Genetic characterization of orthobunyavirus Melao, strains BE AR633512 and BE AR8033, and experimental infection in golden hamsters (Mesocricetus auratus)

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Melao virus (MELV) strains BE AR8033 and BE AR633512 were isolated from pools of Ochlerotatus scapularis mosquitoes in Belém, Pará State (1955), and Alta Floresta, Rondônia State (2000), Brazil, respectively. The aim of the present study was to molecularly characterize these strains and to describe the histopathological, biochemical and immunological changes in golden hamsters (Mesocricetus auratus) following intraperitoneal injection of MELV strains. Hamsters were susceptible to both of the MELV strains studied. Viraemia was observed 3–6 days post-infection (p.i.) for BE AR633512 and only on the second day p.i. for BE AR8033. Neutralizing antibodies against both strains were detected in blood samples obtained at 5 days p.i. and persisted up to 30 days p.i. Aspartate aminotransferase, alanine aminotransferase and blood urea nitrogen were significantly altered in animals infected with the two MELV strains, while creatinine was only altered in animals inoculated with BE AR633512. Histopathological changes were observed in the central nervous system, liver, kidney and spleen of hamsters, and infection was confirmed by detection of specific MELV antigens by immunohistochemistry. Strain BE AR633512 caused more severe tissue damage than strain BE AR8033, showing increased neurovirulence and pathogenicity. Genetic analysis based on the full-length sequences of the glycoprotein (Gn and Gc) and nucleocapsid protein (N) genes revealed high levels of homology between the MELV strains. Interestingly, the greatest genetic divergence was found for the Gn gene of strain BE AR633512, in which several synonymous and non-synonymous mutations causing changes in RNA secondary structure were observed. Further studies will be necessary to investigate the role of Gn and Gc mutations in the MELV pathogenicity.

INTRODUCTION

Melao virus (MELV), prototype strain TRVL9375 belongs to the family Bunyaviridae, genus Orthobunyavirus, California serogroup (Karabatsos, 1985). It was first isolated in 1955 from a pool of Ochlerotatus scapularis (formerly Aedes scapularis) mosquitoes captured in the Melao Forest, in the municipality of St Andrew, Trinidad and Tobago (Spence et al., 1962). In 1957, a MELV strain (BE AR8033) was isolated from a mixed pool of O. scapularis mosquitoes and mosquitoes of the genera Psorophora sp., Sabethes sp. and Ochlerotatus sp. captured in the forest of Instituto Agronômico do Norte, Belém, Brazil (Karabatsos, 1985). In 2000, a new strain, BE AR633512, was isolated from a pool of O. scapularis mosquitoes captured in Alta Floresta do Oeste, Rondônia State, Brazil.

Like other orthobunyaviruses, the genome of MELV consists of three single-stranded RNA molecules of negative polarity and helicoidal symmetry, called large
(L)-, medium (M)- and small (S)-RNA. The L-RNA segment encodes protein L, which possesses viral polymerase activity. The M-RNA segment encodes a single polyprotein that is cleaved into two viral surface glycoproteins, Gn and Gc, and into a non-structural protein, NSm. The S-RNA segment encodes two proteins, a nucleocapsid protein, N, and a non-structural protein, NSs (Elliott, 1990; Fauquet et al., 2005). Gc, of the viral envelope, is important for the determination of MELV virulence in vertebrate hosts. In contrast, Gn seems to play an important role in viral adsorption, serving as a ligand for arthropod cells (Mertz, 1997).

Classically, viruses of the California serogroup have been subdivided into four different complexes (Melao, California encephalitis, Trivittatus and Guaroa), according to their antigenic relationship determined by classical serological tests such as complement fixation, haemagglutination and neutralization test (Bishop, 1985; Calisher & Karabatsos, 1988; Whitman & Shope, 1962). Current molecular studies based on nucleotide sequencing of the S- and M-RNA segments of different viruses of the California serogroup have shown the existence of only three complexes: Melao, California encephalitis and Trivittatus complex, with the Guaroa virus being an evolutionary link between members of the Bunyamwera and California serogroups (Bowen et al., 1995; Huang et al., 1996; Campbell & Huang, 1999).

Studies on tissue pathology contribute to a better understanding of viral dynamics, especially in the case of viruses that present tropism for certain organs, such as California serogroup viruses which show marked tropism for the central nervous system (CNS). In fact, some agents of this serogroup have been responsible for encephalitis in humans, especially in North American countries (Deibel et al., 1983).

