Virology **267,** 80–89 (2000)
doi:10.1006/viro.1999.0118, available online at http://www.idealibrary.com on IDEAL®

V and C Proteins of Measles Virus Function as Virulence Factors *in Vivo*

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Received October 5, 1999; returned to author for revision November 22, 1999; accepted November 30, 1999

The measles virus (MV) P gene encodes three proteins: the P protein and two nonstructural proteins, C and V. Because the functions of both the C and V protein are unknown, we used MV C (C−) and V (V−) deletion recombinants generated by the MV reverse genetics system (F. Radecke, P. Spielhofer, H. Schnieder, K. Kaelin, M. Huber, C. Dotsch, G. Christiansen, and M. A. Billeter 1995. *EMBO J.* 14, 5773–5784). Compared to parental vaccine strain, Edmonston (Ed) MV, both had normal growth and cytopathic effects in Vero cells and showed similar growth kinetics in human neuroblastoma SK-N-MC cells and in primary mouse neurons expressing the MV receptor, CD46. However, *in vivo*, using YAC-CD46 transgenic mice as a model for MV induced CNS disease (M. B. A. Oldstone, H. Lewicki, D. Thomas, A. Tishon, S. Dales, J. Patterson, M. Manchester, D. Homann, D. Naniche, and A. Holz 1999. *Cell* 98, 629–640), C− and V− viruses differed markedly from wt Ed(V^+C^+) virus. Newborn mice inoculated with as little as 10^3 PFU of Ed strain became ill and died after 10–15 days. In contrast, those inoculated with 10^3 or 10^4 PFU of MV C− or MV V− showed significantly fewer and milder clinical symptoms and had a lower mortality. A total of 10^5 PFU V− virus were required to kill most YAC-CD46 mice, and less than half (44%) were killed with a corresponding dose of MV C−. Immunohistochemical staining for MV antigens showed similar extents of spread for MV C− and MV Ed but restricted spread for MV V− throughout the brain. Viral load and transcription were markedly reduced for V− but not for C−. Multiple cytokines and chemokines were equivalently upregulated for all three viruses. Therefore, MV C and V proteins encode virulence functions *in vivo* and likely operate via separate mechanisms.

INTRODUCTION

Measles virus (MV), the prototypic *Morbillivirus*, is encoded by a single genomic RNA of negative sense with six genes that generate eight known proteins (Griffin and Bellini, 1996). The functions of the matrix (M), fusion (F), and hemagglutinin (HA) genes involved in viral entry into cells, assembly, and exit are partially understood. In addition, three gene products are required for replication: the polymerase (L), nucleocapsid (N), and phosphoprotein (P). All MV proteins are derived from single cistrons except for P. The P gene encodes a heavily phosphorylated protein of 60 kDa, which, in association with L, is required for transcription and replication of the ribonucleoprotein (RNP) complex (Griffin and Bellini, 1996). In addition, the P cistron encodes two other proteins, C and V, whose functions are not known but appear not to be required for replication in cultured cells (Radecke and Billeter, 1996; Schneider *et al.,* 1997).

The C protein (21 kDa) is translated from a second open reading frame (ORF) (+1 relative to P ORF) initiated from the second AUG located19 nucleotides (nt) down stream of the P AUG, thus giving rise to a unique amino acid (aa) sequence (Bellini *et al.,* 1985). The C proteins are related amongst the *Morbilliviruses* and are encoded by all *Paramyxoviruses*; however, aa sequences are not conserved within this group. The C protein of Sendai virus (SeV) is thought to downregulate viral transcription by a promoter-specific mechanism (Cadd *et al.,* 1996; Tapparel *et al.,* 1997) and is found in small quantities in nucleocapsids and virions (Portner *et al.,* 1986). A recombinant SeV silenced of C and C′ but not the shorter Y1 and Y2 ORFs was rescued but was greatly attenuated *in vitro* and almost completely incapable of replicating *in vivo* (Kurotani *et al.,* 1998). Furthermore a recombinant SeV devoid of all C ORFs was recovered but highly restricted for growth *in vitro,* clearly showing that the C proteins are not absolutely essential for replication (Kurotani *et al.,* 1998). In contrast, when MV C protein was silenced, the virus grew as well as the wild type in cell culture (Radecke and Billeter, 1996).

Expression of the V protein is driven by transcription and translation of the P gene using the P AUG and ORF. Approximately half of the transcripts undergo an editing process whereby a nontemplated guanosine (G) is cotranscriptionally inserted at P mRNA position 751, leading to an alternative C terminus after aa 231(Cattaneo *et al.,* 1988). Sixty-eight unique aa containing a high proportion of cysteine replace the C terminus of P, giving the V protein a length of 299 aa rather than 507 (Cattaneo *et al.,* 1988). V proteins are not unique amongst the *Morbilliviruses* as they are encoded by all members of subfamily Paramyxovirinae with the exception of human parain-

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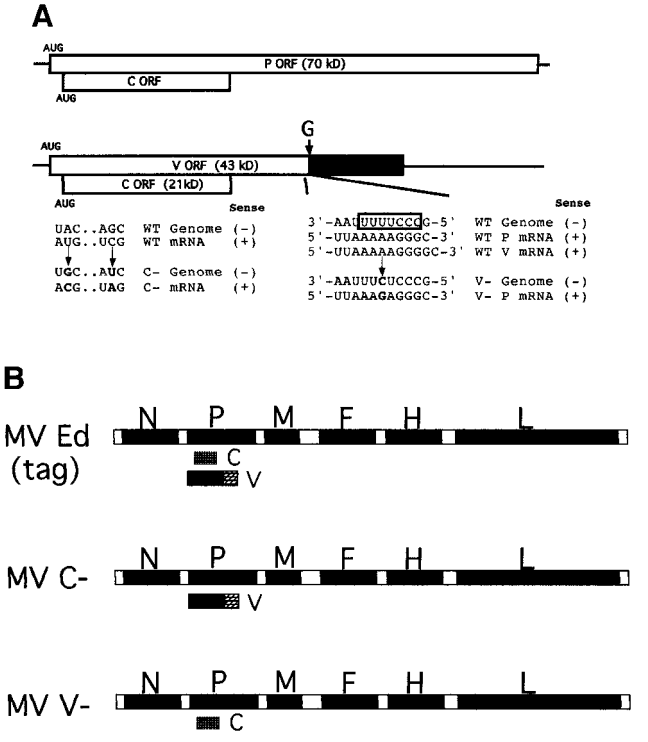


