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Diazepam-Mediated Inhibition of Human Immunodeficiency Virus Type 1 Expression in Human Brain Cells

JAMES R. LOKENSGARD,[1]* GENYA GEKKER,[1] SHUXIAN HU,[1] ANDREW F. ARTHUR,[1] CHUN C. CHAO,[1,2] AND PHILLIP K. PETERSON[1,2]

Institute for Brain and Immune Disorders, Minneapolis Medical Research Foundation,[1] and University of Minnesota Medical School,[2] Minneapolis, Minnesota 55404

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Treatment of acutely infected human brain cell and enriched microglial cell cultures with diazepam inhibited human immunodeficiency virus type 1 (HIV-1) p24 antigen expression. Similarly, diazepam suppressed HIV-1 expression in chronically infected promonocytic (U1) cells and acutely infected monocyte-derived macrophages, and this antiviral activity was associated with decreased activation of nuclear factor kappa B.

Diazepam (Valium) is a benzodiazepine (BDZ) analog that readily crosses the blood-brain barrier (BBB) and is widely prescribed for anxiety disorders (2, 13). Most of diazepam's anxiolytic and sedative attributes are manifested through a central BDZ receptor, an allosteric site on the gamma-aminobutyric acid A receptor located in the central nervous system (CNS) (22, 32). However, diazepam also binds to a pharmacologically distinct peripheral BDZ binding site that has been identified in various tissue and cell types (5, 7, 19). BDZ receptor agonists are classified according to their affinity for one or both of these receptors, either pure central (e.g., clonazepam), pure peripheral (e.g., RO5-4864), or mixed (e.g., diazepam) agonists. In addition to its anxiolytic attributes, diazepam possesses immunomodulatory and anti-inflammatory properties (4, 21, 36).

The exact function of the peripheral BDZ receptors is unclear, but it is believed that they mediate the immunomodulatory properties of BDZs in mononuclear phagocytes (11). Peripheral as well as mixed BDZ ligands show dose-dependent suppressive effects on lipopolysaccharide-induced tumor necrosis factor alpha production by murine peritoneal macrophages (21, 35). In vivo treatment of mice with peripheral (RO5-4864) and mixed (diazepam), but not central, BDZs significantly impairs the ability of peritoneal and splenic macrophages to produce several key mediators of inflammation (36). In addition to central BDZ receptors, peripheral BDZ receptors have recently been found on rodent brain cells including astrocytes (3, 16, 23) and microglia (24). Microglia, the resident macrophages of the brain, are a primary cell type permissive for human immunodeficiency virus type 1 (HIV-1) infection within the human CNS (18, 28, 34). Because of the immunomodulatory properties of BDZs and the presence of peripheral BDZ receptors on glial cells, we tested the hypothesis that diazepam treatment would alter HIV-1 expression in human brain cells.

In mixed glial-neuronal cell cultures (27), a culture system that mirrors the brain microenvironment in that it contains primarily astrocytes and neurons with interspersed microglia and oligodendrocytes, diazepam was found to be a potent inhibitor of HIV-1 strain SF162 (9) p24 antigen (Ag) production with a 50% inhibitory concentration (IC_{50}) of approximately 8 μM (Fig. 1A). Representatives of the other two classes of BDZ receptor ligands, included here for comparison, also inhibited HIV-1 expression in these cultures, but not as potently as diazepam. The pure central ligand (clonazepam) and the pure peripheral ligand (RO5-4864) each inhibited viral p24 Ag production with IC_{50}s of approximately 10 and 27 μM, respectively (Fig. 1A). Similar results were obtained in three separate experiments using glial-neuronal cell cultures derived from different brain specimens. None of these BDZs, at the concentrations used in these experiments, appeared to be cytotoxic, as assessed by trypan blue dye exclusion or by lactate dehydogenase release assay (4.8, 4.3, and 4.5 U/ml for 100 μM diazepam, RO5-4864, and clonazepam, respectively, versus 4.2 U/ml for untreated control).

