Efficacy of individual measles virus structural proteins in the protection of rats from measles encephalitis

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Lewis rats were immunized with recombinant vaccinia virus (VV) expressing the nucleocapsid (N), phospho (P), matrix (M), fusion (F), and haemagglutinin (H) proteins of measles virus (MV). Animals developed humoral as well as cell-mediated immune (CMI) responses to the corresponding MV proteins. Rats immunized with recombinants VVN, VVF or VVH survived a MV challenge infection whereas VVP- and VVM-immunized rats were only partially protected. In vivo depletion of CD8\(^+\) T lymphocytes did not prevent the protective effect of the N, F or H protein-specific CMI response in rats. VVH and VVF immunization induced neutralizing antibodies, but no such antibodies were detected after VVN immunization. Further investigation of the temporal occurrence of the antiviral antibodies indicated that the observed protection provided by VVN and VVF immunization depends on CD4\(^+\) N- or F-specific T cells in the absence of neutralizing antibodies and CD8\(^+\) T cells. A role for neutralizing antibodies induced by VVH cannot be ruled out.

Introduction

Measles virus (MV) is a highly contagious human pathogen with a world-wide prevalence. It is not only responsible for clinical measles but also associated with central nervous system (CNS) disorders. Besides the not uncommon acute MV encephalitis which probably results from an MV-induced autoimmune reaction (Johnson & Griffin, 1986), two chronic CNS disorders, subacute sclerosing panencephalitis (SSPE) and measles inclusion body encephalitis can develop on the basis of a persistent MV infection of brain cells months to years after the acute MV infection (ter Meulen et al., 1983).

Infections with MV are not controlled in all parts of the world. In third world countries in particular, MV infections still cause approximately 2 \times 10^6 childhood deaths per year (Norrby & Oxman, 1990). Immunization strategies with an attenuated MV vaccine have been applied in an attempt to reduce the occurrence of acute measles and its neurological manifestations. However, the reduction of measles has been effectively achieved only in industrialized countries. Moreover, recent epidemiological studies in North America indicate that the current use of live MV vaccination in early childhood does not provide life-long immunity since measles epidemics have occurred there among previously vaccinated children and young adults (Centers for Disease Control, 1989; Anonymous, 1990). These vaccine failures are of great medical concern and necessitate a better understanding of protective immune responses to measles virus infection.

MV is a member of the paramyxovirus family. It is an enveloped negative-stranded RNA virus which encodes at least six structural proteins. The haemagglutinin (H), the fusion (F) and the membrane (M) proteins form part of the viral envelope whereas the nucleocapsid (N), the phospho (P) and the large (L) proteins together with the viral RNA represent the replicative complex. As documented in clinical and experimental studies, H and F proteins are important for the protective humoral immune response since these proteins induce antibodies that neutralize infectious virus and prevent virus spread by cell fusion (Cherry, 1987). However, little is known about cell-mediated immune (CMI) responses. Although a competent CMI response is required to clear the acute viral infection, no data are yet available concerning the role of the individual MV structural proteins for the induction of a protective CMI response in man.

Recently, it has been shown that immunization of mice with recombinant vaccinia viruses (VV) expressing MV H and F proteins or rats immunized with VV expressing MV N protein were protected from measles infection (Drillien et al., 1988; Bankamp et al., 1991). In an extension of these observations the present study compares the ability and efficacy of all MV structural proteins except the L protein to evoke a protective immune response against MV infection in an animal model using VV recombinants expressing individual MV
proteins. Infection with the neurotropic MV CAM/RBH induces an acute lethal encephalitis in weanling rats (Liebert & ter Meulen, 1987). The data obtained reveal that in contrast to immunization of rats with the MV H, F and N proteins, the M and P proteins only partially protect Lewis rats from encephalitis. The protective immune response induced by the N and F proteins obviously depends on MV-specific CD4+ T lymphocytes as suggested by depletion studies of CD8+ T cells and the temporal kinetics of antiviral antibody synthesis, while immunization with VV H protein leads to the early appearance of high titres of virus-neutralizing antibodies.

