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Short Communication

Propentofylline inhibits production of TNFα and infection of LP-BM5 murine leukemia virus in glial cells

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We examined the effects of a xanthine derivative, propentofylline, on TNFα production by glial cells and on infection of glial cells with a murine leukemia virus, LP-BM5, which induces murine AIDS in susceptible mice. Propentofylline suppressed TNFα production in glial cells and also effectively suppressed infection of glial cells with LP-BM5 *in vitro*. Addition of TNFα, but not IL-1 or IL-6, abolished the suppressive effects of propentofylline. Anti-TNFα antibody also suppressed infection of LP-BM5 in these cells. These findings suggest that propentofylline suppressed LP-BM5 infection in glial cells by suppressing TNFα production by these cells. Because propentofylline reportedly passes through the blood-brain barrier, it may be useful in the treatment of central nervous system involvement by HIV infection or neurological diseases in which TNFα plays a causative role, such as multiple sclerosis.

Keywords: TNFα; retrovirus; glia; microglia; mouse; LP-BM5; AIDS

Infection of C57BL/6 mice with a mixture of murine leukemia viruses (MuLV) LP-BM5 leads to the immunodeficiency syndrome called murine acquired immunodeficiency syndrome (MAIDS) (Mosier *et al*, 1985; Hartley *et al*, 1989). Infection of susceptible animals with LP-BM5 induces motor disturbance and cognitive dysfunction, suggesting that this infection model may be of use for studying the pathophysiology of human AIDS-dementia complex (Sei *et al*, 1992a). Sei *et al* (1992b) have shown that LP-BM5 infects astrocytes, and, in a preliminary study, we found that it also infects microglia *in vitro* when they are induced to proliferate with a macrophage colony-stimulating factor. We also found that glial cells were induced to produce various proinflammatory cytokines by LP-BM5 infection (Suzumura, 1993). Among these cytokines, TNFα has been shown to upregulate the expression of retroviruses, via activation of the transcription-activating factor NF-κB, which binds to the long terminal repeat (LTR) of human immunodeficiency virus (HIV) (Duh *et al*, 1989; Osborn *et al*, 1989). The suppression of TNFα successfully decreases the replication of HIV in peripheral blood mononuclear cells and T cells (Fazely *et al*, 1991; Maruyama *et al*, 1993) and also downregulates the expression of HIV in promyelocytic cell line (Peterson *et al*, 1992). Various agents are now used to suppress TNFα production and HIV infection. The xanthine derivative pentoxifylline, a type III phosphodiesterase inhibitor, is one of these agents and reportedly suppresses TNFα production via the elevation of intracellular cyclic AMP level in various cell types. It decreases the serum concentration of TNFα and TNFα mRNA expression in peripheral blood mononuclear cells in AIDS patients (Dezube *et al*, 1993), and also inhibits HIV replication by human microglia *in vitro* (Wilt *et al*, 1995). It also reportedly inhibits experimental allergic encephalomyelitis (EAE) (Nataf *et al*, 1993) in which TNFα is considered to play a causative role as an effector of demyelination (Selmaj *et al*, 1991).

In the CNS, TNFα is produced by both microglia and astrocytes (Sawada *et al*, 1989), and plays

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various roles to regulate the function, proliferation and in some cases degeneration of CNS cells (Lavi *et al*, 1988; Selmaj *et al*, 1990, 1991; Sawada *et al*, 1992). In this study, we examined whether or not another xanthine derivative, propentofylline, which has similar chemical features and similar phosphodiesterase inhibitor activities to pentoxifylline, also suppresses TNFα production by glial cells and whether or not the suppression of TNFα production by propentofylline or pentoxifylline results in the inhibition of LP-BM5 MuLV infection in glial cells.

