VIROLOGY 214, 239–244 (1995)

SHORT COMMUNICATION

Murine Leukemia Virus-Induced Neurodegeneration of Rats: Enhancement of Neuropathogenicity Correlates with Enhanced Viral Tropism for Macrophages, Microglia, and Brain Vascular Cells

MARKUS CZUB,*,[1] STEFANIE CZUB,† MARTINA RAPPOLD,* STEFAN MAZGAREANU,* STEFAN SCHWENDER,‡ MONIKA DEMUTH,* ANDREAS HEIN,§ and RÜDIGER DÖRRIES§

*Institut für Virologie und Immunbiologie, D-97078 Würzburg, Germany; †Pathologisches Institut, D-97080 Würzburg, Germany; ‡Zentrallabor der Medizinischen Univertätsklinik, D-97080 Würzburg, Germany; and §Klinikum Mannheim, Institut für Medizinische Mikrobiologie und Hygiene der Universität Heidelberg, D-68167 Mannheim, Germany

Received July 12, 1995; accepted October 2, 1995

A highly neuropathogenic retrovirus, NT40, was generated by serially passaging an infectious molecular clone of Friend murine leukemia virus, FB29, through F344 Fisher rats. NT40 induced severe neurological signs such as reflex abnormalities and ataxia within 4–6 weeks following neonatal inoculation. FB29 led to only very mild neurological dysfunctions with longer incubation periods. Pathological alterations were characterized by mild (FB29) to extensive (NT40) noninflammatory spongiform degeneration, mainly of brain-stem areas. Infectious center assays revealed that viral titers in brain tissues of NT40-infected rats were 100-fold higher than those of FB29-infected animals. Employing immunohistochemistry, *in situ* hybridization, and flow cytometry, NT40 was found to infect many endothelial cells of brain blood vessels and microglia, whereas FB29 infected only microglia and those to a lower extent. However, when isolated from adult diseased rats, microglial cells turned out in both cases to be nonproductively infected with either FB29 or NT40. Of peripheral organs, we found enhanced levels of NT40 in peritoneal macrophages but not in spleen, thymus, or serum when compared to FB29. Altogether these data suggest that an expanded cellular tropism within the CNS and elevated viral titers in macrophages and microglia correlated with enhancement of neuropathogenicity.

Retroviral infection of the central nervous system (CNS) may result in neurodegenerative disease. A coinciding observation in lentivirus infections, such as human immunodeficiency virus (*1, 2,* reviewed in *3*), simian immunodeficiency virus (*4, 5*), and feline immunodeficiency virus (*6*), as well as in murine leukemia virus (MuLV) models, (*7–12*) is that degenerating neurons do not appear to be infected. Current hypotheses on the pathogenesis of retroviral neurodegeneration are therefore focused on indirectly mediated mechanisms of neuronal injury, probably initiated by retrovirus-infected cells. Within the CNS parenchyma, macrophages and/or microglia are major targets for lentiviruses and MuLV. In primates, emergence of macrophage/microgliatropic virus variants from nonmacrophagetropic virus strains appears to be crucial for the development of neurodegeneration and has been reported to occur after both *in vivo* propagation (*13, 14*) and sequential intracerebral passages (*15*). Here, we addressed a similar question for neuropathogenic MuLV, i.e., whether an enhanced macrophage/microgliatropism would evolve after serial *in vivo* passaging of MuLV and whether this would correlate with enhanced neuropathogenicity.

