Journal of NeuroVirology (1997) 3, 28–37

http://www.jneurovirol.com

Establishment and characterization of conditionally immortalized astrocytes to study their interaction with *ts*1, a neuropathogenic mutant of Moloney murine leukemia virus

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The cytopathic infection of primary astrocytes with *ts*1, a neuroimmunopathogenic mutant of Moloney murine leukemia virus (MuLV), has been correlated to intracellular accumulation of viral precursor envelope protein gPr80^{env}. To further study this specific virus-astrocyte interaction in a homogenous population, several immortal astrocyte lines were established from neonatal FVB/N mice using the temperature-sensitive SV40 tsA58 T antigen. These cells expressed glial fibrillary acidic protein, vimentin and T antigen; appeared nontransformed; were star-shape with long processes. They were susceptible to *ts*1 infection and suffered a cytopathic effect similar to that caused by *ts*1 infection of primary astrocytes. This cytopathic effect was characterized by growth inhibition, loss of cell processes and syncytium formation. Some cells also rounded up, formed mini cells and became detached from the culture dish. As in primary astrocytes, the processing of gPr80^{env} in the immortalized astrocytes was inefficient. Since the envelope proteins interact with the ecotropic MuLV receptor both intracellularly and on the cell surface and since the receptor has been shown to be an arginine transporter, we attempted to determine the effect of *ts*1 on arginine uptake by these cells. Our results showed that in both immortalized and primary astrocytes, *ts*1 infection reduced the uptake of arginine more than did wild-type virus infection. Since arginine localizes predominantly in astrocytes in the CNS and has diverse functions, the decrease of arginine uptake in *ts*1-infected astrocytes may alter the metabolism of these cells, leading to impairment of their functions.

Keywords: astrocytes; retrovirus; envelope processing; arginine uptake

Introduction

*ts*1, a neuropathogenic and lymphocytopathic mutant of Moloney murine leukemia virus (Mo-MuLV), induces a progressive neuroimmunodegenerative disease in susceptible strain of mice which clinically is manifested as hindlimb paralysis, wasting and severe immunodeficiency (reviewed in Wong, 1990; Wong and Yuen, 1992, 1994). The prominent pathological features in the central nervous system (CNS) are spongiform degeneration, neuronal cell loss, demyelination, and gliosis without inflammatory cell infiltration (Wong and Yuen, 1992, 1994). One of our major goals is to elucidate the molecular and cellular events leading to the neurodegenerative disease induced by this virus as a model for human neurodegenerative diseases such as encephalomyelopathy, Cruetzfeldt-Jakob disease, amyotrophic lateral sclerosis, AIDS dementia, scrapie and 'mad cow' disease.

We have previously reported that the neuropathogenic determinants of *ts*1 map to two single-amino-acid substitutions, a Val-to-Ile substitution at position 25 and an Arg-to-Lys substitution at position 430 of the envelope protein (Szurek *et al*, 1988; 1990b). The former substitution renders the precursor envelope protein gPr80^{env} relatively inefficient for transport from the endoplasmic reticulum (ER) to the cell surface, whereas the latter substitution enhances the ability of the *ts*1 virus to replicate in the CNS (Szurek *et al*, 1988, 1990a). In

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Received 9 May 1996; revised 6 August 1996; accepted 27 August 1996

the CNS, the virus is localized in capillary endothelial cells, astrocytes, and microglial cells and also in the extracellular space around spongiform lesions (Stoica *et al*, 1993). Neurons appear not to replicate the virus, although they show most of the severe pathological effects associate with the disease. The earliest lesions within the CNS target areas are characterized by the swelling of astrocytic foot processes around capillaries and neuronal postsynaptic sites. As the disease progresses, vacuolation within astrocytic and neuronal soma in the affected area also occurs. In addition, mitochondrial dissolution and vesicular enlargement of the Golgi complexes and ER are also observed in some astrocytes (Stoica *et al*, 1993). The observations suggest that astrocytes are the primary target of the virus infection with resultant cytopathic effect. The impaired astrocytes may be unable to perform their crucial supportive and protective functions for the nearby neurons, which may in part contribute to neuronal death.

We have also previously shown that infection of primary cultures (PC) of astrocytes with *ts*1 was cytopathic, whereas infection of primary cultures of endothelia is not (Shikova *et al*, 1993). Furthermore, the cytopathic effect of *ts*1 in primary astrocytes correlates with the intracellular accumulation of precursor envelope proteins $gPr80^{env}$ and aberrant virus particles. These data suggest that *ts*1 and astrocytes may interact in a specific manner. However, studies of specific virus-astrocyte interactions in PC are limited by the heterogeneity of the primary cell populations and the limited amounts of tissue available to obtain the cells. Furthermore, a major problem with using primary astrocyte cultures for mechanistic studies is that these cultures are usually contaminated with microglia, which produce many cytokines and other factors. Since this could complicate the interpretation of the results, we tried to circumvent this problem by cloning immortalized cell lines each of which is derived from a single cell and thus provide unlimited amounts of cells from a homogeneous astrocyte population. We therefore attempted to establish immortalized astrocyte cell lines from FVB/N mice, the strain of mouse most susceptible to *ts*1 infection (Wong *et al*, 1991). Cellular preparations of highly enriched astrocytes from the neocortex and brain stem of FVB/N mice were infected with a retrovirus vector containing DNA sequences of the temperature-sensitive mutant tsA58 of SV40 large T antigen (tsA58 TAg) (Jat and Sharp, 1989). An advantage of using the tsA58 TAg is that the mitogenic drive of the T antigen can be removed by simply shifting the incubation temperature of the established astrocyte culture from the permissive (33–34°C) to the restrictive (37–38°C) temperature, which thus allows the cells to differentiate normally (Noble *et al*, 1995).

