Protective immune responses to the E and NS1 proteins of Murray Valley encephalitis virus in hybrids of flavivirus-resistant mice


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The lack of an effective animal model has been a major obstacle in attempts to define the role of humoral and cellular immune responses in protection against flavivirus infection. We have used F1 hybrid mice (BALB/c × C3H/RV) that are heterozygous for the flavivirus resistance allele FlvR and show reduced virus replication in the brain after intracerebral inoculation. F1 hybrid mice challenged by intracerebral inoculation with Murray Valley encephalitis (MVE) virus developed encephalitis 2–3 days later than a genetically susceptible strain (BALB/c) but showed a similar mortality rate. This delay in the onset of disease provided more opportunity for virus clearance by primed immune responses. Using F1 hybrid mice we were able to demonstrate protective immunity induced by structural and non-structural proteins of MVE virus by immunization with pure NS1 protein or recombinant vaccinia viruses that expressed various regions of the MVE genome. These constructs included VV-STR (C-prM-E-NS1-NS2A), VV-AC (prM-E-NS1-NS2A) and VV-NS1 (NS1-NS2A). VV-AC vaccinated mice were completely protected (100% survival) from challenge with 1000 infectious units of MVE virus, while mice inoculated with VV-STR, VV-NS1 or pure NS1 were partially protected (40%, 47% and 85% respectively). Analysis of prechallenge sera and in vivo depletion studies revealed that the solid protection induced by VV-AC was mediated by neutralizing antibody to the E protein and did not require a CD8+ T cell response. The partial protection provided by VV-STR, VV-NS1 and pure NS1 occurred after induction of antibody to NS1. However, depletion of CD8+ cells prior to virus challenge ablated the protection provided by VV-NS1 indicating some requirement for class I restricted cytotoxic T cells.

Introduction

Flaviviruses are small, spherical, enveloped viruses that consist of two envelope associated proteins, E and prM (precursor to M), and a nucleocapsid that comprises the core protein (C) in association with approximately 11 kb of single strand, positive sense RNA (reviewed in Chambers et al., 1990). Genomic RNA provides the only message and represents one long open reading frame containing three structural and seven non-structural genes in the order 5' C-prM/M-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5 3'. Translation of the coding region produces a polyprotein that is co- and post-translationally cleaved by the viral protease and cell signalase to form individual viral proteins.

Several in vivo studies have shown that neutralizing antibodies to the E protein provide solid protection to mice against homologous flavivirus challenge (Gould et al., 1986; Kimura-Kuroda & Yasui, 1988; Hawkes et al., 1988), while antibodies to the NS1 protein protect to a lesser extent apparently through complement-mediated lysis or antibody-dependent cell-mediated cytotoxicity (ADCC) (Schlesinger et al., 1990, 1993; Jacobs et al., 1994). In vitro studies have shown that the flavivirus NS3 protein contains immunodominant cytotoxic T (Tc) cell determinants for several major histocompatibility complex (MHC) class I restricted elements (Kurane et al., 1989, 1991; Parrish et al., 1991; Rothman et al., 1993; Lobigs et al., 1994).

The role of these humoral and cellular immune responses in protection against flavivirus-induced disease has been difficult to demonstrate in vivo with most flaviviruses due to the lack of a good animal model. The common laboratory strains of mice are only fully susceptible to flavivirus infection by peripheral routes of inoculation in the first few weeks of life (Grossberg & Scherer, 1966; Boyle, 1979). As mice mature they become...
more resistant to flavivirus infection by this route of inoculation, even when using high doses of virus, probably due to maturation of the immune system, lack of virus replication at the inoculation site and tightening of the cell junctions in the blood–brain barrier. Although some strains of Japanese encephalitis virus (JE) (Mason et al., 1991) and tick-borne encephalitis virus (TBE) (Jacobs et al., 1994) are still neurovirulent in adult mice by this route of inoculation, most flaviviruses are not. This results in low or unpredictable mortality rates in adult mice infected by intraperitoneal (i.p.) inoculation, rendering this form of challenge unsuitable for protection studies. In contrast, adult mice are highly susceptible to intracerebral (i.c.) inoculation with neurotropic flaviviruses and develop encephalitis within 7–9 days. However, this form of virus challenge is also unsuitable for protection studies as the rapid onset of disease may preclude primary or memory immune responses from an effective role in virus clearance.