A new variant of MuLV, NT40, was isolated after serial passage of a molecular clone of Friend MuLV, FB29 (*16*), through F344 Fisher rats. The inocula were prepared from spleen and brain tissues of neonatally infected donor rats not older than 3 weeks at the time of sacrifice. After the third passage, brain material was used to infect NIH3T3 cells. Virus obtained from the supernatant was cloned by limiting dilutions on Fisher rat embryo cells. Intraperitoneal (ip) inoculation of neonatal Fisher rats with clone NT40 induced severe reflex abnormalities of fore- and hindlimbs and subsequent ataxia within 4–6 weeks postinfection (pi). Later in disease some NT40-infected animals developed priapism. In contrast, neonatal inoculation (ip) with the parental clone FB29 led to very mild reflex abnormalties of hind- and occasionally forelimbs within 8–12 weeks pi. Clinical evaluations involved examinations of the movements of rats in the cage. Reflex functions were tested by lifting each animal by the tail and placing it on the edge of the cage (grabbing reflex) and by short falls (placing reflex). After infec-

[1] To whom reprint requests should be addressed at Institut für Virologie und Immunbiologie, Versbacherstr. 7, D-97078 Würzburg, Germany. Fax: +49-931-2013934. E-mail: Markus.Czub@vax.rz.uni-wuerzburg.d400.de.

0042-6822/95 $12.00

TABLE 1

Infectivity Associated with Various Organs Isolated from Neonatally Inoculated Rats

Virus	Serum[a]	Spleen[b]	Thymus[b]	CNS[c]	P-MO[b]	Microglia[b]
FB29[d]	4.4 ± 0.2 (n = 22)	4.2 ± 0.2 (n = 17)	4.6 ± 0.2 (n = 19)	3.8 ± 0.2 (n = 16)	4.1 ± 0.2 (n = 12)	<1 (n = 3)
NT40[d]	5.0 ± 0.2 (n = 10)	4.3 ± 0.4 (n = 4)	4.3 ± 0.1 (n = 4)	5.7 ± 0.2 (n = 4)	4.9 ± 0.2 (n = 10)	0–2 (n = 4)

[a] Serum titers are expressed as log FFU/ml ± SE.
[b] Titers from spleen, thymus, peritoneal cells (P-MO), or microglial cells are expressed as log FFU/10^6 cells ± SE.
[c] CNS titers are expressed as log FFU/g tissue ± SE.
[d] FB29 or NT40 was administered ip (4×10^4 FFU in 50 μl).

tion with either virus, clinical signs progressed slowly but the affected animals usually did not die before 100 days, a time when all experiments were terminated.