Here we report the establishment and characterization of immortalized astrocyte clones. All the clones tested appeared to express glial fibrillary acidic protein (GFAP) and display the astrocytic morphology. More important, they resembled primary astrocyte cultures infected with *ts*1 in being susceptible to *ts*1 infection and suffering the same resultant cytopathic effect. One of these immortal astrocytic lines, C1, was used to study the specific *ts*1-astrocyte interactions reported here.

Results

Characterization of immortalized astrocyte clones

To develop immortalized astrocytic lines, cellular preparations of highly enriched primary astrocytes from the cerebral cortex and brain stem of FVB/N mice were infected with a retrovirus carrying the gene of the tsA58 T antigen. After 2 weeks of

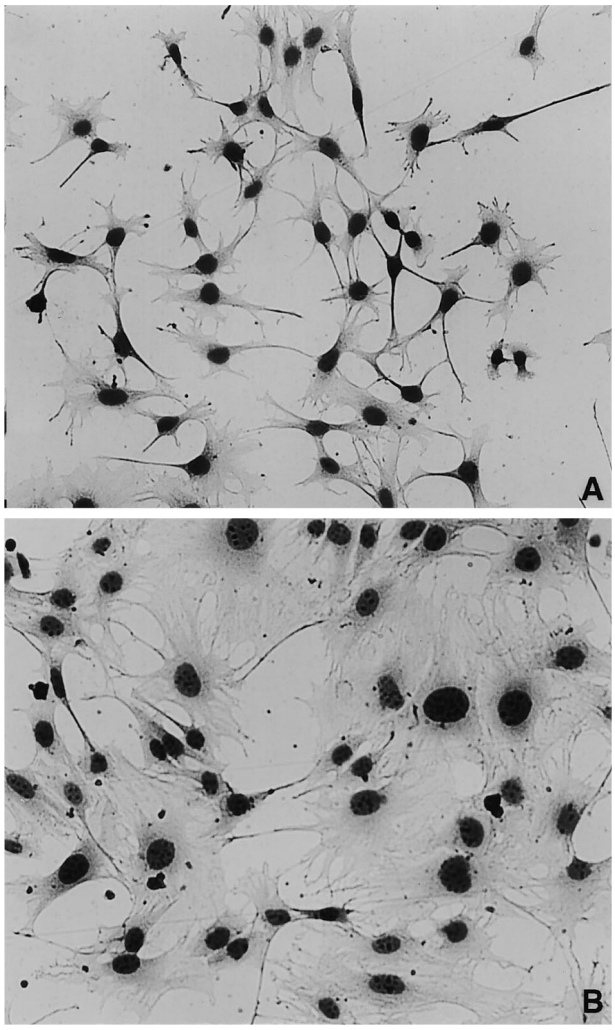

Figure 1 Morphology of two representative astrocyte cell lines: (A) C1 and (B) C6. Both were stained with Giemsa.

selection, six immortal astroglial clones (C1, 2, 7, 11, 14, 15) from cortex and two (B1, 5) from brain stem were established. All the astrocyte clones grew indefinitely *in vitro*. They formed uniform monolayers, were contact inhibited and were anchorage dependent. Although each cell clone varied somewhat in size and morphology, all clonal cells were star-shaped with extended processes and network. Figure 1 shows the morphology of cells from two typical clones. Note the star shape and elongated processes characteristics of astrocytes.

The expression of T antigen was also positive in these clones. Figure 2 shows the analysis of T antigen expression by Western blotting of two of these cell clones (C1 and C11) and the positive (K1–30) as well as negative (TB and PC) controls. Expression of GFAP in these clonal cell lines was also determined. As shown in Figure 3a, all clones expressed GFAP as detected by Western blot analysis. The expression of GFAP is further studied in one of these clonal cell lines, C1. We observed that the levels of GFAP expression in C1 cell line could be enhanced in response to dibutyryl cyclic AMP (dBcAMP) or when the culture became confluent and mature or by a shift in the incubation temperature from 34°C to 37°C (Figure 4). Together these data suggested that the immortalized cells retained the normal differentiation characteristics of astrocytes when stimulated by dBcAMP (Hertz, 1990) or when growth-arrested by contact inhibition (Galiana *et al*, 1990) or when responding to a decrease in the mitogenic activity of large T antigen caused by a temperature shift to restrictive temperature (Whittemore *et al*, 1994). All cell lines also stained positive for GFAP immunochemically. Figure 3b presents the result from one of these cell lines (C1). All the cell clones tested were stained positive for vimentin and negative for galactocerebroside, synaptophysin, mac-1 and DiI-ac-LDL (data not shown). Thus, our immunochemical data for the astrocyte clones are consistent with astroglial cells from the brain.