A combination of genetic, histopathological, serological, and biochemical data becomes necessary to better understand the molecular epidemiology and pathogenesis of MELV. The objectives of the present study were to molecularly characterize MELV strains BE AR633512 and BE AR8033, and to describe the histopathological, biochemical and serological changes that occur in golden hamsters (Mesocricetus auratus) experimentally infected with these strains via intraperitoneal (i.p.) inoculation, in order to contribute to a better understanding of the pathogenesis of this viral agent.

METHODS

Viral strains. The viral strains were kindly provided by the WHO Collaborating Center for Arbovirus Reference and Research at Instituto Evandro Chagas (IEC), Departamento de Arbovirologia e Febres Haemorrágicas, Belém, Brazil, and consisted of virus lyophilized after isolation from newborn mice and one passage in VERO cells.

Animals. Young hamsters (M. auratus) aged 3–4 weeks, as well as newborn (2–3 days) and young (3–4 weeks) mice, were used for this study. All animals were handled in a level 2 biosafety facility and processed in a class II B2 safety cabinet. The study was approved by the Ethics Committee on Animal Research (CEPAN/IEC).

Virus stock preparation and viral titration in mice. A 10 % suspension was prepared from a flask containing lyophilized virus in PBS containing 0.75 % bovine serum albumin (BSA) and antibiotics (100 U penicillin ml \(^{-1}\) and 100 μg streptomycin ml \(^{-1}\)). For virus stock preparation, 0.02 ml of this suspension was inoculated intracerebrally (i.c.) into newborn mice. For titration of the viruses, dilutions of 10\(^{-1}\) to 10\(^{-8}\) were prepared and 0.02 ml of each dilution was inoculated i.c. into newborn mice. The viral titre was calculated using the method described by Reed & Muench (1938) and expressed as LD\(_{50}\) per 0.02 ml.

Experimental infection of hamsters. One hundred and twenty golden hamsters were used in the experiments (96 infected and 24 uninfected controls). Animals were infected via the i.p. route with 0.2 ml 10 % viral suspension containing 10\(^{6.7}\) LD\(_{50}\) per 0.02 ml of strain BE AR633512 or 10\(^{6.5}\) LD\(_{50}\) per 0.02 ml of strain BE AR8033, prepared from mouse brain and diluted in PBS as described above. After infection, five animals (two infected animals for each strain and one uninfected control) were anaesthetized daily from 1 to 7 days post-infection (p.i.) and at 21 days p.i. (Massone, 2003). Blood was collected for the determination of viraemia, antibodies and biochemical markers and a blood count. In addition, the viscera (lung, heart, brain, kidney, spleen and liver) were removed for histopathological analysis and antigen detection by immunohistochemical techniques. For serological analysis, blood was collected from days 1 to 10 p.i. and at 15, 20, 25 and 30 days p.i.

Determination of viraemia. The blood samples collected from the hamsters were diluted (10\(^{-1}\) to 10\(^{-8}\)) in PBS containing 0.75 % BSA and antibiotics (100 U penicillin ml \(^{-1}\) and 100 μg streptomycin ml \(^{-1}\)) and 0.02 ml was inoculated i.c. into newborn mice. Viral titres were calculated and expressed as LD\(_{50}\) per 0.02 ml (Reed & Muench, 1938).

Preparation of hyperimmune serum. Hyperimmune sera against the MELV strains were prepared in young Swiss albino mice (n=12). The immunization scheme consisted of four i.p. injections of 0.2 ml per mouse at intervals of 7 days. The immunizing antigens were prepared as described by Casals (1967) from a suspension of infected mouse brains (1:5 in 0.85 % NaCl). Blood was collected from anaesthetized animals by intracardiac puncture after a minimum interval of 7 days from the last immunizing injection.

Neutralization test. The neutralization test in newborn mice (Casals, 1967) was used for the detection of neutralizing antibodies in sera of hamsters infected with viral strains BE AR633512 and BE AR8033 using the constant serum dilution technique, with a final dilution of 1:8 being obtained for each serum. The virus was serially diluted 10-fold. Sera with a log neutralization index (LNI) ≥1.8, calculated by the method of Reed & Muench (1938), were considered to be positive.

Histopathological analysis. After collection, the organs were fixed in 10 % buffered formalin, dehydrated in increasing (70 to 100 %) ethyl alcohol concentrations, cleared in xylene and embedded in paraffin. The tissue blocks were cut into 4 μm sections with a microtome, the sections were stained with haematoxylin–eosin and examined under a Nikon Eclipse E200 light microscope (Prophet et al., 1992; Michalaney, 1998).

