1 nM gp120 (pmol/mg of protein: controls, 63 ± 6; 1 nM gp120, 66 ± 8; isoproterenol, 174 ± 15; gp120 plus isoproterenol, 117 ± 10; mean ± SEM of three experiments run in duplicate, $P < 0.02$ for isoproterenol versus control or versus gp120 plus isoproterenol). Similar results, with a lower increase in cAMP, were obtained in three other experiments in which 0.1 μM rather than 1 μM isoproterenol was used. These results are qualitatively consistent with those obtained with the rat cultures.

It seemed important to determine whether the interference of gp120 with the β-adrenergic agonist-induced cAMP accumulation resulted in biologically relevant alterations in astroglial and microglial functions. In astrocytes, the activation of β-adrenergic receptors was shown to stimulate the phosphorylation of the two major cytoskeletal proteins vimentin and GFAP (14), to induce the secretion of nerve growth factor (16) and of the neuroactive amino acid taurine (15), and to down-regulate the expression of major histocompatibility complex class II antigens (17). In activated microglia, β-adrenergic receptors were reported to modulate the expression and secretion of inflammatory cytokines, and in particular to down-regulate the production of TNF-α (19), probably through a mechanism involving cAMP (20).

As a test for the biological significance of the gp120 effects on cAMP production, we measured vimentin and GFAP phosphorylation in astrocytes and TNF-α production in microglia. In astrocyte cultures (Fig. 2) the HIV gp120 protein (100 pM) induced, by itself, only a negligible increase in GFAP and vimentin phosphorylation but substantially reduced the β-adrenergic agonist-induced phosphorylation of both proteins. In microglial cell cultures (Fig. 3), 100 pM

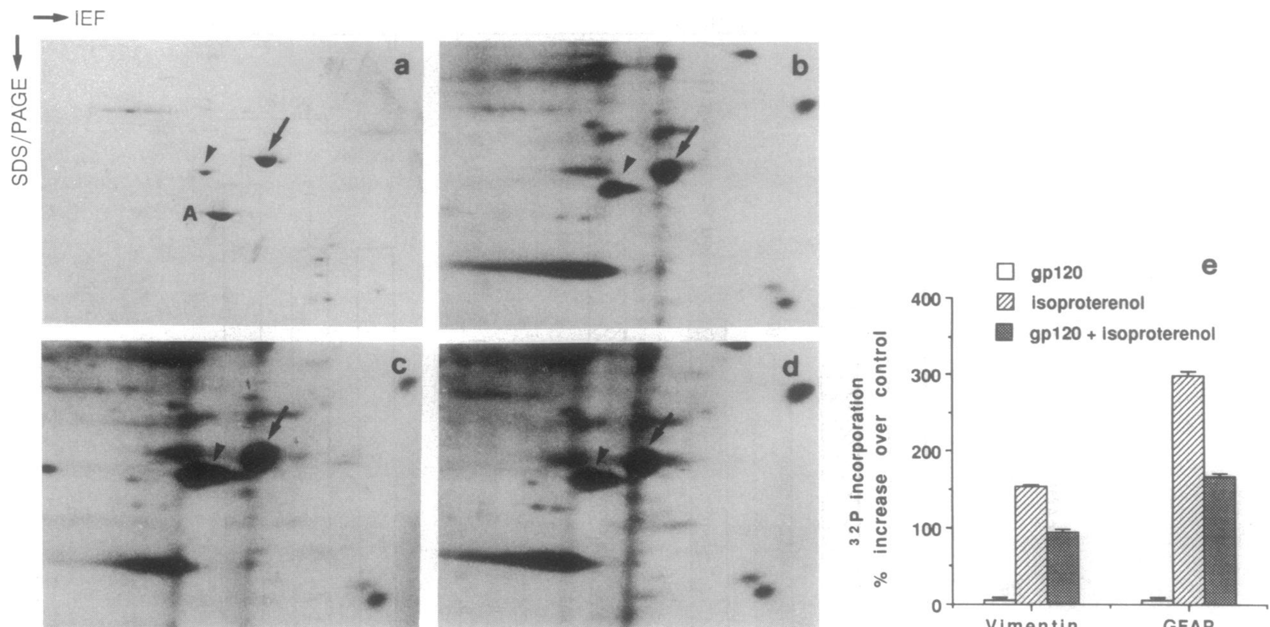

FIG. 2. Effect of gp120 on the phosphorylation of GFAP and vimentin induced by activation of β-adrenergic receptors in cultured rat type 1 astrocytes. (*a*) Coomassie blue-stained gel showing polypeptides of Triton-insoluble extracts of astrocyte cultures, after separation by two-dimensional gel electrophoresis. Spots corresponding to vimentin (arrow), GFAP (arrowhead), and actin (A) are indicated. (*b–d*) Autoradiograms of ^{32}P-labeled peptides from control (*b*), isoproterenol-treated (*c*) and isoproterenol plus gp120-treated (*d*) astrocytes. (*e*) Quantitative analysis of the incorporation of ^{32}P into astroglial vimentin and GFAP in cultures treated with gp120 (100 pM) (open bar) isoproterenol (10 nM) (hatched bar), or both (filled bar) (means ± SEM of eight independent duplicate experiments). The inhibitory effect of gp120 on the isoproterenol-induced phosphorylation was statistically significant, as evaluated with paired Student's *t* test ($P < 0.01$ for both vimentin and GFAP). IEF, isoelectric focusing.

