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Induction of Interleukin-1 and Tumor Necrosis Factor Alpha in Brain Cultures by Human Immunodeficiency Virus Type 1

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Cytokines such as interleukin-1 (IL-1) and tumor necrosis factor alpha (TNFα) are produced by leukocytes and play a role in immune responses. They also function in normal brain physiology as well as in pathological conditions within the central nervous system, where they are produced by brain macrophages (microglia) and brain astrocytes. In this study, we document the ability of human immunodeficiency virus type 1 (HIV-1) to induce TNFα and IL-1 in primary rat brain cultures. While productive infection did not occur in these cells, it was not required for cytokine induction. Using monocyte/macrophage-tropic (JRFL) and T-cell-tropic (IIIB) strains of HIV-1, we were able to induce cytokines in both microglia and astrocytes. In addition to whole virus, recombinant envelope proteins also induced these cytokines. The induction of IL-1 and TNFα could be blocked by a panel of antibodies recognizing epitopes in the gp120 and gp41 areas of the envelope. Soluble recombinant CD4 did not block TNFα and IL-1 production. If TNFα and IL-1 can be induced in brain tissue by HIV-1, they may contribute to some of the neurologic disorders associated with AIDS.

In the brains of AIDS patients, the predominant cell types productively infected with human immunodeficiency type 1 (HIV-1) are macrophages and microglia (brain macrophages). AIDS-associated neurological diseases are correlated with the levels of HIV-1 in the brain (29, 33, 67). Clinical hallmarks of the central nervous system (CNS) disease are fever, ataxia, headaches, pain, cognitive changes, and motor and sensory dysfunction (20). Histopathological changes involve astrogliosis, microglial nodules, giant cell syncytia, and diffuse dysmyelination (20). Such broad clinical and biological manifestations involving cells not infected with virus suggest that such effects may be mediated indirectly by cytokines produced in the CNS. Two such cytokines are interleukin-1 (IL-1) and tumor necrosis factor (TNFα), which have been implicated in astrogliosis, microglial cell proliferation, and demyelination (23, 26, 30, 42, 44, 48), as well as having been shown to induce fever and cachexia (63, 64). Both cytokines are produced by blood macrophages, microglia, and astrocytes and are elevated in AIDS patients (17, 18, 22, 37). Because of the strong implication of IL-1 and TNFα in the disease process and the recent finding that TNFα may lead to an autocrine feedback involving further productive virus replication either directly or indirectly through induction of IL-6 (53), we investigated HIV-1 induction of these two cytokines in brain cultures.

MATERIALS AND METHODS

Primary glial cultures. Primary glial cell cultures were established from neonatal rat cerebra according to previously published methods from this laboratory (46). Meninges were removed prior to generation of a glial cell suspension. After 6 days in culture, microglia were removed by gentle shaking and their purity was assessed by nonspecific esterase staining. These cultures were 96% pure microglia. Pure astrocytes were generated from 2- to 3-week-old cultures which had been passed three times by trypsinization, which removes most contaminating cells. These cultures are 95 to 99% pure astrocytes. In purified cultures of microglia or astrocytes, contaminating cells are usually glial precursor cells which do not produce IL-1 or TNFα (unpublished observations). Mixed glial cell cultures were used at day 6 or 7 of culture. These cultures typically contained 30% microglia, 50% astrocytes, 7 to 10% oligodendrocytes, and 10 to 13% precursors.

Astrocytes were identified by cytoplasmic staining of glial fibrillary acidic protein (GFAP) (rabbit antibody from Accurate Chemicals; 1:50). Oligodendrocytes were identified by surface staining for galactocerebroside (GalC) (rabbit antibody generated in this laboratory; 1:100). Precursor cells were identified by surface ganglioside A2B5 (monoclonal antibody from a mouse hybridoma line, the generous gift of Arthur McMorris; 1:10). Microglia were identified by staining for the macrophage-specific cytoplasmic antigen ED1 (1:100; Serotech, Ltd.), for receptors for acetylated low-density lipoprotein (DiI-αLDL; 1,1′-dioctadecyl-1-3,3,3′3′-tetramethylindocarbocyanine perchlorate-conjugated LDL, 1 μg/ml; BioMedical Technologies, Inc.), and nonspecific esterase. For GFAP staining, a 2-min fixation in cold acetone was performed. Primary antibodies were added for a 30-min incubation at 4°C, followed by fluorescein isothiocyanate-conjugated affinity-purified goat antibodies to rabbit (anti-GFAP, anti-GalC) or mouse (anti-A2B5) immunoglobulins (1:20; Accurate Chemicals). These cultures contained no neurons or fibroblasts and contained 1 to 10 ependymal cells per culture.

Preparation of virus and treatment of cultures. HIV-1_{JRFL}-containing culture supernatants from infected human peripheral blood T cells were clarified by low-speed centrifugation (2,100 rpm, 20 min). HIV-1 virions were pelleted by high-speed centrifugation (26,000 rpm, 0.5 h) through a 25%

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Renografin (diatrizoateimeglumine; Squibb) cushion to purify virions away from culture supernatants containing growth factors from activated T cells. Pelleted virus was resuspended in RPMI medium with 0.25% bovine serum albumin (BSA) and stored at −70°C. Mock material was prepared from uninfected mitogen-stimulated T cells from the same donors in an identical way. Human T-cell leukemia virus type II (HTLV-II) from pH6neo 729 cell lines was also prepared by the same procedure. Heat inactivation of HIV-1 was performed at 60°C for 45 min to remove infectivity. β-Propiolactone (BPL) inactivation was performed by addition of 0.015% (vol/vol) BPL in the dark at room temperature for 24 h. Endotoxin levels in these preparations were determined by the *Limulus* amebocyte lysate assay kit (Whittaker Bioproducts).