**Methods**

**VV recombinants expressing MV structural genes.** VV strain Copenhagen and the temperature-sensitive mutant ts7 were used to generate VV recombinants. Full-length genes coding for the N, P and M structural proteins of the Edmonston strain of MV (obtained from K. Baczko, Universität Würzburg) were excised from Bluescript vectors. The genes were inserted into the transfer vectors pSCC11 (kindly provided by B. Moss, National Institutes of Health, Bethesda, Md., U.S.A.) or p7. SK131 (kindly provided by A. von Brunn, DKFZ, Heidelberg, Germany) under the control of the early/late p7.5 VV promoter. The resulting plasmids were used to introduce the MV genes into the thymidine kinase (TK) gene of wild type VV (VV-wt) by homologous recombination (Mackett et al., 1985). The recombinants are referred to as VVN, VVP and VVM, expressing the N, P and M proteins respectively. The VVH and VVF recombinants cloned from the Hallé isolate of MV which differs from the Edmonston isolate by only a few amino acids (B. K. Rima & K. Baczko, personal communication) were obtained from R. Drilljen, Strasbourg, France (Drilljen et al., 1988). The expression of the inserted MV genes was analysed by immunoprecipitation, immunofluorescence or Western blotting as described previously (Bankamp et al., 1991).

Animals, immunization and in vivo protection assay. Lewis rats, purchased from the Zentralinstitut für Versuchstiere, Hannover, Germany, were kept under specific pathogen-free conditions. For analysis of the immune responses induced by recombinant VV, young adult Lewis rats were infected intraperitoneally (i.p.) with one to three injections of 10^7 p.f.u. at 1 week intervals. Serum antibody titres and the T cell lymphoproliferative response (LPR) were determined at various time points.

To determine the protective effect of individual MV proteins, 2- to 3-week-old suckling rats were immunized either once, twice or three times at 5 to 7 day intervals by i.p. injections of 10^7 p.f.u. of VV recombinants expressing individual structural proteins or VV-wt. Three to 5 days after the last recombinant VV injection, the animals were challenged by intracerebral (i.c.) infection with the neurotropic rat brain-adapted MV strain CAM/RBH as described (0.5×10^3 to 8×10^4 TCID_{50}; Liebert & ter Meulen, 1987; Bankamp et al., 1991). Animals were weighed daily and observed for clinical signs of encephalitis. For histological analysis, brains and spinal cords were fixed in buffered paraformaldehyde, paraffin-embedded, and cut and stained according to standard procedures (Liebert & ter Meulen, 1987).

**Characterization of the MV-specific immune response.** For the determination of humoral immunity, serum samples were taken by tail vein puncture at various time points before and after recombinant VV immunization and MV infection. The titre of antiviral antibodies was determined by an ELISA as described (Liebert & ter Meulen, 1987). Briefly, 10 µg per ml of lysates from uninfected and MV-infected Vero cells, and from TK- cells, uninfected or infected with the various VV recombinants, was coated onto flat bottom microtitre plates especially manufactured for ELISA (Nunc). The sera were absorbed with lysates of VV-wt-infected TK- cells before ELISA testing. A neutralization test based on plaque reduction was used as described previously for the detection of MV-neutralizing antibodies (Liebert & ter Meulen, 1987).

The MV-specific CMI in the presence of purified measles virions or purified MV structural proteins was analysed in single cell lymph node preparations obtained from animals immunized with the different VV recombinants before and after MV infection in a lymphoproliferation assay as described (Liebert et al., 1988). Briefly, single-cell suspensions (10^6 to 3×10^7) from mesenteric lymph nodes were incubated with graded doses of MV antigens and control proteins for 72 h with an 18 h pulse of tritiated deoxythymidine (3HdTh). The lymphoproliferative response (LPR) was determined by the ratio of thymidine incorporation in stimulated and unstimulated cells and expressed as the stimulation index. Short-term CD4+ T cell lines were isolated from bulk populations by expansion of the activated lymphocytes in media containing interleukin 2 followed by restimulation with purified MV proteins as described (Liebert et al., 1988). Their specificity and surface phenotype were analysed by LPR assay and fluorescence-activated cell sorting (FACS) analysis after the second and third in vitro restimulations as described previously (Bankamp et al., 1991).

