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Absence of the p55 Kd TNF-α receptor promotes survival in rabies virus acute encephalitis

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We investigated the role played by inflammation in acute encephalitis following infection with a neurotropic virus by comparing the disease caused by the CVS strain of rabies virus in C57BL/6 and mice deficient for the p55 Kd TNF-α receptor (p55TNFR$^{-/-}$). Morbidity (weight loss and paralysis) and mortality of infected mice were associated with viral propagation, cytokine (IL-6, IL-10, TNF-α and IFN-γ) production, induction of apoptosis and infiltration of inflammatory cells. Mortality occurred later in p55TNFR$^{-/-}$ (than in C57BL/6 mice. In contrast, morbidity and the number of cells undergoing apoptosis were similar in C57BL/6 and p55TNFR$^{-/-}$ mice.) This suggests that morbidity and mortality are independently regulated and that the death of the animal was not due to CNS apoptosis. Delayed mortality correlated with: a reduction in viral load on day 9 p.i., an increase in IFN-γ and IL-10 concentrations and a reduction in inflammatory cell infiltration in the CNS. Thus, these data indicate that CVS infection elicits an inflammatory response within the CNS and suggest that cytokines signaling via the p55 Kd TNF-α receptor is deleterious for the survival of the host. These results strongly suggest that, the modulation of TNF-α and upregulation of IFN-γ would be a powerful anti-virus strategy in cases of viral encephalitis. *Journal of NeuroVirology* (2000) **6,** 507–518.

Keywords: central nervous system; inflammation; cytokines; p55 Kd TNF-α receptor; rabies virus

Introduction

Viral infection of the central nervous system (CNS) leads to the production of cytokines and inflammatory molecules such as IL-1 α/β, IL-6, IL-10, TNF-α, IFNs and nitric oxide (NO), and to the secretion of chemokines (Benveniste, 1997, 1998; Glabinski and Ransohoff, 1999). This inflammatory reaction induces the activation and recruitment in the CNS of inflammatory cells that may control the spread of the virus and participate in viral clearance from the CNS, thereby promoting the survival of mice with viral encephalitis. However, the beneficial or deleterious effects of the inflammatory reaction following viral infection of the CNS are still unclear (Merill and Benveniste, 1996). Many studies have focused on the role played by TNF-α produced during viral infections of the CNS (Morimoto *et al*, 1996; Morris *et al*, 1997; Parra *et al*, 1997; Pearce *et al*, 1994). TNF-α is thought to play a negative role during demyelination following CNS infection with the neurotropic coronavirus mouse hepatitis virus strain JHM (JHMV) Parra *et al*, 1997) and may be responsible for the pathological sleep, fever and wasting syndrome (Leon *et al*, 1998; Probert *et al*, 1993, 1995) induced during rabies virus infection (Gourmelon *et al*, 1986; Marquette *et al*, 1996b; Torres-Anjel *et al*, 1988). However, TNF-α-neutralizing antibodies have no effect on the demyelination induced by JHMV (Stohlman *et al*, 1995) and encephalitis induced by measles virus (Finke *et al*, 1995). Furthermore, the injection of TNF-α-neutralizing antibodies into mice infected with Herpes Simplex Virus Type 1 (HSV-1) was even found to be deleterious (Kodukula *et al*, 1999). Signaling via the p55 Kd TNF-α receptor has been associated with Lymphotoxin and TNF-α (LT/TNF-α) apoptosis (Ware *et al*, 1996) and inflammation induction (Warzocha *et al*, 1995). Moreover, the p55 Kd TNF-α receptor is crucially involved in the development of the experimental autoimmune encepha-

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lomyelitis (E.A.E.) demyelinating disease in mice (Eugster *et al*, 1999; Willenborg *et al*, 1998).

Infection of mice with the highly neurotropic strain of rabies virus, the challenge virus standard (CVS), provokes a fatal encephalitis. Virus propagation from the periphery to the brain by retro-axonal transport (Coulon *et al*, 1989) induces an inflammatory reaction characterised by apoptosis (Jackson and Rossiter, 1997) (Theerasurakarn and Ubol, 1998) and production of IL-1α/β (Marquette *et al*, 1996a,b), IFN (Lodmell *et al*, 1989), NO (Akaike *et al*, 1995; Koprowski *et al*, 1993; Van Dam *et al*, 1995) and TNF-α (Marquette *et al*, 1996b; Theerasurakarn and Ubol, 1998) in the CNS. In order to determine the role played by members of the TNF-α family in survival to an acute viral encephalitis, we compared the disease caused by the CVS strain of rabies virus in C57BL/6 and mice deficient for the p55 Kd TNF-α receptor (p55TNFR$^{-/-}$).

The characteristics of CVS infection, including loss of weight, paralysis, mortality, viral invasion of the CNS, were compared between both strains of mice. Then, the production of TNF-α, IL-6, IL-10 and IFN-γ was followed in the CNS of C57BL/6 and deficient mice by ELISA. Apoptosis within the brain was detected by immunocytochemistry (TUNEL assay) and by the nucleosome detection technique. Finally, the effect of the lack of the p55 Kd TNF-α receptor on inflammatory cell infiltration in the CNS was assessed by comparing the numbers and natures of infiltrating cells in C57BL/6 and p55TNFR$^{-/-}$ mice, as determined by flow cytometry. This study provides evidence that signaling via the p55 Kd TNF-α receptor is involved in rabies virus-induced mortality, but not in morbidity, and suggests that appropriate modulation of cytokine production in the CNS may help to reduce mortality following rabies encephalitis.

Results

Characterisation of CNS invasion and disease caused by the neurotropic highly pathogenic CVS rabies virus strain in C57BL/6

We followed development of the symptoms induced in mice by infection with CVS in association with nervous system invasion. C57BL/6 mice were injected i.m. in both hind legs with 10^7 p.f.u. of the CVS strain of rabies virus. Clinical score, mortality, loss of weight and rabies N protein concentration were followed from day 3 until day 15. Mice began to lose body weight on day 5 p.i. and weight loss continued throughout the course of infection (Figure 1A). Weight loss was severe, with infected mice losing $31.96 \pm 3.92\%$ of their initial body weight by 12 days p.i. The mice started to lose weight as soon as the rabies virus began to invade the spinal cord and medulla (Figure 1D,E). Aggravation of the disease was assessed by mean clinical score (Figure 1B), as described in Materials and methods. Ruffled fur was observed in mice between days 3 and 4 p.i., before rabies N protein was detected in the CNS. The first signs of hind limb weakness appeared as early as day 4 p.i. and paralysis of one hind leg as early as day 5 p.i., when rabies virus started to invade the spinal cord and medulla. Aggravation of paralysis correlated with progress of viral spread, first to the spinal cord (Figure 1D) and then to the medulla (Figure 1E), with the 'cortex' invaded one day later (Figure 1F), suggesting that paralysis reflected progressive infection of the entire CNS. Death was the inevitable prognosis once the animal was paralysed in one hind leg. The cumulative mortality of C57BL/6 mice is shown in Figure 1C. Mice began to die from CVS infection on day 9 p.i. (mean day of 50% mortality was 11.3) and all the animals were dead by day 13 p.i., with N protein concentration in the medulla and cortex increasing and reaching a peak on day 10 p.i. Thus, loss of body weight, paralysis and mortality correlated with the progressive ascending invasion of the CNS by rabies virus.