Glial cell cultures were infected with titrations of 0.5×10^4 to 5×10^4 infectious units of HIV-1_{JRFL} or HTLV-IIIB (50 to 500 ng of p24 titer as measured by the HIV-1-specific enzyme-linked immunosorbent assay (ELISA) (Abbott Laboratories) or with HTLV-II (at the same protein concentrations as HIV-1). HTLV-IIIB/H9 (from Robert Gallo [44, 45, 57]), recombinant gp120 (IIIB), and purified recombinant wild-type gp120 from HIV-1_{SF2} (36, 60) were obtained from the AIDS Research and Reference Reagent Program (ARRRP), Division of AIDS, National Institute of Allergy and Infectious Diseases. HTLV-IIIB/H9 was used at 50 to 300 ng/ml and recombinant gp120 was used at 10 μg/ml in the same way as was HIV-1_{JRFL}. Recombinant gp160 (MicroGeneSys, Inc.) and recombinant gp41 (NEN Dupont) were used at 4 μg/ml. Virus or viral proteins were incubated with glial cells for 1 h in the presence of Polybrene (10 μg/ml) at 37°C. Following incubation, the cells were washed and placed in Iscove's medium supplemented with 0.25% BSA and transferrin (1 μg/ml). In some experiments, 15 μg of polymyxin B per ml and/or 10^{-8} M indomethacin were used. Lipopolysaccharide (LPS) was used at 10 μg/ml. Supernatants were harvested at 2, 6, 12, 24, 48, and 72 h. Times after exposure, however, are not cumulative; supernatants harvested at 6 h reflect cytokine production during the previous 4 h (2- to 6-h time period), supernatants harvested at 12 h reflect cytokine production during the previous 6 h (6- to 12-h period), and so on. Supernatants from HIV-1 or recombinant protein-exposed or unexposed cells were stored at −70°C until testing for IL-1, TNFα, and virus production at the indicated time points.

PCR to detect virus infection. To determine whether HIV-1 can infect rat glial cells, DNA from glial cells exposed to gradient-purified virus was examined by quantitative polymerase chain reaction (PCR) analysis as previously described (68). To accomplish infections, gradient-purified virus stocks were diluted 1:10 in BSA medium, filtered through a 0.2-μm-pore-size filter, and treated with DNase (2 μg/ml; Worthington) for 30 min at room temperature in the presence of 10 mm $MgCl_2$. Polybrene was added to 10 μg/ml, and 0.5 ml of this treated virus was added to confluent 24-well cultures of glial cells or to 10^6 mitogen-stimulated peripheral blood lymphocytes (PBL) (48-h treatment with phytohemagglutinin). Infection was performed for 2 h at 37°C. Cells received either live virus or heat-inactivated virus as a control. DNA was harvested and subjected to phenol-chloroform extraction as previously described (68). The recovered DNA (10%) was subjected to 25 cycles of amplification using the oligonucleotide primer pair M667/M661 (68). These primers amplify a 200-bp fragment specific for the 5′ long terminal repeat (LTR)/*gag* region of HIV-1. Primer M667 was radioactively end labeled, and following amplification, the products were resolved on a 6% polyacrylamide gel and subjected to autoradiography. Quantitation was accomplished by analyzing in parallel a standard curve of cloned HIV-$1_{JR\text{-}CSF}$ (6). DNA was linearized with *Eco*RI.

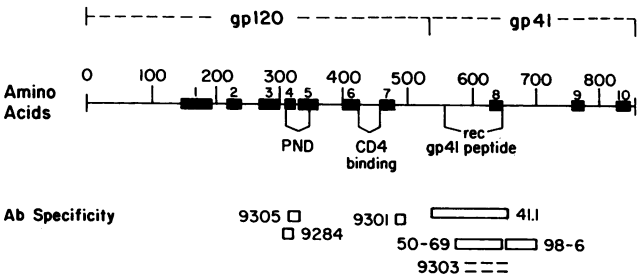

FIG. 1. Map of gp160 showing antibody specificities. PND is the primary neutralizing determinant; rec gp41 peptide is a recombinant gp41 peptide used to induce cytokines and includes the indicated amino acids. Solid boxes labeled 1 to 10 indicate regions of HIV-1_{IIIB} and HIV-1_{JRFL} that are of reduced amino acid homology. The specific area of antibody (Ab) 9303 is not known; the general area is indicated by dashed lines. For each of the other antibodies, the amino acid sequence recognized is indicated by an open box spanning the sequence.