Studies by Sangster et al. (1994) have shown that C3H/RV mice, which possess the flavivirus resistance allele Flv', have reduced virus replication in the brain and are more resistant to i.c. inoculation with flaviviruses than laboratory strains which do not possess this allele. F1 hybrid mice (C3H/RV × BALB/c) and (C3H/RV × C3H/Hej) that are heterozygous for Flv' are less viral than homozygous mice but show significantly lower viral titres in the brain compared with the susceptible strains BALB/c and C3H/Hej. In this study we investigated the suitability of F1 hybrid mice for immunoprotection studies by determining their relative susceptibility to challenge with Murray Valley encephalitis (MVE) virus and the time to onset of disease. We then used this mouse model to demonstrate a protective effect of NS1 by immunofluorescence using NS1 specific MAbs.

**Methods**

**Virus stocks.** MVE strain F/3/51 was used to infect Vero cells (m.o.i. = 1) and virus stocks were prepared as clarified culture medium collected at 72 h post-infection.

**Cell cultures.** 143B, SW13, L929 and CV1 cells were grown in RPMI 1640 supplemented with L-glutamine, antibiotics and 10% FBS at 37 °C in a 5% CO2 atmosphere. Vero and C6/36 cells were cultured in M199 medium (Gibco), and similarly supplemented for growth and incubated at 28 °C in 5% CO2 (C6/36) or 37 °C with 15 mM HEPES (Vero).

**Monoclonal antibodies.** MAbs to the prM, E and NS1 proteins of MVE virus have been described previously (Hall et al., 1988, 1990).

**Construction of recombination plasmids.** The vaccinia virus recombinant plasmid pVPGJ4 was kindly provided by Denis Trent, CDC, Fort Collins, Co., USA. This vector was derived from pGS62 by deleting BamHI and SnaI sites in order to regenerate the original p7.5 promoter sequence (Esposito et al., 1987). A cDNA fragment corresponding to 70 nucleotides at the 3′ end of the prM gene, all of the NS1 and NS2A genes and 70 nucleotides at the 5′ end of the NS2B gene of MVE virus was generated by PCR using Taq polymerase (Biotech). Primers 2411s (TG AAT TCA ATT GCT GCT TCT C) and 4295 (GGAATTC AAT CAC AGT GTC TAG CTC A) incorporating MVE sequence (underlined), and EcoRI sites (italicized), were used to amplify cDNA corresponding to nucleotides 2411–4295 of the MVE genome from the plasmid 2/1/22 (Dalgarano et al., 1986). The PCR product was cloned into the pCR II TA cloning vector (Invitrogen) and the EcoRI fragment excised and cloned into EcoRI-digested pVPGJ4 to produce pVPNS1 so that the ATG, immediately preceding the EcoRI cleavage site, served as the initiation codon.

**Production of recombinant VV.** The methods described by Mackett et al. (1984), with some modifications, were used to transfect wild-type VV-infected Vero cells with pVPNS1 and to isolate thymidine kinase-minus recombinant viruses. Recombinants that were positive for the NS1 gene by hybridization were plaque purified and tested for expression of NS1 by immunofluorescence using NS1 specific MAbs. VV virus recombinants expressing NS1 (VV-NS1) were cultured in CV1 cells by inoculation at an m.o.i. of 5 and cultured for 48 h. Infected cells were then scraped into medium and freeze-thawed three times to release virus; the virus suspensions were then homogenized by repeated aspiration through a Pasteur pipette. Virus titres were determined by plaque assay on CV1 cells. Other vaccinia virus recombinants expressing MVE virus proteins, VV-AC (prM-E-NS1-NS2A) and VV-STR (C-prM-E-NS1-NS2A), have been described previously (Lobigs, 1993).