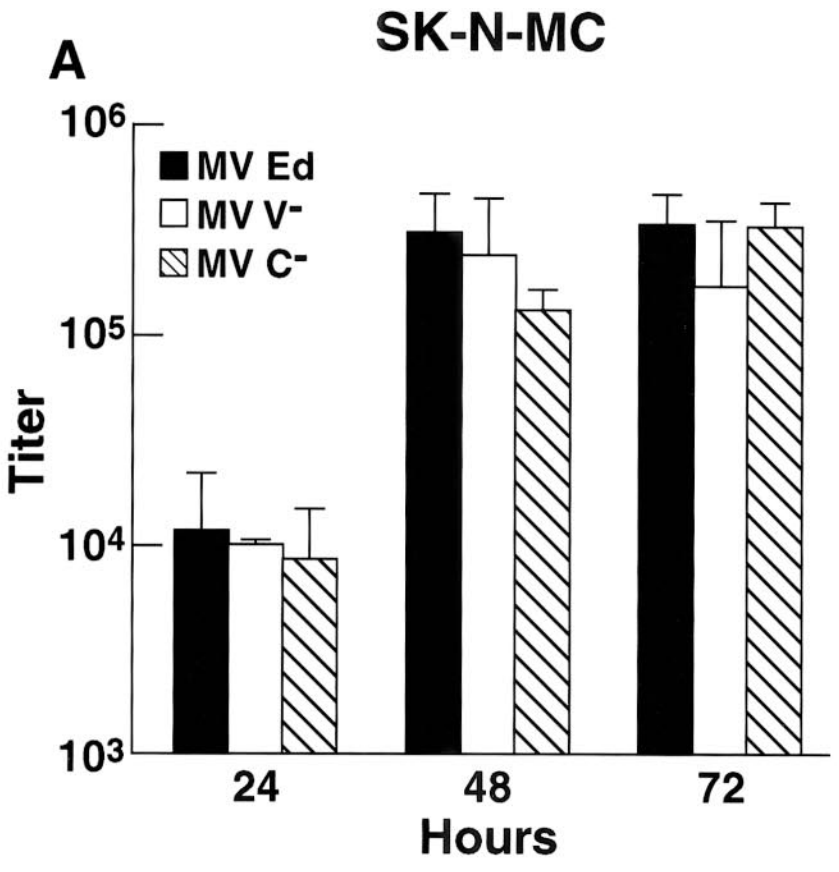
FIG. 1. Construction and generation of recombinant MVs lacking expression of the C or V proteins. (A) Schematic diagram of the P cistron encoding P, C, and V proteins showing alternative ORF for C and shared P and V ORFs proximal to G insertion. Expression of the C protein was eliminated by silencing of the C initiator AUG (met) by mutation of T to C (bold), [p(+)MV position 1830] and placing a stop codon UAG (C to A mutation), [p(+)MV position 1845] downstream in the C ORF. Insertion of the nontemplated G was eliminated by replacing A with G [p(+)MV position 2494], disrupting the *cis*-acting element (box) required for RNA editing and generating a mutant MV lacking the expression of V. (B) Genomic organization of wild-type MV Ed and MVs lacking expression of the C (MV C−) or V (MV V−) proteins used in this study.

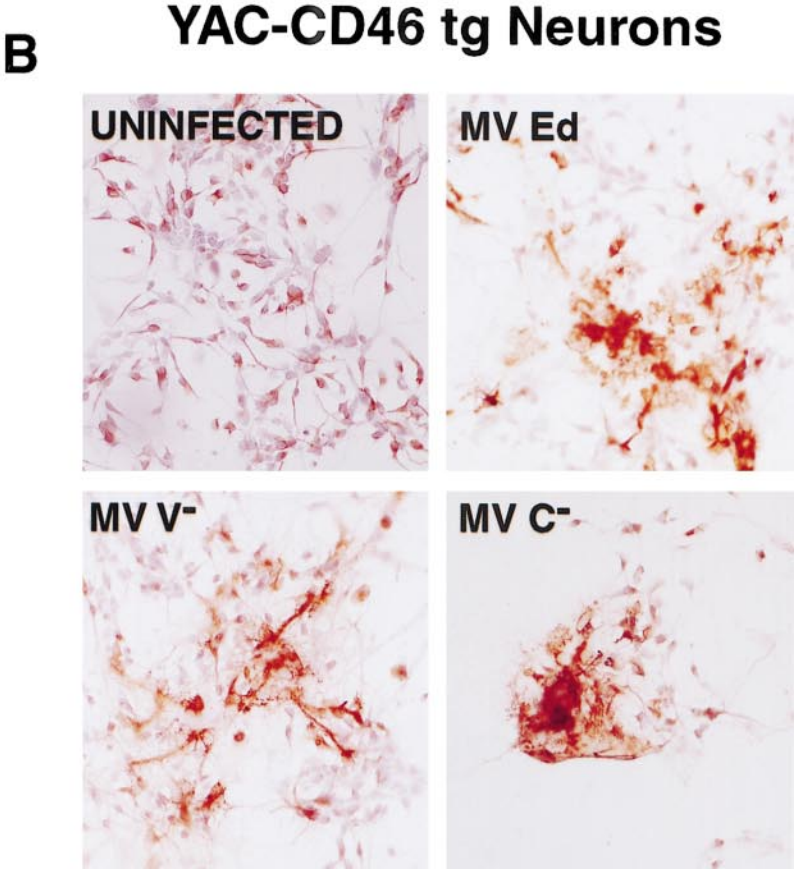
fluenza viruses 1 and 3 (Lamb and Kolakofsky, 1996). Although different strategies for generation of V proteins are utilized by different viruses within the group, the C-terminal cysteine-rich domain of the V protein is highly conserved, whereas the entire P protein is not (Lamb and Kolakofsky, 1996). The seven cysteine residues are exactly conserved within all of the known V proteins, and show a remote similarity to zinc fingers. Zinc binding has been demonstrated for MV V (Liston and Briedis, 1994) and SeV V (Paterson *et al.,* 1995). Other aa are exactly conserved with the most notable stretch being an HRRE motif immediately distal to the nontemplated G insertion site. This degree of conservation suggests that the V proteins have an important function in the life cycle of these viruses. MV V protein is not associated with either MV virions or RNP complexes but rather is found diffusely distributed thoughout infected cells (Wardrop and Briedis, 1991). The N-terminal portion of V binds to unincorporated N protein and may down modulate RNA synthesis (Curran *et al.,* 1991; Horikami *et al.,* 1996; Tober *et al.,* 1998). Studies of V function have been facilitated by the development of reverse genetics, which has allowed the elimination of V expression in MV (Schneider *et al.,* 1997) and SeV (Kato *et al.,* 1997b; Delenda *et al.,* 1998) with few or no observable effects on replication *in vitro*. *In vivo*, SeVs devoid of V were markedly attenuated in the lungs of mice compared to the wild type (Kato *et al.,* 1997b; Delenda *et al.,* 1998). Further, recombinant SeVs deleted of the unique cysteine-rich C-terminal portion of V have also been reported to replicate poorly in ICR outbred mice (Kato *et al.,* 1997a; Delenda *et al.,* 1998).