Unstimulated microglia and astrocytes did not produce a detectable amount of TNFα in the culture supernatants when determined by cytotoxicity assay against L929 cells. When stimulated with LPS, both microglia and astrocytes produced significant amounts of TNFα. Higher doses, 10 μg/ml or more, of propentofylline significantly suppressed the production of TNFα in LPS-stimulated microglia and astrocytes ($P<0.001$) (Figure 1). Pentoxifylline used as a positive control also suppressed LPS-induced TNFα production in a dose-dependent manner. Even the lowest dose (1 μg/ml) of propentofylline and pentoxifylline significantly reduced TNF activity ($P<0.001$) in LPS-stimulated astrocytes. In contrast, these drugs did not significantly suppress the induction of IL-6, though they suppressed IL-1 production moderately (data not shown). The suppression of LPS-induced expression of cytokines was confirmed at the mRNA level examined by RT–PCR. Propentofylline and

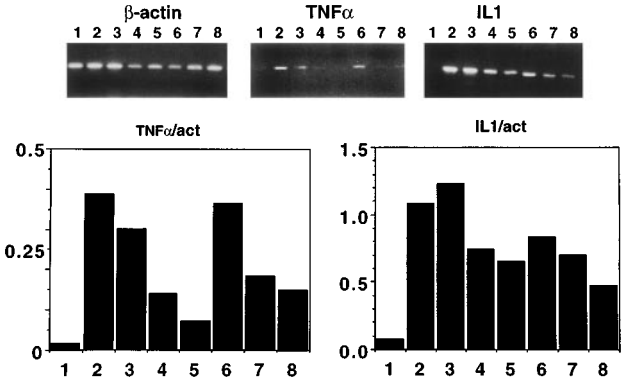

Figure 2 The expression of cytokine mRNA examined by RT–PCR method. Upper: RT–PCR analyses for expression of cytokine mRNA and β-actin in astrocytes treated with LPS and 1–100 μg/ml of propentofylline (Pro) and pentoxifylline (Pxf). 1; non-treated astrocytes, 2; astrocytes treated with 10 μg/ml of LPS, 3–5; LPS plus 1, 10 and 100 μg/ml of Pro, 6–8; LPS plus 1, 10 and 100 μg/ml of Pxf. Lower: Columns indicate mean values of the ratio of cytokine mRNA/β-actin mRNA calculated by each value obtained from the TIAS-200 image analyzer.