Pretreatment of virus or virus-infected cells with antibodies or soluble CD4. HIV-1 was pretreated at 4°C for 45 min prior to addition to glial cell cultures with human or mouse monoclonal antibodies as well as polyclonal antibodies or sera. Antibodies and sera were titrated for maximal neutralizing activity and used at optimal concentrations as indicated. All sera were heat inactivated at 56°C for 30 min prior to use. Some antibody specificities are shown in Fig. 1. Controls included monoclonal immunoglobulin G2K from CESS cells (1:100), normal sheep serum (1:50; obtained through ARRRP), and normal goat serum (1:50; Accurate Chemicals). Polyclonal antibodies included sheep anti-HIV-1 gp120, anti-HIV-1 p17, and anti-HIV-1 p24 (all used at 1:50; obtained through ARRRP; antiserum to HIV-1 gp120 was from Michael Phelan, Food and Drug Administration) and polyclonal goat anti-gp160B and gp160RF (1:50; obtained through ARRRP [43, 58]). Virus was also treated with normal seronegative human serum and seropositive serum (1:50) obtained through ARRRP (human HIV immunoglobulin from Alfred Prince [56]). A human immune serum (gift of David Ho) that is capable of neutralizing HIV-1_{JRFL} to a dilution of 1:1,000 was used at 1:50. Anti-gp41 monoclonal antibodies ALAV 41.1 and ALAV 41.6 (Genetics Systems Corp.) (25) were used at 1:100; human monoclonal anti-gp41 antibodies 50-69 and 98-6 (gift of Susan Zolla-Pazner [24]) were used at 1:10 to 1:100. Anti-gp41 monoclonal antibody 9303 (NEN Dupont) was used at 1:25. Anti-gp120 monoclonal antibodies 9284 (63), 9305, and 9301 (15) (NEN Dupont) were used at 1:25 (4 μg/ml). The specificity maps of some of these antibodies are shown in Fig. 1. Soluble CD4 (gift of David Ho; soluble recombinant CD4 from Jay Raina, American Bio-Technologies, Inc., and ARRRP) was used at 15 to 25 μg/ml as previously described (45).

IL-1 and TNFα bioassays. IL-1 was measured using the technique of Conlon (12), using the LBRM 331A5 conversion assay. Supernatants were incubated overnight at a final concentration of 1:8 on LBRM cells (10^5 per well, in a Falcon 96-well microtiter plate) with 10 μg of phytohemagglutinin P (Difco) per ml. These cell-free supernatants were harvested, and IL-2 production was assessed according to Gillis et al. (21). Previous experience had shown optimal

dilutions for IL-1- and IL-2-containing supernatants in these bioassays (45, 47). CTLL-2 cells were resuspended to 4 × 10^4 cells per ml in Click's medium (Altick Associates), and 100 μl was added to each well of a 96-well, flat-bottom plate (Falcon; Becton Dickinson). IL-2-containing sample supernatants were added in 100-μl quantities at doubling dilutions from 1/8 to 1/512. The plates were incubated for 24 h at 37°C, at which time they were pulsed with 0.5 μCi of [^{3}H]TdR per well (New England Nuclear). The plates were harvested after an additional 18 h. The amount of incorporation was measured with a Beckman liquid scintillation counter. In every assay the [^{3}H]TdR uptake by CTLL-2 cells after response to test samples was compared with the CTLL-2 response to standards, using a probit analysis (21). The dilution point at which the standard gave 30% maximal proliferation ([^{3}H]TdR uptake) was considered 100 U/ml. The IL-1 activity is expressed as units of IL-2 per milliliter according to the following equation: units of IL-2 = (reciprocal titer of test material at 30% maximal cpm of standard/reciprocal titer at 30% maximal cpm of standard) × 100.

TNFα was measured by a modification of the procedure of Aggarwal et al. (1, 47). The murine L929 cell line was retrieved in log growth phase by trypsinization, washed several times, chromated in a total volume of 1.0 ml with 0.25 mCi of ^{51}Cr (Amersham International) for 1 h, and washed three times in medium. Chromated target cells were plated with the test sample in 96-well flat-bottom plates (Costar) at a concentration of 5 × 10^4 cells per 0.2 ml per well with 5 μg of actinomycin D per ml. Eighteen hours later, the plates were harvested with a Skatron harvester (Skatron, Oslo, Norway) and counted in a gamma counter. Experimental values were read off a standard curve generated by using recombinant TNFα standards, a gift from Cetus Corp.

RESULTS

We have previously obtained an isolate of HIV-1 termed HIV-1_{JRFL} from the brain tissue of an individual with severe AIDS dementia. HIV-1_{JRFL} was shown to replicate well in mononuclear phagocytes (34). In this study, HIV-1_{JRFL} induced comparable levels of IL-1 and TNFα in purified populations of microglia and astrocytes. Mixed glial cell cultures gave additive levels of cytokine production and earlier peaks of activity than did individual cell subtypes. Mixed glia were used in the remaining studies (Fig. 2). TNFα was produced in significant amounts in the first 2 h and was still elevated above background at 24 h, while IL-1 peaked in production at 6 to 24 h in the supernatants of the mixed glial cell cultures (Fig. 3 and 4). Untreated or mock-treated cultures did not produce significant amounts of IL-1 or TNFα (Fig. 2 to 4), nor did cultures treated with an unrelated virus, HTLV-II (data not shown). Indomethacin, used to prevent any inhibitory effects that might have occurred in the presence of prostaglandin E, did not affect these results (28; data not shown).