Detection of viral antigen by immunohistochemistry. Immunohistochemistry was performed according to the method described by Hsu et al. (1981). Briefly, the 4 μm histological sections...
obtained from paraffin blocks were mounted on slides previously prepared with 3-aminopropyltriethoxy-silane adhesive (Sigma). The slides were then treated with xylene and decreasing ethanol concentrations (100 to 70%), and blocked with endogenous peroxidase. Next, the specimens were incubated with the primary polyclonal antibodies (anti-BE AR633512 or anti-BE AR8033 hyperimmune serum prepared in young mice) diluted in 1% BSA fraction V supplemented with 0.1% sodium azide in PBS, pH 7.4. After washing in PBS, the slides were incubated with biotin-labelled universal anti-IgG (rabbit, sheep and mouse IgG) (LSAB kit; DAKO), followed by incubation with the streptavidin–biotin peroxidase complex (DAKO). The slides were developed with 0.03% 3,3′-diaminobenzidine (Sigma), as a chromogen, plus 1.2 ml 3% H2O2. Finally, the slides were washed under running water and counterstained with Harris haematoxylin.

Study of hepatic and renal function markers. Serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured to determine hepatic function, and blood urea nitrogen (BUN) and creatinine were measured to evaluate renal function in infected and control hamsters. All measurements were performed with a Cobas Mira Plus automated biochemical analyser (Cobas, São Paulo, Brazil) using Roche diagnostic kits) according to the manufacturer’s instructions.

Statistical analysis. For statistical analysis, the variables were compared between infected and control groups using the BioEstat 4.0 program (Ayres et al., 2005). Student’s t-test for independent samples was applied to determine the level of significance of differences between means, with a significance level of 5% (P<0.05).

Molecular characterization.

Viral propagation in Vero cell culture and viral RNA extraction. Suspensions of MELV strains BE AR633512 and BE AR8033 were inoculated into Vero cells. Supernatants were collected when approximately 75% of the cells showed a cytopathic effect; viral RNA was extracted from supernatants with the Trizol LS reagent (Invitrogen) according to the manufacturer’s instructions.

Amplification of the N, Gn and Gc genes by RT-PCR. Single-tube RT-PCR (Dunn et al., 1994) was employed for the synthesis of cDNA corresponding to the N gene (S-RNA) and to the Gn and Gc genes (M-RNA) using specific primers (Supplementary Table S1, available in JGV Online). RT-PCR was performed in a final volume of 50 μl containing 5 μl RNA (2 to 5 ng), 5 μl 10× PCR buffer, 1.5 μl 50 mM MgCl2, 1 μl 10 mM dNTPs, 1 μl each primer (50 μM), 0.5 μl 1.5 mM DTT, 0.002 μl reverse transcriptase (20 U μl−1), Superscript II; Invitrogen) and 0.2 μl Taq DNA polymerase (40 U; Invitrogen). Reverse transcription was carried out for 60 min at 42 °C, followed by 35 cycles consisting of denaturation at 94 °C for 40 s, annealing at 55 °C for 40 s and elongation at 72 °C for 1 min, in an automatic GeneAmp PCR Systems 9700 thermocycler (Applied Biosystems). The amplicons were visualized on a 1.2% agarose gel stained with GeneAmp PCR Systems 9700 thermocycler (Applied Biosystems). The slides were developed with 0.03% 3,3′-diaminobenzidine (Sigma), as a chromogen, plus 1.2 ml 3% H2O2. Finally, the slides were washed under running water and counterstained with Harris haematoxylin.

Nucleotide sequencing. Purified RT-PCR products were sequenced in an automatic ABI Prism 377 sequencer (Applied Biosystems) using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems) and the same primers as used for RT-PCR amplification.

Sequence analysis and phylogeny. The nucleotide sequences of the N, Gn and Gc genes of the viral strains BE AR633512 and BE AR8033 were first analysed for quality with the SeqMan v.5.0 program (Laser Gene, DNA Star Package) and then mounted in a single consensus sequence. Next, the consensus sequences obtained for each viral strain were compared to each other and to sequences of other orthobunyaviruses belonging to the California serogroup (including the MELV prototype, TRVL9375) available in GenBank, using the CLUSTAL_X program (Altschul & Gish, 1996; Thompson et al., 1997). Phylogenetic trees were constructed by the neighbour-joining (NJ) (Saitou & Nei, 1987), maximum-parsimony (MP) (Swoford, 1999) and maximum-likelihood (ML) (Goldman et al., 2000) methods using the PAUP 4.0 (Swoford, 1999) and MEGA 3.1 (Kumar et al., 2004) programs. For NJ analysis, the distance matrix was calculated from the aligned sequences using the two-parameter formula described by Kimura (1980). For MP analysis, a 4:1 transition/transversion ratio was established. A bootstrap test using 1000 replicates was applied together with the NJ and MP methods to guarantee a higher reliability of the generated trees (Felsenstein, 1985). To root the phylogenetic tree, the Bunyamwera virus was included as an outgroup. The ML method was used to evaluate the topologies obtained for the S and M segments using the Kishino–Hasegawa test (Goldman et al., 2000).