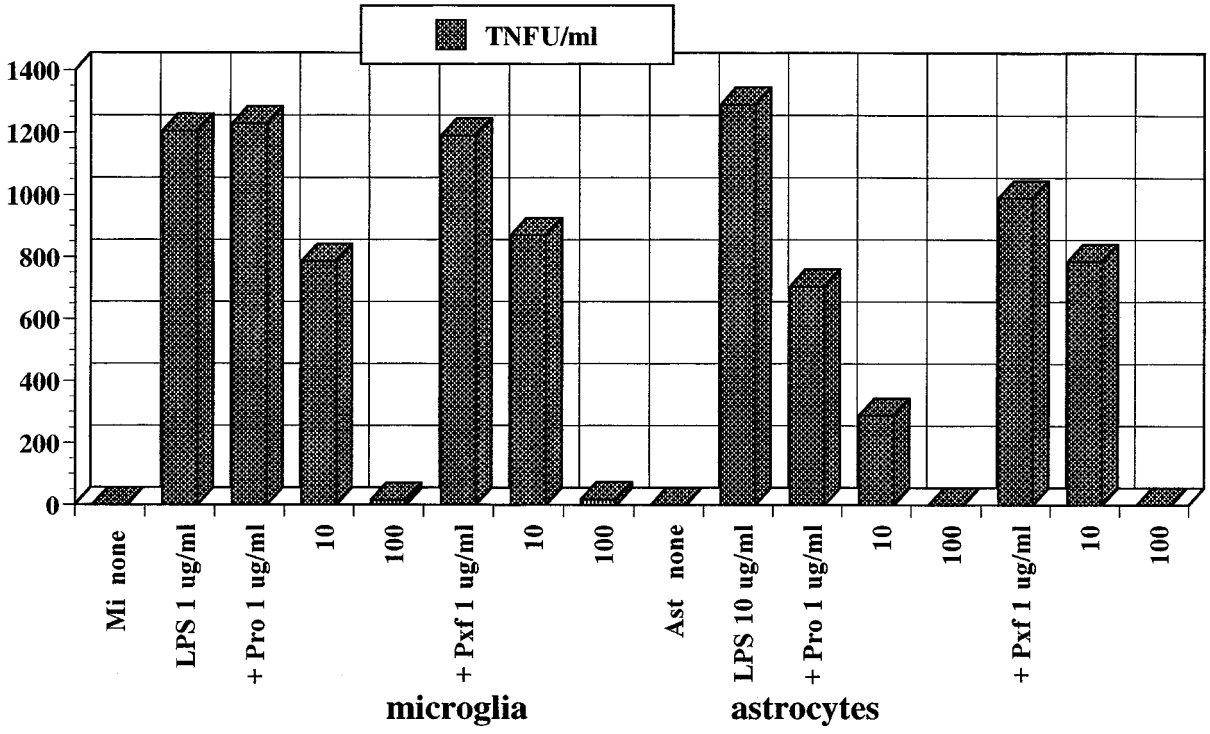

Figure 1 TNFα production by microglia and astrocytes; suppression by propentofylline and pentoxifylline. Microglia and astrocytes were treated with 1–100 μg/ml of propentofylline (Pro) and pentoxifylline (Pxf) in the presence of 1 μg/ml of LPS (10 μg/ml for astrocytes) for 24 h. Each column indicates mean value obtained from six samples (standard deviations were less than 10% of the mean).

pentoxifylline suppressed the ratio of cytokine mRNA to β actin mRNA in a dose-dependent manner (Figure 2).

Although productive infection of LP-BM5 was not observed in unstimulated microglia, ecotropic virus was recovered from cell lysate of microglia 7 days post-infection (p.i.). Both propentofylline (10–100 μg/ml) and pentoxifylline (1–100 μg/ml) completely suppressed the virus recovery from microglia (Figure 3a). In contrast, the productive infection was observed in astrocytes. Ecotropic virus titer increased at the 3rd p.i. day, maximized at 5 p.i. day. Propentofylline suppressed the infection of LP-BM5 in astrocytes in a dose-dependent manner at each time point examined (Figure 3b). Significant suppression ($P<0.001$) was noted in all the samples treated with 10–100 μg/ml propentofylline, and in samples treated with 1 μg/ml for 5 and 7 days. Similar significant suppression was observed in pentoxifylline treated astrocytes (Figure 3b). The expression of defective BM5d genome in astrocytes, was also suppressed by treatment with propentofylline and pentoxifylline (Figure 4). Addition of murine recombinant TNFα diminished the suppressive effects of propentofylline. The doses of 100 U/ml or more completely abolished the suppression by propentofylline, and 1000 U/ml TNF increased the viral recovery from the cultures at 5 and 7 days p.i. (Figure 5a). Addition of IL-6 or IL-1 at the same concentration