**Determination of MV replication in brain material.** Attempts to isolate MV from the brain tissue of infected rats was carried out by cocultivation of freshly obtained CNS material with Vero cells as described (Liebert & ter Meulen, 1987). Briefly, 0.1 to 0.2 g of brain material from cortical hemispheres was chopped into small pieces and seeded in 50 ml culture flasks containing 2×10^6 Vero cells. Thirty-six to 48 h after cultivation (37°C, 5% CO2) non-adherent brain material was removed and the medium replaced. The occurrence of MV-characteristic c.p.e. was recorded daily. When no c.p.e. had occurred 1 week to 10 days later, the flasks were subcultivated. The experiment was terminated after 3 to 4 weeks with a minimum of three subcultivations.

**Depletion of CD8+ T lymphocytes.** In order to define the lymphocyte subpopulation responsible for the restriction of the virus spread in brain tissue, CD8+ T cells were depleted from animals as described previously (Sedgwick, 1988; Bankamp et al., 1991). Briefly, 21-day-old rats were injected i.p. on three consecutive days with monoclonal antibodies directed against the rat CD8 molecule (OX8). This treatment resulted in complete and prolonged (at least 20 days) depletion of the CD8+ T cell population from lymph nodes as shown previously (Bankamp et al., 1991). No CD8+/α/β TCR+ T cells were detected and a compensatory increase of the CD4+/α/β TCR+ subpopulation from about 45% to 60% was apparent. In each animal the efficacy of the antibody treatment was determined retrospectively by FACS analysis at the end of the experiment, and only those with a complete absence of CD8+/α/β TCR+ cells were included in the study.

**Results**

**Cloning and expression of MV genes in VV recombinants**

The entire coding sequences of the various structural genes of MV were inserted into the TK gene of VV strain Copenhagen. TK- cells infected with the recombinant VV exhibited the plaque morphology typical of VV (not shown). The expression of the individual MV proteins
was confirmed by indirect immunofluorescence using monoclonal and monospecific antibodies (Fig. 1). In vivo labelling with [\(^{35}\)S]methionine of Vero or TK\(^{-}\) cells infected with VV recombinants and immunoprecipitation with the appropriate human MV-hyperimmune serum pre-absorbed with lysates of recombinant VV-infected TK\(^{-}\) cells or Western blotting revealed bands comigrating with the respective authentic MV proteins (data not shown).

Induction of humoral immune responses specific for MV proteins

The immunogenic potential of the recombinant VV with regard to the MV structural proteins was determined in young adult Lewis rats immunized with recombinant VV. At various time-points after infection, sera were taken by tail vein puncture. Monospecific anti-MV antibodies were generated by all recombinant VVs. By indirect immunofluorescence on MV-infected Vero cells, rat sera yielded a positive reaction (data not shown). Using ELISA, MV-specific serum antibodies were detected with titres in the range of 1 in 100 to >6400 (Table 1). Neutralizing antibodies were induced by immunization with recombinant VV expressing the F or H protein only (Table 1). The temporal kinetics of the antibody titres were determined in single-shot VVH-

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**Table 1. Immune response in Lewis rats following immunization with recombinant vaccinia virus**