Because microglial cells are a primary site for HIV-1 replication within the CNS (14, 18, 28, 34), and we have previously demonstrated that this is also the only cell type in our mixed glial-neuronal cell cultures that is permissive for viral replication (20), we next evaluated the effect of diazepam on HIV-1_{SF162} expression in highly enriched human microglial cell cultures (8). Diazepam treatment was again found to potently inhibit HIV-1 p24 Ag production in a dose-dependent manner with an IC_{50} of approximately 3 μM (Fig. 1B). The pure central ligand (clonazepam) and the pure peripheral ligand (RO5-4864) each inhibited viral p24 Ag production with IC_{50}s of approximately 65 and 10 μM, respectively (Fig. 1B). Similar results were obtained in three separate experiments using microglia isolated from different brain specimens. No evidence of BDZ-induced toxicity, as assessed by trypan blue dye exclusion, was observed.

Because chronically infected monocytes are thought to be a vehicle for viral entry into the CNS (the so-called "Trojan Horse" hypothesis) and to be a reservoir of HIV-1 within the brain, experiments were also performed with chronically infected promonocytes. U1 cells, a promonocytic line that contains integrated copies of HIV-1 proviral DNA, produce very little HIV-1 p24 Ag under basal conditions; however, HIV-1 expression can be induced by plating these cells at a high density. We found that diazepam treatment also inhibited HIV-1 expression in these chronically infected cells (Fig. 1C). This suggests that the diazepam-induced suppression of HIV-1 occurs after infection. Although diazepam inhibited HIV-1 expression in U1 cells, the pure peripheral agonist RO5-4864 was the most potent among the three ligands tested, with an approximate IC_{50} of 3 μM versus 12 μM for diazepam (Fig. 1C). Also, in contrast to the findings in mixed glial-neuronal

* Corresponding author. Mailing address: Minneapolis Medical Research Foundation, 914 South 8th St., Building D-3, Minneapolis, MN 55404. Phone: (612) 347-6849. Fax: (612) 337-7372.