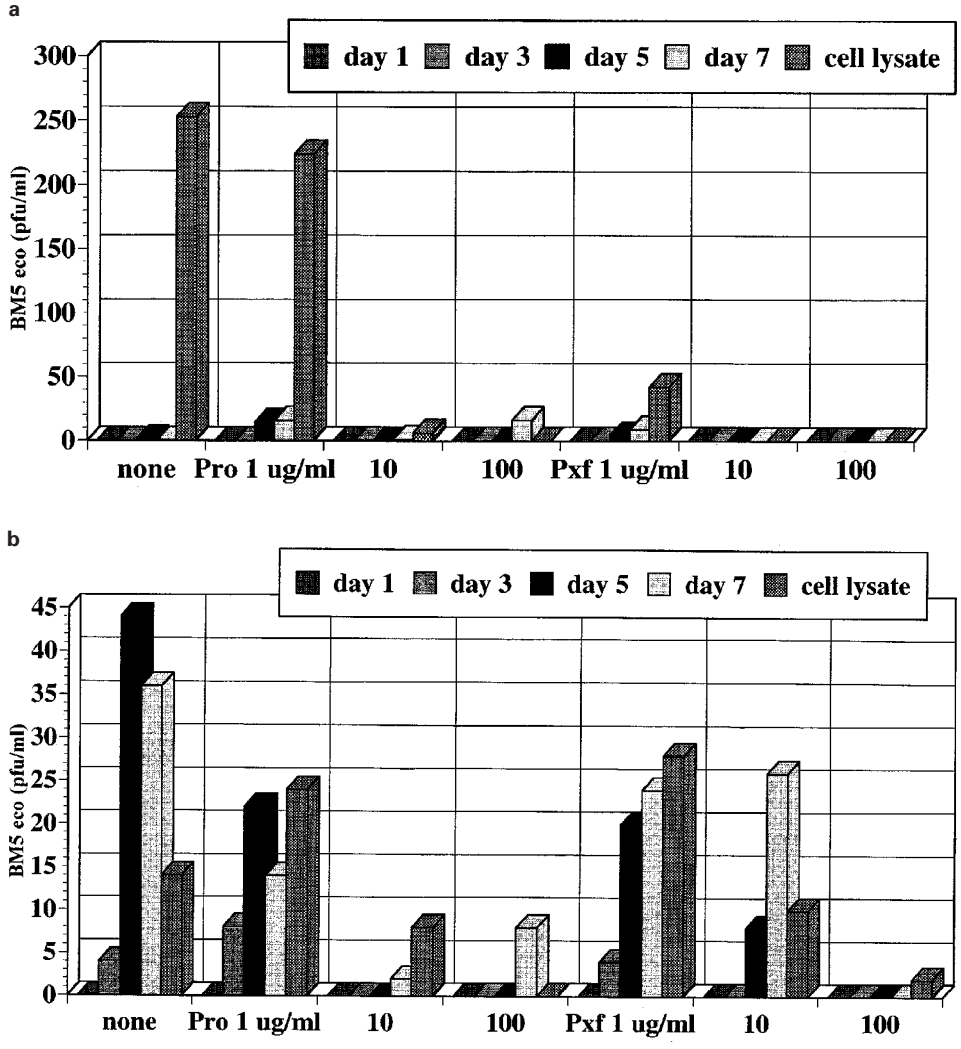

Figure 3 Suppression of LP-BM5 MuLV infection in microglia (**a**) and astrocytes (**b**). (**a**) Ecotropic virus titer could be detected only in the cell lysate of microglia 7 days post-infection. Ten and 100 μg/ml propentofylline (Pro) and 1–100 μg/ml pentoxifylline (Pxf) significantly suppressed the virus titer in the cell lysate ($P<0.001$ as compared to the virus titer of cell lysate in non-treated microglia). (**b**) Ecotropic virus titer in non-treated astrocytes increased on days 3, 5 and 7. Pro at the doses of 10 to 100 μg/ml significantly suppressed the viral recovery during the experimental period. Even 1 μg/ml Pro or, Pxf suppressed the maximum virus recovery significantly at days 3, 5 post-infection.