**Analysis of MVE virus proteins expressed by VV recombinants.** Recombinant viruses VV-STR, VV-AC and VV-NS1 were cultured in CV1 cells (m.o.i. = 5) for 48 h and infected cells pelleted and resuspended in lysis buffer (2% Triton X-100, 1% NP40, 1% sodium deoxycholate, 0.1% SDS in PBS pH 7.4) overnight at 4 °C. Lysates were then clarified, the recombinant protein separated by PAGE and analysed by Western blot with a cocktail of anti-NS1 MAbs (Hall et al., 1990). Recombinant antigens were also analysed by ELISA. VV-WT, VV-STR, VV-AC and VV-NS1 were cultured in CV1 cells in 96-well trays. At 48 h after infection, cell monolayers were rinsed with PBS, fixed with 20% acetone in PBS for 1 h at 4 °C then dried at 37 °C overnight. Twofold dilutions of MAbs to the prM, E or NS1 proteins of MVE virus were then incubated on fixed monolayers for 1 h at room temperature. After washing, bound MAb was detected using goat anti-mouse conjugated horse-radish peroxidase (Bio-Rad) followed by incubation with the chromogenic substrate ABTS.

** Purification of NS1.** Native, dimeric NS1 was immunoaffinity purified from 50 h MVE virus-infected Vero cells using anti-NS1 MAbs and anti-NS1 MAb by visualizing the protein on 10% SDS–PAGE gels after silver staining (Wray et al., 1981).

**Immunization of mice.** Specific pathogen free male (BALB/c × C3H/RV) F1 hybrid mice were immunized with approximately 5 μg of pure NS1 protein with 50% Freund’s complete adjuvant in PBS by the i.p. route at 3–4 weeks of age. A second dose of 5 μg was administered i.p. with incomplete Freund’s adjuvant at 5–6 weeks of age, and the final boost consisted of 5 μg of protein injected intravenously without adjuvant at 8–10 weeks of age. Control mice were inoculated at these times with 5 μg of BSA using the same dose of adjuvant, or sublethally infected with 100 TCID50 of MVE virus by the i.p. route concurrent with the second dose of NS1. In VV experiments hybrid mice were immunized by i.p. injection with 107 p.f.u. of wild-type or recombinant VV at 5–6 weeks of age. This was repeated 3 weeks later. Two weeks after the final immunization all mice were bled for serology or adoptive transfer. Two days later animals
were challenged with 1000 TCID$_{50}$ of MVE virus in 5 µl by the i.c.
route and monitored for signs of encephalitis for 21 days.

**Depletion of CD8$^+$ T cells.** At 5 and 2 days prior to virus challenge
mice were injected i.p. with a dose of anti-Lyt-2 antibody YTS 169.4
(Cobbold et al., 1984) sufficient to deplete CD8$^+$ T cells. Depletion
was confirmed on the day of challenge and at 10 days post-infection by
FACscan analysis of fluorescently labelled CD8$^+$ splenic lymphocytes.

**Adoptive transfer.** Serum (250 µl) was transferred i.p. to susceptible
18–20-day-old male BALB/c or 10-week-old F$_1$ hybrid male recipients
2 h prior to an i.p. (BALB/c) or i.c. (F$_1$ hybrid) challenge with
1000 TCID$_{50}$ of MVE virus. At 12–16 h after challenge a further 250 µl
of serum was transferred i.p. and mice were observed for 21 days.

**Serology.** Doubling dilutions of prechallenge sera from immunized
mice were tested for antibody to MVE viral proteins by ELISA on
immunofluorescence at 24 h post-infection. Cells were trypsinized for
route and monitored for signs of encephalitis for 21 days,
confirmed on the day of challenge and at 10 days post-infection by
SW 13, L929, Vero and C6/36 cells were infected with a sufficiently high
m.o.i. of MVE virus to produce 100% infection as determined by
plaques by 70% compared with that in control wells were taken as the
end point.

