MONOCLONAL ANTIBODY-SELECTED VARIANTS OF MHV-4 CONTAIN SUBSTITUTIONS AND DELETIONS IN THE E2 SPIKE GLYCOPROTEIN

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ABSTRACT

Selection and analysis of MHV-4 (strain JHM) variants resistant to E2-specific neutralizing MAbs was performed. Two types of variation in the E2 spike glycoprotein were found. From minimally passaged stocks of MHV-4, putative point mutants were obtained. These mutants were resistant only to the MAb used to select them. In contrast, multiply passaged stocks were found to harbor variants uniformly resistant to two selecting MAbs. Northern and Western blot analysis of the viral RNAs and proteins synthesized by these doubly-resistant variants showed that they contained large deletions in both mRNA 3 and its E2 translation product, localized to a 15 kilodalton region within the amino terminal 90B post-translational fragment. The selective advantage of this second class of variants lacking sequences within E2 90B was a result of their reduced cytopathology, thereby allowing cultures to support virus production for prolonged periods.

INTRODUCTION

Variants of neurotropic murine coronaviruses have received much attention in the past decade because they serve as useful tools for the correlation of viral genotype and virus-induced disease. Methods for variant generation and selection have included chemical mutagenesis followed by isolation of temperature sensitive virus (1) or multiple passaging in vivo and purification of resulting small plaque virus (2). In contrast to the fatal encephalitis caused by MHV-4 infection of murine brain, infection with these variants typically results in a non-fatal demyelinating disease.

MHV-4 variants have also been selected for their ability to escape the effects of neutralizing MAbs (3-5). In most cases neutralizing MAbs directed against the 180 kilodalton spike glycoprotein E2 have been used as selecting agents. A neuroattenuation phenotype has often been observed among the

Coronaviruses and Their Diseases
Edited by D. Cavanagh and T.D.K. Brown
Plenum Press, New York, 1990

resulting neutralization-resistant mutants (3-5) indicating that at least one determinant of neurovirulence resides on E2.

In our laboratory, variants uniformly resistant to neutralization by two E2-specific MAbs 4B11.6 and 5A13.5 have been isolated from stocks of MHV-4 passaged in Sac- culture (3). Although these variants were shown to be neuroattenuated (3) the precise nature of the genetic lesion in E2 correlating with this attenuation was not determined. Because one source of heterogeneity among strains and variants of MHV involves extensive deletion and insertion of sequences within the E2 gene (6-8) we sought to determine whether any of these neutralization-resistance mutations were deletions. We found that these original variants were indeed deletion mutants lacking sequences from the middle of E2 post-translational fragment 90B.

METHODS

Cells and Virus

All cell lines were grown as monolayer cultures in DMEM supplemented with either calf or fetal calf serum. Infections were performed as described by Sturman et al. (9) and progeny virus infectivity was determined by plaque assay on DBT cells.

Selection of MAb-Resistant Variants

The properties of the MAbs used in this study have been described previously (10). Selection of MAb-resistant mutants followed those described (3) except that in most cases resistant virus populations rather than individual plaque isolates were selected for subsequent analysis.

Analysis of Viral RNA and Protein

Electrophoretic analysis of phenol-extracted RNA from infected Sac- cells was performed on denaturing agarose gels. Following transfer of separated RNAs to nitrocellulose, Northern blot hybridization with a [^{32}P] RNA complementary to nucleotides 636 to 840 of MHV-A59 RNA 7 was utilized to visualize all seven MHV-4 mRNAs.

For detection of the E2 proteins of MHV-4 and variants, [^{35}S] methionine labeled infected cell proteins were immune precipitated with antiserum directed against E2 and subjected to SDS-polyacrylamide gel electrophoresis. Further analysis by Western blotting was performed according to standard methods (11) using antibodies raised against peptides represented in the known MHV-A59 E2 sequence (6) as detection agents.