P55kd TNF-α receptor-deficient mice died later than C57BL/6 mice following rabies virus infection

We investigated the role of LT/TNF-α in the CNS during rabies virus encephalitis by comparing the course of CVS infection in C57BL/6 and p55Kd TNFR$^{-/-}$ mice. The p55 Kd TNFR$^{-/-}$ mice died a mean of 2 days later than C57BL/6 mice (Figure 2A). The mean day of 50% mortality was 10.4 days for C57BL/6 and 12.3 days for p55TNFR$^{-/-}$ mice. However, there was no significant difference in the timing of onset of signs of illness, weight loss or progression of paralysis, between the C57BL/6 and p55Kd TNFR$^{-/-}$ mice (data not shown). We investigated whether the delayed mortality was due to differences in CNS infection, by analysing the spread of the virus within the CNS. We did this by testing for rabies N protein in the spinal cords and brains of infected mice by immunocapture ELISA. Between days 3 and 7 p.i. no difference in viral load was detected between C57BL/6 and p55Kd TNFR$^{-/-}$ (data not shown). Thus, the delay in mortality of the p55Kd TNFR$^{-/-}$ mice cannot be accounted for solely by a delay in the early invasion of the CNS by the virus. However, later in infection (day 9 p.i.), the concentration of rabies N protein in the brain was lower in p55TNFR$^{-/-}$ than in C57BL/6 mice (Figure 2B). This suggests that the delay in mortality of the p55TNFR$^{-/-}$ mice may be partially due to late control of the viral load in the CNS.

Overall, these results suggest that LT/TNF-α is deleterious to the survival of mice infected with CVS. In contrast, signaling via the p55Kd TNF-α receptor has no impact on rabies-induced morbidity. Finally, absence of the p55Kd TNF-α receptor resulted in late control of viral spread and may have helped to clear rabies virus from the CNS.

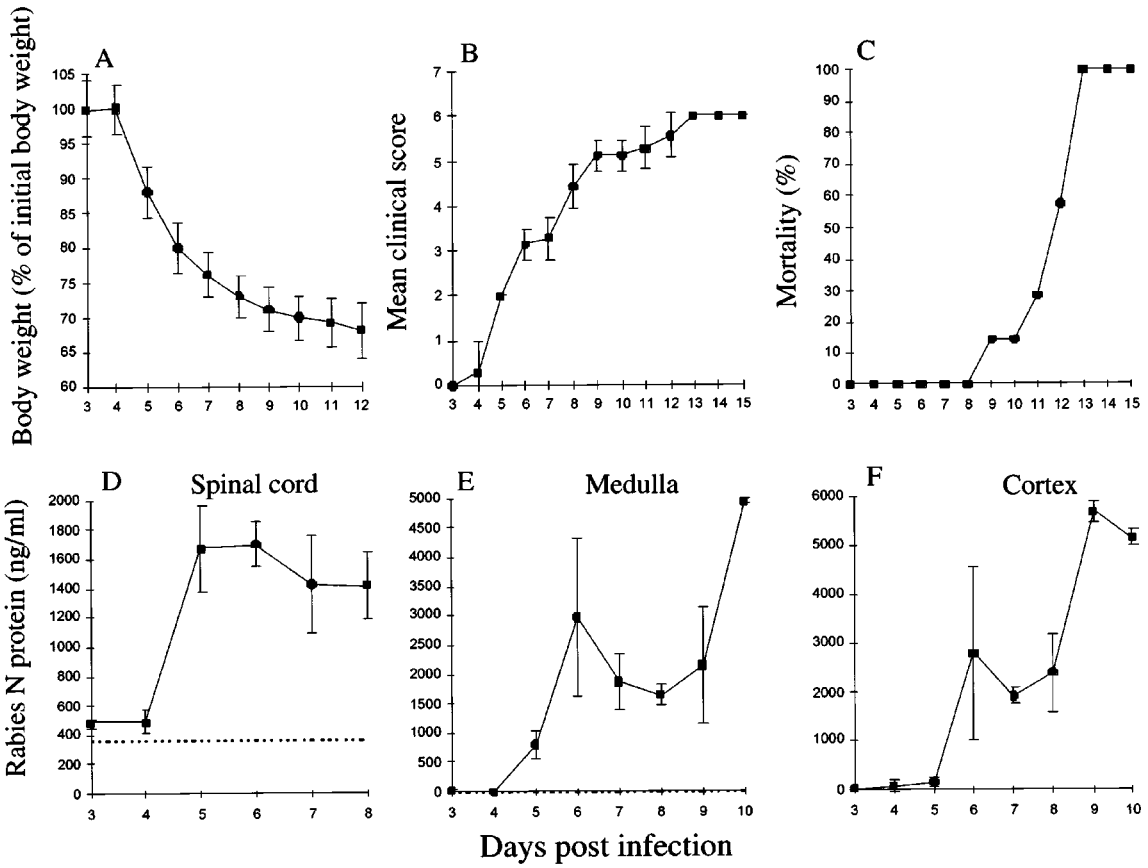

Figure 1 Characterisation of rabies virus (CVS) infection in C57BL/6 mice. After infection with 10^7 p.f.u. of rabies virus strain CVS by i.m. injection into both hind-legs, (**A**) body weight (n=12), (**B**) morbidity (n=7) and (**C**) mortality (n=7) were recorded daily. Individual body weights were transformed into percentages, taking the weight at day 0 of infection as 100%. All figures are representative of at least three experiments. Rabies virus invasion of various parts of the nervous system: (**D**) spinal cord, (**E**) medulla, and (**F**) 'cortex', was studied in homogenates by determining the rabies virus N protein by immunocapture ELISA. Results are expressed in ng/ml as mean determinations for three mice per time point (except on day 10 where n=2). Error bars indicate the s.d.