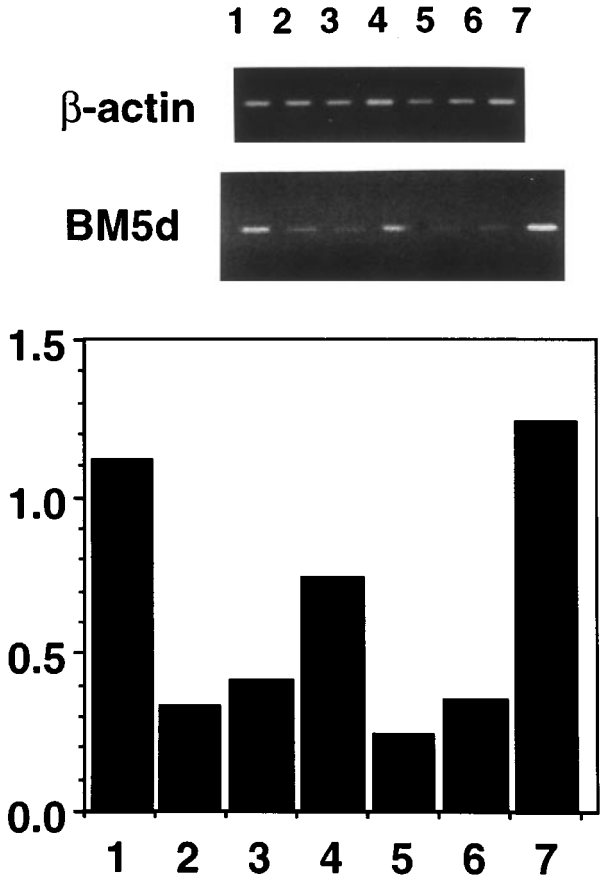

Figure 4 Suppression of the expression of BM5d defective viral genome. The expression of BM5d genome in non-treated astrocytes (1), treated with 100, 10, and 1 μg/ml of propentofylline (2–4) and 100, 10, and 1 μg/ml of pentoxifylline (5–7). Both drugs dose-dependently suppressed the expression of BM5d when examined by RT–PCR method. Lower columns indicate mean values of the ratio of BM5d/β-actin mRNA calculated by each value obtained from the TIAS-200 image analyzer.

did not alter the suppressive effects of propentofylline (data not shown). In addition, anti-TNFα antibody by itself also suppressed the productive infection of LP-BM5. The final concentrations higher than $1:10^6$ significantly suppressed viral recovery from infected astrocytes (Figure 5b). The expression of BM5d in these infected cultures was also suppressed by anti-TNFα antibody, and upregulated by addition of recombinant TNFα (Figure 6).

In a previous study, we have shown that both astrocytes and microglia produce TNFα in response to LPS stimulation (Sawada *et al*, 1989). We also found that LP-BM5 infection of glial cells induced production of cytokines including TNFα in glial cells (Suzumura, 1993). Although the precise roles of the produced TNFα in the CNS are yet unclear, it has been shown that TNFα induces proliferation of, the expression of major histocompatibility complex antigen and cytokine production in glial cells (Selmaj *et al*, 1990; Lavi *et al*, 1988; Sawada *et al*, 1992). TNFα exerts cytotoxic effects on oligodendrocytes and induces degradation of myelin *in vitro* (Selmaj *et al*, 1991), suggesting possible roles of TNFα on demyelinating processes. In fact, suppression of TNFα by anti-TNFα antibody inhibits the development of inflammatory demyelination in experimental allergic encephalomyelitis (Selmaj *et al*, 1991) and virus-induced demyelination (Inoue *et al*, 1996). In this *in vitro* study, we showed for the first time that propentofylline as well as pentoxifylline effectively suppressed TNFα production by glial cells and also suppressed productive infection of LP-BM5, as well as the expression of defective viral genome, BM5d, which is important for the development of MAIDS pathology. Addition of recombinant TNFα, but not IL-1 or IL-6, dose dependently diminished the suppressive effects of both drugs. Anti-TNFα antibody alone could suppress productive infection of LP-BM5 in astrocytes. These observations suggest that the modulation of a cytokine, such as TNFα, may affect the infection of murine AIDS virus in CNS cells. Since TNFα has been shown to increase HIV expression via activating the HIV LTR (Duh *et al*, 1989; Osborn *et al*, 1989), suppression of TNFα is considered to result in suppression of HIV replication. It is possible that propentofylline and pentoxifylline suppressed LP-BM5 infection in astrocytes by similar mechanisms. However, the precise role of TNFα on murine AIDS virus infection remains to be investigated.