Humans are the only natural host for MV, which, in addition to targeting the respiratory tract, regularly at-

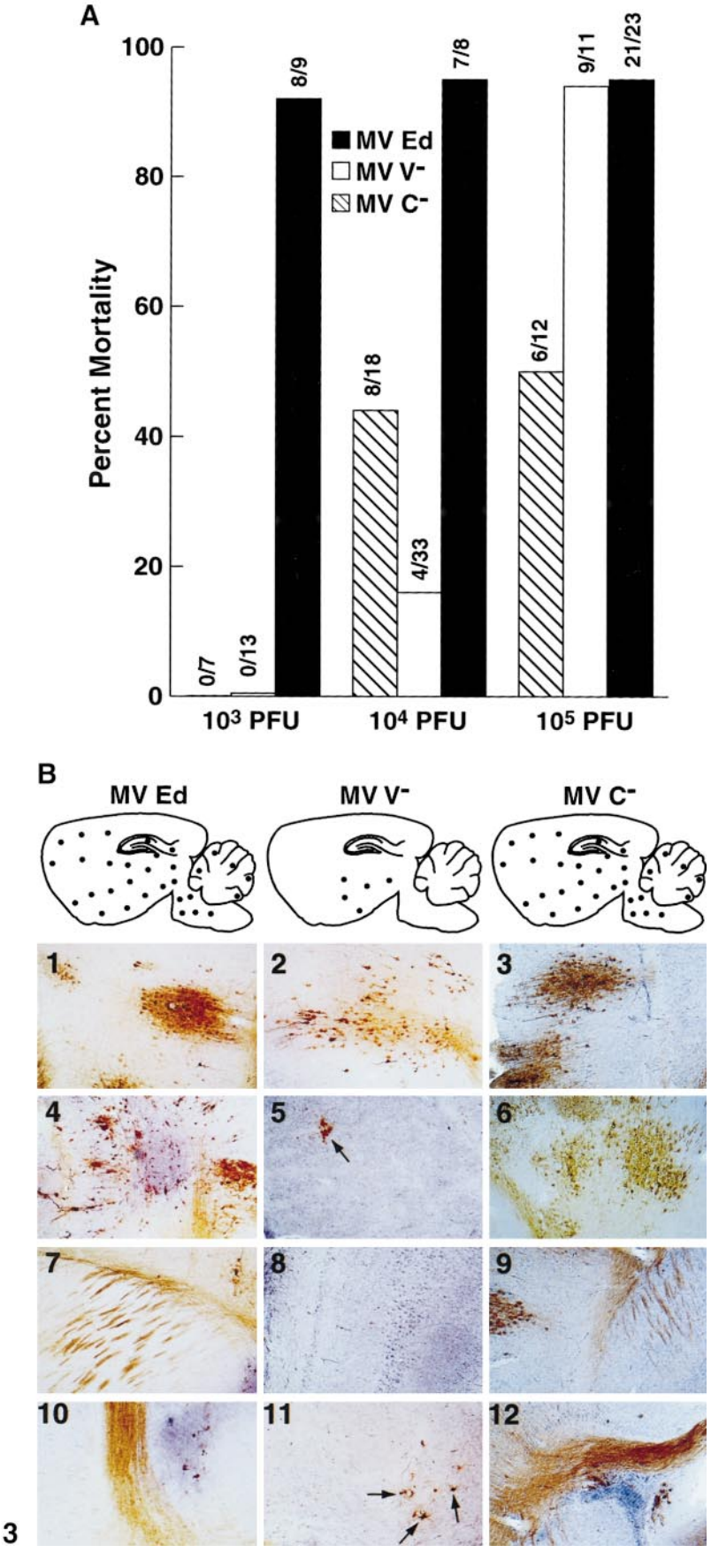
FIG. 2. Replication and spread of MV Ed, MV C−, and MV V− in neuronal cells *in vitro*. (A) Two-step growth analysis of the three viruses in human neuroblastoma SK-N-MC cells. At least three different experiments were performed for each virus with SK-N-MC cells. Infected at m.o.i. of 0.1. One, 2, and 3 days later, cells were harvested, freeze thawed at −80°C and titered on Vero cells by plaque assay. (B) Primary neurons were taken from fetal (P16) YAC-CD46 transgenic mice (Rall *et al.,* 1997) and infected with MV Ed, MV C−, and MV V− (m.o.i. of 3). Seven days later coverslips were stained with human antisera to MV. The number of infected neurons and spread of the viruses were similar for all three viruses.