The level of apoptosis is similar in the CNS of C57BL/6 and p55TNFR$^{-/-}$ rabies-infected mice

We investigated whether rabies virus acute encephalitis was linked to apoptosis of CVS-infected cells, by performing double immunostaining for TUNEL and rabies N protein on brain slides. Very few TUNEL-positive cells were detected in the brains of uninfected animals (see Figure 3Aa,C). In contrast, both CVS-infected neurons and TUNEL-positive cells were observed on sections of brains from CVS-infected C57BL/6 mice 8 days p.i. (Figure 3Ab). Thus infected CNS cells can undergo apoptosis during rabies virus acute encephalitis. However, infected Purkinje neurons, which do not undergo apoptosis (white arrowhead) and uninfected cells positive for DNA fragmentation (open arrowhead) were also observed in brain (Figure 3Ab). Similar observations could be made in spinal cord (data not shown). Thus, the apoptosis observed during the course of rabies virus encephalitis is not strictly restricted to infected neurons. The detection of apoptosis using the Cell Death ELISA^{plus} kit showed (Figure 3B) a positive correlation between apoptosis and viral antigen accumulation in the brains of infected mice ($R^2 > 0.86$) (but not in the spinal cords ($R^2 < 0.23$).) Cytokines induced signaling via the p55 Kd TNF receptor is known to promote apoptosis. If the p55Kd TNF-α receptor is involved in the induction of apoptosis in the CNS of CVS-infected mice, then the absence of this receptor in p55TNFR$^{-/-}$ mice may reduce the number of cells undergoing apoptosis, accounting for the greater resistance of these mice. We investigated this hypothesis by comparing the number of TUNEL-positive cells in the cerebellum for C57BL/6 and p55TNFR$^{-/-}$ rabies virus-infected mice. There were more TUNEL-positive cells in the cerebellum in infected C57BL/6 and p55TNFR$^{-/-}$ mice (Figure 3C) than in uninfected mice (control) (3.45 ± 2.85 TUNEL-positive cells/section for uninfected mice *versus* 83.58 ± 51.43 for infected C57BL/6 mice, $P < 0.05$ and 61.8 ± 27.89 TUNEL-positive cells/section for p55TNFR$^{-/-}$ mice, $P < 0.05$). However there was no difference in the number of TUNEL-

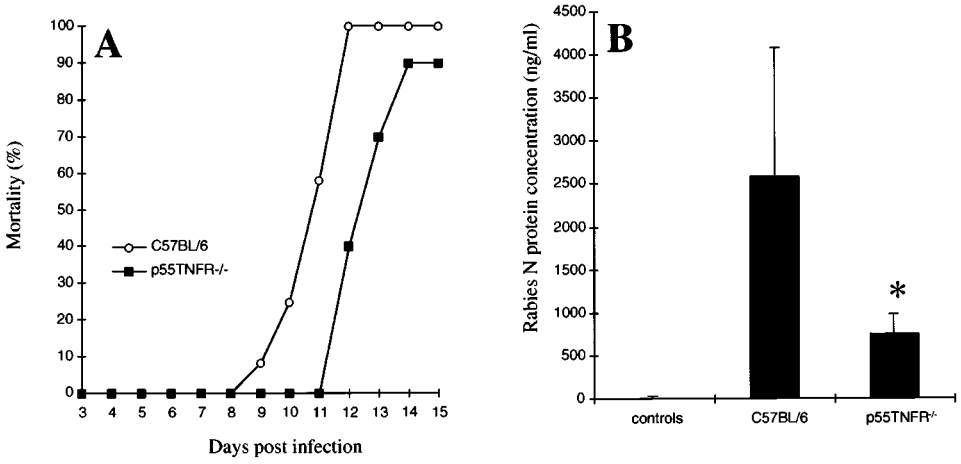

Figure 2 Characterisation of CVS rabies virus infection in p55TNFR$^{-/-}$ mice. (**A**) The pattern of mortality was compared from day 3 to day 15 for p55TNFR$^{-/-}$ (closed squares) (n=10) and C57BL/6 mice (open circles) (n=12). Data are representative of two separate experiments. (**B**) Rabies N protein concentrations were determined by ELISA, from homogenates of brains from p55TNFR$^{-/-}$ mice and C57BL/6 mice. Each bar is the mean of duplicate determinations from two mice, 9 days p.i. Error bars show the s.d. Asterisks (*) indicate statistical significance of Student's t-test ($P<0.05$).

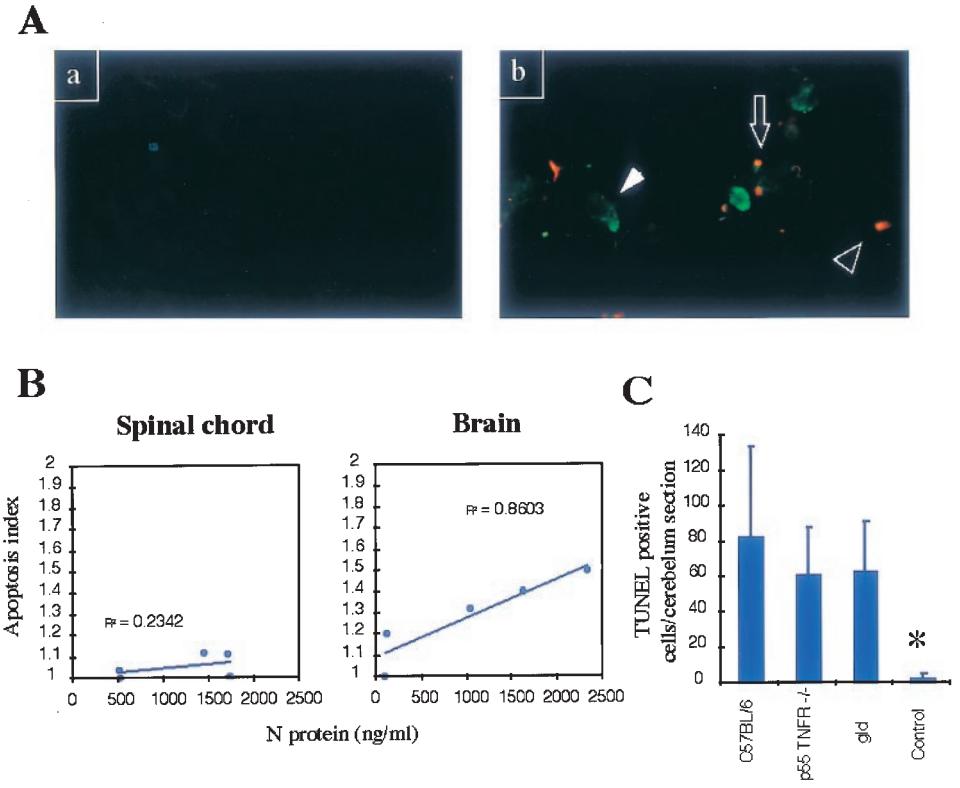

Figure 3 Apoptosis in the CNS of CVS-infected mice. (**A**) Immunofluorescence codetection of apoptosis (TUNEL red) and viral antigens (green) in the cerebellum of (a) uninfected mice and (b) CVS-infected mice 8 days p.i. The white arrowhead shows an infected Purkinje neuron. Some apoptotic bodies contain viral N protein antigen (open arrow), consistent with the disintegration of infected neurons, whereas others do not (open arrowhead). Original magnification ×40. (**B**) Analysis of the correlation between DNA fragmentation detected by ELISA, expressed as apoptosis index, and rabies N protein concentration in homogenates of brains and spinal cords from infected C57BL/6 mice (n=5). The regression coefficient R^2 is given. $R^2>0.8$ can be regarded as evidence of correlation. (**C**) Measure of apoptosis by the TUNEL technique. Bars represent the mean number of TUNEL-positive cells per cerebellum section (15–20) from infected C57BL/6, p55TNFR$^{-/-}$ and gld mice, 8 days p.i. and uninfected mice (n=3). Error bars show the s.d. Asterisks (*) indicate statistical significance in comparisons between infected and uninfected mice, by Student's t-test ($P<0.05$).