Determination of the secondary structure of the Gn and Gc genes. The secondary structure of the M-RNA molecules of strains BE AR633512 and BE AR8033 and of prototype TRVL9375 was established with the MFOLD program (http://mfold.burnet.edu.au/dna_form) using the complete nucleotide sequences of the Gn (897 nt) and Gc (2907 nt) genes.

RESULTS

Susceptibility of mice and hamsters to the MELV strains

Newborn mice were susceptible to infection with MELV strains BE AR633512 and BE AR8033, showing signs of disease such as paralysis, shivering and prostration, and dying within 2–3 days p.i. Golden hamsters were also found to be susceptible to infection. Hamsters inoculated with BE AR633512 developed the disease within 6 days p.i., which was characterized by the absence of motor coordination, shivering and hind limb paralysis. In addition, 4 of 38 BE AR633512-infected hamsters died between 6 and 7 days p.i. None of the hamsters infected with BE AR8033 presented signs of the disease or died.

Viraemia caused by BE AR633512 was observed in hamsters between 3 and 6 days p.i., with a maximum titre of 10^4.5 LD50 per 0.02 ml at 3 days p.i. The titre of this viral strain in the brain of stock mice was 10^6.7 LD50 per 0.02 ml. For BE AR8033, viraemia was only observed on the second day p.i., with a titre of 10^2.5 LD50 per 0.02 ml. The titre of this strain in the brain of mice was 10^6.5 LD50 per 0.02 ml. Fig. 1(a) shows the viraemia titres obtained for the two MELV strains.

Neutralizing antibodies against strains BE AR633512 and BE AR8033 were detected in the sera of infected animals between 5 and 30 days p.i. (Fig. 1b).

Evaluation of hepatic and renal function markers

Comparison of infected and uninfected (control) animals showed changes in biochemical hepatic (AST and ALT) and renal (BUN and creatinine) function markers of
infected animals. Differences in the results were also observed between the MELV strains BE AR8033 and BE AR633512, with many of the biochemical alterations being statistically significant (Fig. 1). Significantly higher values of all biochemical markers were observed in sera of hamsters infected with BE AR633512 when compared with the control group. Sera of hamsters infected with BE AR8033 presented significantly higher BUN levels; however, these were more discrete than those observed for BE AR633512. There was also a significant increase in AST and ALT and a discrete, but non-significant, increase in creatinine.

Comparison of mean BUN levels between each group (infected with strain BE AR633512 or BE AR8033) and the control group (Fig. 1c) revealed a significant difference ($P<0.05$). Analysis of mean creatinine levels showed a significant difference ($P<0.05$) between animals infected with BE AR633512 and the control group, whereas no significant difference was observed between BE AR8033 and the control (Fig. 1d). Significant differences ($P<0.05$) in mean AST and ALT levels were observed between animals infected with BE AR633512 and BE AR8033 and the control group (Fig. 1e, f).

**Histopathological analysis**

Histopathological analysis of tissue samples at different post-infection times revealed alterations that were characteristic of infection, especially in the CNS, liver, spleen and kidney (Fig. 2). Discrete changes were observed in the other organs studied (lung and heart), mainly at 7 days p.i. The lesional spectrum varied in intensity and amplitude between the days analysed (1–7 and 21 days p.i.), with peak intensity being observed between 6 and 7 days p.i. in the different organs of sick animals. For the two MELV strains studied, the histopathological alterations were more intense in organs of hamsters infected with BE AR633512 compared with animals infected with BE AR8033, in agreement with the viraemia titres (Fig. 2).

**Strain BE AR633512.** The CNS of hamsters infected with BE AR633512 was characterized by the presence of congested meninges containing lymphomononuclear cells associated with oedema. The neural parenchyma presented features of congested tissue, especially in areas of the hippocampus, brain and cerebellum, and frequently contained a lymphomononuclear inflammatory infiltrate. Vacuolization of the parenchyma was also observed, sometimes conferring a sponge-like aspect to the tissue. Neurons were frequently apoptotic, showing characteristic nuclear pyknosis (Fig. 2a).