We had previously demonstrated that a productive infection of mononuclear phagocytes was not necessary for HIV-1_{JRFL} to induce IL-1 and TNFα (45). To determine whether HIV-1_{JRFL} was capable of infecting rat glial cells, we performed in vitro infections with gradient-purified virus and subsequent quantitative PCR analysis (68). As a control, mitogen-stimulated PBL were exposed to virus in parallel. To control for background viral DNA contamination in the virus stock (arising from lysis of infected cells during cultivation of the virus), heat-inactivated virus was also used to infect cells. Figure 5 illustrates that while the virus preparation is highly infectious for human PBL, no virus-specific amplified product was seen following PCR analysis of virus-treated glial cells. The primer pair used in these studies detects essentially the last region of the virus synthesized during reverse transcription. We also tested the samples with a different primer pair (specific for the HIV LTR), which detects initiation of reverse transcription even in the absence of full-length viral DNA (68). These studies gave similar results and indicated that reverse transcription does not initiate in infected glial cells (not shown). These results indicate that HIV-1 does not productively infect rat glial cells.

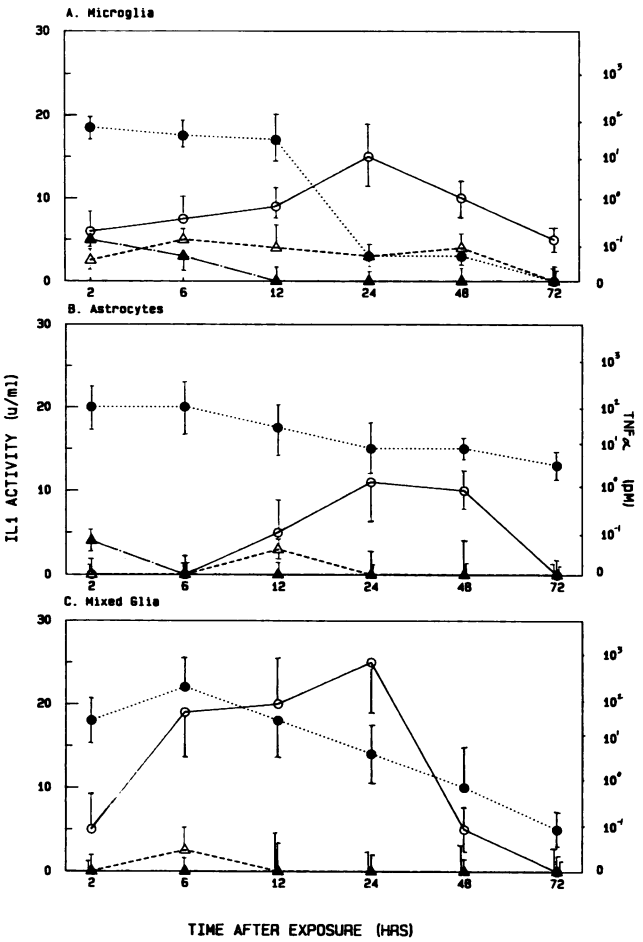

FIG. 2. Kinetics of IL-1 and TNFα production after exposure of rat glia to HIV-1. Symbols for IL-1: △, mock; ○, live HIV-1_{JRFL} (100 ng/ml). Symbols for TNFα: ▲, mock; ●, live HIV-1_{JRFL}. Data are means ± standard deviations of eight experiments. Time after exposure is not cumulative (see Materials and Methods).

Since the rat cells were not infected, we tested the possibility that cytokines could be induced in glial cells by heat- or BPL-inactivated HIV-1_{JRFL} or HIV-1_{IIIB}. Production by both strains was assayed by measurements of viral p24*gag* antigen in a specific ELISA (Abbott); there was no detectable p24 antigen following infection with inactivated HIV-1 preparations (45; data not shown). The heat- and BPL-inactivated HIV-1_{JRFL} were both as effective in inducing TNFα in mixed glial cell cultures at the same level as was live virus. Heat-inactivated HIV-1_{JRFL} was better than live virus at induction of IL-1 (Fig. 3 and 4). Heat-inactivated

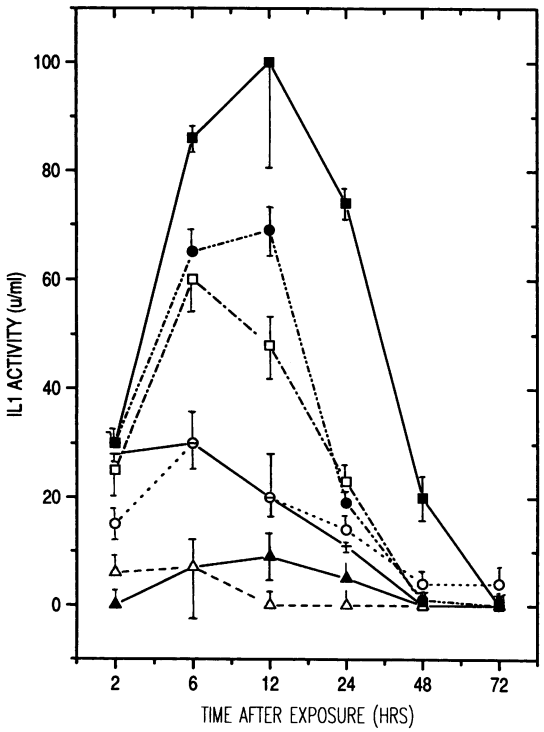

FIG. 3. Rat mixed glial cell response to inactivated HIV-1: IL-1. Symbols: ▲, no treatment; △, mock; ○, live HIV-1_{JRFL} (100 ng/ml); ●, heat-inactivated HIV-1_{JRFL}; ■, inactivated BPL; □, live HIV-1_{IIIB}; ■, heat-inactivated HIV-1_{IIIB}. Data are means ± standard deviations of six experiments.