positive cells between C57BL/6 and p55TNFR$^{-/-}$, suggesting that the p55 Kd TNF-α receptor is not required for the induction of apoptosis in the CNS of rabies virus-infected mice. This result was confirmed by nucleosome detection in the brains and spinal cords of C57BL/6 and p55TNFR$^{-/-}$ mice (data not shown). The similar level of DNA fragmentation in the CNS of C57BL/6 and p55TNFR$^{-/-}$ mice suggests that survival is not related to the tendency of cells in the CNS to die by apoptosis. We investigated the mechanism of apoptosis induction in the CNS of rabies virus-infected mice by comparing the number of TUNEL-positive cells in the cerebellum of C57BL/6 mice and *gld* mice expressing a nonfunctional form of the Fas ligand (FasL) such that cell apoptosis by the Fas/FasL pathway does not occur. CVS infection triggered a level of apoptosis in the cerebellum of *gld* mice significantly higher than that in uninfected mice (3.45 ± 2.85 TUNEL-positive cells/section for uninfected mice *versus* 63.5 ± 29 TUNEL-positive cells/section for *gld* mice, $P < 0.05$). However no difference in apoptosis was observed in infected C57BL/6 and *gld* mice, indicating that the apoptosis induced in the cerebellum in CVS-infected mice does not require interaction between Fas and its ligand. Finally, neither the FasL mutation in *gld* mice nor the absence of p55 Kd TNF-α receptor signaling reduced the number of TUNEL-positive cells in the cerebellum of CVS-infected mice, suggesting that another mechanism is involved or that these two apoptosis induction pathways are redundant during CVS infection.

Greater resistance to rabies-induced encephalitis correlates with higher levels of TNF-α, IFN-γ and IL-10 and lower levels of IL-6 within the brain

We investigated whether the difference in rabies mortality was due to modification of the cytokine profiles in the CNS, using immunocapture ELISA to compare the concentrations of IL-6, IL-10, TNF-α and IFN-γ in brain homogenates from infected C57BL/6 and p55Kd TNFR$^{-/-}$ mice between days 3 and 7 of infection. IL-6 production began in both strains of mice by day 4 and increased up to day 6 p.i. but there was a drastic drop in IL-6 concentration on day 7 of infection in p55Kd TNFR$^{-/-}$ (5414 ± 1901 pg/ml for C57BL/6 *versus* 1493 ± 364 pg/ml for p55Kd TNFR$^{-/-}$ mice on day 7 p.i. $P < 0.05$) (Figure 4). TNF-α production peaked in both strains of mice on day 5 p.i. but significantly more TNF-α was produced in p55TNFR$^{-/-}$ mice than in C57BL/6 mice (661 ± 398 pg/ml for C57BL/6 mice *versus* 1497 ± 290 for p55TNFR$^{-/-}$ mice, $P < 0.05$). Similarly, the levels of IFN-γ detected in the brains of p55TNFR$^{-/-}$ mice were higher than those found in C57BL/6 mice on days 6 and 7 p.i. (831 ± 931 pg/ml *versus* 8030 ± 3055 pg/ml for C57BL/6 and p55TNFR$^{-/-}$ mice respectively on day 6 p.i., $P < 0.05$). Furthermore, whereas IL-10 concentration was below the detection threshold of ELISA in C57BL/6 mice, IL-10 was detected in p55TNFR$^{-/-}$ mice, 7 days p.i. (31 ± 23 AU/ml *versus* 114 ± 21 AU/ml for C57BL/6 and p55TNFR$^{-/-}$ mice respectively, $P < 0.05$). This late IL-10 production in p55TNFR$^{-/-}$ mice is consistent with the concentrations of TNF-α and IFN-γ in the brains of these mice between days 5 and 7 p.i., because these cytokines are well known to control the production of anti-inflammatory cytokines such as IL-10. Thus, during rabies infection, the absence of the p55 Kd TNF-α receptor had a major impact on brain cytokine levels, increasing TNF-α, IFN-γ and IL-10 levels and decreasing late IL-6 production.

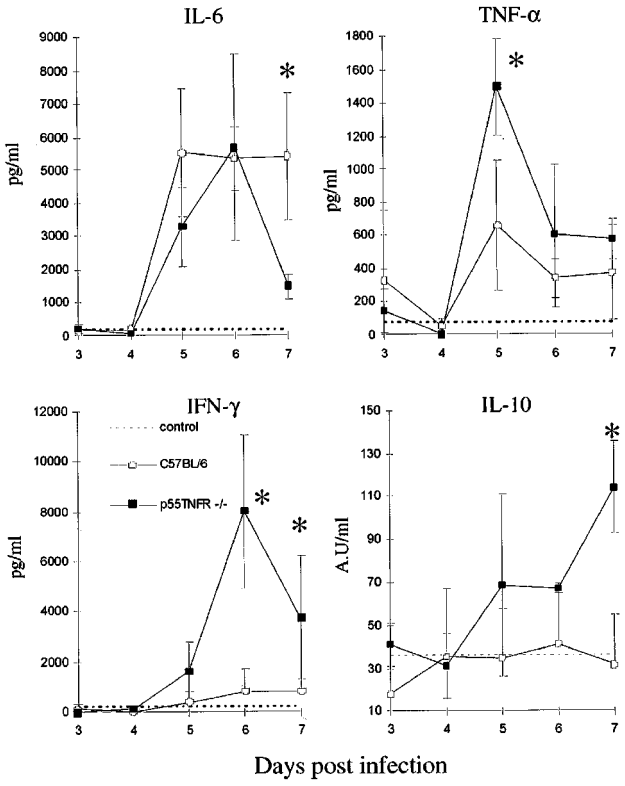

Figure 4 Cytokine production in the brain of p55TNFR$^{-/-}$ mice in the course of rabies virus infection. IL-6, TNF-α, IFN-γ and IL-10 concentrations were determined by immunocapture ELISA from the supernatants of homogenized brains from three mice per time point, between days 3 and 7 p.i. Results are expressed as means of duplicate determinations with s.d. indicated by error bars. Dashed lines indicate the basal level in uninfected mice. Asterisks (*) indicate statistical significance in Student's *t*-test ($P < 0.05$).