FIG. 3. Mortality profiles and analysis of viral replication and spread in the brains of YAC-CD46 transgenic mice. (A) Percent mortality caused by the three viruses at differing log dilutions of MV inoculation. Numbers at the end of each bar refer to the number of inoculated mice dead by Day 30 over the total number of mice inoculated per experimental group. (B) Comparison of the replication and spread of MV Ed, MV C−, and MV V− in the brains of YAC-CD46 transgenic mice inoculated with 1×10^4 PFU of the three different viruses. Cartoons display typical results obtained by comparing spread of MV Ed to that of MV V− and MV C− in which the expression of the V or C proteins have been eliminated in brains of YAC-CD46 transgenic mice 5–10 days after inoculation. The information shown is from four mice in each group. Similar differences in spread of virus were noted in YAC-CD46 transgenic mice receiving 10^5 PFU. The lower panels show replication and tracking of MV and the mutants throughout neurons and axon tracts in the cerebral cortex of YAC-CD46 brains as illustrated in the cartoons. Tissues were harvested 6–7 days after viral inoculation, fixed with 4% paraformaldehyde, and sectioned in a vibratome, and 40-μm sections stained for the expression of MV antigens using polyclonal human antibody to MV. Axon tracts of the extra pyramidal system are shown in panels 7–9, whereas those of the corticospinal pyrmidal tract are shown in 10–12. Panels 2, 5, 8, and 11 are from a MV V− inoculated mouse with the heaviest viral replication in neurons seen in panel 2 but little spread in axon tracts (panels 5, 8, and 11). Only occasional neurons and axons throughout the CNS expressed viral antigens (panels 5 and 11). Arrows point to neurons expressing viral antigens (panels 5 and 11). Similar patterns of low level viral expression and lack of axonal spread were seen in three other YAC-CD46 transgenic mice inoculated with MV V− (10^4 PFU) and in two additional transgenic mice inoculated with 10-fold more MV V− (10^5 PFU). By comparison, panels 1, 4, 7, and 10 show commonly observed virus replication and spread at corresponding sites with MV Ed inoculated mice. This pattern of MV replication and spread was uniformly observed in four additional mice inoculated with 10^3 and 10^4 PFU of virus. MV Ed and MV C− staining were similar in matched sections of the brain (panels 3, 6, 9, and 12) in three mice inoculated with 10^4 PFU and two mice inoculated with 10^5 PFU (data not shown).


A
SK-N-MC
MV Ed
MV V-
MV C-
Titer
10^6
10^5
10^4
10^3
24
48
72
Hours


B
YAC-CD46 tg Neurons
UNINFECTED
MV Ed
MV V-
MV C-

2


A
Percent Mortality
100
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60
40
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MV V⁻
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0/7
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MV Ed
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1
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3

tacks the immune and occasionally the nervous systems. Infection of the immune system causes a transient immune suppression that can lead to severe complications. Infection of the central nervous system (CNS) can induce an acute encephalopathology or rarely a chronic CNS disease, subacute sclerosing panencephalitis (SSPE), that ultimately results in death.

To study the pathogenesis of MV, we developed a transgenic mouse model in which expression of the MV receptor, CD46, largely mimics its natural expression in humans. These mice are highly susceptible to CNS infection with the standard laboratory strain of MV, Edmonston (Ed) (Oldstone *et al.,* 1999). By using a reverse genetics system for MV (Radecke and Billeter, 1996), we are now able to analyze host and viral genes contributing to replication, spread, and pathogenesis of MV *in vivo*.

From these experiments, we have learned recombinant MVs lacking expression of the C (MV C−) or V (MV V−) protein replicate equivalently to the parental vaccine Ed strain *in vitro* but display markedly different replication and mortality profiles *in vivo*; both MV C− and MV V− kill far fewer mice relative to MV Ed at equivalent doses. *In vivo*, MV V− undergoes limited transcription and replication and fails to spread within the CNS, whereas MV C− replicates and spreads as well as MV Ed. These studies show that both C and V are required for MV CNS pathogenesis but suggest the pathogenetic activity is functionally distinct.

RESULTS

In vitro growth kinetics

Previous reports indicated that both MV C− and MV V− had similar growth characteristics in cell culture relative to the parental strain MV Ed (tag) (Radecke and Billeter, 1996; Schneider *et al.,* 1997). To extend these findings to neuronal cells and analyze the growth characteristics of these viruses, such mutants (Fig. 1) were used to infect human neuroblastoma SK-N-MC cells. Multistep growth analysis showed identical rates of replication for MV C−, MV V−, and MV Ed (Fig. 2A). The CPE was similar for all three viruses. Growth rates in HeLa cells were also similar for all these viruses although peak titers reached 1 log higher than the neuroblastoma cells (data not shown). After infection of primary mouse CD46+ neurons (Fig. 2B), replication and spread were similar for all three viruses. Infection of CD46 minus (CD46−) mouse neurons did not support infection (data not shown) (Rall *et al.,* 1997). Culture supernatants from infected CD46+ mouse neurons did not contain infectious virus; however, when such infected neurons were cocultivated with Vero cells, the three viruses formed similar numbers of plaques 7 days later.

Mortality rates

To assess the mortality profiles for MV Ed, MV C− and MV V−, viruses were injected IC into 1-day-old CD46+ mice, and mortality was quantitated. When injected with as little as 10^3 PFU MV Ed, most mice died by Day 15 (Oldstone *et al.,* 1999) (Fig. 3A), yet none or 27% of the mice injected with 10^3 PFU of MV V− or MV C−, respectively, died. Thirteen percent (4/32) of CD46+ mice died from 10^4 PFU of MV V−, and 48% (15/31) died from infection with MV C− compared to 90% mortality with the MV Ed control (Fig. 3A). Increasing the dosage to 10^5 PFU resulted in death of 17 of 21 CD46+ mice receiving MV V− comparable to the killing of the control virus. When mice received 10^5 PFU of MV C−, only 7 of 16 (44%) of the mice died.