Because propentofylline is shown to pass through the blood-brain barrier (Ohtake *et al*, 1990), it is possible that orally administered propentofylline could suppress the cytokine production by glial cells *in vivo*, and may be useful for the treatment of neurological disorders. It is currently unknown whether propentofylline exerts the above effects by the same mechanisms as does pentoxifylline. However, since propentofylline has similar phosphodiesterase inhibitor activity as that of pentoxifylline (Nagata *et al*, 1985), it is possible that propentofylline may also increase intracellular cyclic AMP resulting in inhibition of cytokine mRNA expression in glial cells.

Since propentofylline and pentoxifylline suppressed TNFα production by glial cells, they may also play a protective role against processes of demyelination. Both of these drugs have been safely used for the treatment and prevention of cerebrovascular diseases. However, the doses of propentofylline and pentoxifylline which significantly suppressed TNF production were higher than the maximum serum concentration observed in ordinary therapeutic use. The maximum serum concentration of propentofylline after the therapeutic dose of oral administration was 74.3 ± 51.9 ng/ml (Naka-

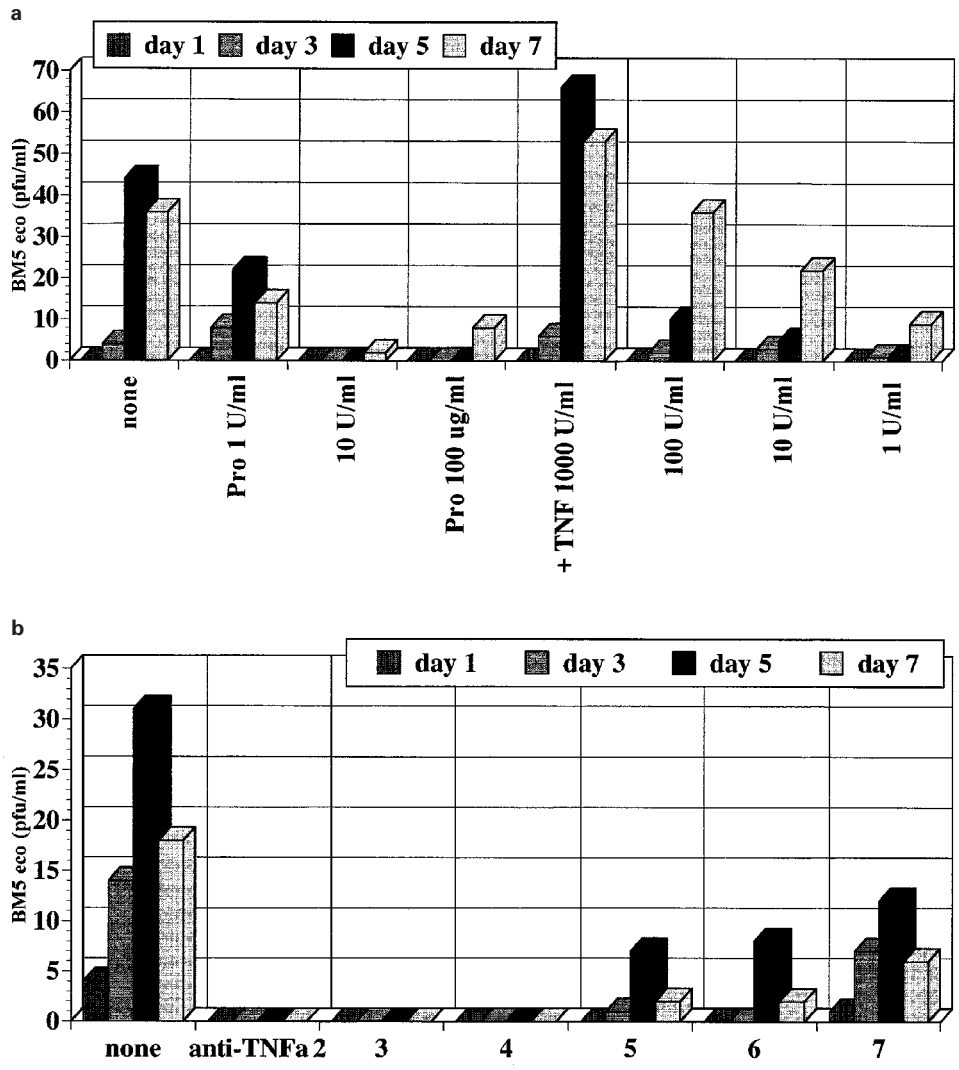

Figure 5 (**a**) Effect of recombinant TNFα on LP-BM5 infection in astrocytes. Addition of recombinant TNFα dose-dependently diminished the suppressive effects of propentofylline on LP-BM5 infection in astrocytes. 