Absence of the p55Kd TNF-α receptor reduces the number of CNS-infiltrating cells

Modification of the cytokine profile in the CNS of p55TNFR$^{-/-}$ mice may decrease the number and change the nature of inflammatory cell influx into the CNS of CVS-infected mice. We investigated this possibility by counting and comparing, by flow

cytometry, the nature of infiltrating cells recovered from the CNS of C57BL/6 and p55TNFR$^{-/-}$ mice infected with CVS. The same cell populations, including CD3, (CD4 and CD8), T lymphocytes, B lymphocytes, monocytes and PMN, were present in the CNS of both C57BL/6 and p55TNFR$^{-/-}$ mice on day 6 p.i., indicating that the nature of the infiltrating cells was not affected by the lack of the p55KD TNF-α receptor (Figure 5).

In contrast, the number of inflammatory cells in each of the populations recovered from infected p55TNFR$^{-/-}$ mice was slightly smaller than that for infected C57BL/6 mice. For instance, p55TNFR$^{-/-}$ mice had about half as many CD3 T lymphocytes as C57BL/6 mice ($22\times10^3\pm4.25\times10^3$ cells for C57BL/6 mice *versus* $9.84\times10^3\pm1.67\times10^3$ cells for p55TNFR$^{-/-}$ mice; $P<0.05$). Similar differences were observed for other markers including CD4, CD5 and CD8-positive T cells, Gr-1-positive neutrophils, CD11b-positive cells and B220-positive B lymphocytes in C57BL/6 and p55TNFR$^{-/-}$ mice; $P<0.05$ for each cell type). The absence of the p55KD TNF-α receptor also reduced the number of CNS-infiltrating cells expressing CD69; ($25.1\times10^3\pm4.8\times10^3$ cells for C57BL/6 mice *versus* $12.4\times10^3\pm2.11\times10^3$ cells for p55TNFR$^{-/-}$ mice; $P<0.05$). The presence of activated cells was confirmed by detection of CD25, the α-chain of the IL-2 receptor, in immuno-fluorescence analysis of brain slides from infected C57BL/6 mice (data not shown). Thus fewer lymphocytes and monocytes were recovered from the CNS of CVS-infected p55TNFR$^{-/-}$ mice than CNS-infiltrating leukocytes from C57BL/6 mice ($P<0.05$ for each cell type). However, the proportion of each cell population was similar in the two strains of mice. This suggests that the absence of the p55Kd TNF-α receptor induces a quantitative, but not qualitative, modification of inflammatory cell influx into the CNS of rabies-infected mice.

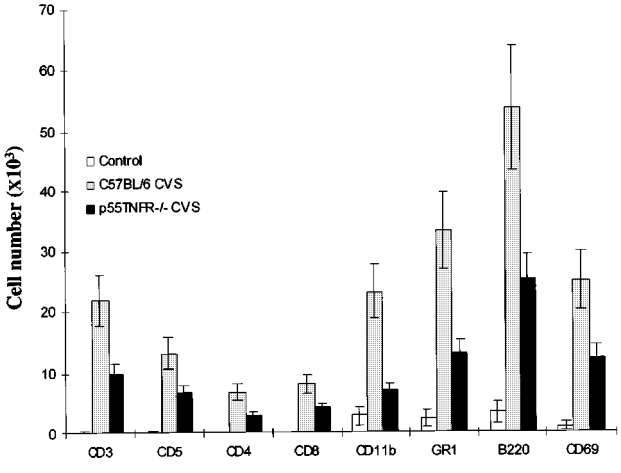

Figure 5 Phenotyping of CNS-infiltrating lymphocytes and monocytes by flow cytometry in C57BL/6 and p55TNFR$^{-/-}$ mice. The nature of infiltrating cells (CD3, CD4, CD5, CD8, CD11b, Gr-1, B220 and CD69) was determined by flow cytometry, after Percoll isolation from homogenates of brains and spinal cords from uninfected mice (white bars) (n=3), and infected C57BL/6 (gray bars) (n=4) and p55TNFR$^{-/-}$ mice (black bars) (n=4), 6 days p.i. Numbers of each cell type were calculated as follows: total cell number in the CNS of each mouse × percentage for that cell type as determined by flow cytometry. Bars show the s.d.

Discussion

To identify the survival factors controlling acute rabies virus encephalitis, we investigated the role of LT/TNF-α, in host survival, by comparing the various features of CVS infection in C57BL/6 and p55TNFR$^{-/-}$ mice. We found that the receptor-deficient mice died later and presented a reduction in viral invasion late in the infection. This suggests that signaling via the p55 Kd TNF-α receptor is detrimental to host survival. Delayed mortality was associated with equal numbers of apoptosis cells in the cerebellum, lower inflammatory cell infiltration and higher concentrations of TNF-α, IFN-γ and IL-10 within the CNS. These observations suggest that mortality is not related to apoptosis in the cerebellum but may be linked to the decreased recruitment of inflammatory cells and that the production of TNF-α, IFN-γ and IL-10 which is downregulated in normal mice, may be involved in viral clearance and host survival.

LT and TNF-α have pleiotropic effects. Therefore the absence of the p55 Kd TNF-α receptor may affect rabies virus acute encephalitis at several levels. First, TNF-α, also called cachectin, is thought to be involved in weight loss during sepsis (Leon *et al*, 1998). Torres-Anjel *et al*, have suggested that TNF-α is responsible for the paralysis, weight loss and wasting syndrome that occur during CVS infection (Torres-Anjel *et al*, 1988). However, in our mouse model, the absence of the p55Kd TNF-α receptor had no effect on these symptoms. Thus, in contrast to its effects on mortality, signaling via the p55Kd TNF-α receptor is probably not involved in morbidity. This result also suggests that the weight loss observed during rabies virus acute encephalitis may be regulated by the p75Kd TNF-α receptor, which is functional in p55TNFR$^{-/-}$ mice or that molecules other than LT/TNF-α are involved in these morbid symptoms. However, none of the other inflammatory factors studied (IL-6, prostaglandins) were found to be involved in rabies morbidity in mice (data not shown).