The liver of these BE AR633512-infected animals presented intense histopathological alterations compared with uninfected hamsters (Supplementary Fig. S1, available in JGV Online), consisting, at the cellular level, of characteristic...
hepatocyte swelling accompanied by hepatocyte ballooning. These cells frequently presented a pale cytoplasm containing transparent vacuoles (steatosis) adjacent to reactive nuclei which often showed areas of regeneration of the double plaque type. The portal spaces showed characteristic expansion involving lymphomononuclear inflammatory proliferation, oedema and congestion of the portal vein and arteriole. No vascular wall or cholangiolar injuries were observed. There were also no signs of congestion, sinusoidal cholestasis, or portal, portal–portal or portal–centrilobular fibrous proliferation with consequent subversion of hepatic architecture (Fig. 2b). The splenic alterations observed in these animals included prominence of the red and white pulp and frequent areas of congestion and parenchymal haemorrhage (Fig. 2c).

Marked histopathological alterations were also observed in the kidney of animals infected with BE AR633512 and consisted of glomerular congestion and swelling of renal tubular cells. In addition, areas of lytic and coagulative necrosis associated with a discrete to moderate lymphomononuclear infiltrate and congestion of the renal interstitium were noted (Fig. 2d). Analysis of the lung showed only mild congestion and thickening of the alveolar walls. No viral pneumonitis was observed during any phase of infection (Fig. 2e). The heart of infected animals was characterized by mild lymphocyte infiltration among myocardial fibres at 7 days p.i., although no changes in cardiomyocytes were observed by conventional microscopy (Fig. 2f).

**Strain BE AR8033.** The tissue lesions found in the organs of BE AR8033-infected hamsters were similar to, but less intense than those observed for BE AR633512 (Supplementary Fig. S2, available in JGV Online).

**Immunohistochemistry**

Overall, the immunostaining pattern of viral antigens did not differ between infection with the two MELV strains in any of the organs analysed. Immunoreactivity to viral antigen was more intense in the CNS and liver, with a peak intensity at 3 days p.i. (Fig. 3a, b; Supplementary Fig. S3a, b, available in JGV Online) and a mild decline up to 7 days p.i. Interestingly, positive immunostaining persisted in the CNS and liver up to the end of the experiment at 21 days p.i. In the kidney, immunoreactivity to viral antigens showed a peak intensity at 4 days p.i. (Fig. 3c; Supplementary Fig. S3c), followed by a mild decline up to day 6 and remaining unchanged until the end of the experiment (21 days p.i.). Positive immunostaining was also detected in spleen samples, although no variation in staining intensity was observed between the strains analysed. No viral antigens were detected in the heart or lung (data not shown).
Genetic analysis of N, Gn and Gc genes

The sequence of the N gene (5-RNA segment) of MELV strains BE AR8033 and BE AR633512 comprised 708 nt and contained two ORFs corresponding to the N (708 nt) and NSs (294 nt) genes that encode a structural N protein (235 aa) and NSs protein (97 aa), respectively. The Gn and Gc genes (M-RNA fragment) comprise 897 and 2907 nt, respectively, and encode the Gn (299 aa) and Gc (969 aa) glycoproteins, respectively. Analysis of genetic similarity demonstrated a high degree of nucleotide identity between the N and Gc genes (>93.6%) between MELV strains BE AR8033 and BE AR633512 and prototype strain TRVL9375. The N (98.6%) and Gn (97.1%) genes were more similar between strains BE AR8033 and TRVL9375, whereas the Gc gene showed 94.8% nucleotide identity between BE AR633512 and BE AR8033. The greatest genetic divergence was observed for the Gn gene of BE AR633512, which presented 86.1% and 87.8% nucleotide identity with prototype strain TRVL9375 and strain BE AR8033, respectively. In addition, the last two strains showed a high degree of homology (97.1%) in their nucleotide sequences (Table 1).

Multiple alignment of the N, Gn and Gc gene sequences of strains BE AR633512, BE AR8033 and prototype TRVL9375 revealed the presence of multiple nucleotide substitutions (Supplementary Table S2, available in JGV Online). Most of these substitutions were silent, with a predominance of transition mutations (si) (334) compared with transversions (sv) (117) and a mean si/sv ratio of 2.8. Compared to the MELV prototype strain TRVL9375, BE AR633512 showed the largest number of substitutions, mainly in the Gc ($n=125$, $si=106$, $sv=19$) and Gn ($n=182$, $si=130$, $sv=52$) genes.

Table 1. Genetic relationship between MELV strains BE AR8033, BE AR633512 and prototype TRVL9375 according to genes N, Gn and Gc

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<th>Gene</th>
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<th>BE AR8033</th>
<th>TRVL9375</th>
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<td>95.8</td>
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<tr>
<td></td>
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*100% nucleotide identity.