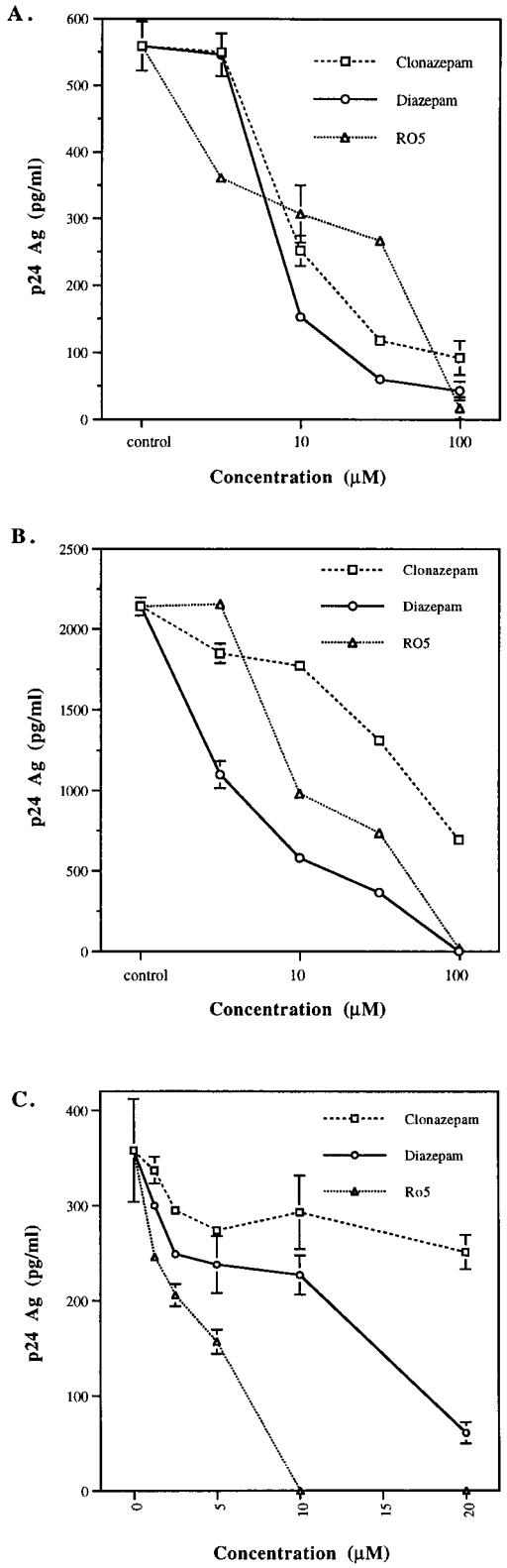

FIG. 1. Concentration-dependent inhibition of HIV-1 p24 Ag expression by diazepam. (A) Mixed glial-neuronal cell cultures (10^5 cells/well) were infected with HIV-1_{SF162} in the presence or absence of each class of BDZ receptor ligand at the indicated concentrations for 14 days. Supernatants were then assayed for p24 Ag levels. Results shown are representative of three independent experiments using different brain cell specimens. (B) Highly enriched (>99%) human microglial cells (10^5/well) were infected with HIV-1_{SF162} in the presence or absence of BDZ receptor ligands. Following infection, cultures were incubated at the indicated concentrations for 14 days; the supernatants were then assayed for p24 Ag levels. Results shown are representative of three independent experiments using microglial cells derived from different specimens. (C) Chronically infected promonocytes (U1 cells), seeded at 2×10^4/well, were incubated with the various classes of BDZs at the indicated concentrations. U1 cells normally express low levels of virus but produce abundant amounts of HIV-1 when plated at this relatively high cell density. Culture supernatants were harvested 4 days later and assayed for HIV-1 p24 Ag levels. Results are representative of three independent experiments.

and enriched microglial cell cultures, the pure central ligand (clonazepam) had a nominal antiviral effect in U1 cells (Fig. 1C). Similar results were obtained when HIV-1 expression was induced by treating the U1 cells with 20 pg of tumor necrosis factor alpha per ml (i.e., diazepam inhibited p24 expression with an IC_{50} of approximately 12 μM, and clonazepam treatment had little effect).

Taken together, the data from mixed glial-neuronal and enriched microglial cell cultures suggest that diazepam inhibits HIV-1 expression during acute infection of human brain cells. The presence of multinucleated giant cells (syncytia), which result from fusion of microglia, is the histopathological hallmark of HIV-1 encephalitis (6, 10). Hence, we next investigated the effect of diazepam on multinucleated giant-cell formation in HIV-1-infected microglial cell cultures. In contrast to uninfected control cells (Fig. 2A), microglial cells formed syncytia in response to HIV-1 infection (Fig. 2B), and immunofluorescence staining with an anti-p24 monoclonal antibody (Dako, Carpinteria, Calif.) demonstrated that these syncytia were active sites of viral replication (Fig. 2B). Infected microglial cell cultures treated with diazepam (20 μM) displayed marked reduction in syncytium formation as well as p24 Ag staining (Fig. 2C).

The mechanism underlying BDZ-induced inhibition of HIV-1 expression is unclear. It is well known that cellular activation is a key requirement for HIV-1 replication in infected cells, and transcription factors that participate in HIV-1 expression could be key therapeutic targets for antiviral agents. Nuclear factor kappa B (NF-κB) is an important transcriptional activator of inflammatory mediators, including certain cytokines, chemokines, and adhesion molecules (30), and it is well established that activation of this transcription factor is necessary for self-perpetuated HIV-1 replication in mononuclear phagocytes (1, 17, 26, 29, 33). Thus, we hypothesized that the mechanism of diazepam's anti-HIV-1 effect involved an inhibition of NF-κB activation. Nuclear extracts (31) were obtained from 5×10^6 chronically infected U1 cells which were induced by high-density plating and treated with diazepam, as well as the other prototypical classes of BDZs, to determine the effect on NF-κB activation. Although equal amounts of nuclear extract (2 μg) were added for different binding reactions, a decrease in NF-κB binding was observed in the diazepam- and RO5-4864-treated cultures, whereas clonazepam treatment had little effect (Fig. 3A and B). This differential effect of the three BDZs was consistent with their effects on p24 Ag production in U1 cells, presented above.