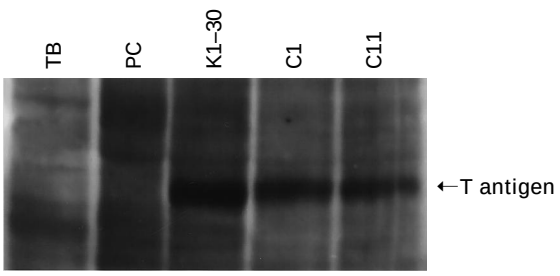

Figure 2 Western blot analysis of T antigen expression. Cells were plated and grown for 1 week before lysis with protein lysis buffer. Cell lysates (50 μg/per well) were subjected to SDS–PAGE and then transferred to nitrocellulose membrane. Blot was probed with anti-T antigen (1 μg/ml).

Infection of immortalized astrocyte clone C1 with ts1 or wild type (WT) MoMuLV

All astrocyte clones so far tested could be productively infected by *ts*1 MoMuLV although the amount of virus produced from different clones varied (Table 1). Among the clones tested, *ts*1-infected C1 cells had the highest proportion of dead cells at 3 to 5 days post infection (data not shown). The C1 was therefore chosen for further characterization and study. As shown in Figure 5, the growth curve of *ts*1- and WT-infected C1 astrocytes during the first day post infection (dpi), were relatively similar. By 2 dpi, both cultures showed retarded growth when

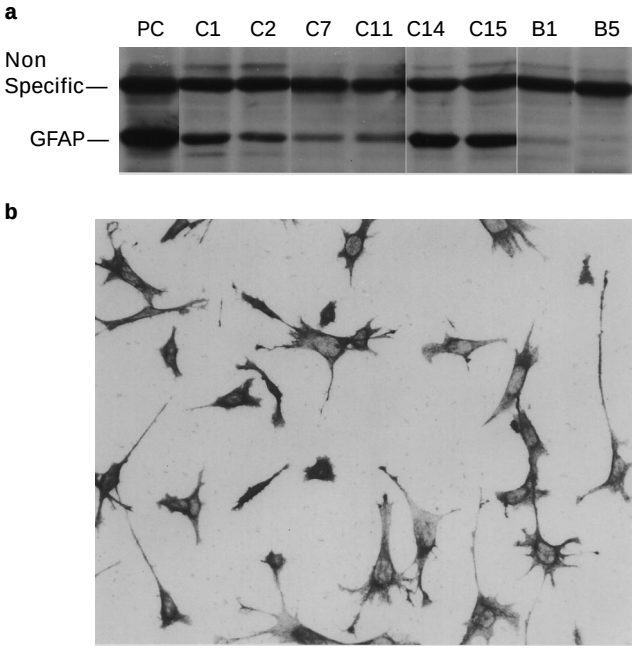

Figure 3 Expression of GFAP. Western blot analysis was as described in Figure 2. (**a**) Analysis with Western blot in primary culture and in different immortalized astrocyte cell lines. The blot was probed with anti-GFAP (1 : 2000 dilution). (**b**) Immunocytochemical staining of C1 cell lines with anti-GFAP (1 : 200 dilution).

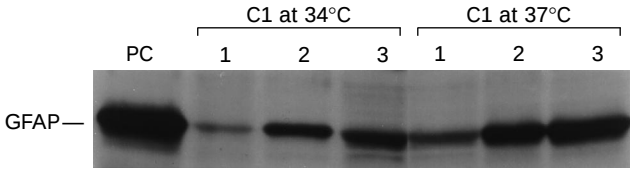

Figure 4 Regulation of GFAP expression in C1 cells by dibutyryl cyclic AMP, temperature, and cell confluency. Western blot analysis was as described in Figure 2. For subconfluent culture, cells were plated and grown for 7 days at a density of 2×10^5 cells/60 mm dish before lysis in lysis buffer. For confluent culture, 6×10^5 cells were plated. (1) subconfluent cultures (2) subconfluent cultures + dBc AMP (1 mM), (3) confluent cultures.

compared to an uninfected control. However, by 3 dpi, when the cytopathic effect became evident, the number of viable cells in *ts*1-infected C1 was several times less than in WT-infected C1. The cytopathic effect was manifested by shrinkage of processes, syncytium formation, and rounding up of cells (Figure 6), uninfected C1 cells did not show this (Figure 1a). By 5 dpi, the number of *ts*1-infected cells was 10-fold less than that of uninfected controls. These results were consistent with our previous findings in primary astrocyte cultures (Shikova *et al*, 1993) the astrocytes are the target of *ts*1 and WT MoMuLV and that *ts*1 apparently causes more severe cytopathic effect than WT in astrocytes. However, in both *ts*1- or WT-infected astrocyte cultures, a portion of the cells survived and upon passage became resistant to the virus-infected cell-death.

Table 1 Titers of virus produced by different immortalized astrocyte clones infected with *ts*1

Cell clones	*Virus titer* IU/ml* 3 dpi	5 dpi
C1	7.5×10^5	1.8×10^6
C2	2.3×10^5	7.0×10^5
C11	2.0×10^6	5.0×10^6
C15	1.6×10^6	2.5×10^6

*Titers of virus were determined by the 15F assay at 34°C. Titers are the average of two experiments.