For complement-mediated cytolytic (CMC) assays, monolayers of
SW13, L929, Vero and C6/36 cells were infected with a sufficiently high
m.o.i. of MVE virus to produce 100% infection as determined by
immunofluorescence at 24 h post-infection. Cells were trypsinized for
10 min then resuspended, washed in serum-free RPMI, and 10$^5$ cells
were labelled with 1 mCi of $^{51}$Cr (sodium chromate) in a volume of
200 µl for 30 min at 37 °C. Labelled cells were then washed in RPMI,
and the supernatant was removed. Inoculum was then added to each well
of a 96-well plate and 10$^5$ cells in 25 µl were added to each well of a 96-well plate and
incubated with 25 µl of serum dilutions (1/5 and 1/50) on ice for
15 min. Twenty-five µl of guinea-pig complement (CSl, Melbourne,
Australia) diluted in RPMI was added to test wells and plates were
incubated at 37 °C for 45 min. Complement-mediated lysis of a similar
number of BALB/c thymic cells in the presence of anti-Thy-1 antibody
30H12 (ATCC TIB 107) was used as the positive control and to
determine the optimum dilution of complement. Culture medium from
each well was then harvested and analysed for radioactivity on a
gamma counter (Packard). Percent lysis was calculated by the formula:

\[
\text{% release} = \frac{(\text{test} - \text{SR}) \times 100}{(\text{TR} - \text{SR})}
\]

where test is the c.p.m. of test supernatant, SR is spontaneous release
by labelled cells that received no serum or complement, and TR is total
release by labelled cells lysed by 1% Triton X-100.

**Results**

**Analysis of purified NS1 and recombinant VV expressed
MVE viral proteins**

Purified NS1 was analysed by protein specific MAbs in
ELISA and Western blot, and by silver stain of the
preparation resolved on SDS–PAGE. NS1, pre-
dominantly in dimer form, was the only protein detected
(Fig. 1). NS1 protein expressed by VV-NS1, VV-STR
and VV-ΔC cultured in CV1 cells was compared to
native viral protein produced in MVE virus infected cells
by Western blot using a cocktail of anti-NS1 MAbs. This
revealed that VV-ΔC, VV-NS1 and VV-STR expressed
dimeric NS1 that co-migrated with authentic NS1
produced in MVE-infected cells (Fig. 2). ELISA analysis
revealed that all MAbs produced to the native NS1
protein of MVE virus reacted equally well with cells
infected with VV-NS1, VV-STR and VV-ΔC antigens
(Table 1). All anti-E MAbs reacted with cells infected
with VV-STR or VV-ΔC, but failed to react with VV-
NS1 infected cells. Two anti-prM MAbs (M-B11 and
M2-1E7) bound strongly to VV-ΔC infected cells but
failed to react with cells infected with VV-STR and VV-
NS1, consistent with the absence of prM in VV-NS1 or
greatly reduced cleavage of C-prM in VV-STR (Lobigs,
1993).

![Fig. 1. Silver stain of purified NS1 separated on 10% SDS-PAGE.](image)

**Susceptibility of resistant, susceptible and F$_1$ hybrid mice
to MVE virus challenge**

Susceptible, resistant and F$_1$ hybrid strains were ex-
aminied for their response to i.c. inoculation with MVE
virus. The results, summarized in Table 2, indicated that
BALB/c and (BALB/c × C3H/RV) F$_1$ hybrid mice
showed a similar mortality rate; however, hybrid mice
exhibited symptoms of disease 2–3 days later than did
BALB/c mice. A similar delay of onset of encephalitis
was observed between the susceptible strain C3H/Hej
and (C3H/Hej × C3H/RV) F$_1$ hybrids (results not
shown). C3H/RV mice, on the other hand, were
significantly less susceptible. The (BALB/c × C3H/RV)
Fig. 2. Western blot analysis of recombinant VV-MVE virus proteins using a pool of anti-NS1 MAb. (a) boiled; (b) unboiled. Con, control.