RESULTS

Original Neuroattenuated and Neutralization-Resistant Variants Contain Deletions in E2

Electrophoretic analysis of the viral RNAs induced by infection with MHV-4 and two representative variants

revealed a difference in the sizes of the largest three of the seven 3' coterminal viral mRNAs (Figure 1, Panel A). Such shifts indicated the presence of deletions within mRNA 3, the gene encoding E2. The increased mobility of variant mRNA 3 species (8.3 kb) suggested that about 400 nucleotides were deleted in each of the variants. These deletions were similarly reflected in the E2 protein. Radiolabeling of E2 in the presence of the glycosylation inhibitor tunicamycin followed by immune precipitation and electrophoresis revealed that the variant polypeptides lacked about 150 amino acids relative to MHV-4 (Figure 1, Panel B).

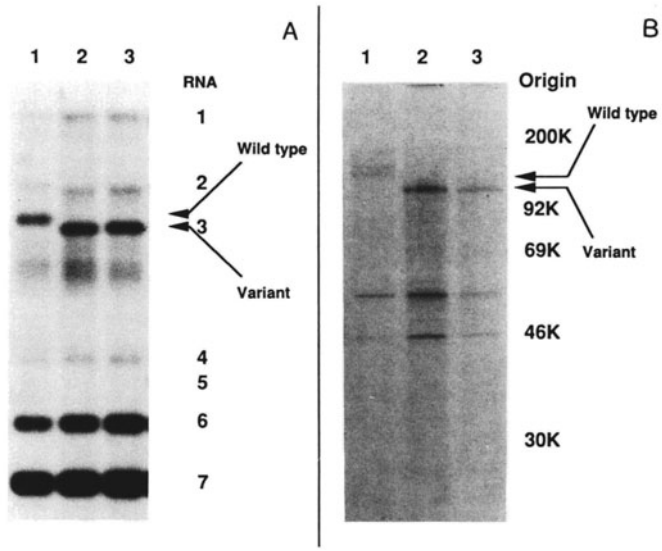

Fig. 1. Electrophoretic profiles of viral RNAs (A) and E2 polypeptides (B) from infected Sac- cells. Infected cell RNAs were denatured with formamide and electrophoresed on formaldehyde-agarose gels prior to detection by hybridization with [^{32}P] complementary RNA 7 transcripts. Infected cell proteins were radiolabeled with [^{35}S] methionine in the presence of tunicamycin (1 ug per ml) then immune precipitated with antiserum against E2 and electrophoresed on Laemmli slab gels. Lanes represent RNAs and proteins from wild type MHV-4 (1), V4B11.3 deletion mutant (2) and V5A13.1 deletion mutant (3) infection.

Antipeptide antibodies directed against selected portions of the E2 polypeptide were used on Western blot analyses to localize these deletions to one of the two post-translational products of E2. To this end, virus was partially purified and concentrated by sedimentation through sucrose gradients and prepared for Western blotting. Immunoblotting of the immobilized protein showed that antiserum against peptide A, which corresponds to residues near the middle of the MHV-A59 90B fragment, recognized E2 90B from MHV-4 but not the variants V4B11.3 and V5A13.1 (Figure 2, Panel A). Specific binding to a previously unidentified band at 75 K (p75) was also observed, as was non-specific binding to nucleocapsid. Antipeptide B serum, directed against the carboxyl terminus of E2 cleavage

produce 90B, reacted with distinct 90 K proteins from MHV-4 and the variants (Figure 2, Panel B). The different increases in electrophoretic mobility of the variant 90B chains was indicative of varying extents of deletion. Serum reactive to the sequence present on the amino terminus of E2 cleavage product 90A (peptide C) bound to two bands at 90 K and 270 K. The co-electrophoresis of MHV-4 and variant 90A-specific proteins, which represent monomers (90 K) and trimers (270 K), indicated that no large deletions were present in this fragment. Together these results indicate that the major deletions in the neuroattenuated variants V4B11.3 and V5A13.1 are localized to the middle of the E2 cleavage product 90B.