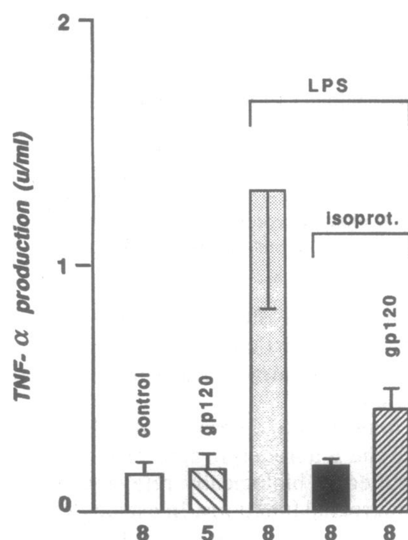

FIG. 3. gp120 partially antagonizes the inhibitory effect of isoproterenol on the production of TNF-α by LPS-activated microglia. Rat microglia subcultures were prepared as for Table 1 and used after 3 days. The following substances were added to the culture medium for 4 hr: (*i*) gp120 (100 pM); (*ii*) LPS (10 ng/ml); LPS (10 ng/ml) plus isoproterenol (isoprot., 100 nM) LPS (10 ng/ml) plus isoproterenol (100 nM) plus gp120 (100 pM). Aliquots of the medium were then utilized to measure TNF-α [units (u)/ml] by a biological assay (see *Materials and Methods*). Means ± SEM are presented. The number of independent experiments (each run in quadruplicate) is shown at the bottom of each bar. The TNF-α values measured in the presence of isoproterenol and gp120 were significantly higher than those measured in the presence of isoproterenol alone ($P < 0.01$, paired Student's *t* test). In five experiments not shown, gp120 did not affect the LPS-induced secretion of TNF-α in the absence of isoproterenol. Moreover, propranolol antagonized by 80% the effect of isoproterenol, and isoproterenol did not influence TNF-α production in unstimulated microglia.

gp120 did not affect the low basal production of TNF-α or the TNF-α production induced by LPS (10 ng/ml) but did partially prevent the inhibitory effect of isoproterenol on the LPS-induced secretion of TNF-α (Fig. 3). These results are in accord with those reported above for cAMP formation. In a paper published while this manuscript was in preparation (21), TNF-α and interleukin 1β production by rat astrocytes and microglia was reported to be unaffected by gp120 even at high concentrations and to be enhanced by nanomolar concentrations of HIV gp41 and gp160 and by intact or heat-inactivated HIV-1. Interestingly, these effects could be prevented by monoclonal antibodies raised against selective epitopes of gp120.

The results obtained with astrocyte-enriched cultures cannot be due to the small amounts of contaminant fibroblastic or microglial cells. In fact, although vimentin is present in all these cell types, GFAP is expressed only in astrocytes. Moreover, the isoproterenol-induced stimulation of cAMP accumulation was much more robust in astrocytes than in microglia. Conversely, the effects observed in microglial cultures cannot be accounted for by the negligible astroglial contamination present (<0.5%).

The implications of the results reported here may be far-reaching, for several reasons. (*i*) An inhibition by gp120 of the β-adrenergic regulation of the phosphorylation of astroglial intermediate-filament proteins may interfere with those plastic changes occurring in reactive gliosis. (*ii*) Inhibition of the β-adrenergic stimulation of astroglial nerve growth factor expression (16) and release of the neuroactive amino acid taurine (15) may alter astrocyte–neuron interactions. (*iii*) Depression of the β-adrenergic down-regulation of major histocompatibility complex class II antigen expression (17) in astrocytes might facilitate autoantigen presentation to T cells and the establishment of autoimmune reactions that may be important in the pathogenesis of AIDS encephalopathy (22).

Our results may also be relevant to the elucidation of microglial function in AIDS. The inhibition of the β-adrenergic regulation of the microglial secretion of inflammatory cytokines (19) may facilitate a cascade of events important for the development of brain damage. For example, the observed decrease of the β-adrenergic control of TNF-α production may lead to an excessive extracellular level of this cytokine, which was recently found to be elevated in plasma (23), cerebrospinal fluid (24) (however, see ref. 25), and microglia (26) of AIDS patients with neurological involvement. TNF-α has been shown to be toxic for oligodendrocytes and myelin (27), to induce major histocompatibility complex antigen expression in astrocytes (12, 28), to stimulate the astroglial expression and production of interleukin 6 (29, 30) and of colony-stimulating factors (30, 31), to stimulate astrocyte proliferation (32), to synergize with other inflammatory cytokines such as interferon γ (12), to facilitate the passage of T cells across the blood–brain barrier (33), and to stimulate HIV replication in infected cells (see ref. 4). In conclusion, by interfering with brain β-adrenergic receptors, the HIV envelope protein gp120 may contribute to the pathogenesis of brain damage by altering astroglial reactivity and neuron–glia interactions and by upsetting the delicate cytokine network responsible for the defense against viral as well as opportunistic infections. It seems legitimate to hypothesize that also the β-adrenergic regulation of peripheral immunocompetent cells expressing β-adrenergic receptors (34, 35) may be affected by the viral protein.

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