Table 1. Reaction of anti-MVE virus MAb with VV recombinant MVE viral proteins in ELISA

<table>
<thead>
<tr>
<th>MAb</th>
<th>Reactive protein</th>
<th>WT</th>
<th>NS1</th>
<th>STR</th>
<th>ΔC</th>
<th>MVE virus</th>
</tr>
</thead>
<tbody>
<tr>
<td>MB11</td>
<td>prM</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1E7</td>
<td>prM</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>8E7</td>
<td>E</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1H5</td>
<td>E</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3H6</td>
<td>E</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2D8</td>
<td>E</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3B2</td>
<td>E</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>ME6</td>
<td>NS1</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>8C4</td>
<td>NS1</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>10C6</td>
<td>NS1</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4D6</td>
<td>NS1</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1B6</td>
<td>NS1</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>7F12</td>
<td>NS1</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

F₁ hybrids were chosen for further studies because BALB/c mice had been used in preliminary immunization studies with purified NS1.

Protection of hybrid mice against MVE challenge

When F₁ hybrid mice were immunized with pure NS1 protein and challenged i.c. with MVE virus, mortality (15%) was greatly reduced compared with controls (94%) and time to death of nonsurvivors prolonged (Table 3). Hybrid mice immunized with recombinant VV were also protected. All (10/10) mice immunized with VV-ΔC (prM-E-NS1-NS2A) survived, as did 47% (9/19) of those immunized with VV-NS1 (NS1-NS2A) and 40% (4/10) with VV-STR (C-prM-E-NS1-NS2A). Only 20% (4/20) of mice immunized with wild-type vaccinia virus (VV-WT) and 20% (2/10) of unimmunized mice survived the challenge. Mice that succumbed to virus challenge after vaccination with VV-NS1 or VV-STR survived significantly longer (12.9 and 13.6 days respectively) than control mice (11.7) or mice immunized with VV-WT (11.5).

Analysis of prechallenge sera

Mice inoculated with pure NS1, VV-NS1 or VV-STR produced a high titre of antibody to MVE in ELISA (Table 3). Western blot analysis of these sera revealed

Table 2. Comparative susceptibility of mouse strains to i.c. inoculation with MVE virus

<table>
<thead>
<tr>
<th>Virus dose (TCID₅₀/5 µl)</th>
<th>BALB/c</th>
<th>C3H/RV</th>
<th>(BALB/c×C3H/RV) F₁</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Percentage mortality</td>
<td>Mean time to death (days)</td>
<td>Percentage mortality</td>
</tr>
<tr>
<td>1000</td>
<td>100% (9/9)</td>
<td>8.2±0.2 (range 7-9)</td>
<td>60% (6/10)</td>
</tr>
<tr>
<td>100</td>
<td>90% (9/10)</td>
<td>9.0±0.3 (8-11)</td>
<td>30% (3/10)</td>
</tr>
<tr>
<td>10</td>
<td>50% (5/10)</td>
<td>10.0±0.6 (8-13)</td>
<td>10% (1/10)</td>
</tr>
<tr>
<td>1</td>
<td>0% (0/10)</td>
<td>-</td>
<td>0% (0/8)</td>
</tr>
<tr>
<td>0.1</td>
<td>0% (0/10)</td>
<td>-</td>
<td>0% (0/9)</td>
</tr>
</tbody>
</table>

* Mice were inoculated i.c. with 5 µl of dilutions of MVE virus and euthanased when symptoms of encephalitis were obvious.
Table 3. Effect of MVE virus challenge on mice immunized with purified NS1, live MVE virus or recombinant VV