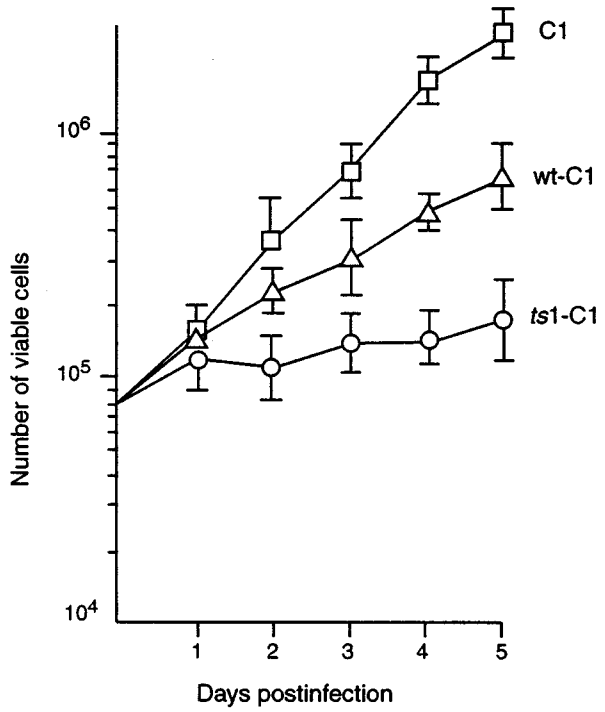

Figure 5 Survival curves of cultured C1 after WT and *ts*1 virus infection. Cell cultures were infected as described in Materials and methods. At the indicated times, viable cells were counted in a hemocytometer by trypan blue exclusion. Data are mean ± standard deviation from three experiments.

Processing of gPr80^{env} in ts*1-infected C1 astrocytes*
We have previously shown that *ts*1 is relatively inefficient in processing gPr80^{env} to gp70 and p15E in primary astrocyte cultures (Shikova *et al*, 1993). We therefore attempted to determine whether a similar effect occurs in immortalized astrocytes. As shown in Figure 7, processing of gPr80^{env} in *ts*1-infected C1 astrocytes, as in *ts*1-infected primary astrocytes, was relatively less efficient than in TB cells (the degree to which gPr80^{env} was processed into gp70 is indicated by the intensity of the gp70 band). Also, the processing of gPr80^{env} in the *ts*1-infected C1 was less efficient than that in the WT-infected C1 cells similar to our observation in PC. So far, in all the astrocytic clones tested, the processing of gPr80^{env} of *ts*1 has been inefficient, although this

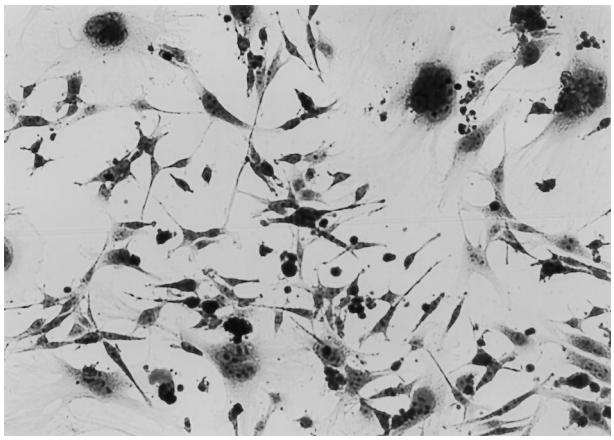

Figure 6 Cytopathic effects in *ts*1-infected C1 cells. Cells were infected as described in Materials and methods. The infected cells were incubated at 37°C for 4 days. Cell cultures were then fixed, stained with Giemsa, and photographed (For uninfected control referred to Figure 1A).

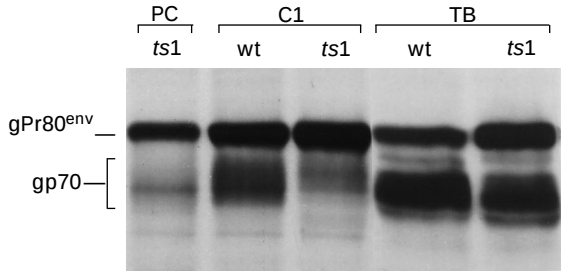

Figure 7 Western blot analysis of the processing of gPr80^{env}. The cells were infected and grown as described in Materials and methods for 5 days before lysed with lysis buffer. Thirty μg of cell lysate were subjected to SDS–PAGE and transfer. Anti-gp70 (1 : 5000 dilution) was used to analyse the processing of precursor gPr80^{env} to gp70.

result might vary somewhat from clone to clone (data not shown). Overall, these results showed that the processing of $gPr80^{env}$ is not efficient in astrocytes.

Amino acid uptake in primary and immortalized astrocyte cultures infected with ts*1 and WT*

The murine cationic amino acid (arginine and lysine) transporter (MCAT-1) has been shown to act as the cellular receptor for ecotropic MuLV (White, 1981; Kim *et al*, 1991). Moreover, infection of mouse cells with ecotropic MuLV result in a partial downregulation of receptor expression on the cell surface (Wang *et al*, 1992). We therefore attempted to evaluate the possible effect of *ts*1 infection on the uptake activity of MCAT-1 in astrocytes. Arginine uptake was measured by uptake assay in both infected and uninfected primary astrocyte culture and C1 cells with leucine uptake in infected and uninfected cells as a control.