<table>
<thead>
<tr>
<th>Immunization</th>
<th>No. of immunizations</th>
<th>Serum taken (days after immunization)</th>
<th>ELISA §</th>
<th>NT §</th>
<th>Specificity for MV protein*</th>
</tr>
</thead>
<tbody>
<tr>
<td>VV-wt</td>
<td>1</td>
<td>7</td>
<td>&lt;50</td>
<td>&lt;20</td>
<td>H - P + N - F - M -</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>4</td>
<td>&lt;50</td>
<td>&lt;20</td>
<td>H - P + N - F - M -</td>
</tr>
<tr>
<td>VVN</td>
<td>1</td>
<td>8</td>
<td>400</td>
<td>&lt;20</td>
<td>H - P + N - F - M -</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>4</td>
<td>1600</td>
<td>&lt;20</td>
<td>H - P + N - F - M -</td>
</tr>
<tr>
<td>VVP</td>
<td>1</td>
<td>8</td>
<td>100</td>
<td>&lt;20</td>
<td>H + P - N - F - M -</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>5</td>
<td>200</td>
<td>&lt;20</td>
<td>H + P - N - F - M -</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>4</td>
<td>400</td>
<td>&lt;20</td>
<td>H + P - N - F - M -</td>
</tr>
<tr>
<td>VVM</td>
<td>1</td>
<td>8</td>
<td>≤100</td>
<td>&lt;20</td>
<td>H + P - N - F - M -</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>5</td>
<td>≤100</td>
<td>&lt;20</td>
<td>H + P - N - F - M -</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>4</td>
<td>100</td>
<td>&lt;20</td>
<td>H + P - N - F - M -</td>
</tr>
<tr>
<td>VVF</td>
<td>1</td>
<td>8</td>
<td>400</td>
<td>&lt;20</td>
<td>H - P - N - F - M -</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>5</td>
<td>400</td>
<td>&lt;20</td>
<td>H - P - N - F - M -</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>4</td>
<td>800</td>
<td>&lt;20</td>
<td>H - P - N - F - M -</td>
</tr>
<tr>
<td>VVH</td>
<td>1</td>
<td>8</td>
<td>100</td>
<td>&lt;20</td>
<td>H - P - N - F - M -</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>5</td>
<td>800</td>
<td>&lt;20</td>
<td>H - P - N - F - M -</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>4</td>
<td>&gt;6400</td>
<td>320</td>
<td>H - P - N - F - M -</td>
</tr>
</tbody>
</table>

* The specificity of MV antiviral antibodies was investigated by indirect immunofluorescence analysis.
† Rats were immunized by i.p. infection with 10\(^7\) p.f.u. of the respective VV recombinants. Where there were two or three immunizations, the interval between each was 6 to 8 days.
‡ Time point of bleeding after the last injection with the VV recombinant.
§ The reciprocal titres shown were obtained in an ELISA using whole purified MV virions as coating antigens, and in an MV neutralization test (NT) (Liebert & ter Meulen, 1987).
VVF-immunized rats. Low titres of neutralizing antibody were detected as early as 4 days after VVH immunization but not until 9 days after injection with VVF (Fig. 2).

**MV-specific CMI response**

The MV protein-specific CMI response was analysed 4, 8 and 12 days after a single i.p. injection of recombinant VV into weanling Lewis rats (Table 2a). A low to moderate MV-specific T cell response was demonstrated in the polyclonal lymphocyte cultures from spleen and mesenterial lymph nodes. No significant increase in the $^{3}$HdT uptake could be observed when the results obtained at different times p.i. were compared. The LPR in the presence of MV and the respective protein determined after multiple injections of the various recombinant VV increased, although however insignificantly statistically, after two injections of VV (Table 2b). When complete inactivated MV was used as an in vitro restimulating antigen, the stimulation indices ranged from 2.2 after VVH immunization to 4.1 following VVP injection. The MV protein-specific cellular immune response somewhat exceeded the MV-specific one. Stimulation indices were in the range of 3-3 for M and 7-4 for N if MV-specific proteins were used for in vitro restimulation of the T cells. Control rats immunized with VV-wt developed no MV-specific antiviral immune response. After a second in vitro restimulation with the respective structural proteins, specific T cell populations with much higher stimulation indices of between 6 and 38 were obtained (Table 2c). The determined LPS was specific for the antigen used for immunization.