Viral spread in the CNS

To determine the extent of CNS infection by MV V−, MV C−, and MV Ed, the infected mouse brains were stained for MV antigen with a monospecific antibody to measles virus. Immunohistochemistry of brain sections from MV V−, MV C−, or MV Ed infected YAC-CD46 mice 6–7 days p.i. showed significant differences between the three viruses. MV Ed and MV C− revealed similar staining patterns throughout several regions of the brain when corresponding neuronal tracts and foci were assessed for at least five animals of each virally treated group (Fig. 3B). In comparison, MV V− infected brains (5 of 5 mice) showed greatly reduced numbers of foci and neuronal tracts throughout (Fig. 3B), although the intensity of staining in a few infected foci resembled that from MV Ed and C− (Fig. 3B).

Viral load and replication in the CNS

Because the spread of MV V− was greatly reduced compared to MV Ed when analyzed by immunohistochemistry, we sought to quantify the total viral load in the brain of these animals. Viral RNAs from infected YAC-CD46 mouse brains were then analyzed by Northern blot. MV Ed and MV C− had similar steady-state levels and timing of viral transcripts whereas MV V− yielded lower levels at later times. Within 2 days p.i., MV N transcripts were comparable if not more abundant in three mice inoculated with MV V− when compared to three mice inoculated with MV Ed (Fig. 4). Massive increase of viral load was noted by Day 10 for MV Ed when compared to Day 2. Upon analysis of total viral transcripts, the relative abundances of P, M, H, and F mRNAs were similar among individual mice; however, at Days 5 and 10 p.i., the total viral RNA for MV Ed and MV C− was equivalent but was significantly reduced for MV V− (Fig. 4).

Inflammatory cytokines and chemokines

Previously, we described cytokine and chemokine induction, apoptosis, activation of microglia, astrocy-

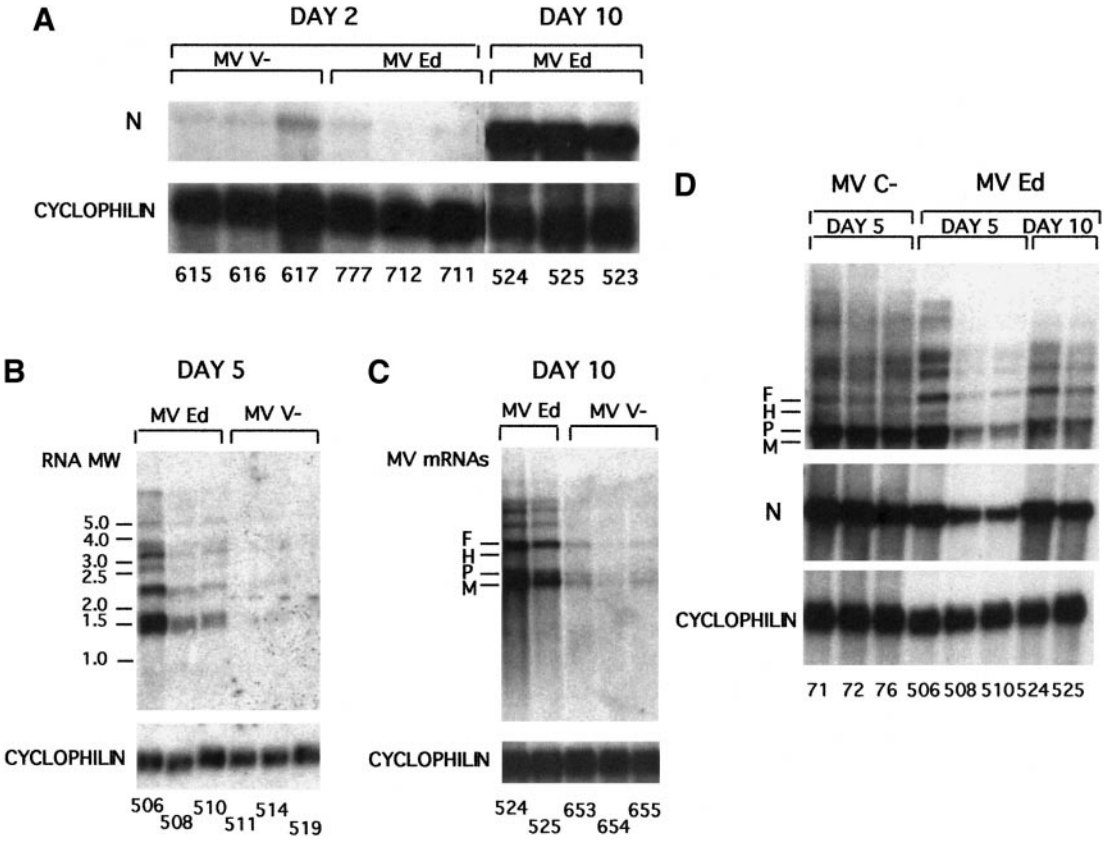

FIG. 4. Replication and transcription of MV Ed, MV V−, and MV C− in the CNS of YAC-CD46 transgenic mice inoculated with 10^4 PFU. Northern blots are arranged so that viral load and transcription can be compared for all three viruses in each individual mouse (number at bottom of each lane). (A) Northern blot analysis of total RNA (5 μg per lane) isolated from the brains of three mice each infected with MV Ed or MV V−. Blot was probed with MV N gene (top) showing comparable levels of N transcript at Day 2 in the brain of MV Ed or MV V− infected mice and massive expansion of N transcript by Day 10 of MV Ed infection. Cyclophilin was used as a probe showing equal loading of the gel (bottom).(B) Comparison of the amount of MV transcripts 5 days p.i. between three mice infected with MV Ed or MV V− showed greatly reduced levels of MV transcripts for MV V−. Northern gel blot (5 μg total RNA per lane) was probed with entire MV genome, p(+) MV (Radecke *et al.,* 1995), excluding the N gene (top), and reprobed with cyclophilin (bottom). (C) Northern gel blot showing reduced levels of MV transcripts (M, P, H, and F) in the brains of three mice infected with MV V− relative to two mice infected with MV Ed at Day 10 p.i. (D) Comparison of mRNAs from N, M, P, H, and F genes of mice infected with MV Ed or MV C−. Transcript levels were similar if not greater in brains of three mice infected with MV C− at Day 5 p.i. compared to brains infected with MV Ed 5 or 10 days p.i. Northern blots were first probed with MV N gene (1.7 kb) (middle) and clyclophilin (1.0 kb) (bottom), stripped, and then repobed with MV genome p(+) MV deleted of the N gene (top).