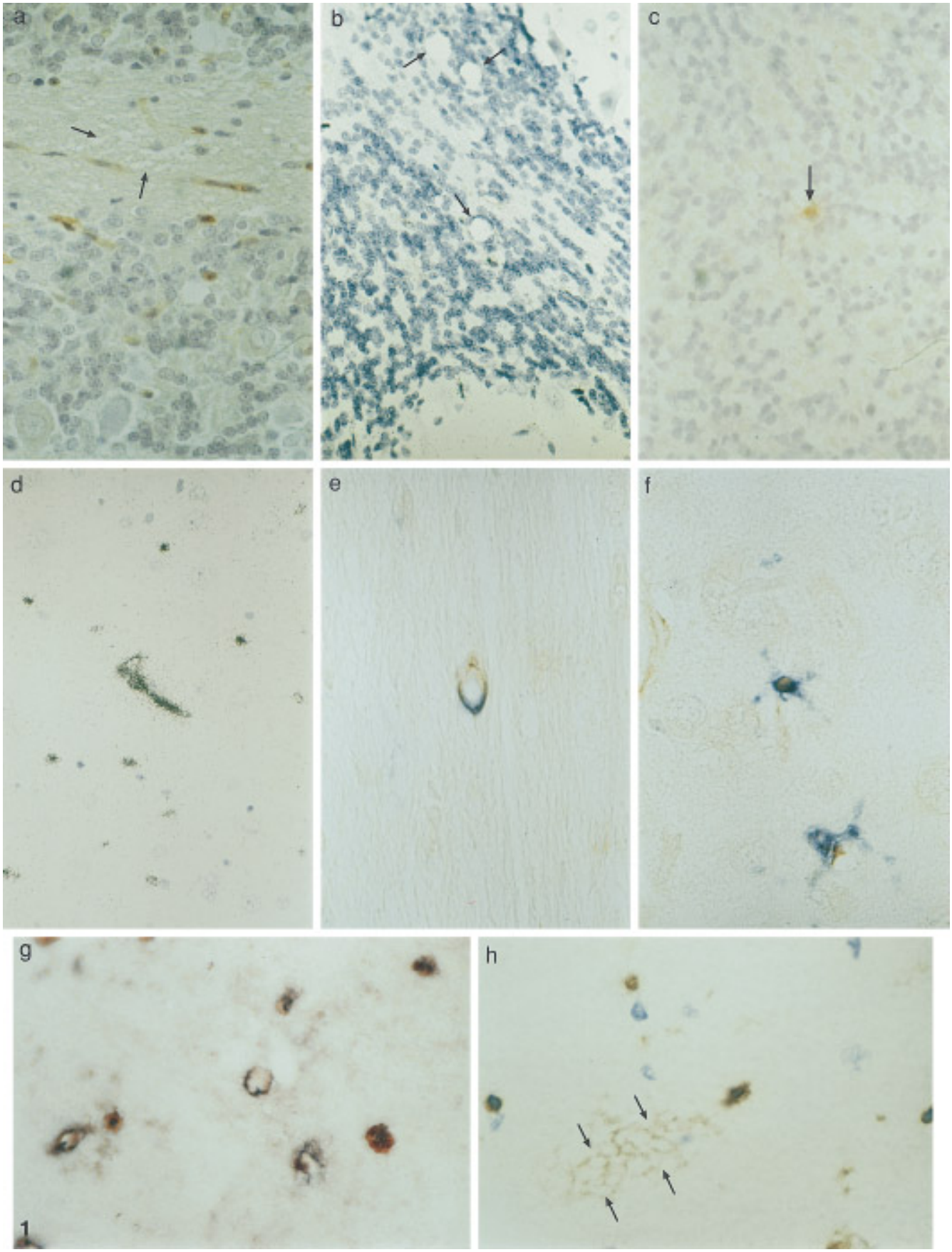
Histopathological examination of brain tissues revealed mild (FB29) and extensive (NT40) spongiform degeneration, predominantly of brain-stem areas. The inner molecular layer (Fig. 1a) and to a lower extent the granular cell layer (Fig. 1b) of the cerebellum displayed vacuolization in brains from NT40- but not FB29 (not shown)-infected rats. A critical determinant of neuropathogenicity is the level of CNS infection. Single-cell suspensions made from brains of adult rats (>5 weeks of age) neonatally inoculated with NT40 contained 100-fold more productively infected cells than cell suspensions from the CNS of FB29-infected rats ($P < 0.001$, two-tailed t test; Table 1) as revealed by means of an infectious center assay (*16*).

Previous studies utilizing various neuropathogenic MuLVs in rats and mice suggested that infection of brain vascular elements may play a key role (*10, 17–20*) or may even be sufficient (*17*) for the induction of neurological disease. Employing virus-specific immunohistochemistry using a virus-specific hyperimmune serum (*16*), as well as *in situ* hybridization on brain tissues, we found no evidence for the presence of either FB29 RNA (*16*) or FB29 proteins (Fig. 1c) in cells associated with blood vessels. FB29-specific signals obtained independently by both techniques were rare, were scattered predominantly over brain-stem areas, and were associated with small round cells with scanty cytoplasms and small, polymorphic, rather hyperchromatic nuclei. These cells were regarded as microglia (*16*).