**Significance of the MV-specific immune response for the course of experimental measles encephalitis in rats**

In order to evaluate the protective effect of the MV-specific immune response for measles CNS infection, suckling Lewis rats were vaccinated i.p. with one of the recombinant VVs or with VV-wt prior to i.c. infection with CAM/DBH neurotropic MV. The infectious dose used for challenge with MV was $0.5 \times 10^4$ and $8 \times 10^4$ TCID$_{50}$ per animal. All control rats as well as rats immunized with VVP and VVM exhibited an acute encephalitis with incubation periods of 12 ± 2 days and they either died or were killed when they showed severe neurological symptoms. In contrast, animals vaccinated with VVN, VVF or VVH did not exhibit symptoms even with the highest possible MV challenge dose (Table 3a).

In an attempt to increase the protective efficiency of VVM and VVP immunization, rats were injected twice with the respective recombinant VV (Table 3b), partial protection was achieved and about 25% of the VVM- or VVP-immunized rats developed no symptoms after the i.c. MV infection. The protected rats had higher antiviral antibody titres and a higher stimulation index than rats immunized only once with VVM or VVP (data not shown).

The isolation of MV from fresh brain material by co-cultivation with Vero cells was successful only in unprotected rats immunized with either VV-wt, VVP or VVM. MV could be reisolated from seven out of nine VVM-immunized rats and from six out of six VVP-immunized rats (Table 3b). After double-shot VVM and VVP immunization, infectious MV could be isolated from all diseased rats tested, as well as from one of two VVP- and two of three VVM-immunized rats which were devoid of clinical symptoms after MV infection. In contrast, infectious MV could not be isolated from any of the rats vaccinated with VVN, VVH or VVF (Table 3a, b).

**Histopathological examination**

Histopathological examination of the CNS revealed uniform disseminated encephalitis in all unprotected rats, i.e. animals which had been immunized with VV-wt, and most rats immunized with VVM and VVP. Severe inflammatory and degenerative lesions involved both grey and white matter of the cerebral hemispheres, basal ganglia, and to a lesser degree the brain stem. In contrast, almost all of the VVF- and VVH-protected rats and approximately 50% of the VVN-immunized rats had no detectable neuropathological lesions (Table 3). The histopathological analysis of brain sections of the
Table 2. Lymphoproliferative response of rats following immunization with recombinant VV

(a) Kinetics of the polyclonal T cell response after VV immunization using inactivated MV to stimulate the cells in vitro

<table>
<thead>
<tr>
<th>VV recombinant used for i.p. immunization</th>
<th>Day after immunization</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) Kinetics of the polyclonal T cell response after VV immunization using inactivated MV to stimulate the cells in vitro</td>
<td>4</td>
</tr>
<tr>
<td><strong>VV-wt</strong></td>
<td>ND*</td>
</tr>
<tr>
<td><strong>VVN</strong></td>
<td>2.8</td>
</tr>
<tr>
<td><strong>VVP</strong></td>
<td>2.5</td>
</tr>
<tr>
<td><strong>VVM</strong></td>
<td>2.0</td>
</tr>
<tr>
<td><strong>VVF</strong></td>
<td>1.4</td>
</tr>
<tr>
<td><strong>VVH</strong></td>
<td>2.0</td>
</tr>
</tbody>
</table>