Signaling via the p55Kd TNF-α receptor may also cause death via its destructive effects on CNS cells, either directly by triggering neuron apoptosis, as has been shown during HIV dementia (Petito *et al*, 1999; Westmoreland *et al*, 1996) or indirectly by inducing neuronal dysfunction due to the activation of microglia (Adle-Biassette *et al*, 1999)

or the apoptosis of oligodendrocytes (Akassoglou *et al*, 1998; Selmaj *et al*, 1991). Both neuronal dysfunction (Bouzamondo *et al*, 1993; Ceccaldi *et al*, 1997; Gourmelon *et al*, 1986; Koschel and Halbach, 1979; Koschel and Munzel, 1984; Tsiang, 1982) and apoptosis have been suggested to play a role in rabies acute encephalitis-induced morbidity and mortality (Jackson and Rossiter, 1997; Theerasurakarn and Ubol, 1998). In our model, we detected apoptotic cells in the CNS of rabies virus-infected mice. However, codetection of apoptotic cells by the TUNEL technique and of rabies viral antigen in the cerebellum indicated that both infected and uninfected cells underwent apoptosis. Similar findings were observed in the cortex (data not shown). This suggests that uninfected neurons or other cells such as astrocytes, oligodendrocytes, microglia and infiltrating immune system inflammatory cells, may undergo apoptosis in the CNS of rabies virus-infected mice. Resistance of Purkinje neurons to apoptosis is in agreement with previous observations reported by Jackson (1999). We investigated the involvement of apoptosis on rabies mortality by comparing apoptosis induction in the cerebellum of C57BL/6 and p55TNFR$^{-/-}$ mice infected with CVS. The number of TUNEL-positive cells in the cerebellum was similar for these two strains of mice. This suggests that apoptosis is not a major factor in rabies mortality. The lack of involvement of apoptosis in encephalitis mortality is consistent with results obtained following infection with a neurotropic coronavirus (Wu and Perlman, 1999). However, our observations do not exclude the possibility that morbidity, including paralysis, may be linked to apoptosis, as shown by Galelli *et al* in mice infected with a rabies virus strain with attenuated pathogenicity (PV) (Galelli *et al*, 2000). The equal number of cells undergoing apoptosis in the cerebellum of p55TNFR$^{-/-}$ and C57BL/6 mice indicates that the transduction of apoptotic signals via the p55Kd TNF-α receptor is not an important pathway for the induction of apoptosis in the CNS of rabies-infected mice. The Fas ligant (FasL) is essential for the induction of apoptosis in cells expressing Fas, a member of the corresponding receptor family and immune privilege requires FasL expression (Bechmann *et al*, 1999) in the CNS. Mice with generalized lymphoproliferative disorder (gld), are homozygous for a point mutation in FasL, leading to the expression of a nonfunctional form of FasL on the cell surface and to the failure of the Fas/FasL apoptotic pathway. We used these mice to determine whether FasL expression was essential to apoptosis induction in the CNS of CVS-infected mice. Similar numbers of apoptotic cells were detected in the cerebellum of C57BL/6 and gld mice, suggesting that apoptosis induced by CVS infection in mice was independent of Fas/FasL interaction. Thus the mechanisms underlying CNS apoptosis in rabies acute encephalitis depend neither on the TNF-α/p55 Kd TNF-α receptor nor FasL/Fas interactions and remain to be elucidated.

LT/TNF-α may also affect survival during rabies virus-induced acute encephalitis by controlling the inflammatory response. Cytokines signalling via the p55Kd TNF-α receptor is known to promote leukocyte recruitment (Peschon *et al*, 1998). We found that significant numbers of T cells, B cells, neutrophils and cells expressing the activation marker CD69 infiltrated the CNS of rabies virus-infected mice. As expected, the number of infiltrating inflammatory cells was slightly lower in the CNS of p55TNFR$^{-/-}$ mice than in that of C57BL/6 mice. Thus, delayed mortality was associated with there being fewer T cells, B cells, and neutrophils in the CNS, suggesting that the infiltrating cells have a negative effect on survival after rabies virus encephalitis. However, it seems unlikely that T cells play a negative role because athymic Nude mice lacking T cells had a cumulative mortality curve similar to that of immunocompetent BALB/c mice infected with CVS (unpublished data). Therefore LT/TNF-α may have a deleterious effect by recruiting monocytes and neutrophils, which may themselves exert negative effects.

The delayed mortality of p55TNFR$^{-/-}$ mice also correlated with modified cytokine profiles in the brains of CVS-infected mice. Although the lower levels of IL-6 in the brains of p55TNFR$^{-/-}$ mice can be regarded as a good prognostic indicator for the survival of mice, the course of CVS infection was similar in IL-6$^{-/-}$ mice and IL-6$^{+/+}$ mice (unpublished data). Thus IL-6 *per se* is not a key factor in rabies mortality. However, caution in interpretation is necessary due to the redundancy of cytokines inducing a signal via gp130, the constant chain of the IL-6 receptor (Hirano, 1998). Thus, the potential effects of IL-6 deficiency may be compensated by ciliary neurotrophic factor (CNTF), oncostatin M (OSM), leukaemia inhibitory factor (LIF) or IL-11 production.

Larger amounts of TNF-α were found in the brains of p55TNFR$^{-/-}$ mice than in those of C57BL/6 mice. Similarly, an increase in TNF-α concentration has also been observed in p55TNFR$^{-/-}$ mice after LPS injection (Rothe *et al*, 1993). Thus, this seems to be an intrinsic characteristic of p55TNFR$^{-/-}$ mice. It is unclear whether the difference in TNF-α concentration between C57BL/6 and p55TNFR$^{-/-}$ mice is due to greater TNF-α production in these mice or to lower consumption due to the absence of the p55 Kd TNF-α receptor. It is well known that TNF-α may exert an antiviral effect via the p75Kd TNF-α receptor which is still functional in p55TNFR$^{-/-}$ mice (Ruby *et al*, 1997). In our model, however, it is unlikely that TNF-α could play a direct antiviral effect *in vivo* since it is unable to inhibit rabies virus replication *in vitro* (unpublished data).

A higher concentration of IFN-γ, was also observed in the CNS of p55TNFR$^{-/-}$ mice. IFN-γ has been shown to be an important factor for reducing the severity of measles virus encephalitis (Finke *et al*, 1995) and for the clearance of mouse hepatitis virus from the CNS (Parra *et al*, 1999). Similarly, higher IFN-γ concentrations in p55TNFR$^{-/-}$ mice may be responsible for the reduction in viral load in the brains of p55TNFR$^{-/-}$ mice. IFN-γ may control CVS propagation in the CNS of infected mice by mediating the production and isotype switching from IgM to IgG2a of rabies-neutralizing antibodies. However, only a low concentration of rabies-neutralizing antibodies and no IgG2a rabies-specific antibodies were found in the CNS of CVS-infected mice (unpublished). Furthermore as the infection of IL-6$^{-/-}$ mice, which are unable to produce IgG (Bluethmann *et al*, 1994), did not result in delayed mortality, antibodies cannot be involved in viral clearance from the brains of CVS-infected mice. It is therefore more likely that IFN-γ had a direct viricidal effect against rabies virus either alone or in combination with TNF-α or IFN-α and β (Oleszak and Stewart, 1985), in the CNS of CVS-infected mice (Lodmell *et al*, 1989), as has been shown for other viruses such as Venezuelan equine encephalitis virus (VEE) and type 2 herpes simplex virus (HSV-2) (Pinto *et al*, 1990). IFN-γ may also have an indirect anti-viral effect by upregulating the expression of MHC molecules on glial cells (Benveniste *et al*, 1989, 1991), or by inducing NO production. This second possibility is more likely because T lymphocytes are ineffective at controlling rabies virus acute encephalitis and because NO, which has antiviral properties *in vivo* (Komatsu *et al*, 1999; Lin *et al*, 1997), has been detected in the CNS of rabies virus-infected mice (Akaike *et al*, 1995; Koprowski *et al*, 1993; Van Dam *et al*, 1995).