tosis, and lymphocyte infiltration following infection of CD46+ mice with MV Ed (Manchester *et al.,* 1999; Oldstone *et al.,* 1999). To understand the underlying mechanisms responsible for the differences in the mortality and spread of MV Ed, MV V−, and MV C−, we analyzed these markers of CNS pathogenesis by RNase protection assay (RPA). Similar upregulation was observed for the inflammatory cytokines IL-12, LT-β, TNF-α, IL-1α, IL-1β, and TGF-β and the IL-1 receptor between Days 5 and 10 p.i. (Fig. 5). No IFN-β or γ was detectable. The chemokines RANTES, MIP-1α, MIP-1β, MCP-1, IP-10, and TCA-3 were upregulated in three brains each infected with MV Ed, MV V−, or MV C− (Fig. 5). However, MV C− infected brains showed less RANTES expression relative to MV Ed and MV V− infected brains. RPAs showed that steady-state levels of F4/80 mRNA were at least fivefold higher for all mice infected with any of the three viruses than those of uninfected YAC-CD46 mice. TUNEL staining, assaying for apoptosis, showed that the numbers of apototic neurons were similar in brains infected with C− and Ed viruses (data not shown).

Because SeV V− and MV V− had an attenuated phenotype *in vivo*, it could be that the V protein may modulate the IFN system (Kato *et al.,* 1997b). We thus compared the type I IFN sensitivity of MV Ed and MV V−. No differences were found even with concentrations of 1000 IU on HeLa cells infected at m.o.i. of 0.1. Titers reached 10^2 and 10^3 PFU/ml after 24 h of infection and 2×10^5 to 1×10^6 PFU/ml after 48 h when cells were pretreated with 1000 IU type I IFN.

Viral clearance or persistence

The majority of YAC-CD46 + mice infected with 1×10^4 PFU of MV V− or MV C− survived, whereas nearly

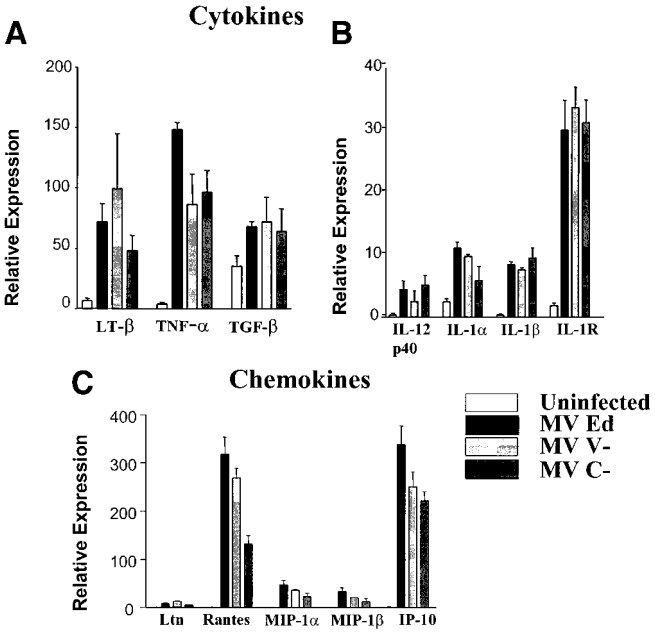

FIG. 5. RNase protection assays of cytokines and chemokines expressed in the brains of YAC-CD46 mice following inoculation with MV Ed, MV C− and MV V−. RPA analysis of specified cytokine or IL-1 receptor (IL-1R) from the brains of three mice each infected with 10^4 PFU of either MV Ed, MV C− or MV V− or uninfected YAC-CD46 control mice (uninifected) from total brain RNA harvested 6–8 days p.i. (B) is shown separately from (A) because relative expression levels were much higher for LT-β, TNF-α, and TGF-β. (C) RPA analysis of relative chemokine mRNA levels in the brains of the same mice analyzed for cytokines in the above panels. Bars represent the mean normalized values ± standard error of specified cytokine or chemokine mRNA species to housekeeping gene mRNAs L32 and GAPDH. ^{32}P-labeled protected RNA fragments were quantified using a molecular dynamics phoshor screen, and storm 860 phosphorimager.

all mice similarly infected with MV Ed died. To determine whether MV V− or MV C− persisted or were cleared in surviving mice, we assessed the amount of viral RNA in brains of four mice infected with MV C− or MV V−. In Fig. 6 we compared the viral load of one mouse infected with MV Ed 10 days after infection with that in MV V− or MV C− infected mice ≥30 days after infection. The brain of only one MV V− infected mouse contained significant levels of MV N mRNA, and no N mRNA was detectable in the MV C− infected mice. When the same brains were analyzed by RT–PCR for MV RNA, three of four MV V− infected mice had detectable levels of MV RNA and two of four MV C− brains contained detectable viral levels.

DISCUSSION

Herein we report that the C and V proteins of MV function as virulence factors in a transgenic mouse model of MV induced CNS disease. MV V− replicated and spread less efficiently than the parental virus MV Ed, resulting in a lower mortality rate for the V− recipients. However, in the case of MV C−, replication and spread appeared similar to that of MV Ed, although MV C− caused a lower mortality rate. This outcome suggests novel and disparate functions for the C and V proteins of MV.

Previously, C and V proteins of *Paramyxoviruses* were thought to play a role in viral transcription and replication (Lamb and Kolakofsky, 1996). Although no function has been established for the MV C protein, the SeV C protein has been shown to have an inhibitory effect on viral transcription *in vitro* by a promoter-specific mechanism (Cadd *et al.,* 1996; Tapparel *et al.,* 1997). The C proteins of *Morbilliviruses* and other *Paramyxoviruses* are related by gene position but not by sequence (21% aa identity between MV C and SeV C proteins), yet they are all basic and relatively small (180–209 aa). It is possible that the functions of MV C and SeV C are not or only partially conserved. Using reverse genetics, C proteins have been silenced for both MV and SeV, and the MV C− recovered had no observable defects *in vitro*, while the replication of SeV C− was greatly restricted. This demonstrated that although C and the related C′, Y1 and Y2, proteins are not absolutely required for replication, they are important for efficiency of replication and pathogenesis (Kurotani *et al.,* 1998).