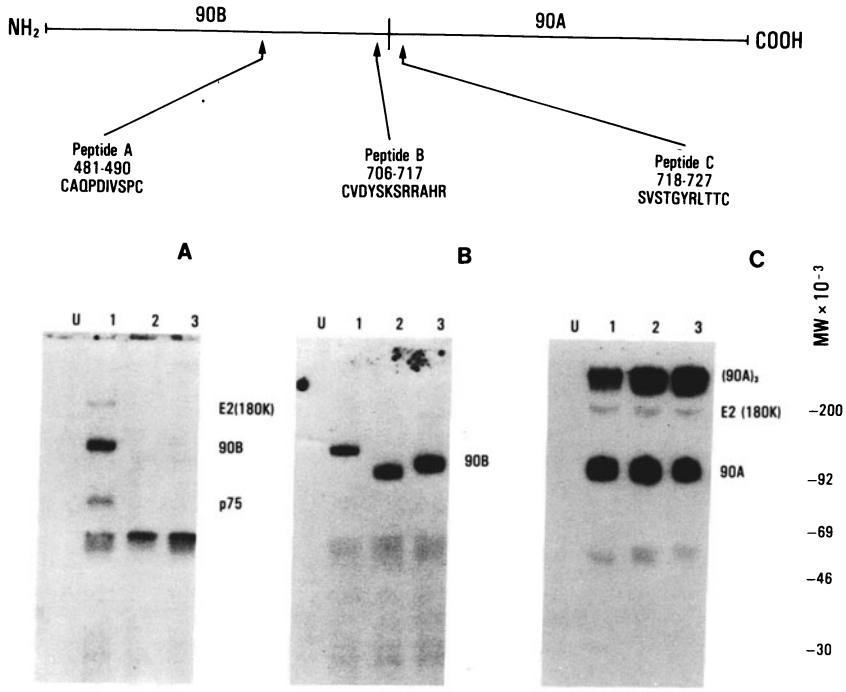

Fig. 2. Western blot analysis of MHV-4 and deletion variant virion proteins. Virions were purified from infected Sac- cells and electrophoresed on SDS-polyacrylamide gels prior to transfer to nitrocellulose. Proteins were then incubated in the presence of rabbit anti-peptide antibodies A, B and C, directed against the indicated portions of the MHV-A59 E2 protein. Bound antibodies were detected with [^{125}I] protein A. U; uninfected control, 1; MHV-4, 2; V4B11.3, 3; V5A13.1.

Isolation of Deletion Mutants Requires Multiple Passaging of Virus Stocks in Cell Culture

To assess the frequency with which deletions in E2 arise in MHV-4 stocks we isolated pure MHV-4 from a single well-isolated plaque on a DBT cell lawn and prepared passage 2 virus by two short 12 hour growth cycles in Sac- culture. The resulting stock was then subjected to neutralization by

each of three E2-specific MAbs 4B11.6, 5A13.5 and 5B19.2. In all three neutralizations, resistant virus capable for forming plaques on DBT cells was observed. However, only small plaque virus (less than 0.1 cm diameter) escaped the initial round of neutralization by MAbs 4B11.6 and 5B19.2; amplification of these small plaque variants under selecting MAbs resulted in restoration of normal (0.5 cm diameter) plaque size. Thus disabling mutations conferring neutralization resistance are followed by additional compensating mutation(s) in the viral genome. All of the large plaque variants were neurovirulent in Balb/c mouse brain (data not shown).

Table 1. Frequencies of MAb-Resistant Variants in Low and High Passage MHV-4 Stocks[a]

MHV-4 Passage	Selecting MAb	Percent Resistance
2	4B11.6	0.0027
	5A13.5	0.0027
	4B11.6 + 5A13.5	<0.00045
10	4B11.6	12
	5A13.5	20
	4B11.6 + 5A13.5	13

[a]MHV-4 stocks of known titer were subjected to MAb ascites fluids (1:20) and plated onto DBT cells. Plaque development was for 3 days in the presence of 1:50 dilutions of MAb.

All three of these variants from MHV-4 passage 2 synthesized viral RNAs and proteins electrophoretically identical to MHV-4-specific RNAs and proteins (data not shown) indicating that large deletions were not present in the variant genomes. Evidence that two of these variants likely harbored point mutations was suggested by their 10^{-4} to 10^{-5} selection frequency (Table 1) which is the expected frequency of point mutation in RNA virus populations (12). In addition, each of the three variants displayed resistance only to the MAb used to select them (Table 2). Resistance to both MAbs 4B11.6 and 5A13.5 was not observed, indicating that deletion mutants possessing this double-resistance character were below the limits of detection.