<table>
<thead>
<tr>
<th>Immunogen</th>
<th>Western blot</th>
<th>ELISA</th>
<th>PRNT</th>
<th>CMC</th>
<th>HI</th>
<th>Prechallenge serology</th>
<th>Percentage survival*</th>
<th>Mean time to death (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>–</td>
<td>&lt; 10^2</td>
<td>&lt; 5</td>
<td>–</td>
<td>–</td>
<td>20 (2/10)</td>
<td>11:7 (range 11-12)</td>
<td></td>
</tr>
<tr>
<td>VV-WT</td>
<td>–</td>
<td>&lt; 10^4</td>
<td>&lt; 5</td>
<td>–</td>
<td>–</td>
<td>20 (4/20)</td>
<td>11.5 (10-13)</td>
<td></td>
</tr>
<tr>
<td>VV-STR NS1</td>
<td>–</td>
<td>–</td>
<td>2 x 10^4</td>
<td>40</td>
<td>–</td>
<td>40 (4/10)</td>
<td>13:6 (11-21)</td>
<td></td>
</tr>
<tr>
<td>VV-AC E/NS1</td>
<td>3-2 x 10^4</td>
<td>50</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>100 (10/10)</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>MVE E/NS1</td>
<td>3-2 x 10^4</td>
<td>&lt; 5</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>47 (9/19)</td>
<td>12.9 (11-15)</td>
<td></td>
</tr>
<tr>
<td>BSA NS1</td>
<td>–</td>
<td>&lt; 10^5</td>
<td>&lt; 5</td>
<td>–</td>
<td>–</td>
<td>93 (27/29)</td>
<td>14 (12-16)</td>
<td></td>
</tr>
<tr>
<td>Pure NS1</td>
<td>6 x 10^4</td>
<td>&lt; 5</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>6 (1/15)</td>
<td>11:5 (10-15)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>85 (17/20)</td>
<td>13 (13)</td>
<td></td>
</tr>
</tbody>
</table>

* Mice were challenged i.c. with 1000 infectious units of MVE virus and euthanased when symptoms of encephalitis were obvious.

Table 4. Effect of CD8+ T cell depletion on survival of vaccinated mice

<table>
<thead>
<tr>
<th>Immunogen</th>
<th>CD8 depletion</th>
<th>Percentage survival*</th>
<th>Mean time to death (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>–</td>
<td>6:6 (1/15)</td>
<td>9:5 (range 8-10)</td>
</tr>
<tr>
<td>VV-AC</td>
<td>–</td>
<td>100% (11/11)</td>
<td>–</td>
</tr>
<tr>
<td>VV-AC</td>
<td>+</td>
<td>100% (10/10)</td>
<td>–</td>
</tr>
<tr>
<td>VV-NS1</td>
<td>–</td>
<td>80% (12/15)</td>
<td>11 (9-12)</td>
</tr>
<tr>
<td>VV-NS1</td>
<td>+</td>
<td>20% (3/15)</td>
<td>12 (10-14)</td>
</tr>
</tbody>
</table>

* Mice were challenged i.c. with 1000 infectious units of MVE virus and euthanased when symptoms of encephalitis were obvious.

that the response was specific for NS1. Sera from mice sublethally immunized with live MVE virus or those immunized with VV-AC also produced high levels of antibody which reacted with E and NS1 proteins with equal intensity in Western blot (results not shown). Control mice immunized with BSA or VV-WT produced no antibody reactive to MVE viral proteins.

Prechallenge sera from immunized mice were tested for the ability to neutralize MVE virus, to inhibit haemagglutination (HI) by MVE antigen and to lyse infected cells in the presence of complement (Table 3). Antibody in antisera reactive to NS1 was unable to neutralize MVE virus nor did it inhibit haemagglutination. In contrast, sera produced to live MVE virus or VV-AC possessed neutralizing and HI activity. All sera produced less than 10% lysis of 24 or 48 h MVE virus-infected L929, Vero, SW13 or C6/36 cells in the presence of complement (Table 1), which was similar to levels observed with uninfected cells. However, anti-Thy-1 MAb (30H12) induced complement-mediated lysis of 95–100% of BALB/c thymic cells in the same assay (results not shown).