Previous reports have stated that MV C− grew relatively poorly in human PBMC *in vitro* (Escoffier *et al.,* 1999) and reached lower peak titers *in vivo* in mice receiving human thymic xenografts (Valsamakis *et al.,* 1998). In contrast to these reports, we have shown that MV C− replicated and spread similarly to MV Ed in the CNS of YAC-CD46 transgenic mice; however, the mortality due to MV C− was greatly reduced. A possible explanation for this discrepancy is that in our studies replication was studied in the CNS, whereas the others

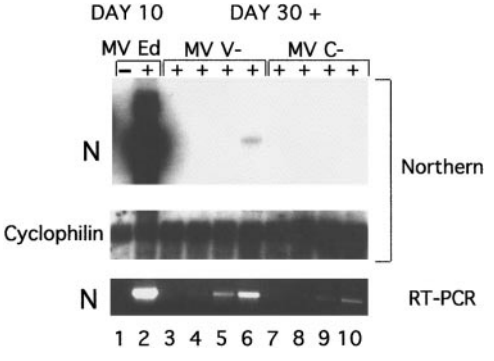

FIG. 6. Analysis of viral clearance or persistence in the brains of YAC-CD46 mice 30 days p.i. Mice were infected with 10^4 PFU MV C− or MV V− and killed 30 days later. Northern analysis of four mice from each group showed only minimal amounts of N transcript in one mouse infected with MV V− (lane 6) compared to heavy viral load of MV Ed (10^4 PFU) infected mouse at Day 10 (lane 2). RT–PCR analysis of the same brains with MV N gene specific primers showed amounts of N transcript in the other mice infected with MV V− (lanes 4 and 5) or MV C− (lanes 9 and 10) and no detectable N transcript in one mouse infected with MV V− (lane 3) and two infected with MV C− after 40 cycles of amplification.

infected cells of the immune system *in vitro* (Escoffier *et al.,* 1999) or in mice lacking an immune system (Valsamakis *et al.,* 1998). Investigation of markers for CNS pathology demonstrated that inflammatory cytokines such as TNF-α, which has been described as mediators of neuronal cell death (Campbell *et al.,* 1998), were upregulated in MV Ed, MV V−, and MV C− infected brains. Mice receiving 10^3 or 10^4 PFU of MV C−, typically did not die and eventually cleared or greatly reduced viral content. Because MV Ed and MV C− infected mice had similar viral loads, extents of spread, and cytokine/chemokine profiles, the reason for differences in mortality are not yet clear. Preliminary analysis of lymphocyte markers by RPA showed higher levels of TCRδ, TCRα, CD3ϵ, CD4, CD8α, CD8β, F4/80, and CD45 for three brains infected with MV C− compared to those infected with MV Ed or MV V− at Day 5 p.i., suggesting a possible role for the innate or adaptive immune response in limiting MV infection in the absence of C. This possibility is currently under investigation.

In the case of MV V− pathogenesis, mortality was related to viral load and spread, and both were greatly reduced for MV V− compared to the parental MV Ed. Interestingly, cytokine, chemokine and lymphocyte markers were at equivalent levels for MV V− and MV Ed, even though at 5 and 10 days after infection, the viral load was greatly reduced in MV V− infected mice. Although the MV V protein is clearly not required for regulation of transcription or replication in some cell types (Schneider *et al.,* 1997), the V protein may control those processes in other cells. (Tober *et al.,* 1998; Valsamakis *et al.,* 1998).

Our work is in agreement with studies in other models of MV and SeV pathogenesis whereby deletion of V attenuates pathogenesis. That is, MV V− grew more slowly than wild type in the lungs of cotton rats (Tober *et al.,* 1998) and in human thymuses implanted into SCID mice where the incidence of thymocyte apoptosis decreased (Valsamakis *et al.,* 1998). Similarly, a SeV V− mutant was hampered in the ability to replicate and to cause pathogenesis in the lungs of mice; this mutant replicated for 1 day and was cleared by Day 9, whereas the wild-type SeV killed all infected mice by Day 9 (Kato *et al.,* 1997b). We also found that MV V− and MV Ed had a similar viral load at Day 2, but after Day 5, the quantity of MV V− was markedly reduced compared to MV Ed.

Many viruses interfere with the IFN system, and Kato *et al.,* (1997b) suggested such a scenario for SeV V protein. However, treatment of HeLa cells with 1000 IU of IFN showed no difference in IFN sensitivity between MV Ed or MV V−. Similarly, no differences in type I IFN production followed MV Ed and MV V− infection of human PBMC (D. Naniche *et al.,* unpublished observations). Therefore abrogation of the IFN system probably does not play a role in the greater spread of MV Ed as compared to MV V−. In the case of SeV, reports have shown that C protein rather than V protein (Garcin *et al.,* 1999) is responsible for inhibition of IFN-induced antiviral effects (Didcock *et al.,* 1999).

Surprisingly, little if any IFN-γ was detected during the immune response to any of the MVs, and induction of IFN-β was not observed. Our preliminary data from YAC-CD46 mice crossed with type I IFN receptor- or IFN-γ-knock-out mice suggest that type I IFNs are protective, whereas IFN-γ plays little if any role (Patterson *et al.,* manuscript in preparation). Conceivably, at least one of the numerous IFN-αs are being produced *in vivo*.

Because of the lack of IFN-γ induction or protection of MV-infected YAC-CD46 mice, an interesting question that arises from this study is how are MV C− and MV V− cleared from the CNS because CD8+ T cells are unable to recognize and directly kill virus infected neurons due to lack of MHC class I expression (Joly *et al.,* 1991) Mice that cannot produce B or T cells are much more susceptible to CNS infection by MV (Lawrence *et al.,* 1999), indicating that adaptive immunity may be responsible for protection of neurons from MV infection; however, the mechanism of clearance remains to be established.