Because acutely infected monocytes may also serve as a vehicle for HIV-1 entry into the brain, we next examined the effect of diazepam treatment on HIV-1-induced NF-κB activation in acutely infected monocyte-derived macrophages (MDM). Using nuclear extracts from these cells (2×10^6), we again found evidence of decreased activation of NF-κB after diazepam treatment (Fig. 3C). The reduced activation of NF-κB in MDM was associated with an 84% suppression of p24 Ag production at 14 days after infection, following treat-

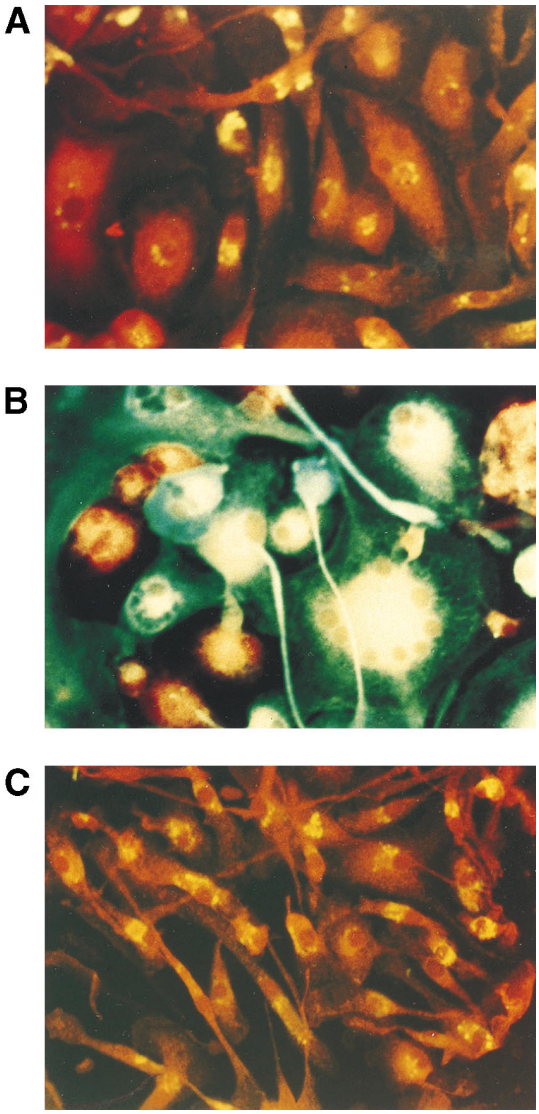

FIG. 2. Effect of diazepam treatment on the formation of multinucleated giant cells (syncytia). (A) Uninfected human microglia display no positive staining for viral p24 Ag and no syncytium formation after 14 days in culture. (B) Immunofluorescence staining with a monoclonal antibody to HIV-1 p24 capsid protein shows that the syncytia formed by fusion of human microglia, 14 days after infection with HIV-1_{SF162}, are active sites of viral expression (intense green fluorescence). (C) Absence of syncytia and decreased p24 Ag levels in the presence of 20 μM diazepam.