The higher concentration of IFN-γ in the brains of p55TNFR$^{-/-}$ mice suggests that the p55Kd TNF-α receptor inhibits IFN-γ production. A similar observation has been made for *Leishmania major* infection, in which p55TNFR$^{-/-}$ mice had more inflammatory and necrotic lesions and higher levels of IFN-γ production (Vieira *et al*, 1996). It is possible that in C57BL/6, use of the p55Kd TNF-α receptor may induce the production of immunosuppressive molecules (Nicod *et al*, 1996) able to inhibit IFN-γ production, such as prostaglandin E2 (Betz and Fox, 1991).

If inflammation has a negative effect on survival, then the higher levels of IL-10, an anti-inflammatory molecule, detected in the brains of p55TNFR$^{-/-}$ mice on day 7 p.i. may help to delay the death of these mice. However, IL-10 production starting on day 8 may be too late to have a major effect on the outcome of the infection. It is more likely that the higher IL-10 concentration results from the high level of production of IFN-γ between days 5 and 7 p.i. in the brains of p55TNFR$^{-/-}$ mice but not in C57BL/6 mice.

Although it has been previously shown that TNF-α is produced in the CNS of rabies infected mice (Marquette *et al*, 1996b) it is not known whether cytokines are produced either by CNS resident cells like microglia, astrocytes and endothelial cells or by infiltrating macrophages, neutrophils and T lymphocytes. The fact that higher cytokine concentrations were observed in the brain of p55TNFR$^{-/-}$ (mice) in spite of the reduction of the number of infiltrating cells suggests that CNS resident cells may be the major source of cytokine production in the CNS of rabies virus infected mice.

In summary, although the mechanisms of viral clearance in our model are unknown, our results suggest that IFN-γ may have a beneficial effect on host survival during acute encephalitis induced by CVS infection. Thus, molecules able to potentiate the production of IFN-γ during CNS infection may increase host survival following viral encephalitis.

Materials and methods

Virus

The rabies laboratory strain Challenge Virus Standard, CVS, obtained from the American Type Cell Collection, Rockville, MD (Vr959) was propagated on BSR cells, a baby hamster kidney (BHK-21)-derived cell line. Cell culture supernatant was used as the inoculum.

Mice, infection and assessment of clinical symptoms

Experiments were performed with 6-week-old female mice, C57BL/6 (H-2b, 1-E^{-}) mice were purchased from Janvier (St. Berthevin, France). gld mice with generalized lymphoproliferative disease due to a point mutation in the gene coding for Fas ligand (FasL), were obtained from Jackson Laboratories. P55 Kd TNF-α-receptor-deficient mice (p55TNFR$^{-/-}$) (H-2b, I-E^{-}) kindly provided by Dr Werner Lesslauer of Hoffman-Laroche Ltd (Rothe *et al*, 1993), were bred at the Pasteur Institute in OPS-free conditions in A3L2-type animal colonies. Mice were injected (day 0) intramuscularly (i.m.) in both hind legs with 1×10^7 infectious particles of rabies virus. The progression of the disease was evaluated by scoring several symptoms such as ruffled fur, limb paralysis, and hunchback incidence. Groups of two or three mice were killed 3, 4, 5, 6, 7, 8 and 9 days p.i. Morbidity and mortality were scored as follows: 0=normal mice, 1=ruffled fur, 2=loss of agility, 3=one paralysed hind leg, 4=two paralysed hind legs, 5=total paralysis (defined as a total loss of mobility) and 6=death. Cachexia was assessed following infection by daily determination of animal weight.

Reagents
Capture (nonbiotinylated) and detection (biotinylated) mAb anti-Il-6, anti-IL-10, anti-TNF-α, and anti-IFN-γ for cytokine ELISA, recombinant TNF-α, IL-6 and INF-γ used as references in ELISA capture, FITC-conjugated mAb against TNP, CD3, CD4, CD11b, B220 and Ly-6G (Gr-1), PE-conjugated mAb against CD5 (a gift from Bernardo Reina-San Martin), CD8 and CD69, the biotinylated mAb directed against pan NK and the purified mAb directed against CD16/CD32 FcIIγ/III receptors (Fc block®), used for phenotyping and immunocytochemistry were all obtained from Pharmingen-Becton-Dickinson. Biotinylated mAb directed against F4/80 was purchased from Serotec. PVA3 is an ammonium sulphate-purified mAb from the laboratory (Lafon and Wiktor, 1985). Streptavidin-conjugated horseradish peroxidase was obtained from Amersham. Streptavidin-conjugated PE was obtained from Dako (Denmark), DTAF-streptavidin was obtained from Immunotech-Beckman Coulter (France). Hypnorm was purchased from Janssen (Oxford, UK). RPMI 1640 was obtained from GIBCO–BRL (Cergy-Pointoise, France). PMSF and aprotinin were purchased from Sigma Chemical Co (St Louis, MO, USA). Percoll was purchased from Amersham-Pharmacia (Sweden). ABTS (2.2′-Azino-di[3-ethylbenzthiazoline-sulphonate), Cell Death detection ELISA^{plus} and TUNEL (terminal deoxynucleotidyltransferase-mediated dUTP-tetramethylrhodamine-conjugated nick-end labelling) reagents were purchased from Boehringer Biochemicals (Mannheim, Germany).

Preparation of nervous system samples for ELISA
Mice were transcardially perfused with 50 ml PBS under terminal anaesthesia with Hypnorm. Brains (total weight 500 mg) were dissected free of the cranium, homogenised in 0.8 ml of ice-cold RPMI 1640 containing 10% MEM, 1% BSA, 1% Gentamicin, 1 mM PMSF, 1 mM aprotinin and centrifuged at 11 000 × g for 10 min at 5°C. For detection of N protein concentration, the CNS was separated into three parts: spinal cord, medulla and the rest of the brain noted as 'cortex', including the cerebellum and the hippocampus. Pellets and supernatants were separately collected and stored at −80°C until use.