These results were in marked contrast to those observed upon selection of MAb-resistant variants from MHV-4 passaged ten times in Sac- cells. In these passaged stocks the frequency of virus resistant to either of two MAbs (4B11.6 and 5A13.5) was as high as 20% (Table 1). In addition, variants selected by MAbs 4B11.6 were resistant to MAb 5A13.5, and vice versa (Table 2). Western blot analyses using antipeptide antibodies (not shown) revealed that these doubly-resistant populations contained large deletions in 90B whose size and location were similar to those seen in the original neuroattenuated mutants. Thus serial passage of MHV-4 in Sac- culture resulted in the selective amplification of deletion mutants.

Table 2. Neutralization Resistance Patterns for Selected Variants[a]

Virus	Origin of Variant	Neutralizing MAb 4B11.6	5A13.5	5B19.2
MHV-4 (P2)		+	+	+
V4B11	MHV-4 (P2)	-	+	+
V5A13	"	+	-	+
V5B19	"	+	+	-
V4B11	MHV-4 (P10)	-	-	+
V5A13	"	-	-	+

[a]Virus samples were treated as described in Table 1. A (+) indicates >99% neutralization while a (-) represents <30% neutralization relative to untreated controls.

A Selective Advantage for Deletion Mutants Exists Only in Cell Lines Susceptible to Virus-Induced Cytopathic Effects

We found that high multiplicity MHV-4 infection of a number of cultured cell lines, including DBT, Sac-, Cl300 and Neuro 2A, resulted in extensive cell-cell fusion after about 8 hours. Destruction of cell monolayers was complete by 16 hours post-infection. In contrast, 4 out of 4 independently isolated deletion mutants exhibited a delayed induction of fusion in these cell lines. Although cytopathic, these deletion mutants typically induced fusion 4 to 6 hours later than MHV-4. That this reduced cytotoxicity permitted cells to support virus replication for prolonged periods was shown by pulse labeling of infected cultures with [^{3}H] uridine in the presence of actinomycin D, an inhibitor of host but not coronavirus transcription (13). The relative amount of radiolabel incorporation into acid insoluble material showed that Sac- cells supported equivalent amounts of wild type and variant RNA for the first 10 hours of infection. However between 10 and 16 hours post-infection, variant RNA accumulated whereas wild type RNA synthesis rapidly declined (Figure 3, Panel A). This profile of viral RNA synthesis correlated well with the onset of cytopathic effects. In addition the fact that 5 to 10 times more variant-specific RNA was produced throughout the time course was consistent with the 10-fold higher yields of infectious variant progeny typically obtained from Sac- cultures (Table 3).

Two continuous neuronal cell cultures derived from central nervous system olfactory bulb, OBL 21 and OBL 21A, supported MHV-4 replication without suffering from any virus-induced cytopathic effect. With the OBL 21A line a slight preference for MHV-4 replication relative to the deletion mutant was observed (Figure 3, Panel B). MHV-4 progeny yields were slightly higher in the OBL 21A line (Table 3). Thus in the fusion-negative OBL 21A line, where no selective advantage for diminished viral cytotoxicity exists, wild type virus with its full-length E2 was favored. In this regard the OBL 21A line mimicked *in vivo*

infection in murine brain, which consistently produced more wild type virus (Table 3).

Table 3. Yields of Wild Type and Deletion Mutant Viruses in Selected Neuronal and Non-neuronal Cultures and in Murine Brain[a]

Host	Fusion	Virus Yields MHV-4	V4B11.3	V5A13.1
DBT	+	2.2[b]	208	188
Sac-	+	320	3000	2800
C1300	+	192	1280	800
Neuro 2A	+	148	1760	1480
OBL 21	-	1.6	0.6	1.2
OBL 21A	-	2.2	1.3	1.0
Balb/c brain		57[c]	14	2

[a]Inoculation multiplicities were 0.01 PFU per cell for cultures and 100 PFU per mouse for intracerebral injections.
[b]Progeny PFU x 10^{-4} per ml at 24 hours post-infection.
[c]Progeny PFU x 10^{-3} per gram at 4 days post-infection.