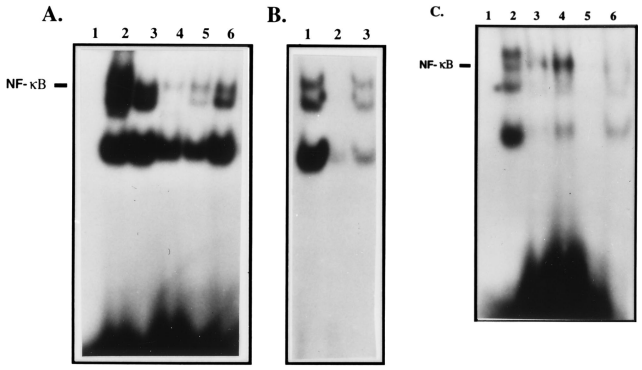

FIG. 3. Effect of diazepam treatment on activation of NF-κB. (A) Electrophoretic mobility shift assay using U1 cells. Nuclear extracts (2 μg) were probed for transcription factor binding to a [^{32}P]NF-κB-specific oligonucleotide. Lane 1, probe alone; lane 2, HeLa cells (control for NF-κB binding); lane 3, no treatment; lane 4, RO5-4864 (10 μM); lane 5, diazepam (10 μM); lane 6, clonazepam (10 μM). (B) Specificity controls. Lane 1, no treatment; lane 2, no treatment, with 50-fold excess unlabeled NF-κB probe; lane 3, no treatment, with 50-fold excess unlabeled mutant NF-κB binding site. (C) Band shift assay using extracts from MDM. Nuclear extracts (1 μg) were probed for NF-κB binding activity. Lane 1, probe alone; lane 2, HeLa cell extract; lane 3, uninfected MDM cell extract; lane 4, 3 days after HIV-1 infection; lane 5, 3 days after HIV-1 infection in the presence of 20 μM diazepam; lane 6, 3 days after HIV-1 infection (same extract as that in lane 4), with anti-p65 antibody.

ment with 20 μM diazepam, compared to untreated HIV-1 infected MDM. These data suggest that the anti-HIV-1 effects observed with diazepam are mediated, at least in part, through inhibition of activation of the NF-κB transcription factor.

The prototypical BDZ drugs used in this study (clonazepam, diazepam, and RO5-4864) differ from the BDZ-derived TIBO [tetrahydroimidazo(4,5,1-jk)(1,4)-benzodiazepin-2(1H)-one and -thione) compounds (e.g., R14458) and the BDZ-derived Tat inhibitors (i.e., RO5-3335 and RO5-24-7420], whose anti-HIV-1 activity has been well studied in cell lines (reviewed in references 15 and 25). TIBO compounds inhibit HIV-1 reverse transcriptase yet are devoid of any BDZ-like pharmacological effects (25). Previous experiments have shown that TIBOs and other reverse transcriptase inhibitors (e.g., zidovudine) do not suppress HIV-1 expression in chronically infected promonocytes (U1 cells) which already contain two copies of integrated provirus. Regarding Tat inhibitors, the affinity of RO5-3335 for the BDZ receptor is less than 1% of that of diazepam (15), and HIV-1 replication during chronic infection of macrophages is not inhibited by BDZ-derived Tat inhibitors (12). It is currently unknown if diazepam possesses any anti-RT or anti-Tat activity.

Because many of the presently used antiretroviral drugs do not readily cross the BBB, an urgent need exists to identify novel approaches for stopping HIV-1 replication within this viral sanctuary. These in vitro studies show that diazepam, which is currently one of the most widely prescribed medications in the world (13), markedly inhibits HIV-1 expression in human brain cells, as well as in cells that may carry HIV-1 into the brain. This inhibition occurs at clinically achievable concentrations (2) and appears to be mediated through a cellular mechanism involving decreased activation of NF-κB. Interruption of HIV-1 expression by psychotropic agents such as diazepam, which readily cross the BBB and interfere with activation of cellular transcription factors, could offer novel therapeutic approaches to the management of HIV-associated neurological disease.

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REFERENCES

1. **Bachelerie, F., J. Alcami, F. Arenzana-Seisdedos, and J. L. Virelizier.** 1991. HIV enhancer activity perpetuated by NF-kappa B induction on infection of monocytes. Nature **350:**709–712.
2. **Baldessarini, R. J.** 1996. Drugs and the treatment of psychiatric disorders: psychosis and anxiety, p. 399–430. *In* J. G. Hardman, L. E. Limbird, P. B. Molinoff, R. W. Rudden, and A. Goodman Gilman (ed.), Goodman and Gilman's The Pharmacological Basis of Therapeutics. McGraw-Hill, New York, N.Y.
3. **Bender, A. S., and L. Hertz.** 1987. Pharmacological characteristics of diazepam receptors in neurons and astrocytes in primary cultures. J. Neurosci. Res. **18:**366–372.
4. **Bessler, H., R. Weizman, M. Gavish, I. Notti, and M. Djaldetti.** 1992. Immunomodulatory effect of peripheral benzodiazepine receptor ligands on human mononuclear cells. J. Neuroimmunol. **38:**19–25.
5. **Braestrup, C., and R. F. Squires.** 1977. Specific benzodiazepine receptors in