(b) Polyclonal T cell response after multiple immunizations

<table>
<thead>
<tr>
<th>VV recombinant used for immunization</th>
<th>Antigen used for restimulation of T cells</th>
<th>Number of immunizations</th>
</tr>
</thead>
<tbody>
<tr>
<td>(b) Polyclonal T cell response after multiple immunizations</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td><strong>VV-wt</strong></td>
<td><strong>MV</strong></td>
<td>0.7</td>
</tr>
<tr>
<td><strong>VVN</strong></td>
<td><strong>MV</strong></td>
<td>2.8</td>
</tr>
<tr>
<td><strong>VVP</strong></td>
<td><strong>N</strong></td>
<td>5.1</td>
</tr>
<tr>
<td><strong>VVM</strong></td>
<td><strong>Control†</strong></td>
<td>&lt; 2.0</td>
</tr>
<tr>
<td><strong>VVF</strong></td>
<td><strong>MV</strong></td>
<td>2.5</td>
</tr>
<tr>
<td><strong>VVH</strong></td>
<td><strong>P</strong></td>
<td>5.4</td>
</tr>
<tr>
<td><strong>VV-wt</strong></td>
<td><strong>MV</strong></td>
<td>2.0</td>
</tr>
<tr>
<td><strong>VVN</strong></td>
<td><strong>M</strong></td>
<td>2.9</td>
</tr>
<tr>
<td><strong>VVP</strong></td>
<td><strong>Control</strong></td>
<td>&lt; 2.2</td>
</tr>
<tr>
<td><strong>VVM</strong></td>
<td><strong>MV</strong></td>
<td>1.4</td>
</tr>
<tr>
<td><strong>VVF</strong></td>
<td><strong>F</strong></td>
<td>3.3</td>
</tr>
<tr>
<td><strong>VVH</strong></td>
<td><strong>Control</strong></td>
<td>&lt; 1.3</td>
</tr>
<tr>
<td><strong>VV-wt</strong></td>
<td><strong>MV</strong></td>
<td>2.0</td>
</tr>
<tr>
<td><strong>VVN</strong></td>
<td><strong>H</strong></td>
<td>2.8</td>
</tr>
<tr>
<td><strong>VVP</strong></td>
<td><strong>Control</strong></td>
<td>&lt; 1.6</td>
</tr>
</tbody>
</table>

(c) Secondary in vitro restimulation

<table>
<thead>
<tr>
<th>Antigen used for LPR assay‡</th>
<th>VV recombinant used for i.p. immunization</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>VVN</strong></td>
<td>VVP</td>
</tr>
<tr>
<td><strong>Complete MV virions</strong></td>
<td>19.5 ± 7.6</td>
</tr>
<tr>
<td><strong>Specific MV protein</strong></td>
<td>37.6 ± 7.4</td>
</tr>
<tr>
<td><strong>Control§</strong></td>
<td>≤ 4.6 ± 2.5</td>
</tr>
</tbody>
</table>

§ Lysate from bacteria without measles sequences or from bacteria expressing irrelevant MV sequences was used as a control.

* ND, Not done.
† MV proteins considered non-relevant were used to stimulate T cells (control).
‡ The LPR of three to five experiments is expressed as the mean (+ S.D.) of the stimulation index (ratio of 3HdT uptake in the presence and the absence of purified MV or individual structural proteins). The following antigens were used for in vitro restimulation: MV (inactivated purified MV as described by Liebert et al., 1988), or MV structural proteins N, P, M, F and H expressed as bacterial (Escherichia coli) recombinant proteins and purified by urea extraction from bacterial lysates.
§ Lysate from bacteria without measles sequences or from bacteria expressing irrelevant MV sequences was used as a control.

remaining VVN-immunized and the few surviving VVM- or VVP-immunized rats revealed minor residual non-inflammatory lesions (gliotic scars, no mononuclear cell infiltrates) 3 to 4 weeks after infection with MV. In brain sections sampled up to approximately 15 days p.i., only minor focal lesions predominantly near the site of injection in the left brain hemisphere were seen. Similarly to the controls, VVM- or VVP-
Table 3. Protection from measles encephalitis of rats immunized with recombinant VV expressing single MV proteins