Analysis of NT40 brains revealed a widespread distribution of both viral RNA (Figs. 1b and 1d) and viral proteins (Figs. 1a and 1e–1g). Positive signals were found in association with vascular structures as well as with glial cells but not with neurons (Figs. 1a, 1b, and 1d–1g). Of positive blood vessels, the inner cellular lining was stained with antiviral antibodies, indicating infection of endothelial cells. This result was confirmed by double staining using hyperimmune serum and Griffonia simplicifolia B_4 isolectin (Sigma) specific for endothelial cells of blood vessels (Fig. 1e) and for microglia (*21–23*) (Fig. 1f). Some cells positive for NT40 also reacted with antiferritin antibodies (Biogenex) indicative of infection of microglial cells (*24*) (Figs. 1f and 1g). Of infected glial cells, some possessed a highly ramified morphology (Fig. 1h), suggesting that they were nonactivated microglial cells. These results showed that NT40 possessed an expanded cellular tropism within the CNS compared to FB29. Infection of vascular elements by NT40 possibly contributed to both enhanced levels of CNS infection and facilitation of viral spread into CNS parenchyma. However, both viruses induced spongiosis, and the extent of spongiform lesions correlated with the level of glial infection. These cells were identified as microglia. For further verification, FACS analyses were performed.

Microglia were isolated from adult rat brains by means of a method previously developed at our institute (*25*). We could recover $0.8–1.7 \times 10^6$ cells from a brain and spinal cord of an adult rat. The majority of these cells (55–70%) expressed CD11b/c and low-to-moderate levels of CD45 as revealed by FACS analysis (*16*) and were regarded as microglial cells (*25*). We saw no differences in total cell numbers nor in the quantitative distribution of the subpopulations when comparing preparations from uninfected, FB29-infected, or NT40-infected rats (data not shown). For additional antiviral staining, cells were fixed

FIG. 1. Microscopic examination of infected rat brains. Spongiform changes (arrows) in (a) the molecular layer (antiviral immunostaining brown) and (b) the granule cell layer, respectively, of a rat cerebellum infected with NT40. (c) Immunohistochemical staining of a single cell (arrow) infected with FB29. (d) *In situ* hybridization of a NT40 brain-stem area exhibiting positive signals associated with single cells as well as with a blood vessel. (e) Double labeling employing GSA (blue) and antiviral staining (brown) indicates NT40 infection of an endothelial cell. (f–h) Dual immunolabeling showing positive reactions for ferritin (blue) and viral proteins (brown or red) in NT40-infected microglia. Note that not all cells were positive for both signals. (h) Ramified morphology stained by antiviral antibodies (arrows) indicating NT40 infection of nonactivated microglial cell(s). Original magnifications: a, c, h, 250×; b, d, 150×; e, f, g, 480×.