Fig. 3. Immunohistochemistry of liver (a, d), CNS (b, e) and kidney (c, f) sections of a hamster infected with MELV strain BE AR633512 (a–c) and uninfected controls (d–f). Positivity is characterized by brown staining of the tissue (arrows) seen in (a–c). All images are ×400 magnification.
Phylogenetic analysis

Both the NJ and MP methods generated trees with a similar topology; however, slightly lower bootstrap values were obtained for trees generated by MP analysis. Thus, in the present study, trees constructed using the NJ method were used for final analysis. All phylogenetic trees obtained for the N gene showed segregation into the three main clades corresponding to the Melao, California encephalitis and Trivittatus groups, which are considered to be polyphyletic, i.e. they do not share a common ancestor. Within the Melao group, strains BE AR633512, BE AR8033 and prototype TRVL9375 represent a separate clade (clade A), which is more genetically related to Keystone virus (clade B) but distinct from clade C consisting of South River virus, Inkoo virus and Jamestown Canyon virus, and clade D represented by Serra do Navio virus. The California encephalitis group was subdivided into four clades: clade E, San Angelo virus; clade F, Tahyna virus and Lumbo virus; clade G, California encephalitis virus; and clade H, La Crosse virus and snowshoe hare virus (Fig. 4a).

The trees constructed for the Gn and Gc genes (M-RNA segment) had topologies that differed from those obtained for the N gene. Using both the NJ and MP methods, strains TRVL9375, BE AR633512 and BE AR8033 were more phylogenetically related to Keystone virus based on the S-RNA segment (N gene), but were more closely related to Serra do Navio virus when the M-segment (Gn and Gc genes) was used (Fig. 4b, c). It should be emphasized that for all trees, the bootstrap values obtained for the MELV cluster were significantly strong (99–100%). Analysis of the N and Gn genes revealed that BE AR633512 was genetically more distinct from BE AR8033 and TRVL9375. However, phylogenetic analysis of the Gc gene showed that for this gene, strain BE AR633512 was more closely related to BE AR8033.

ML analysis was used to evaluate the topologies obtained for MELV segments S and M using the Kishino–Hasegawa test. Evolutionary models were optimized using genomic segments as well as their respective topologies. Regardless of the model selected, the topology generated for a certain segment (N gene for the S-RNA segment and Gn and Gc genes for the M-RNA segment) by NJ and MP analysis was more significantly related ($P < 0.001$) compared with the topology obtained between it and the other segment. These results suggest that the topologies obtained for the genomic segments of MELV are significantly different.

Structural analysis of Gn and Gc genes

The secondary structures of the Gn and Gc genes were found to differ between the three MELV strains studied. However, the greatest difference was observed for BE

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Fig. 4. Phylogenetic analysis of N (a), Gn (b) and Gc (c) genes of MELV prototype TRVL9375 and strains BE AR8033 and BE AR633512 using the NJ method. Bootstrap values are indicated above each main node of the tree. Bunyamwera virus was used as outgroup. A value of 0.05 (a, b) and 0.1 (c) below the bar corresponds to 5 and 10% nucleotide divergence between genetic sequences, respectively. Clades or phylogenetic groups are indicated by the letters A to H.
AR633512, probably because of the presence of synonymous and non-synonymous mutations along its genome. The Gn gene (Fig. 5a; Supplementary Table S2) of this strain contained different synonymous nucleotide substitutions that were not dependent on the region of the gene. In addition, eight non-synonymous mutations were observed, four in the extracellular domain and four in the cytoplasmic domain. The G→A nucleotide substitution (position 172) in the extracellular domain was considered to be important because it changed residue N58 at the NNTG glycosylation site present in strains TRVL9573 and BE AR8033 from Asp→Asn and, consequently, the biochemical character of the amino acid (strongly acidic amino acid—polar amino acid). With respect to the Gc gene (Fig. 5b), a smaller total number of substitutions was observed when compared with the Gn gene, including eight important non-synonymous substitutions all located in the extracellular domain, four of which changed the biochemical character of the amino acid.

DISCUSSION

Golden hamsters have frequently been used to study the tissue injury caused by viral tropism, the pathogenicity and the virulence of arboviruses. These animals are easy to handle, of low cost and are commonly susceptible to most viruses listed in the International Catalogue of Arboviruses (Karabatsos, 1985). In addition, these animals serve as an experimental model for the study of many arboviruses, some of which, such as yellow fever and Oropouche viruses, play an important role in public health (Pinheiro et al., 1981; Saeed et al., 2001; Tesh et al., 2001; Xiao et al., 2001).