1000 U/ml recombinant TNFα completely abolished the suppressive effects of Pro, and increased the productive infection of astrocytes by LP-BM5. (**b**) Effect of anti-TNFα antibody on LP-BM5 infection by astrocytes. Anti-TNFα antibody by itself when applied to non-treated astrocytes at the final concentration of $1:10^2$ (2), $1:10^3$ (3), $1:10^4$ (4), $1:10^5$ (5), $1:10^6$ (6), and $1:10^7$ (7) suppressed productive infection of ecotropic helper virus.

jima *et al*, 1986). Thus, more than ten times the ordinary therapeutic dose is needed to suppress cytokine production in the CNS. Combination of these two drugs may decrease the doses of each drug. Further evaluation of these drugs is necessary before a clinical trial, but it is possible that these inhibitors of TNFα production in both peripheral mononuclear cells and glial cells may be useful for future therapeutic strategies against retrovirus-related CNS disorders, as well as demyelinating disorders.

Recombinant murine IL-1β, TNFα and human IL-6 were obtained from Genzyme (Boston, MA, USA). Propentofylline and pentoxifylline were provided by Hoechst Japan (Tokyo, Japan). *Escherichia coli* LPS was obtained from Difco (Detroit, MI, USA). LP-BM5 MuLV stocks were cell-free supernatants of SC-1 cells, containing C-type B-tropic retroviruses including ecotropic, mink cell focus-inducing MuLV (Harley *et al*, 1989), and a replication-defective genome (BM5d) that is required for induction of the disease (Aziz *et al*, 1989).

Microglia were isolated from the primary mixed glial cell cultures of newborn C57BL/6 mice on the 14th day, by the 'shaking off' method previously described (Suzumura *et al*, 1987); the purity of the cultures was 97 to 100% as