a
b
c
d
e
f
g
h
1

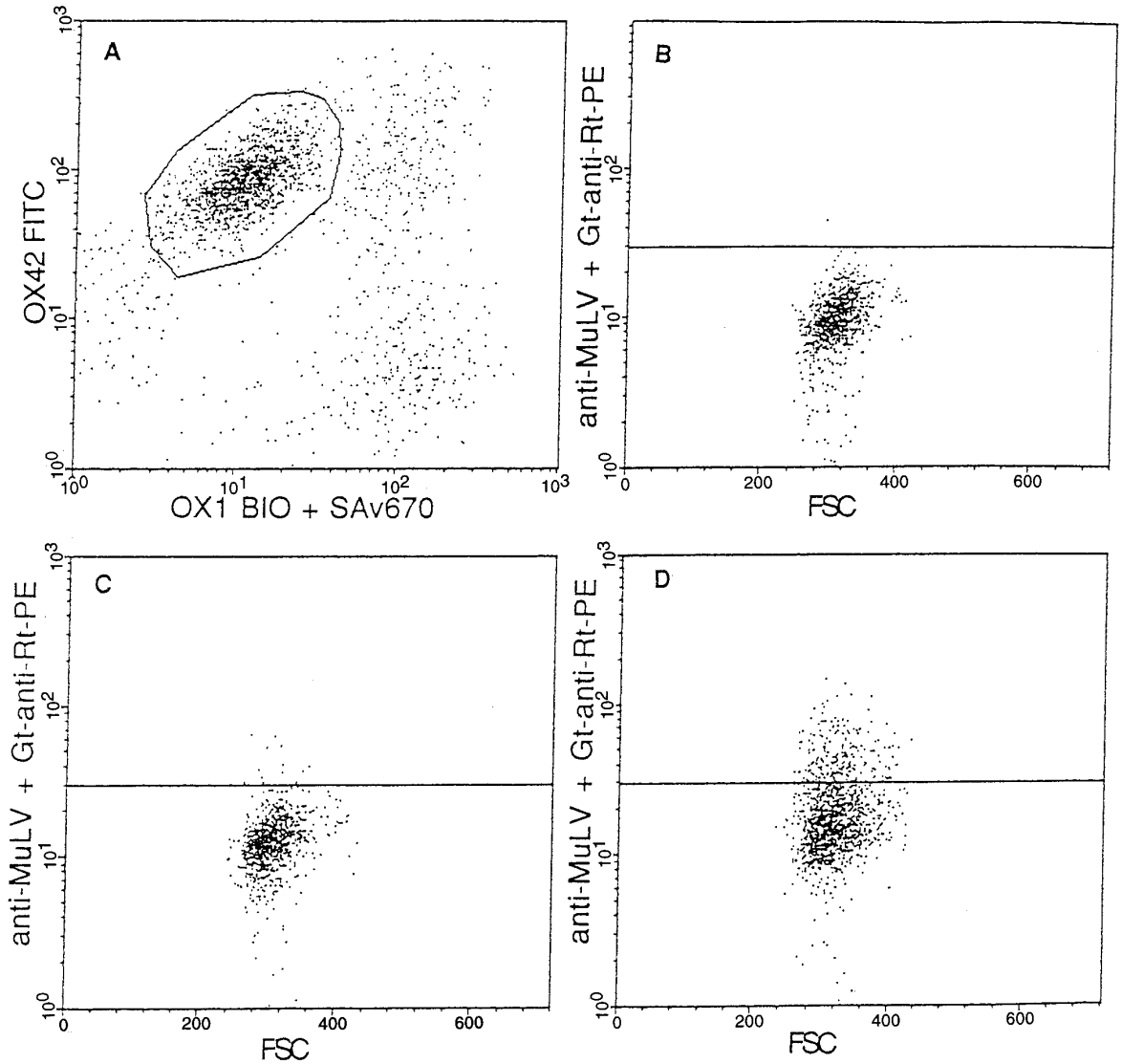


FIG. 2. Cytofluorographic analysis of microglial cells. After density gradient centrifugation, brain cells were stained with monoclonal antibodies OX42 and OX1 and with virus-specific hyperimmune serum. CD11b/c^+/$CD45^{low}$ were regarded as microglial cells, gated (as seen in A), and further analyzed for expression of viral proteins. (B) No antiviral staining could be detected in microglia from uninfected rats, (C) less than 3% of all microglia expressed viral proteins in FB29 infected animals, and (D) around 12% in NT40-infected rats.

in 3.7% formaldehyde–PBS for 30 min and permeabilized with 0.3% Triton X-100 (Sigma) for 30 min after cell surface labeling. Employing triple immunostaining, 12% of microglial cells (CD11b/c^+/$CD45^{low}$) isolated from NT40-infected rats were found to react specifically with antiviral antibodies (Fig. 2D). This indicated an infection of microglia with NT40 and confirmed our histological data. Microglial cells from FB29-infected rats could also be stained with antiviral antibodies, yet to a lower degree ($>$3%) compared to microglia from NT40-infected rats (Fig. 2C). Among nonmicroglial cells purified with this technique, no specific staining with antiviral antibodies was observed (data not shown).