Fig. 5. Schematic representation of the RNA secondary structures of the Gn (a) and Gc (b) genes of MELV strains BE AR633512 (i), BE AR8033 (ii) and prototype TRVL9375 (iii). Red dots, synonymous mutations; black dots, non-synonymous mutations (change in amino acids); arrows indicate an amino acid change altering the biochemical character of the amino acid. ED, Extracellular domain; TMD, transmembrane domain; CD, cytoplasmic domain.
Young golden hamsters were found to be susceptible to infection with MELV strains, especially BE AR633512, with 10.5% of infected animals showing signs of disease and with the occurrence of death of 4 of 48 animals. Manifestations of disease were not observed in hamsters infected with BE AR8033, suggesting that BE AR633512 is probably more neurovirulent and/or more pathogenic to hamsters than BE AR8033. Conversely, although hamsters infected with BE AR8033 showed no signs of the disease, susceptibility of these animals to this strain could be confirmed by the detection of viraemia and neutralizing antibodies, as well as by histopathology and immunohistochemistry, which demonstrated the presence of tissue lesions and viral antigens in the liver and brain, respectively. The lack of experimental studies involving orthobunyaviruses in hamsters, excluding those developed by Pinheiro et al. (1981) and Saeed et al. (2001), impairs discussion of infection in these animals. Orthobunyaviruses are pathogenic to mice and some of them, including MELV, are also pathogenic to hamsters, (Karabatsos, 1985; Travassos da Rosa et al., 1997).

The viral titres found in newborn mice infected i.c. with strains BE AR633512 and BE AR8033 were larger than the largest viral titre observed in blood of hamsters infected with these strains. Differences in viral titres obtained for different animals (newborn mice and young hamsters) might be explained by the greater susceptibility of newborn mice to viral infection compared with young golden hamsters, due to the difference in age and also because the immune system of newborn mice is still not well developed, a fact facilitating viral infection. Another variable is the route of inoculation used for the two animal species. Mice were infected i.c. and hamsters were inoculated i.p., a fact that commonly results in differences in viral titres, with titres generally being higher when the virus is inoculated by the central route (i.c.) compared with peripheral inoculation (Gonzalez-Scarano & Nathanson, 1996).

Neutralizing antibodies against the two MELV strains, BE AR633512 and BE AR8033, were detected from 5 days p.i. to the end of the experiment (30 days p.i.), thus indicating that these antibodies can be detected in serum at approximately 5 days p.i. and remain elevated for at least 1 month after inoculation, the maximum period used in the present experiment for serum collection and serological analysis by neutralization. The neutralizing antibody titre against BE AR633512 at 5 days p.i. was higher than that observed for BE AR8033. Nevertheless, the titre of the latter apparently exceeded that of the former from 10 to 30 days p.i., indicating that BE AR8033 is probably more immunogenic to young golden hamsters than BE AR633512, although the latter is more pathogenic and virulent. This difference in immunogenicity might be due to mutations in the viral glycoproteins Gn and Gc of strains BE AR633512 and BE AR8033. Unfortunately, no data regarding this virus or other orthobunyaviruses are available in the literature.

The MELV strains BE AR633512 and BE AR8033 promoted pantoletic infections, causing histological lesions in the CNS, liver, kidney and spleen of hamsters infected i.p. Several bunyaviruses cause neurological disease in humans, especially La Crosse virus (California serogroup), which has been implicated as a causal agent of encephalitis in naturally infected individuals (Gonzalez-Scarano & Nathanson, 1996). Thus, the typical neurotropism of arboviruses was also observed in young hamsters infected with MELV strains BE AR633512 and BE AR8033. Curiously, virulence and pathogenicity were more intense in animals infected with the BE AR633512 strain. Indeed, infected hamsters presented intense CNS lesions, both in the meninges, where the virus caused meningitis accompanied by a large inflammatory infiltrate, and in the cerebral parenchyma, where the two viral strains caused intense neuronal injury (Fig. 3a). Since peripheral (i.p.) inoculation was used and the virus reached the CNS, it seems clear that MELV was able to enter the CNS by the haematogenic route and was possibly introduced through the meninges.

After the CNS, the liver seemed to be the most frequent target organ of arboviruses in newborn Swiss albino mice and hamsters (Dias, 1986; Tesh et al., 2001; Xiao et al., 2003). This was also seen for MELV strains in hamsters, which showed neurotropism and viscerotropism, with the liver being the most affected organ and exhibiting intense tissue lesions. A pattern of hepatitis similar to that found in yellow fever was observed after i.p. infection of hamsters with strains BE AR633512 and BE AR8033, with the former showing apparently more intense viscerotropism for the liver. The liver pathologies obtained from hamsters infected with the MELV’s strains indicated acute involvement of the hepatic parenchyma that does not result in a fibrosing process, as observed in persistent chronic liver infections of the hepatitis C-type, but rather resembles the lesions caused by Yellow fever virus (Xiao et al., 2001; Quaresma et al., 2005, 2007).