Our studies clearly demonstrate that MV V and C proteins operate by different mechanisms *in vivo* and suggest the involvement of an interaction with host factors that may be in part neuron specific, as neurons constitute 99% of MV infected cells in the CNS of YAC-CD46 mice (Oldstone *et al.,* 1999). The V protein of simian virus 5 and MV interact with the damage specific DNA binding protein, DDB, (Lin *et al.,* 1998); however, it is difficult to explain how such an interaction would account for the differences we observe in mortality and spread in the developing CNS. Nonstructural viral proteins could conceivably interact with one or more host components because many such viral proteins have multiple physiological functions. For example, the influenza A NS1 protein is thought to be involved with pre-mRNA splicing, retention of polyA (+) RNA within the nucleus, enhanced viral mRNA translation (Ortin, 1998) and inhibition of the antiviral action of IFN (Garcia-Sastre *et al.,* 1998). These functions are likely mediated by NS1 binding to RNA (Wang *et al.,* 1999) and interacting with at least four unique cellular proteins (Tan and Katz, 1998; Chen *et al.,* 1999; Marion *et al.,* 1999). All animal viruses have been selected for replication and spread in the whole organism that they infect. To do so, they must infect multiple cell types and deal with a vigorous and diverse immune response. It is entirely possible that both C and V have numerous functions, both tissue and cell specific in their natural host. A better understanding of CNS infection and immune suppression by MV in humans likely rests on determining the host cellular factors involved.

MATERIALS AND METHODS

Viruses and cells

MV Ed (tag), MV C−, and MV V− were generated using the MV reverse genetics system as described in Fig. 1 (Radecke *et al.,* 1995; Radecke and Billeter, 1996; Schneider *et al.,* 1997), amplified, and passaged in Vero cells. Virus stocks were prepared by low-multiplicity infection of Vero cells, harvested as supernatants after low-speed centrifugation to remove cellular debris and stored at −70°C. Each stock was retitered on Vero cells.

Screening of transgenic mice

YAC-CD46 transgenic mice were screened by tail biopsy and purification of genomic DNA, followed by dot–blot hybridization with a ^{32}P-labeled CD46 cDNA probe as previously described (Oldstone *et al.,* 1999). YAC-CD46 mice were maintained by mating them with FVB/N mice obtained from the Scripps Rodent Breeding Colony.

Infection of mice with recombinant MVs

FVB/N mice heterozygous for the YAC-CD46 transgene were bred to nontransgenic FVB/N, and the offspring of these matings were infected with the different recombinant MVs intracranially (IC). At least 30 one-day-old mice were inoculated IC with each virus (MV Ed tag, MV C− and V−) with 1×10^3, 1×10^4, or 1×10^5 PFU. Cages were monitored daily; deaths were recorded, and tails were taken for confirmation of the CD46 transgene.

Immunohistochemistry

Brains were harvested from mice at various times postinfection, fixed with 4% paraformaldehadye, and cut into 40-μm sections in a vibratome. Sections were blocked with normal goat serum, biotin, and streptavidin (Vector Laboratories, Burlingame, CA). Immunostaining was performed with the Vectastain Elite kit (Vector Laboratories) as described (Rall *et al.,* 1997; Oldstone *et al.,* 1999). Anti-MV antibody was a human polyclonal antiserum from an SSPE patient and was previously shown to recognize MV antigens (Manchester *et al.,* 1999; Oldstone *et al.,* 1999).

Northern blot analysis of MV replication

At the time of death, brains were removed from the mice and divided along the midline. One hemisphere was immediately frozen in liquid nitrogen, whereas the other half was frozen in OCT compound on dry ice for preparing cryostat sections. RNA was isolated from fresh-frozen brains using Tri-reagent (Molecular Research Center, Cincinnati, OH). Total RNA (5 μg/sample) was run on a 1.5% formaldehyde-agarose gel and transferred to a Nytran membrane (Micron Separations, Westborough, MA). For detection of MV mRNA, a MV−N probe was prepared by random primer labeling the 1.7-kb cDNA fragment from the MV N-gene (plasmid peN1). Blots were probed with a random-primed, ^{32}P-radiolabeled DNA probe for MV nucleoprotein RNA or the entire genomic cDNA for MV, p(+)MV, deleted of N (Radecke *et al.,* 1995) by hybridizing at 65°C in QuickHyb (Stratagene, La Jolla, CA) for 3 h. Blots were washed at high a stringency with 0.2× SSC/0.1% SDS at 65°C

Quantitation of cytokines and chemokines by RNase protection assay

Measurement of steady-state levels of cytokine and chemokine mRNAs was performed using the Riboquant RPA assay system according to the manufacturer's instructions (Pharmingen, La Jolla, CA). Total RNA (20 μg/sample) was used. ^{32}P-labeled probe sets were prepared and hybridized to the total brain RNA overnight. Following RNase digestion of hybridized RNAs, samples were separated on an 8% polyacrylamide gel, dried for 1 h, and exposed to a phosphorimager screen (Molecular Dynamics Systems, Sunnyvale, CA). L32 and GAPDH standards were included in each lane. The Pharmingen probe sets mCK-2 and mCK-3b were used for cytokine analysis, probe set mCK-5 was used for chemokine analysis.

RT–PCR analysis of MV persistence

RT–PCR amplification of MV RNA was carried out using MV N gene specific primers 5′-GGCCACACTTTTAAGGAGCTTAGC-3′ and 5′-GGGCCGTAACCGCCTTTGCG-3′, 5′, and 3′ relative to the N mRNA, respectively. The 553 nt N fragment was generated using standard conditions with *Taq* polymerase (Boehringer Mannheim, Germany) and 40 cycles of amplification

ACKNOWLEDGMENTS

We thank Denise Naniche for the critical reading of the manuscript and Michael Chu for technical assistance. This is Publication Number 12628-NP from the Department of Neuropharmacology, The Scripps Research Institute, La Jolla, California. This work was supported in part by USPHS Grant AI-36222 and Training Grants MH-19185 and AI-39466 and the Swiss National Science Foundation Grant 31.43475.95.

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