In previous studies on neuropathogenic MuLV in Fisher rats, apparently conflicting results on the cellular tropism within the CNS were presented. Whereas Kai and Furuta found viral proteins in glial cells (*26*), Hoffman *et al.* reported this same virus, PVC 211, to be restricted to endothelial cells of brain blood vessels (BVEC) as revealed by dual (immuno)labeling using virus-specific antibodies and RCA-1 (*17*). However, RCA-1 is specific for both BVEC and microglial cells (*8, 27, 28*). Acknowledging all these data including ours, microglial infection has to be considered as an inevitable event in MuLV-induced neurodegeneration of rats. In brains of NT40-infected rats, infection of microglia was abundant, both in degenerated and in healthy brain areas. FB29-infected glia cells were rather rare and more distant from diseased regions. Neurons failed to show viral infection, in regions both with and without spongiform alterations.

Altogether, these findings support hypotheses suggesting that the pathogenesis of spongiform change is indirectly mediated through microglial infection.

However, after seeding microglial cells from NT40- or FB29-infected diseased rats as infectious centers, only very few cells were found to produce virus (Table 1). Similarly, release of infectious virus particles (NT40) into the supernatants of cultured microglia preparations was negligible as titers found were below 20 focus-forming units/ml supernatant (data not shown). Our data demonstrate that a high proportion of microglia cells was infected with NT40 and a lower proportion with FB29, yet, under the experimental conditions given here, this infection was nonproductive.

Although the significance of this finding is not clear, it is consistent with observations made in related mouse models of retroviral neurodegeneration (*12, 29*). It has been shown that microglial infection with neurovirulent MuLV resulted in defective processing of viral envelope protein (*29*), intracellular budding of virus particles (*12, 29*), and only limited release of infectious virus into supernatants of cultivated microglia, yet infected microglia were highly infectious *in vivo* (*29*). These viral–host interactions may represent functional disturbances consequently leading to disruption of normal brain functions. To find out whether nonproductive infection was unique to microglial cells, we analyzed viral–host interactions in another cell type of the monocytic lineage, peritoneal macrophages.

For the isolation of peritoneal cells, 10 ml of a 10% sucrose–PBS solution was administered ip into the peritoneal cavity of killed rats. Animals were carefully rotated to achieve a thorough lavage. About 7 ml of the administered solution was recovered from the abdomen and spun at 250 *g* for 10 min at 4°. The pelleted cells were suspended in 3 ml of erythrocyte-lysis buffer (0.155 *M* NH_4Cl, 0.01 *M* $KHCO_3$, 0.1 m*M* EDTA in H_2O) for 3 min, washed in PBS, and counted in a Neubauer chamber before further analyses. Of the peritoneal cells collected by lavaging peritoneal cavities of adult rats, 70–80% expressed both CD45 and CD11b/c (data not shown) and were thus regarded as macrophages. When seeded as infectious centers, peritoneal cells isolated from FB29-infected rats were found to contain six times less productively infected cells than those isolated from NT40-infected rats ($P < 0.02$, two-tailed *t* test; Table 1). It was also noted that neither the total numbers of peritoneal cells nor the profile of the cellular compositions appeared to be influenced by infection with either virus (as revealed by FACS analysis, data not shown), indicating that the infection was nonlytic. Thus, enhanced neuropathogenicity of NT40 correlated with enhanced tropism not only for microglial and vascular brain cells but also for peritoneal macrophages.

Enhanced numbers of NT40-infected cells of the monocytic lineage and of BVEC might have resulted from