Effect of CD8 depletion

To evaluate whether primary or memory Tc cell mediated immunity contributed to protection of hybrid mice, vaccinated mice were depleted of CD8+ cells prior to virus challenge. After depletion the protection provided by VV-AC was unaffected (Table 4). In contrast, the protection provided by VV-NS1 was reduced from 80% in undepleted to 20% in depleted mice. However,

Table 5. Protection of weanling BALB/c mice and adult F1 hybrid mice from challenge with MVE virus by passive transfer of pooled immune sera

<table>
<thead>
<tr>
<th>Recipient mouse</th>
<th>Serum transferred*</th>
<th>ELISA titre of serum</th>
<th>Percentage survival</th>
<th>Mean time to death (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALB/c†</td>
<td>Mouse anti-BSA</td>
<td>&lt; 100</td>
<td>0% (0/10)</td>
<td>8.5 (range 7-11)</td>
</tr>
<tr>
<td>BALB/c†</td>
<td>Mouse anti-NS1</td>
<td>2 x 10^5</td>
<td>0% (0/10)</td>
<td>11:2 (9-15)</td>
</tr>
<tr>
<td>BALB/c†</td>
<td>Mouse anti-MVE</td>
<td>1-2 x 10^4</td>
<td>100% (10/10)</td>
<td>–</td>
</tr>
<tr>
<td>F1 hybrid‡</td>
<td>Mouse anti-BSA</td>
<td>&lt; 100</td>
<td>30% (3/10)</td>
<td>8.9 (8-11)</td>
</tr>
<tr>
<td>F1 hybrid‡</td>
<td>Mouse anti-NS1</td>
<td>2 x 10^5</td>
<td>40% (4/10)</td>
<td>12.5 (9-16)</td>
</tr>
<tr>
<td>F1 hybrid‡</td>
<td>Mouse anti-MVE</td>
<td>1-2 x 10^4</td>
<td>90% (9/10)</td>
<td>15</td>
</tr>
</tbody>
</table>

* Serum raised in F1 hybrid mice.
† BALB/c mice were challenged i.p. with 1000 TCID<sub>50</sub> of MVE virus.
‡ F1 mice were challenged i.c. with 1000 TCID<sub>50</sub> of MVE virus.
depleted mice succumbed to infection significantly later than unvaccinated controls.

Adaptive transfer of immune serum

When F1 hybrid mice received 0.5 ml of anti-MVE serum, they were solidly protected from a lethal i.c. challenge, while a similar transfer of anti-NS1 serum failed to provide protection (Table 5). However, the recipients of anti-NS1 serum survived, on average, 48 h longer than mice receiving control serum. Similar results were observed in weanling BALB/c recipients prior to lethal i.p. challenge with MVE virus (Table 5).

Discussion

Investigations of the susceptibility of heterozygous resistant [(BALB/c x C3H/RV) F1] and susceptible (BALB/c) mice to i.c. inoculation with MVE virus revealed that F1 and BALB/c mice showed similar mortality rates (Table 2). However, F1 hybrid mice developed symptoms of encephalitis 2-3 days later than susceptible mice. This was consistent with findings of Sangster et al. (1994) who showed that F1 mice produce significantly lower virus titres in the brain than BALB/c mice after i.c. inoculation with MVE virus. When F1 hybrid mice were immunized with purified NS1 protein, the majority were protected from i.c. challenge with MVE virus. This is in contrast to the high mortality seen in BALB/c mice that had been immunized and challenged in an identical fashion (results not shown). Presumably the delay in onset of the disease in F1 mice and/or the lower virus titres in the brain allowed the NS1-specific immune response to play some protective role.

The level of protective immunity generated in mice by vaccination with recombinant VV was also examined in this study. The solid protection induced by VV-ΔC was apparently mediated by neutralizing antibodies to the E protein although some additional contribution of NS1 in this construct to protection cannot be excluded. Infection of CV1 cells with this construct results in the secretion of subviral particles that contain prM/M and E embedded in lipid envelope derived from the host cell (Lobigs, 1993). Similar particles have also been observed in the culture fluid of cells infected with recombinant VV expressing corresponding regions of the JE genome (Mason et al., 1991). Immunization with these recombinant viruses or purified preparations of the secreted particles has also been shown to protect mice from i.p. challenge with JE virus (Konishi et al., 1992).