(a) Single-shot immunization

<table>
<thead>
<tr>
<th>Immunization</th>
<th>Diseased/total rats</th>
<th>MV isolation from CNS</th>
<th>Number of rats</th>
<th>Type of lesion</th>
</tr>
</thead>
<tbody>
<tr>
<td>VV-wt</td>
<td>3/3</td>
<td>+</td>
<td>3</td>
<td>AE</td>
</tr>
<tr>
<td>VVN</td>
<td>0/17</td>
<td>-</td>
<td>5</td>
<td>No lesions</td>
</tr>
<tr>
<td>VVP</td>
<td>6/6</td>
<td>+</td>
<td>6</td>
<td>AE</td>
</tr>
<tr>
<td>VVM</td>
<td>11/11</td>
<td>+</td>
<td>11</td>
<td>AE</td>
</tr>
<tr>
<td>VVF</td>
<td>0/8</td>
<td>-</td>
<td>7</td>
<td>No lesions</td>
</tr>
<tr>
<td>VVH</td>
<td>0/9</td>
<td>-</td>
<td>9</td>
<td>No lesions</td>
</tr>
</tbody>
</table>

(b) Double-shot immunization

<table>
<thead>
<tr>
<th>Immunization</th>
<th>Diseased/total rats</th>
<th>MV isolation from CNS</th>
<th>Number of rats</th>
<th>Type of lesion</th>
</tr>
</thead>
<tbody>
<tr>
<td>VV-wt</td>
<td>18/18</td>
<td>+</td>
<td>18</td>
<td>AE</td>
</tr>
<tr>
<td>VVN</td>
<td>0/20</td>
<td>-</td>
<td>13</td>
<td>No lesions</td>
</tr>
<tr>
<td>VVP</td>
<td>10/13</td>
<td>+</td>
<td>2</td>
<td>No lesions</td>
</tr>
<tr>
<td>VVM</td>
<td>14/18</td>
<td>+</td>
<td>1</td>
<td>No lesions</td>
</tr>
<tr>
<td>VVF</td>
<td>0/21</td>
<td>+</td>
<td>13</td>
<td>AE</td>
</tr>
<tr>
<td>VVH</td>
<td>0/23</td>
<td>-</td>
<td>22</td>
<td>No lesions</td>
</tr>
</tbody>
</table>

Neuropathology*:

- AE: acute encephalitis with severe disseminated lesions;
- MFE: mild focal encephalitis;
- residual lesions: microglial nodules and patches of gliosis in the absence of inflammatory cell infiltrates.

* All rats were investigated histopathologically. AE, acute encephalitis with severe disseminated lesions; MFE, mild focal encephalitis; residual lesions, microglial nodules and patches of gliosis in the absence of inflammatory cell infiltrates.

† Weanling Lewis rats were immunized once with VV constructs and were infected with MV 4 days later (infectious dose 0.5 × 10^4 to 8 × 10^4 TCID_{50}). For part (b), rats were immunized twice with VV constructs.

‡ MV could be isolated from the brain tissue of the diseased animal 14 days p.i.

In general, the histopathological lesions were less severe in rats immunized twice compared to those immunized once, particularly in the VVM- and VVP-injected animals.

Effect of depletion of CD8+ T cells

In order to analyse the significance of the CMI responses against N, H and F proteins of MV for protection against MV encephalitis, CD8+ T cells were depleted by injection of the OX8 monoclonal antibody 4 days after recombinant VV immunization. In the absence of CD8+ T cells VVN-, VVF- or VVH-immunized rats survived an i.c. challenge infection with 5 × 10^4 TCID_{50} without the appearance of clinical symptoms (Table 4). MV-specific humoral and cellular immune reactions showed no difference in comparison with non-depleted rats.

Histopathologically mild focal encephalitic lesions were detected in only two out of 10 rats which had been immunized with VVN and residual non-inflammatory lesions were apparent in three of 10 VVN- as well as one of eight VVH-treated rats. The remainder were free of neuropathological lesions.