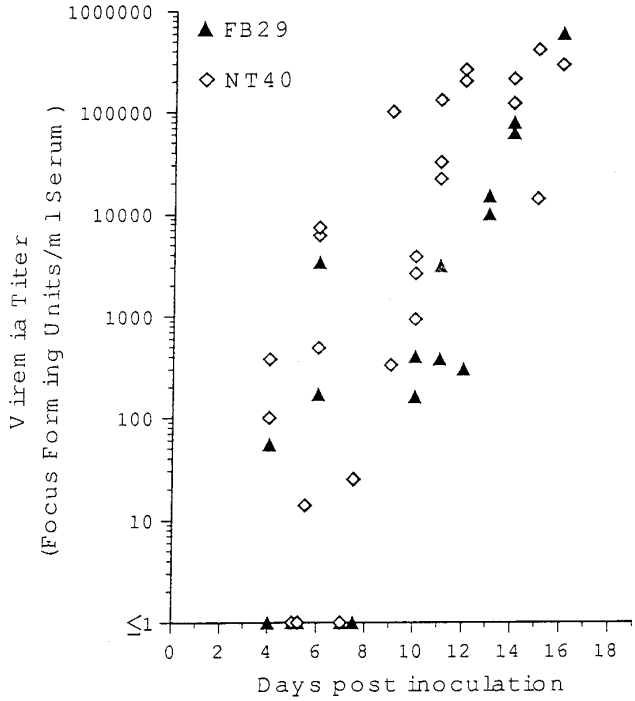


FIG. 3. Viremia kinetics of FB29 and NT40. Each symbol represents a titration of an individual animal.

an enhanced viral spread in peripheral tissues before a progressively developing, age-dependent restriction of retroviral replication was established. The dynamic interaction of the efficiency of viral spread and the development of age-dependent restriction of retroviral infection is a key factor that controls the level of CNS infection (*30–32*). A reliable indicator of the efficiency of replication and/or viral spread are the kinetics of viremia (*33, 34*). A comparison of viremia titers for both viruses, NT40 and FB29, revealed similar kinetics (Fig. 3) and almost identical viremia levels ($P < 0.02$, two-tailed *t* test; Table 1). Among other peripheral organs, i.e., spleen and thymus, no significant (when $P < 0.02$, two-tailed *t* test) differences in their respective levels of infection (FB29 versus NT40) were found when compared with each other (Table 1). Therefore, after infection with either NT40 or FB29, BVEC as well as peritoneal macrophages and microglial cells were probably exposed to similar viral loads at similar time points. The enhanced tropism of NT40 for these cells is probably independent of the establishment of age-dependent susceptibility to retroviral infection.

In summary, our results confirm previous studies by Kai and Furuta (*26*) in that a highly neuropathogenic MuLV variant could be generated by serial *in vivo* passages. We further demonstrate that enhancement of MuLV-associated neuropathogenicity correlated with an expanded cellular tropism within the CNS and elevated viral titers in cells of the monocytic lineage, macrophages and microglia. Nonproductive infection appeared to be

confined to microglial cells and may represent an initial step of cellular dysfunction of these cells, subsequently leading to CNS damage.

ACKNOWLEDGMENTS

The authors thank I. Johnston, J. Portis, and V. ter Meulen, for critical review of the manuscript, and S. Schimmer and E. Bachmann, for excellent technical assistance. This work was supported by grants from the Deutsche Forschungsgemeinschaft (Cz 56/1-1) and from the Bundesministerium für Forschung und Technologie (01/KI 9210/B2).