MELV strains BE AR633512 and BE AR8033 also caused changes in the kidney and spleen of infected hamsters, with some animals presenting splenomegaly between 6 and 7 days p.i. (data not shown). With respect to the lung and heart, discrete tissue alterations were observed that were more characteristic of changes resulting from systemic disturbances secondary to infection rather than as tissue injury resulting from viral tropism. This finding was confirmed by immunohistochemistry which showed no reactivity to MELV antigen in these organs.

The histopathological and immunohistochemical results obtained for the liver and kidney of hamsters infected i.p. with both MELV strains agreed with the findings of the hepatic (ALT and AST) and renal (BUN and creatinine) function tests, since tissue damage to these organs usually alters biochemical markers. Significant alterations in all biochemical markers analysed were observed for BE AR633512, which caused more marked histopathological damage to the liver and kidney than BE AR8033. In addition, BUN, creatinine and AST levels were more altered in animals infected with strain BE AR633512.
compared with strain BE AR8033. Only ALT was found to be higher in animals infected with the BE AR8033 strain, but the reasons for these higher levels remain unknown.

Analysis of the N, Gn and Gc genes showed that the genes encoding the surface glycoproteins of the viral envelope were more genetically variable, indicated by the observation of a large number of synonymous and some non-synonymous mutations. The greatest genetic divergence was observed for BE AR633512, in which these mutations occurred more frequently in the extracellular domain, irrespective of the gene analysed (Gn or Gc). This domain seems to play a critical role in the process of binding of the virus to susceptible cells and also exerts a function in the fusion of the viral envelope to the cell membrane (Pekosz & Gonzalez-Scarano, 1996). In addition, in strain BE AR633512, important mutations were observed at two glycosylation sites, one in the Gn gene (residue N58, NDTG) and the other in the Gc gene (residue N667, NSSL), which may have contributed to the distinct biological behaviour of this strain.

The results of the phylogenetic analysis of RNA segments S (N gene) and M (Gn and Gc genes) agree with those reported by Bowen et al. (1995) and Campbell & Huang (1999), as well as with serological data reported by Bishop (1985) and Calisher & Karabatsos (1988). The phylogenetic trees generated for the S and M segments of MELV presented distinct topologies. Using the S segment, a relationship with the North American Keystone orthobunyavirus was observed; whereas analysis based on the M segment revealed a closer similarity to the Brazilian Serra do Navio orthobunyavirus. The reasons for these differences are still unknown. However, the results obtained by ML analysis suggest distinct evolutionary origins of the RNA segments of MELV, a fact probably related to genetic rearrangements that may have occurred in nature between parental viruses, as previously described for other orthobunyaviruses (Gerrard et al., 2004; Nunes et al., 2005).

There was a correlation between the genetic data and those obtained in the experimental study (histopathological lesions). The Gc gene encodes glycoprotein Gc which is possibly responsible for viral adsorption to the vertebrate host (hamster) cell and is important in the neuropathogenesis of La Crosse virus (Plassmeyer et al. 2007). Although strain BE AR633512 was found to be more closely related to BE AR8033 at the level of the Gc gene, the more intense histopathological alterations observed in hamsters infected with BE AR633512 compared with BE AR8033 might be explained by mutations along the Gc gene. Indeed, although these mutations occurred in a smaller number than was observed in the Gn gene, they were highly significant because many of them were non-synonymous mutations, i.e. they altered the biochemical character of the amino acid. These mutations might have promoted an increase in the pathogenicity and virulence of BE AR633512 in young golden hamsters, since the substitution of amino acids (Fig. 5) was accompanied by changes in their biochemical function, suggesting an association with the higher pathogenicity of BE AR633512 in hamsters, as previously suggested for other orthobunyaviruses (Plassmeyer et al., 2005). However, reverse genetics and further studies regarding the pathogenesis of MELV are necessary to effectively evaluate the role of these alterations in the exacerbation of virulence of BE AR633512.

ACKNOWLEDGEMENTS

We are grateful to all researchers, technicians and trainees of the WHO Collaborating Center for Arbovirus Reference and Research and the Department of Arbovirology and Haemorrhagic Fevers at Instituto Evandro Chagas, for direct and/or indirect contributions to this work. In particular we are grateful to Creusa Lima, Geraldo Medeiros and Basílio Buna, and Daniele Medeiros for helping with the construction of figures. We are also grateful for the CAPES master’s grant (to V.L.C.) for financial support.

REFERENCES