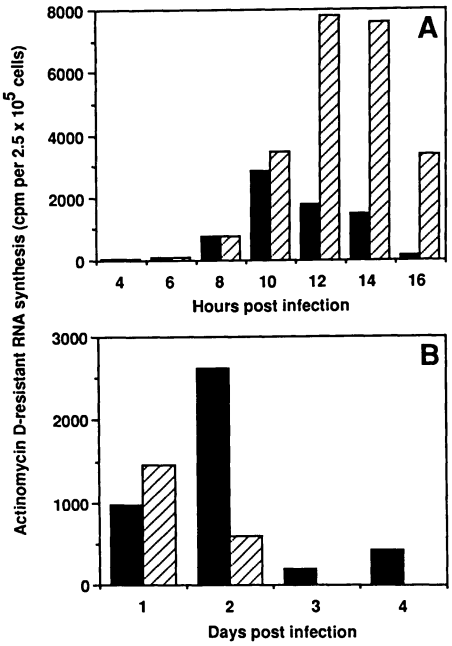

Fig. 3. Time courses of viral RNA synthesis in Sac- (A) and OBL 21A (B) cell lines. Monolayers were infected with MHV-4 (solid bars) or deletion mutant V5A13 (hatched bars) at 2 PFU per cell. RNA synthesis was monitored at the indicated times after infection by 0.5 hour (Sac- cells) or 2 hour (OBL 21A cells) [^{3}H] uridine labeling periods in the presence of actinomycin D (5 ug per ml). Acid insoluble radioactivity was counted immediately after labeling.

DISCUSSION

Point mutations represent the expected outcome of MAb-selection on RNA virus populations. Thus the result of MAb selection on minimally passaged stocks of MHV-4 is typical. We have no evidence that mutants selected from these stocks contain any gross alteration in E2 structure. In fact, nucleotide sequence analysis which is currently in progress has identified single base substitutions in both V4B11 and V5B19. We anticipate that complete variant sequences will not only clearly identify neutralization epitopes on E2 but also point to sites important in receptor binding and/or fusion, as both V4B11 and V5B19 initially displayed a small plaque phenotype in culture.

Viable deletion mutants are unexpected among RNA viruses, although defective interfering (DI) deletion mutants are common among all virus populations (14). Like DI genome generation, viable deletion mutant MHV-4 formation required multiple passaging in culture. Previously described "copy-choice" mechanisms (15,16) provide the most attractive explanation for generation of these mutants. Copy-choice involves transfer of RNA polymerase and nascent genome to alternate site(s) on a template followed by replication reinitiation and elongation. Non-homologous transfers would result in deletion or insertion mutants.

Viable deletion mutants of MHV-4 are rare but are selectively amplified in some cell cultures. In fact, MAbs are not required for selection of deletions in Sac- cells. Continual passaging eventually results in relatively pure stocks of virus with a smaller E2. This is because infected Sac- cells can survive beyond 12 hours post-infection to support the low-cytotoxicity mutants but not the more potent MHV-4. Thus far our comparative analyses of MHV-4 and deletion mutant E2 synthesis, processing and transport have revealed no differences at early stages of infection. Therefore we believe that E2 lacking a region within the middle of 90B is less fusogenic in culture than MHV-4 E2.

What is the *in vivo* function of these dispensable sequences in E2? There does not appear to be any absolute *in vitro* or *in vivo* requirement for this part of E2 90B -- we have yet to find a cell line or murine host that will support the growth of MHV-4 but not the mutants. However these variants do not induce the fatal encephalitis seen with MHV-4. Whether or not this is due to a lack of infection of essential brain cells by the deletion mutants is currently under investigation.

ACKNOWLEDGMENTS

This is Publication Number 5970-IMM from the Department of Neuropharmacology, Scripps Clinic and Research Foundation, La Jolla, CA 92037. This work was supported in part by USPHS grants AI-25913 and NS-12428. TMG was supported by NIH training grant 5-T32-AG-00080.

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