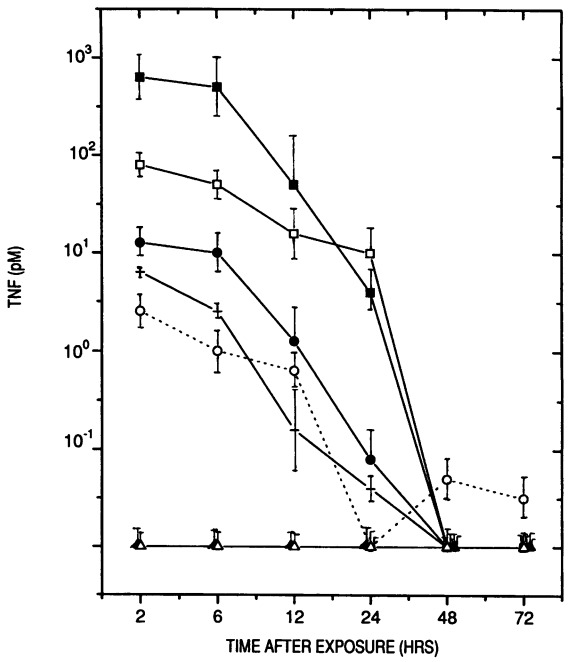

FIG. 4. Rat mixed glial cell response to inactivated HIV-1: TNFα. Symbols are as in Fig. 3. Data are means ± standard deviations of the same six experiments tested for IL-1 in Fig. 3.

HIV-1_{IIIB} was a better inducer of cytokines than was live HIV-1_{IIIB} and better than heat-inactivated or live HIV-1_{JRFL} (Fig. 3 and 4). Consistent with the PCR data, this result suggests that infection is not required for maximal cytokine production. Indeed, heat inactivation seems to improve the induction of these cytokines, suggesting that denatured viral proteins may be more efficient at induction.

Concentrations of virions equivalent to those in the cytokine-containing samples were tested directly in the LBRM conversion assay and produced IL-1 activity of less than 2 U/ml; similar preparations had no effect in the TNFα assay. Treatment of the positive supernatants with anti-IL-1 (1:50; Cistron) reduced IL-1 activity to 3 U/ml. Treatment of these supernatants with antibody to TNFα (1:100; Endogen) reduced TNFα activity by 95%.

To rule out the effects of endotoxin as an explanation for our findings, we measured endotoxin contamination of our virus and mock preparations and performed HIV-1 and LPS cytokine induction in the presence of polymyxin B. The mock preparations and HIV-1_{IIIB} and HIV-1_{JRFL} prepared by this laboratory contained amounts of endotoxin below the detectable limits of 7 pg/ml. Furthermore, as Table 1 demonstrates, polymyxin B can dramatically reduce or eliminate LPS-induced cytokine production while having no significant effect on HIV-1 cytokine induction.

To determine which portion of the virus was inducing IL-1 and TNFα, soluble recombinant envelope proteins were tested. Soluble, purified recombinant gp120 from HIV-1_{SF_2} or HIV-1_{IIIB} at 0.01, 0.1, and 1 μg/ml did not induce TNFα or IL-1 (Fig. 6 and data not shown). However, recombinant gp41 and gp160 gave a small but significant induction of IL-1 at 12 and 24 h and a much larger induction of TNFα which resembled intact IIIB at 2, 6, and 12 h (Fig. 6).

To elucidate the role of viral proteins in induction of TNFα and IL-1, we pretreated the HIV-1_{IIIB} with a variety of antibodies and sera. The purified gamma globulin fraction (98% polyclonal immunoglobulin G) from pooled human plasma of HIV-1-seropositive donors completely inhibited the induction of TNFα and reduced IL-1 production by 50%, while control seronegative serum had no effect. We tested a battery of antisera to determine the virion epitopes involved in induction. Polyclonal anti-gp160 and anti-gp120 had modest inhibitory effects on IL-1 and TNFα. However, monoclonal antibodies recognizing amino acid determinants within the primary neutralizing determinant (antibodies 9305 and 9284 [35, 63]) significantly inhibited both cytokines. Antibody 9301, recognizing a highly conserved gp120 region 3′ to CD4 (15), almost completely eliminated IL-1 and TNFα induction. Finally, while IL-1 production was not inhibited by the majority of anti-gp41 monoclonal antibodies, TNFα was significantly inhibited by these antibodies (Fig. 1, 7, and 8). Soluble human CD4 did not inhibit induction of either cytokine (Fig. 9). Neither the sera or antibody controls nor the anti-p17 or anti-p24 antibody significantly inhibited either IL-1 or TNFα production (Fig. 7 and 8).