In our assay system, uptake of arginine or leucine was linear for at least 3 min at 37°C (data not shown). This linear uptake, however, cannot differentiate between binding or transport. To measure binding in these cells, we have carried out the uptake experiment at 4°C to minimize the transport activity which is temperature dependent. Under this condition, only about 0.57% of the total amount observed at 37°C was bound. Therefore, >99% of the uptake at 37°C is due to active transport. The minimal contribution of binding to the total uptake activity was also the result of extensive washing with large volume of ice cold buffer in this assay (see Materials and methods). Under this condition, the majority of the labelled arginine has either been already transported inside the cells or is washed off the cells.

As shown in Figure 8, arginine uptake of WT-infected PC was about 16% less than in uninfected controls. However, arginine uptake in *ts*1-infected PC was about 31% less than in controls. This indicated that *ts*1 downregulated more uptake activity than WT. In the case of leucine uptake, no significant changes were observed. We further examined the uptake activity of *ts*1-infected C1 cells. As shown in Figure 8, uptake of arginine in *ts*1-infected C1 cells was about 54% less than control and about 16% less than WT infected C1 cells. The leucine uptake in *ts*1 infected-PC and C1 cells was not significantly lower than WT infected PC and C1 cells.

To support our observation that reduction in arginine uptake is not due to virus-mediated cell killing, we carried out time point experiments to compare uptake of leucine and arginine in *ts*1-infected and control C1 cells. As shown in Figure 9, the uptake of leucine in *ts*1-infected cells remained fairly closed to that of uninfected control at all time points up to 5 days post-infection. In contrast, at day 1 post infection, arginine uptake in *ts*1-infected C1

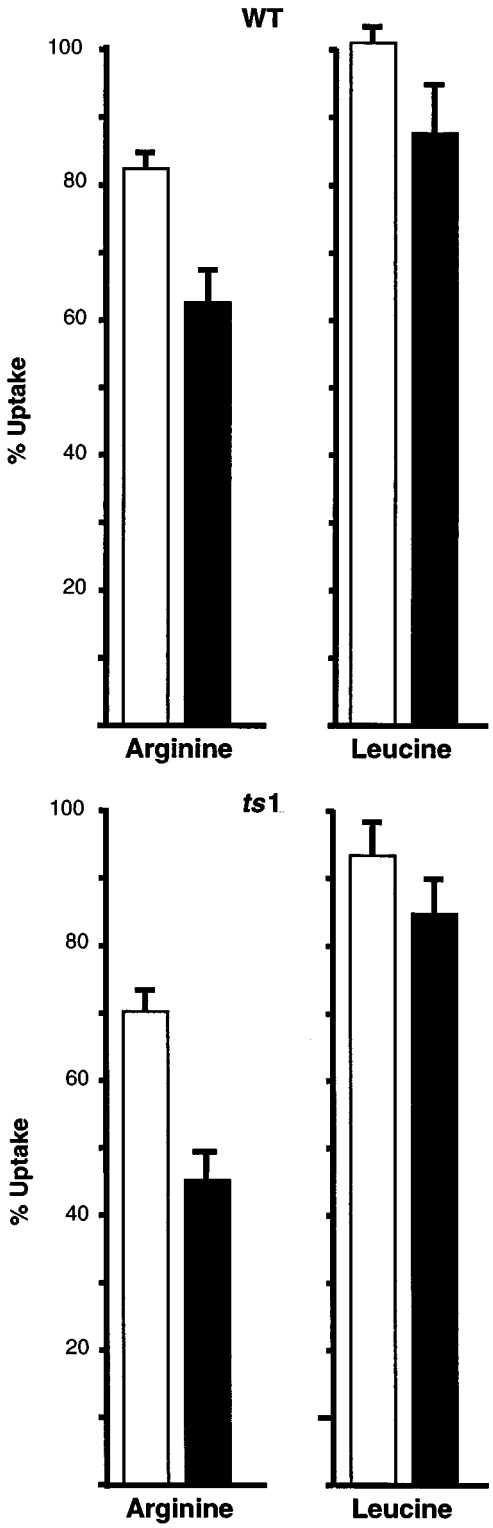

Figure 8 Effect of *ts*1 and WT infection on the uptake of L-arginine and L-leucine in primary astrocyte culture and C1 cells at 5dpi. Detailed transport assay was described in Materials and methods. Data are means ± standard deviations from three independent experiments. □, Primary astrocyte cultures; ■, immortalized C1 cultures. Uptake by uninfected control is considered as 100%.

cells was already reduced to about 75% of that of the control. At one day time point, cell death was not apparent and the number of viable cells in *ts*1-infected culture was not significantly different when compared to control cultures.

Although increasing cell death was observed in virus-infected C1 cells at day 3 and 5 post infection, the data presented in Figures 8 and 9 for arginine and leucine uptake were primarily by living cells. By trypan blue exclusion assay, we observed that in both infected and uninfected cultures, living cells are mainly the cells which remained adherent on the plastic plates after the extensive washing and buffer change before lysis for scintillation count and protein content assay as described in Materials and methods. Furthermore, to ensure that the protein content that we used to standardize the assay corresponds to the number of living cells, we counted the number of viable cells from the *ts*1-infected and the uninfected cultures and determined the protein content from the same number of cells from each culture at 3 to 5 days post infection. Our results (data not shown) indicate that the protein content is closely correlated to the number of cells from each of these cultures at different days pi. Taken together, the above observations indicate that the dramatic decrease in arginine uptake in *ts*1-infected C1 cells is unlikely the result of virus-mediated cell death but truly reflects the decreased ability of *ts*1-infected cells to take up arginine.