rat brain characterized by high-affinity (3H) diazepam binding. Proc. Natl. Acad. Sci. USA **74:**3805–3809.
6. **Budka, H.** 1986. Multinucleated giant cells in the brain: a hallmark of the acquired immunodeficiency syndrome (AIDS). Acta Neuropathol. **69:**253–256.
7. **Canat, X., P. Carayon, M. Bouaboula, D. Cahard, D. Shire, C. Roque, and G. Le Fur.** 1993. Distribution profile and properties of peripheral-type benzodiazepine receptors on human hemopoietic cells. Life Sci. **52:**107–118.
8. **Chao, C. C., G. Gekker, S. Hu, and P. K. Peterson.** 1994. Human microglial cell defense against Toxoplasma gondii: the role of cytokines. J. Immunol. **152:**1246–1252.
9. **Cheng-Mayer, C., and J. Levy.** 1988. Distinct biological and serological properties of human immunodeficiency viruses from the brain. Ann. Neurol. **23**(Suppl.)**:**S58–S61.
10. **Dickson, D. W.** 1986. Multinucleated giant cells in acquired immunodeficiency syndrome: origin from endogenous microglia? Acta Pathol. Lab. Med. **110:**967–968.
11. **Drugan, R. C.** 1994. Are the nonmitochondrial peripheral benzodiazepine receptors on leukocytes a novel intermediary of brain, behavior, and immunity? Lab. Invest. **70:**1–5.
12. **Dunne, A. L., H. Siregar, J. Mills, and S. M. Crowe.** 1994. HIV replication in chronically infected macrophages is not inhibited by the Tat inhibitors Ro-5-3335 and Ro-24-7429. J. Leukocyte Biol. **56:**369–373.
13. **Giri, A. K., and S. Banerjee.** 1996. Genetic toxicology of four commonly used benzodiazepines: a review. Mutat. Res. **340:**93–108.
14. **He, J., Y. Chen, M. Farzan, H. Choe, A. Ohagen, S. Gartner, J. Busciglio, X. Yang, W. Hofmann, W. Newman, C. R. Mackay, J. Sodroski, and D. Gabuzda.** 1997. CCR3 and CCR5 are co-receptors for HIV-1 infection of microglia. Nature **385:**645–649.
15. **Hsu, M.-C., and S. Tam.** 1993. A new approach to antiviral chemotherapy: intervention in viral gene expression by Tat antagonists, p. 153–177. *In* J. Adams and V. J. Merluzzi (ed.), The search for antiviral drugs. Birkhauser, Boston, Mass.
16. **Itzhak, Y., L. Baker, and M. D. Norenberg.** 1993. Characterization of the peripheral-type benzodiazepine receptors in cultured astrocytes: evidence for multiplicity. Glia **9:**211–218.
17. **Jacqué, J.-M., B. Fernández, F. Arenzana-Seisdedos, D. Thomas, F. Baleux, J.-L. Virelizier, and F. Bachelerie.** 1996. Permanent occupancy of the human immunodeficiency virus type 1 enhancer by NF-κB is needed for persistent viral replication in monocytes. J. Virol. **70:**2930–2938.
18. **Koenig, S., H. E. Gendelman, J. M. Orenstein, M. C. Dal Canto, G. H. Pezeshkpour, M. Yungbluth, F. Janotta, A. Aksamit, M. A. Martin, and A. S. Fauci.** 1986. Detection of AIDS virus in macrophages in brain tissue from AIDS patients with encephalopathy. Science **233:**1089–1093.
19. **Le Fur, G., M. L. Perrier, N. Vaucher, F. Imbault, A. Flamier, J. Benavides, A. Uzan, C. Renault, M. C. Dubroeucq, and C. Gueremy.** 1983. Peripheral benzodiazepine binding sites: effect of PK 11195, I-(2-chlorophenyl)-N-methyl-N-(I-methylpropyl)-3-isoquinolinecarboxamide. I. In vitro studies. Life Sci. **32:**1839–1847.
20. **Lokensgard, J. R., G. Gekker, L. C. Ehrlich, S. Hu, C. C. Chao, and P. K. Peterson.** 1997. Proinflammatory cytokines inhibit HIV-1_{SF162} expression in acutely infected human brain cell cultures. J. Immunol. **158:**2449–2455.