DISCUSSION

IL-1 and TNFα are produced and utilized in the brain (2, 16–18, 22, 37). In the CNS, these cytokines influence normal physiological conditions by regulating temperature, controlling corticotropin-releasing factor production, inducing pro-opiomelanocortin gene expression, and stimulating nerve growth factor production (5, 38, 61). During disease and trauma, both cytokines have been associated with lesioned white matter and astrogliosis as well as vascular changes, including vascular permeability, neovascularization, and ne-

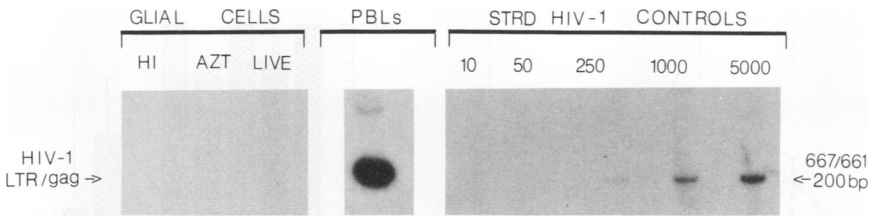

FIG. 5. Quantitative PCR analysis of HIV-1 infection of rat glial cell DNA from glial cell cultures exposed to live HIV-1_{JRFL} in the presence or absence of zidovudine (AZT) or heat-inactivated (HI) virus. This analysis was compared with analysis of infection of stimulated PBL. A primer pair specific for the 5′ viral LTR/*gag* junction of HIV which results in a 200-bp amplified fragment (M667/M661) was used. STRD, standard.

crosis of blood vessels (23, 26, 30, 42). Changes in myelin, astrogliosis, cerebral vasculitis, fever, and chachexia are all associated with AIDS (reviewed in reference 20). Thus, IL-1 and TNFα production within the CNS is of interest in relation to those symptoms and pathological findings. In addition, it has been shown that TNFα stimulates HIV-1 transcription in T cells and macrophages (32, 53). These data, added to our data showing HIV-1 induction of TNFα and IL-1 in blood macrophages (45) and brain cells (this study), suggest positive autocrine and paracrine feedback loops to perpetuate HIV-1 replication in the brains of AIDS patients.

It is clear that CD4 is the HIV-1 receptor in human mononuclear phagocytes and T cells, but whether CD4 is the only receptor for infection of human brain cells is still in question. There have been reports demonstrating HIV-1 infection of CD4-negative human brain cells, skin cells, muscle cells, and B cells (7–10, 19, 34, 39, 59). In addition, HIV-1 tropism for mononuclear phagocytes is determined by regions outside the major CD4-binding domain in gp120, implicating additional epitopes in infection (31, 50, 62, 66). Previous work from this laboratory has demonstrated low levels of HIV-1 infection in short-term cultures of human astroglioma cells, which were devoid of microglia. These glioma cells did not stain for surface CD4 with OKT4 or OKT4A (34). Whether human microglial cells can be infected in vitro by HIV-1 is still unclear, since two recent studies arrived at different conclusions (52, 64). The role of CD4 in these studies was not discussed. Rat glia do not express CD4, and they are not infectable by HIV-1. The interaction of the virus with the rat glial cells must therefore terminate at a step in the viral life cycle prior to reverse transcription. From our data, it seems likely that the virus interacts with the surface of the glial cells but does not enter the cell.

CD4 is involved in cytokine induction in human monocytes (45). However, since rat microglia have low to undetectable CD4 in vitro (51), and astrocytes have no CD4 and rat CD4 is only partially homologous to human CD4 (11), this surface molecule does not account for cytokine induction in these cells. The inability of soluble CD4 to block the HIV-1-induced cytokine production also suggests that the CD4-binding domain of gp120 is not the viral ligand binding to rat glia.

The role of non-CD4 molecules on brain cells in HIV-1 interactions has been suggested. Harouse et al. recently demonstrated a role for GalC in entry and infection of two human brain cell lines by HIV-1 (28). The cultures used in this study contained mostly astrocytes and microglia, which are GalC negative (46, 48). Oligodendrocytes and precursors do not produce IL-1 or TNFα (17, 18, 22, 37). Thus, we do not feel GalC is involved in glial cell cytokine production.

TABLE 1. Effect of polymyxin B on HIV-1- or LPS-induced cytokines[a]

Stimulus	Time (h) after exposure	IL-1 activity (U/ml)		TNFα concn (pM)	
		−PB	+PB	−PB	+PB
LPS	2	93 ± 4	22 ± 3	2,350 ± 250	2.5 ± 1.0
	6	110 ± 5	0 ± 0	2,500 ± 300	0.9 ± 0.1
	12	100 ± 10	17 ± 1	150 ± 25	1.0 ± 0.1
	24	90 ± 3	10 ± 0	35 ± 30	1.0 ± 1.0
	48	39 ± 12	0 ± 0	6 ± 3	0 ± 0
	72	19 ± 3	0 ± 0	0.8 ± 0.2	0 ± 0
HIV-1_{IIIB}	2	33 ± 2	20 ± 4	685 ± 150	550 ± 500
	6	86 ± 3	60 ± 3	500 ± 400	600 ± 500
	12	100 ± 10	93 ± 10	75 ± 10	70 ± 11
	24	69 ± 15	56 ± 11	6 ± 2	1 ± 1
	48	22 ± 5	8 ± 6	4 ± 1	0 ± 0
	72	10 ± 5	0 ± 0	0 ± 0	0 ± 0
HIV-1_{JRFL}	2	10 ± 3	10 ± 1	7.5 ± 5	80 ± 3
	6	65 ± 14	45 ± 3	7.0 ± 4	5.0 ± 2
	12	53 ± 6	80 ± 9	3.0 ± 2	5.0 ± 1
	24	15 ± 10	10 ± 3	0.5 ± 0	1.0 ± 0.5
	48	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	72	0 ± 0	0 ± 0	0 ± 0	0 ± 0

[a] Concentrations used were as follows: polymyxin B (PB), 15 μg/ml; LPS, 10 μg/ml; HIV-1_{IIIB}, 50 ng/ml; HIV-1_{JRFL}, 100 ng/ml. Data are means ± standard deviations of three experiments.