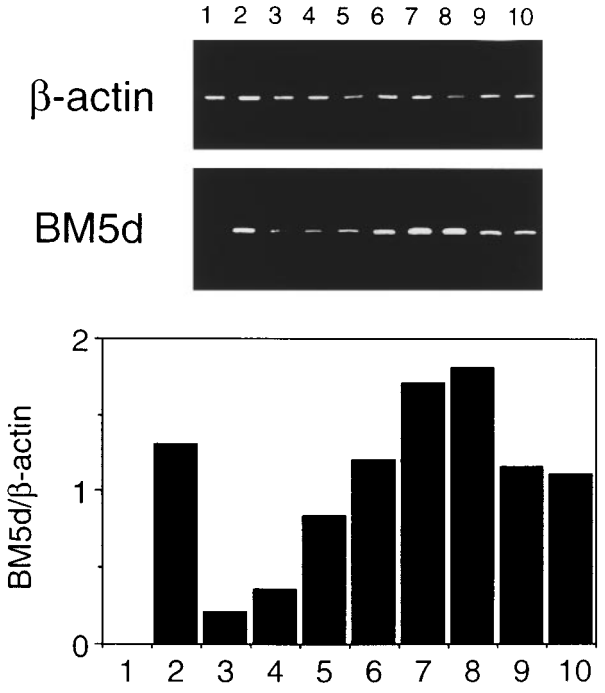

Figure 6 The effect of anti-TNFα antibody and recombinant TNFα and BM5d genome expression in astrocytes. Astrocytes, non-infected (1), infected with LP-BM5 for 7 days without anti-TNFα antibody (2), with anti-TNFα antibody at the concentration of $1:10^2$ (3), $1:10^3$ (4), $1:10^4$ (5), $1:10^5$ (6). Anti-TNFα antibody dose-dependently suppressed the expression of BM5d genome in astrocytes. Addition of recombinant TNFα at the concentration of 1000 U/ml (7), and 100 U/ml increased the expression of BM5d genome in the LP-BM5-infected astrocytes, while 10 U/ml (9) or 1 U/ml (10) had no effect. Lower columns indicate mean values of the ratio of BM5d/β-actin mRNA calculated by each value obtained from the TIAS-200 image analyzer.

determined by immunostaining of Fc receptor (Suzumura *et al*, 1987). Astrocyte-enriched cultures were prepared from the primary mixed glial cell cultures by repetitive exposure to trypsin and replating; the purity of the cultures exceeded 95% as determined by indirect immunofluorescence staining with antibodies to glial fibrillary acidic protein.

In order to see the effects of propentofylline on cytokine production by glial cells, microglia and astrocyte-enriched cultures were plated in 2.5 cm diameter culture dishes (Falcon 3001, Beckton Dickinson, Lincoln Park, NJ, USA) at a density of 5×10^5/ml in Eagle's minimum essential medium supplemented with 10% fetal calf serum, 5 μg/ml insulin and 0.2% glucose and incubated for 24 h in the absence or presence of LPS (1 μg/ml). One to 100 μg/ml of propentofylline or pentoxifylline were applied to these cultures. In the preliminary experiments, we confirmed that the two drugs at these concentrations did not alter the morphology and survival or proliferation of glial cells. The cell supernatant was then collected and stored at −70°C until monokine activities were assayed. Total RNA was isolated from the cells and the expression of cytokine mRNA was analyzed by RT–PCR method as described (Sawada *et al*, 1992). TNF, IL-1 and IL-6 activities were determined by bioassay as described (Sawada *et al*, 1989, 1992).

Effects of propentofylline and pentoxifylline on infection of LP-BM5 in glial cells was examined using microglia and astrocyte-enriched cultures. They were infected with a 40 μl/dish of stock LP-BM5 mixture (containing $10^{4.4}$ plaque forming unit per ml of ecotropic, $10^{2.5}$ focus forming unit per ml of mink cell focus-inducing MuLVs) with 2 μg/ml of polybrene. At the same time, 1–100 μg/ml of propentofylline and pentoxifylline were applied to these cultures and cultured for 24 h. Supernatants were then collected and the fresh culture medium containing the same amount of propentofylline or pentoxifylline were added. Similarly, the supernatant was collected on days 3, 5 and 7. After collecting the supernatant at day 7, cells were washed three times with PBS, collected by rubber policeman and sonicated to get cell lysate. Titers of ecotropic MuLV were determined in SC-1 cells by the XC plaque test (Rowe *et al*, 1970). BM-5 defective genome was also examined in microglia and astrocyte 24 h after inoculation of LP-BM5 by RT–PCR method described above. The sense and antisense primers were as follows: BM5d sense: 5′-ATTCCGCCCCTTTCCCCTGA, antisense: 5′-CCTGTCTATCCTGTCTCTGA (Aziz *et al*, 1989).

Graded doses of TNFα were applied with the maximum dose of propentofylline to see whether addition of TNFα abolished the suppressive effects of propentofylline. In some experiments, antibody against TNFα was applied instead of propentofylline. Then the supernatants were collected and assayed for the virus titer as indicated above.

All experiments were performed at least in triplicate. Data are presented as means ± s.d. and were analyzed by Student *t*-test. A P value of < 0.05 was considered statistically significant.

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