Determination of rabies virus N protein by immunocapture ELISA
Microtiter plates were coated overnight with 5 mg per well of the specific mouse anti-N protein mAb PVA3 in 0.05 M carbonate buffer, pH 9.6, at 4°C. Plates were washed with PBS/Tween, and blocked with 10% goat serum in PBS-Tween. Dilutions (1 : 10 and 1 : 30) of homogenised brain samples from infected and uninfected mice and serial dilutions of a recombinant N protein (gift from André Aubert, Virbac, France), used as a standard, were incubated for 2 h at 37°C. Plates were thoroughly washed with PBS/Tween, incubated with biotinylated anti-N protein PVA3 mAb, and bound antibody was then detected with streptavidin-horseradish peroxidase and ABTS. Concentrations were determined using the linear portion of the curve obtained with recombinant N protein standard and expressed in ng/ml after subtracting the background OD of uninfected mouse brain.

Determination of cytokines by immunocapture ELISA
Supernatants (50 μl) and serial dilutions of cytokine standards were incubated overnight at 4°C on ELISA plates previously blocked with 10% goat serum in PBS/Tween and coated with a mAb for cytokines capture (1 μg/ml for IL-10 detection and 4 μg/ml for IL-6, TNF-α and IFN-γ). Plates were washed three times with PBS/Tween, 50 μl of diluted biotinylated detection mAb (1 μg/ml) was added to each well and the plates were incubated for 1 h at room temperature. Plates were washed with PBS/Tween and incubated with streptavidin-horseradish peroxidase for 30 min at room temperature. The plates were again washed with PBS/Tween, ABTS substrate was added and the plates were incubated at room temperature until the color reaction was complete. OD was read at 405 nm in a spectrophotometer. The OD of the control cell supernatant was subtracted and results expressed in pg/ml when recombinant cytokines were used as standard or in arbitrary units (ml/AU) if the supernatant of IL-10-secreting cells was used as the reference standard. ELISA sensitivity, calculated as the lowest cytokine concentration giving a signal at least two standard deviations above the mean background signal was: 92 pg/ml for IL-6, 35 pg/ml for IL-10, 15 pg/ml for MCP-1, 233 pg/ml for TNF-α and 200 pg/ml for INF-γ.

Purification of CNS-infiltrating mononuclear cells and lymphocytes
After transcardiac perfusion, brains and spinal cords were dissected and homogenised as described above. We centrifuged the homogenates at 500 × g at 4°C for 5 min and the pellet resuspended in 4 ml of 70% Percoll was then overlaid with 4 ml of 37% Percoll and 4 ml of 30% Percoll. The gradient was centrifuged at 1000 × g at 20°C for 20 min. CNS mononuclear cells were collected from the 30%/37% Percoll interface, washed and resuspended in 1% FCS, 0.1% NaN_3 in PBS for counting and flow cytometry.

Flow cytometry
Purified mononuclear cells were double-stained with the following pairs of mAb: FITC-conjugated anti-TNP mAb isotype control and PE-conjugated streptavidin as negative control, FITC-conjugated anti-CD3 mAb and biotinylated anti-NK mAb

detected with PE-conjugated streptavidin, FITC-conjugated anti-CD4 mAb and PE-conjugated anti-CD8 mAb, FITC-conjugated anti-B220 mAb and biotinylated anti-F4/80 mAb detected with PE-conjugated streptavidin, FITC-conjugated anti-CD11b mAb and PE-conjugated anti-CD69 mAb and finally FITC-conjugated anti-Gr-1 mAb and PE-conjugated anti-CD5 mAb. Cells were then analysed by flow cytometry in a FACScan cytofluorimeter using PC lysis II software (Becton Dickinson, CO, Mountain View, CA, USA).

Preparation of nervous system sections
Nervous system tissue sections were prepared for immunostaining to detect virus infection and apoptosis. To prevent contamination with red blood cells, mice were perfused as before with PBS and then with 50 ml of 4% PFA. Spinal cords were removed and incubated overnight in 4% PFA. Tissues were incubated for a further 24 h in 15% sucrose in PBS and were then snap frozen in liquid nitrogen-cooled isopentane. Cryostat sections (20 μm) were cut and mounted on permafrost slides.

Immunocytochemistry
For detection of infected cells by immunochemistry cryostat sections of OCT-embedded samples were incubated with 10% FCS in PBS overnight and then permeabilized with 3% FCS and 0.5% Triton-100 in PBS. The slides were incubated for 5 min at 4°C with purified Fc block®, to prevent nonspecific binding and then for 30 min at 37°C with PVA3 FITC-coupled mAb. The sections were washed by incubation overnight in 1% FCS in PBS and were then examined by fluorescence microscopy.

Detection of apoptosis

(a) by ELISA: DNA fragmentation in brain homogenates was assessed using the Cell Death detection ELISA^{plus} kit. Briefly, 20 μl of CNS lysate was placed in a streptavidin-coated flat-bottomed microtiter plate and incubated for 2 h at room temperature with biotin-labelled anti-histone and peroxidase-conjugated anti-DNA mAbs (80 μl). The plate was washed with PBS-Tween 0.05% and the quantity of nucleosomes fixed to the plates by the biotin-labelled anti-histone mAb and recognised by the peroxidase-conjugated anti-DNA mAb was determined photometrically using ABTS as substrate, measuring absorbance at 410 nm in a spectrophotometer (Dynatech MR5000).

(b) by the TUNEL method: DNA fragmentation was detected on CNS sections by labelling of the 3′OH DNA terminus (TUNEL technique) as previously described (Gavrieli *et al*, 1992). Nervous tissue sections mounted on slides were fixed in pure ethanol for 30 min at −20°C and then dried. The sections were rehydrated and permeabilised by incubation with 10 μg/ml of proteinase K diluted in PBS for 15 min at room temperature, the sections were then washed twice in PBS. CNS sections were incubated for 30 min at 37°C with 25 μl per slot of the labelling preparation containing 12.5 U of terminal deoxynucleotidyltransferase, 2.5 mM $CoCl_2$, 0.2 M potassium cacodylate, 25 mM Tris-HCl and 0.05 nmol of tetramethylrhodamine-conjugated 6-dUTP. The slides were incubated for 15 min at room temperature in 4 × saline sodium citrate (buffer (30 mM trisodium citrate and) 0.3 M NaCl). Slides were then processed for double immunostaining following the protocol described above.

Image analysis
Slides were examined using the appropriate fluorescent filters on a Leica DMRB. Images were processed using Adobe Photoshop and were printed on a color printer (Epson Stylus Color 800).

Statistical analysis
Data were analysed by Student's *t*-test. Results are expressed as means ± s.d. A value of $P<0.05$ was considered significant.

Abbreviations

CNS, central nervous system; CVS, challenge virus standard; p55TNFR$^{-/-}$ mice, p55 Kd TNF-α receptor-deficient mice.

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