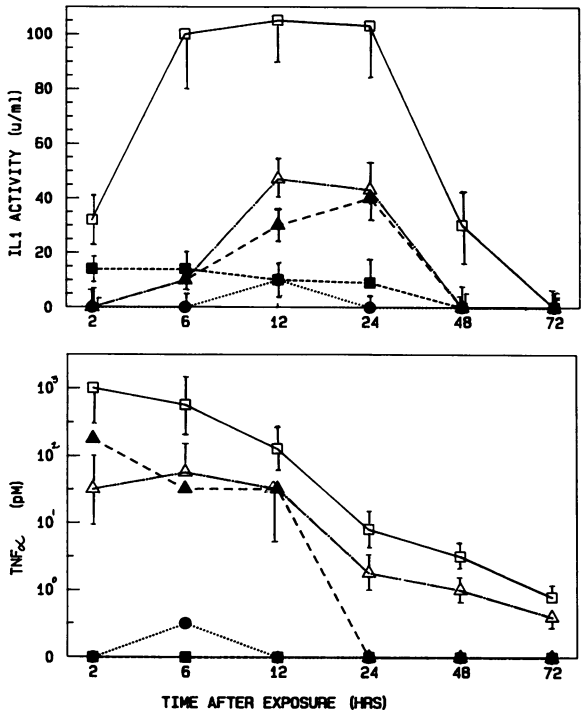

FIG. 6. Induction of cytokines in mixed glial cell cultures by recombinant proteins. Symbols: □, heat-inactivated HIV-1_{IIIB} (50 ng/ml); ■, recombinant gp120SF; ●, recombinant gp120IIIB; △, recombinant gp41; ▲, recombinant gp160. Data are means ± standard deviations of three experiments.

To rule out the possibility of endotoxin contamination of our virus preparations inducing IL-1 and TNFα, we specifically used HIV-1 and mock preparations that had minimal endotoxin; in our experiments, this level was <7 pg/ml. Rat glia (astrocytes and microglia) require concentrations of LPS at 1 ng/ml or greater to produce either cytokine (40, 41). Addition of polymyxin B effectively blocked LPS induction but not HIV-1 induction of cytokines in our cultures. We conclude that the effects we are seeing are virus specific.

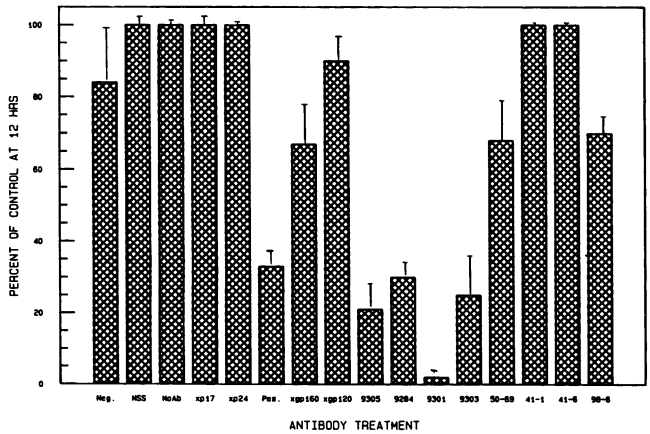

FIG. 7. Antibody effect on HIV-1 induction of IL-1 in mixed glial cells. HIV-1_{IIIB} (50 ng/ml) was pretreated with heat-inactivated antibodies. IL-1 data are those obtained at 12 h. Data are means ± standard deviations of four experiments.

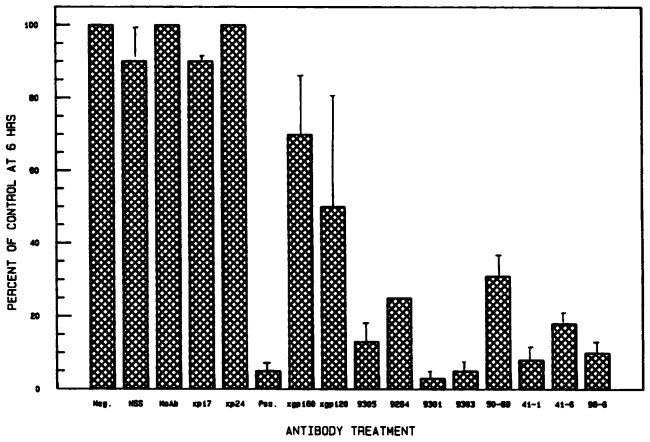

FIG. 8. Antibody effect on HIV-1 induction of TNFα in mixed glial cells. Symbols are as in Fig. 7. TNFα data are those obtained at 6 h. Data are means ± standard deviations of the same four experiments tested for IL-1 in Fig. 7.