In addition to primary astrocyte cultures and C1 cells, arginine uptake was also measured in other astrocyte clones with *ts*1 infection or without *ts*1 infection. Reduction of arginine uptake was observed in all *ts*1-infected clones examined albeit the degree of reduction varied from clone to clone (Table 2). Among the five clones examined, arginine uptake in *ts*1-infected C1 astrocytes is most reduced. This may explain the greater extent of arginine uptake inhibition of C1 by *ts*1 infection when compared to that of *ts*1-infected primary culture which consists of a heterogeneous population of astrocytes.

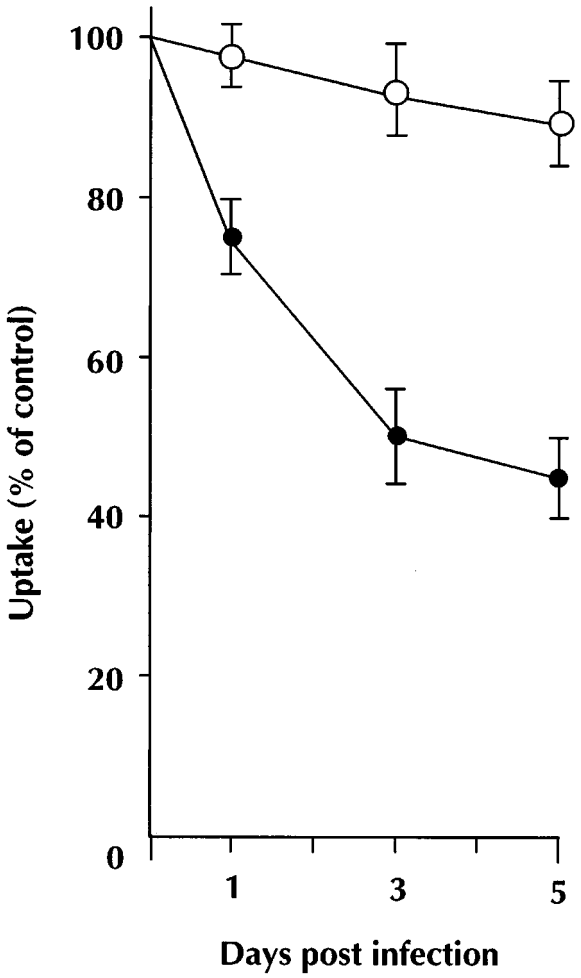

Figure 9 Effect of *ts*1 infection on the uptake of L-arginine and L-leucine in C1 cells at 1, 3, 5 dpi. Transport assay was described in Materials and methods. Data are means ± standard deviations from three independent experiments. ○, uptake of L-leucine; ●, uptake of L-arginine.

Discussion

Astrocytes are considered to be intimate partners with neighbouring neurons, providing nutrients and growth factors and aiding in ion regulation as well as neurotransmission (Hertz, 1993; Shao and McCarthy, 1994; Hansson and Rönnbäck, 1995). On the other hand, astrocytes are immunocompetent, responding to insults and stimuli by expressing cytokines and MHC molecules that may interact with microglia in the CNS to coordinate the immune response (Mucke and Eddleston, 1993). Since astrocytes are susceptible to *ts*1 infection *in vivo* (Stoica *et al*, 1993) and *in vitro* (Shikova *et al*, 1993), it is important to define what roles these cells play in the neuropathology induced by *ts*1. The establishment of immortalized cell lines reported here now affords us the means to prepare homogeneous cells in sufficient numbers for a variety of molecular and cellular studies involving interaction of the *ts*1 retrovirus and astrocytes.

As our present results show, our immortalized astrocyte lines not only display the characteristics of primary astrocyte cultures but also, when

Table 2 Effect of *ts*1 infection on arginine uptake in different astrocyte clones

	Percent of arginine uptake of control				
Virus	*C1*	*C2*	*C11*	*C15*	*B1*
*ts*1	45.6±4.0	61.3±3.9	54.0±2.2	55.5±2.1	60.1±5.0

infected with *ts*1, correlate well in many ways with *ts*1 infected primary astrocyte cultures. More important, our results clearly demonstrate that *ts*1 infection of astrocytes results in the structural and functional alteration of these cells.

Our results on the reduction of arginine uptake in the primary and immortalized astrocytes suggest that this reduction is most likely modulated by virus infection probably through the interaction between envelope proteins and receptors intracellularly or on the cell surface. These results also showed that *ts*1 apparently interfered with the activity of the arginine transporter more than did the WT in astrocytes. In addition, our results also showed that the downregulated arginine uptake by *ts*1 in immortalized astrocytes appeared to be more than in primary astrocytes. This could be due to the heterogeneity of the primary cultures. The heterogeneity itself could be due to a mixture of different cell types (e.g., astrocytes mixed with microglia) or a mixture of astrocyte subtypes in the cultures. We observed that among five clonal cell lines of astrocytes, the reduction of arginine as a result of *ts*1 infection varied from clone to clone. C1 cells, however, showed the most reduction. This may explain why reduction of arginine uptake in *ts*1-infected C1 cells was more than that observed in *ts*1-infected PC.