21. **Matsumoto, T., M. Ogata, K. Koga, and A. Shigematsu.** 1994. Effect of peripheral benzodiazepine receptor ligands on lipopolysaccharide-induced tumor necrosis factor activity in thioglycolate-treated mice. Antimicrob. Agents Chemother. **38:**812–816.
22. **Mohler, H., and T. Okada.** 1977. Benzodiazepine receptor: demonstration in the central nervous system. Science **198:**849–851.
23. **Oh, Y. J., J. W. Francis, G. J. Markelonis, and T. H. Oh.** 1992. Interleukin-I-beta and tumor necrosis factor-alpha increase peripheral-type benzodiazepine binding sites in cultured polygonal astrocytes. J. Neurochem. **58:**2131–2138.
24. **Park, C. H., E. Carboni, P. L. Wood, and K. W. Gee.** 1996. Characterization of peripheral benzodiazepine type sites in a cultured murine BV-2 microglial cell line. Glia **16:**65–70.
25. **Pauwels, R.** 1993. Discovery of TIBO, a new family of HIV-1-specific reverse transcriptase inhibitors, p. 71–104. *In* J. Adams and V. J. Merluzzi (ed.), The search for antiviral drugs. Birkhauser, Boston, Mass.
26. **Paya, C. V., R. M. Ten, C. Bessia, J. Alcami, R. T. Hay, and J. L. Virelizier.** 1992. NF-kappa B-dependent induction of the NF-kappa B p50 subunit gene promoter underlies self-perpetuation of human immunodeficiency virus transcription in monocytic cells. Proc. Natl. Acad. Sci. USA **89:**7826–7830.
27. **Peterson, P. K., G. Gekker, S. Hu, W. R. Anderson, F. Kravitz, P. S. Portoghese, H. H. J. Balfour, and C. C. Chao.** 1994. Morphine amplifies HIV-I expression in chronically infected promonocytes cocultured with human brain cells. J. Neuroimmunol. **50:**167–175.
28. **Price, R. W., B. Brew, J. J. Sidtis, M. Rosenblum, A. C. Scheck, and P. Cleary.** 1988. The brain in AIDS: central nervous system HIV-1 infection and AIDS dementia complex. Science **239:**586–592.
29. **Roulston, A., P. Beauparlant, N. Rice, and J. Hiscott.** 1993. Chronic human immunodeficiency virus type 1 infection stimulates distinct NF-κB/*rel* DNA binding activities in myelomonoblastic cells. J. Virol. **67:**5235–5246.
30. **Roulston, A., R. Lin, P. Beauparlant, M. A. Wainberg, and J. Hiscott.** 1995. Regulation of human immunodeficiency virus type 1 and cytokine gene expression in myeloid cells by NF-κB/Rel transcription factors. Microbiol. Rev. **59:**481–505.
31. **Schreiber, E., P. Matthias, M. M. Muller, and W. Schaffner.** 1989. Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells. Nucleic Acids Res. **17:**6419.
32. **Squires, R. F., and C. Braestrup.** 1977. Benzodiazepine receptors in rat brain. Nature **266:**732–734.
33. **Suzan, M., D. Salaun, C. Neuveut, B. Spire, I. Hirsch, P. Le Bouteiller, G. Querat, and J. Sire.** 1991. Induction of NF-κB during monocyte differentiation by HIV type 1 infection. J. Immunol. **146:**377–383.
34. **Watkins, B. A., H. H. Dom, W. B. Kelly, R. C. Armstrong, B. J. Potts, F. Ntchaels, C. V. Kufta, and M. Dubois-Dalcq.** 1990. Specific tropism of HIV-1 for microglial cells in primary human brain cultures. Science **249:**549–553.
35. **Zavala, F., J. Haumont, and M. Lenfant.** 1985. Interaction of benzodiazepines with mouse macrophages. Eur. J. Pharmacol. **106:**561–566.
36. **Zavala, F., V. Taupin, and B. Descamps-Latscha.** 1990. In vivo treatment with benzodiazepines inhibits murine phagocyte oxidative metabolism and production of interleukin 1, tumor necrosis factor and interleukin 6. J. Pharmacol. Exp. Ther. **255:**442–450.