HIV-1 has been demonstrated to induce metabolic changes in rodent neural cells in vitro. Dreyer et al. have recently shown gp120 to increase intracellular free calcium and to cause injury to rat retinal ganglion cells and hippocampal neurons in culture (14). Brenneman et al. demonstrated neurotoxicity by gp120 on fetal mouse hippocampal cultures (4). Thus, it was interesting that antibody-inhibitable determinants in gp120 and gp41 triggered IL-1 and TNFα in our studies on rat glia. The gp120 epitopes responsible for inducing both cytokines occur (i) in a highly

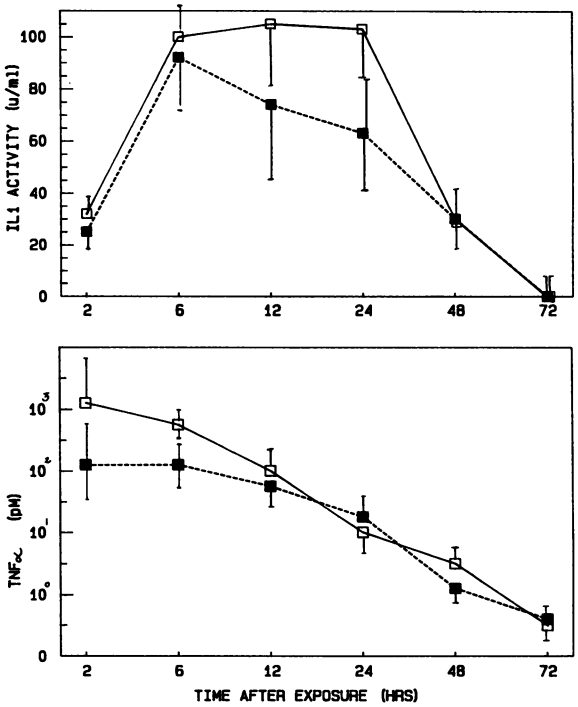

FIG. 9. Effect of soluble CD4 on HIV-1 induction of cytokines in mixed glial cells. Symbols: □, HIV-1_{IIIB} (50 ng/ml); ■, HIV-1_{IIIB} plus 15 μg of soluble recombinant CD4 per ml. Data are means ± standard deviations of three experiments.

conserved, hydrophilic, conformational determinant (recognized by antibody 9301 [15]) whose blockade provides an almost complete inhibition of IL-1 and TNF and (ii) within the area of the primary neutralizing determinant (antibodies 9284 [63] and 9305), which, while being the third hypervariable region disulfide-bridged loop, is nevertheless associated with neutralizing activity by a majority of human HIV-1$^+$ sera and thus may contain conserved amino acids (35). These latter two antibodies recognize a region of gp120 responsible for macrophage tropism (31, 50, 62, 66). Within the gp41 protein, one epitope recognized by antibodies 9303 and 50-69 is involved in both IL-1 and TNFα production. This determinant is a highly conserved, immunogenic site (27). A second epitope recognized by 98-6 is involved in induction of both cytokines, TNFα more than IL-1. Other sites in gp41, however, are not involved in IL-1 production but are significantly involved in TNFα production. One of these determinants recognized by ALAV 41.1 is a highly conserved linear epitope, not recognized as a conformational determinant (25).

These data suggest that IL-1 and TNFα can be induced independently of each other and by several triggering mechanisms, possibly via different activation pathways. This laboratory has evidence for such events in rat glia IL-1 and TNFα induction by substance P, which directly induces IL-1 but not TNFα in rat astrocytes. Substance P does not directly induce production of either cytokine in rat microglia but amplifies IL-1 production induced by LPS in these cells (40, 41). Lieberman et al. have shown that Newcastle disease virus triggers TNFα but not IL-1 in vitro in rat astrocyte cultures (37). Cross-linking of cell surface epitopes by antibodies also causes IL-1 and TNFα production. Anti-LFA-3 induces both cytokines, but antibodies to CD44 or CD45 induce only TNFα in human monocytes (65). We could also clearly demonstrate induction of TNFα by recombinant gp160 and gp41. From our antibody inhibition studies, both gp41 and gp120 had a significant inductive effect on both IL-1 and TNFα. Furthermore, additional epitopes in gp41 may regulate additional TNFα activation pathways. The inability of soluble, purified gp120 to induce cytokines, while gp160 did induce them, suggests that gp120 determinants are altered in the presence of gp41 to become biologically active in cytokine induction. This effect might either be through altered conformational folding within gp120 or due to epitopes composed of both gp120 and gp41 amino acids.

The fact that rat brain cells bind HIV-1 which then triggers biological events suggests the possibility of unique mechanisms for virus interaction with brain cells, possibly through evolutionarily conserved cell surface determinants. The kinetics and magnitude of production of TNFα and IL-1 in rat glial cells is very similar to the kinetics of cytokine production in HIV-1-stimulated normal human blood monocyte/macrophages (45). The triggering of IL-1 and TNFα in the absence of infection is identical to that which we have demonstrated in human PBL. The ability of heat-inactivated virus to more effectively induce cytokines may suggest an alteration in Env protein conformation and exposure of determinants required for triggering these events. It suggests that such cells may, in the absence of infection, still participate in CNS pathology in AIDS. It further suggests that immune system-inactivated virus, HIV-1 virions in which gp41 and gp120 are dissociated (49), or soluble viral proteins all may continue to contribute to pathology and clinical symptomatology.

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