We previously postulated that specific virus-astrocyte interactions lead to inefficient transport and intracellular accumulation of gPr80^{env}, which could damage astrocytes (Shikova *et al*, 1993). Consistent with our previous findings, we observed a high level of virus expression together with an inefficient processing of gPr80^{env} in immortalized astrocytes in this study. We also previously observed, by confocal microscopy, that the perinuclear accumulation of gPr80^{env} of *ts*1 was localized around the ER of astrocytes (Shikova *et al*, 1993). In addition, the unprocessed gPr80^{env} continued to associate with the cellular protein immunoglobulin heavy-chain binding protein (Lin and Wong unpublished data), a known ER chaperone (Munro and Pelham, 1986). Although the association of envelope protein with ecotropic retrovirus receptor has not been directly demonstrated in *ts*1-infected cells, MuLV infection in another system has been shown to interfere with the arginine transport activity (Wang *et al*, 1992). We further hypothesize that the accumulation of *ts*1 envelope proteins in the ER might lead to formation of complexes with the receptor and thus downregulate the expression of the arginine transporters on the cell surface. A similar observation was made by Crise and coworkers (Crise *et al*, 1990; Crise and Rose, 1992) who noted that intracellular HIV gp160-CD4 complexing downmodulated the expression of CD4 at the cell surface. More recently, arginine uptake by the transport system y+ has been demonstrated in primary astrocyte cultures, suggesting that similar MCAT-1 viral receptors exist in astrocytes (Schmidlin and Wiesinger 1994, 1995). Our present data showing that the arginine uptake of both primary and immortalized astrocytes was downregulated by WT and *ts*1 infection, albeit to different degrees, are consistent with the notions that both the *ts*1 virus and MoMuLV use the same arginine transporter of astrocytes as viral receptor. *ts*1 infection appears to reduce the uptake of arginine further than does WT infection. Since *ts*1 gPr80^{env} is less efficient in transport and processing upon synthesis than WT gPr80^{env}, if gPr80^{env} forms complex with MCAT-1 in the ER, it may explain why there is larger reduction of arginine uptake in *ts*1-infected than WT infected astrocytes.

Arginine is an important part of many cellular processes. It is required for normal growth and development in growing animals (Borman *et al*, 1946). In certain circumstances, such as severe stress or trauma, it becomes indispensable for adequate nitrogen balance and physiologic responses (Kirk and Barbul, 1990). Arginine also has multiple potent effects on hormonal secretagogue activity, protein catabolism and immune responses (Barbul, 1990a, b). Arginine can also be converted to ornithine, which is further involved in polyamines, collagen and glutamate synthesis. Polyamines and collagen have been shown to associate with cellular proliferation, differentiation as well as tissue repair.

The effects of arginine on specific cell types like neurons, astrocytes and microglia have not been carefully studied. However, in recent years the arginine-nitric oxide (NO) pathway with special emphasis on NO, has been intensely investigated in the CNS (Moncada and Higgs, 1993; Kerwin and Heller, 1994). Arginine is the only substrate for NO synthase to produce NO. Nitric oxide is considered a very important messenger in the brain as well as many other systems and has been implicated in many of normal physiological functions and disease pathogenesis. Arginine/NO are associated with signal transduction, neurotransmission and cytostasis. NO can also generate an inhibitory effect on replication virus and other infectious agents (Karupiah *et al*, 1993; Moncada and Higgs, 1993; Akarid *et al*, 1995).

In summary, arginine as well as its products are important in the study of *ts*1 pathogenesis in mice because the course of the disease covers the fast developmental stage of growing mice. Since arginine is predominantly localized in astrocytes in the CNS (Aoki *et al*, 1991; Pow, 1994), we speculate that astrocytes are either major uptake and storage sites for arginine or require a high level of arginine for normal physiologic function. Our data show that the uptake of arginine by primary and immortalized astrocytes is severely limited by *ts*1 infection. In turn, the chronic cumulative effect of the down-regulation of arginine uptake may lead to arginine deficiency and may influence the well-being of the

astrocytes as well as limit their NO production. Since astrocytes are the major supportive and protective cells for the neurons as well as other cells in the CNS, their impairment could affect the neuronal survival. In support of our results, significant reduction was recently shown in the CNS of *ts*1-infected mice (Stoica *et al*, submitted). In addition, we have begun using some of these immortalized astrocyte clones to investigate other membrane transport activities, ion exchange activities and the expression of cytokines, in particular interleukin 1 and tumor necrosis factor, as well as cell growth/death factors to determine their role in the neuronal death seen in our *ts*1 model.