While neutralising antibody is thought to prevent infection of the CNS by virus entering from the peripheral route, infection of the CNS via i.c. inoculation may also require a cytolytic T lymphocyte (CTL) response for virus clearance and protection (Monath, 1986). When VV-ΔC vaccinated mice were depleted of CD8+ T cells for up to 10 days post-infection, protection remained complete. This indicates that in the presence of a primed antibody response to the E protein of MVE virus, a CD8+ T cell response is not required for clearance of virus from the CNS. This is consistent with the findings of Levine et al. (1991) who demonstrated that passive transfer of MAbs to the E2 protein of Sindbis virus cleared virus from the neurons of persistently infected SCID mice in the absence of a cellular immune response.

In contrast to VV-ΔC, VV-STR, which expresses E but does not produce the prM/E heterodimer necessary for secretion (Lobigs, 1993), did not induce a humoral response to E in mice. However, like VV-NS1 and ΔC, this construct expresses NS1 that acquires its native dimeric form and is secreted into the extracellular fluid. This resulted in a strong antibody response to NS1 which apparently provided partial protection in mice vaccinated with VV-STR, VV-NS1 or pure NS1. Pre-challenge sera from these mice failed to activate complement-mediated cytolysis of MVE-infected cells, a proposed mechanism of protection induced by antibodies to NS1 of YF virus (Schlesinger et al., 1990). However, our results are consistent with recent findings by Jacobs et al. (1994) who demonstrated that complement-deficient mice immunized with recombinant adenovirus expressing TBE NS1 were still protected from virus challenge suggesting that another mechanism, such as antibody-dependent cell-mediated cytotoxicity (ADCC), was involved. However, our attempts to demonstrate ADCC of MVE-infected L929 cells by BALB/c spleen cells in the presence of sera from these mice were inconclusive (results not shown).

Passive transfer of NS1-immune sera to recipient mice provided no protection, but delayed the onset of symptoms and death for an average of 48 h. This indicated that primed B or T cells were also required to achieve significant protection. Indeed depletion of CD8+ cells in F1 hybrid mice vaccinated with VV-NS1 completely ablated protection, indicating a requirement for class I restricted T cells to clear infection. However, findings by Lobigs et al. (1994) that only NS3 contains immunodominant Tc epitopes and that the MVE-specified polyprotein expressed by VV-STR (C-prM-E-NS1-NS2A) contained no epitopes for Tc cells generated in MVE-infected (CBA) H-2k mice indicates that a cell-mediated immunity response is not the primary mechanism of protection in F1 hybrid mice (H-2k-H-2a) immunized with VV-NS1 or VV-STR. While it is possible that vaccination with recombinants that do not express the dominant NS3 Tc epitopes primes for subdominant
immunization with purified NS1 protein is mediated by primed class I restricted T cells. A likely explanation for the protection provided by immunization with pure NS1 antibody to NS1 and that produced by primed B cells induce immune cytolysis of infected cells, slowing down virus replication in the host. This allows time for a primary class I restricted Tc cell response to virus challenge and clearance of the virus before symptoms occur. This is supported by results of Schlesinger et al. (1993), who showed that protection of mice against YF challenge by transfer of NS1 MAb was ablated in animals treated with cyclophosphamide.

The use of F1 hybrid mice possessing genetic resistance to flavivirus infection proved useful in our studies and overcame the problem of inconsistent mortality rates in adult mice challenged by peripheral routes. Although i.c. inoculation is an unnatural route of entry for the virus, the severity of this form of challenge with a neurotropic virus such as MVE was significantly reduced in these mice due to the lower levels of virus replication in the brain. This has provided a more sensitive animal model to measure the protective response induced by purified viral protein and vaccinia recombinants expressing viral proteins.

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References


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