REFERENCES

1. Dickson, D. W., Mattiace, L. A., Kure, K., Hutchins, K., Lyman, W. D., and Brosnan, C. F., *Lab. Invest.* 64, 135–156 (1991).
2. Peudenier, S., Hery, C., Montagnier, L., and Tardieu, M., *Ann. Neurol.* 29, 152–161 (1991).
3. Budka, H., *Brain Pathol.* 1, 163–175 (1991).
4. Chakrabarti, L., Hurtrel, M., Maire, M. A., Vazeux, R., Dormont, D., Montagnier, L., and Hurtrel, B., *Am. J. Pathol.* 139, 1273–1280 (1991).
5. Hurtrel, B., Chakrabarti, L., Hurtrel, M., and Montagnier, L., *Res. Virol.* 144, 41–46 (1993).
6. Lackner, A. A., Dandekar, S., and Gardner, M. B., *Brain Pathol.* 1, 201–212 (1991).
7. Baszler, T. V., and Zachary, J. F., *Am. J. Pathol.* 138, 655–671 (1991).
8. Gravel, C., Kay, D. G., and Jolicoeur, P., *J. Virol.* 67, 6648–6658 (1993).
9. Hoffman, P. M., Pitts, O. M., Bilello, J. A., and Cimino, E. F., *Rev. Neurol. (Paris)* 144, 676–679 (1988).
10. Kay, D. G., Gravel, C., Robitaille, Y., and Jolicoeur, P., *Proc. Natl. Acad. Sci. USA* 88, 1281–1285 (1991).
11. Lynch, W. P., Czub, S., McAttee, F. J., Hayes, S. F., and Portis, J. L., *Neuron* 7, 365–379 (1991).
12. Stoica, G., Illanes, O., Tasca, S. I., and Wong, P. K., *Lab. Invest.* 69, 724–735 (1993).
13. Desrosiers, R. C., Hansen-Moosa, A., Mori, K., Bouvier, D. P., King, N. W., Daniel, M. D., and Ringler, D. J., *Am. J. Pathol.* 139, 29–35 (1991).
14. Kodama, T., Mori, K., Kawahara, T., Ringler, D. J., and Desrosiers, R. C., *J. Virol.* 67, 6522–6534 (1993).
15. Sharma, D. P., Zink, M. C., Anderson, M., Adams, R., Clements, J. E., Joag, S. V., and Narayan, O., *J. Virol.* 66, 3550–3556 (1992).
16. Hein, A., Czub, S., Xiao, L. X., Schwender, S., Dörries, R., and Czub, M., *Virology* 211, 408–417 (1995).
17. Hoffman, P. M., Cimino, E. F., Robbins, D. S., Broadwell, R. D., Powers, J. M., and Ruscetti, S. K., *Lab. Invest.* 67, 314–321 (1992).
18. Pitts, O. M., Billelo, J. A., Powers, J. M., and Hoffman, P. M., *Lab. Invest.* 56, 401–409 (1987).
19. Zachary, J. F., Knupp, C. J., and Wong, P. K. Y., *Am. J. Pathol.* 124, 457–468 (1986).
20. Masuda, M., Hoffman, P. M., and Ruscetti, S. K., *J. Virol.* 67, 4580–4587 (1993).
21. Kivela, T., and Tarkkanen, A., *Cell Tissue Res.* 249, 277–288 (1987).
22. Streit, W. J., and Kreutzberg, G. W., *J. Neurocytol.* 16, 249–260 (1987).
23. Ashwell, K. W. S., Holländer, H., Streit, W., and Stone, J., *Visual Neurosci.* 2, 437–448 (1989).
24. Kaneko, Y., Kitamoto, T., Tateishi, J., and Yamaguchi, K., *Acta Neuropathol.* 79, 129–136 (1989).
25. Sedgwick, J. D., Schwender, S., Imrich, H., Dörries, R., Butcher, G. W., and ter Meulen, V., *Proc. Natl. Acad. Sci. USA* 88, 7438–7442 (1991).
26. Kai, K., and Furuta, T., *J. Virol.* 50, 970–973 (1984).
27. Michaels, J., Price, R. W., and Rosenblum, M. K., *Acta Neuropathol.* 76, 373–379 (1988).
28. Colton, C. A., Abel, C., Patchett, J., Keri, J., and Yao, J., *J. Histochem. Cytochem.* 40, 505–512 (1992).
29. Lynch, W. P., Brown, W. J., Spangrude, G. J., and Portis, J. L., *J. Virol.* 68, 3401–3409 (1994).
30. Czub, M., Czub, S., McAtee, F. J., and Portis, J. L., *J. Virol.* 65, 2539–2544 (1991).
31. Czub, M., McAttee, F. J., and Portis, J. L., *J. Virol.* 66, 3298–3305 (1992).
32. Park, B. H., Lavi, E., and Gaulton, G. N., *Virology* 203, 393–396 (1994).
33. Czub, M., McAtee, F. J., Czub, S., Lynch, W. P., and Portis, J. L., *Virology* 206, 372–380 (1995).
34. Portis, J. L., Spangrude, G. J., and McAtee, F. J., *J. Virol.* 68, 3879–3887 (1994).