Materials and methods

Virus and cell lines
Molecularly cloned *ts*1 and its parental wild type virus MoMuLV-TB were described previously (Yuen *et al*, 1985). Viruses were propogated in TB cells, a thymus-bone marrow cell line derived from CFW/D mice, and the virus titer was determined using 15F cell assay as described previously (Wong *et al*, 1981). The producer cell line PA317U19tsA58 which produces a replication-defective retrovirus called U19tsA58 was a gift from Dr JW Jacobberger of Case Western Reserve University. U19tsA58 is a viral DNA construct contains sequences encoding the temperature-sensitive mutant of the 58 kDa SV40 large T antigen and the aminoglycoside phosphotransferase coded for by the Tn5 transposon (Neo^r). To generate a retrovirus vector for immortalization, the U19tsA58 construct was transfected into the PA317 cells, an amphotropic retrovirus packaging cell line (Miller and Baltimore, 1986). This cell line contains a multiple altered helper virus genome whose packaging signal is deleted and therefore does not produce a replication-competent helper virus. K1-30, a T antigen-immortalized astrocyte line derived from C57/BL, and also a gift from Dr JW Jacobberger (Frisa *et al*, 1994), was used as positive control for expression of GFAP and T antigen.

Infection of cells
For viral infection of cell, 8×10^4 cells were plated in 60 mm tissue dishes overnight before infection. Cells were treated with 6 μg/ml of polybrene for 2 h and then infected at a multiplicity (MOI) of three with WT or *ts*1. After incubation for 4 h at 37°C, the viral supernatant was removed and cells were washed and replenished with fresh medium. The cultures were maintained at 37°C (this temperature is permissive to *ts*1 replication) and virus production and cytopathic effects were monitored for 5 days.

Preparation of primary astrocytes
Primary astrocyte cultures were isolated essentially as described (Cole and de Vellis, 1992) with slight modification. Briefly, neonatal cerebral cortex and brain stem from 1 to 3-day-old FVB/N mice were dissected free of meninges, and minced in supplemented Eagle's basal medium. Cells were then dissociated by trituration in 0.25% trypsin and 0.2% DNase buffer and incubated at 37°C with constant agitation for 20 min. The digested tissues were triturated again between a pipette and a 130 μm screen until a single-cell suspension was achieved. Tissue debris was removed by low-speed centrifugation (100 g). Cells were then plated in 75 cm^2 polylysine-coated tissue culture flasks containing 10 ml DMEM/F-12 medium with 10% fetal bovine serum and N2 supplement at a density of 2×10^6 cells/ml. To immortalize astrocytes, cells were grown for 48 h before shaking flasks vigorously for 6–12 h (250 rpm in a reciprocal shaker) to remove loosely adherent cells. Astrocytes normally remained attached to the flasks. The highly enriched primary astrocytes were then used for immortalization.

Immortalization of astrocytes
The highly enriched primary astrocyte cultures described above were plated at 5×10^5 cells/100 mm dish in DMEM/F-12 complete medium overnight. The culture was infected with virus-containing supernatant from PA317U19tsA58 at 37°C for 2 h in the presence of 4 μg/ml polybrene. Repeat infection and occasional rocking of the dish increased infection efficiency. The cultures were then incubated at 34°C for 48 h. The infected cells were selected in medium containing 400 μg/ml G418 (Sigma). Surviving and well-isolated cell colonies, which appeared about 2 weeks later, were picked with cloning rings and cloned.

Immunocytochemistry and Western blot analysis of neural cell-specific markers, T antigen and viral envelope protein expression
Anti-GFAP (DAKO), anti-vimentin (Sigma), anti-galactocerebroside (Sigma), anti-synaptophysin (Boehringer), anti-mac-1 (Boehringer), DiI-conjugated acetylated low density lipoprotein (DiI-ac-LDL) (Biochemical Technologies), anti-T antigen (Oncogene Sciences), and anti-gp70 (Quality Biotech) were used for immunochemistry staining to characterize the cloned cell lines. Cells were lysed, denatured, subjected to SDS–PAGE and transferred to nitrocellulose membranes for Western blot detection of viral envelope protein, GFAP, vimentin and T antigen. Bands were visualized with an enhanced chemiluminescent (ECL) system (Amersham).

Amino acid uptake
To determine whether infection of astrocytes by *ts*1 reduced their ability to take up arginine and leucine, initial transport activity was assayed as described (White, 1981) with some modification. In

brief, astrocytes were infected with *ts*1 or WT at an MOI of three as described above. Uninfected cells acted as controls. All cultures were incubated at 37°C. One to two days before assay, cells were plated on 4-well plates at a density of $4-6\times10^4$ cells/per well. Cells were washed twice with 1 ml of PBS and then incubated with 200 μl Earle's balanced salt solution containing 5 μCi/ml L-[2,3,4,5-^{3}H]-arginine (59 Ci/mmol), or L-[4,5-^{3}H(N)]-leucine (52 Ci/mmol) for 2–3 min at 37°C. The initial uptake in our astrocytes was linear with time for at least 3 min. At the end of incubation, the radioactive buffer was removed quickly, and cells were washed four times with 1 ml of ice-cold PBS. Cells were dissolved in 200 μl of 0.5 N NaOH. Samples from this solution were measured for incorporated radioactivity in a liquid scintillation counter. Protein content was determined by the Bio-Rad DC protein assay. The amino acid uptake assay was standardized to protein content. To differentiate binding from transport activity, the amino acid uptake assay was also performed at 4°C to minimize the transport activity.

Acknowledgements

We gratefully acknowledge the superb technical assistance of D Hollowell and Xia Li. We thank C McKinley for patient preparation of the manuscript and R Wilcox and W Lynn for reviewing. This work was supported by Public Health Service Grants AI 28283 and CA 45124 and also by National Cancer Institute Core Grant CA 16672.

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