Table 4. Effect of CD8+ T cell depletion* on MV encephalitis in rats

<table>
<thead>
<tr>
<th>Immunization</th>
<th>Diseased/total rats</th>
<th>MV isolation from brain</th>
<th>LPR</th>
<th>Histopathology</th>
</tr>
</thead>
<tbody>
<tr>
<td>VV-wt</td>
<td>12/12</td>
<td>+</td>
<td>1.6 ± 0.6</td>
<td>Severe disseminated encephalitis (12)</td>
</tr>
<tr>
<td>VVN</td>
<td>1/10</td>
<td>-</td>
<td>4.6 ± 1.3</td>
<td>No lesions (5), residual lesions (3), focal encephalitis (2)</td>
</tr>
<tr>
<td>VVF</td>
<td>0/7</td>
<td>-</td>
<td>4.3 ± 0.9</td>
<td>No lesions (7)</td>
</tr>
<tr>
<td>VVH</td>
<td>0/8</td>
<td>-</td>
<td>3.7 ± 1.2</td>
<td>No lesions (7), residual lesions (1)</td>
</tr>
</tbody>
</table>

* Depletion of CD8+ T cells started in weanling rats aged 22 ± 1 days 4 days after single-shot immunization with recombinant VV. Animals were infected with MV 1 day later. The LPS was determined 13 to 16 days after infection using inactivated MV as the restimulating antigen.

† Number of animals investigated.

‡ MV could be isolated from the brain tissue of the diseased animal 14 days p.i.

In vivo evaluation of the virus-neutralizing humoral immune response after MV challenge

The CMI response of polyclonal T cells was low in the presence of MV. The specific response against the MV protein with which the rats had been immunized was marginally higher after MV challenge infection.

Serum samples collected from rats after the i.c. challenge with MV were analysed in an MV neutralization assay and in an ELISA using purified MV antigen. The ELISA titres obtained were usually two to four times higher than those in rats not challenged by MV infection (Table 5). VVN immunization, as well as injection of VV-wt, VVM and VVP, did not lead to the generation of virus-neutralizing antibodies. Such antibodies did develop following VVF and VVH immunization and MV challenge infection, to an increased titre and earlier (Table 5 and Fig. 2). However, whereas VVH
induced low titres of virus-neutralizing antibodies at the
time of MV challenge infection (i.e. 4 days after single-
shot immunization), a lag phase was observed after VVF
immunization without MV infection and its rise was
delayed until 9 to 11 days p.i. Even after MV challenge
infection at 4 days p.i., 3 further days elapsed before the
titre of neutralizing antibody increased. In contrast,
MV infection following VVH immunization induced a
dramatic increase in the antibody titre within 1 day (Fig.
1).

The contribution of MV neutralizing antibodies to
protective immunity was tested by passive transfer of
neutralizing antibodies. As shown in Fig. 2, rats develop
an average neutralizing antibody titre of 1:20 after VVF
immunization at the time of MV challenge infection.
This antibody response was mimicked by passive transfer of anti-H monospecific serum (0.5 ml i.p. of
monoclonal antibody cocktail; MV neutralization titre of 1:1280). The antibody titre measurable in rats after a
single injection was 1:20 within 24 h of passive antibody
transfer and increased to about 1:80 after three antibody
injections. However, this antibody treatment was insuf-
ficient to prevent completely the occurrence of fatal acute encephalitis in rats after infection with
CAM/RBH. Only a prolonged incubation period was
observed (15 to 18 days).

**Discussion**

In recent years recombinant VVs have been used as
powerful and versatile eukaryotic expression systems to
investigate the immune response to a variety of different
infectious agents and the protective capacity at the level
of single proteins (for review see Moyer & Turner, 1990).

Because of the cytoplasmic virus replication that
prevents cryptic splicing of mRNAs and enables full-
length translation, VV produces high yields of recombi-
nant protein.

Although for most viruses the components responsible
for the induction of protective immunity have been
shown to be membrane-bound glycoproteins, clinical
protection from systemic viral disease following vacci-
nation with VV recombinants expressing internal and
even non-structural proteins has been described for a
number of other viruses such as Lassa fever virus and
influenza virus in different animal species (Hany et al.,
However, comparative analyses of the efficacy of
different individual virus proteins in the induction of
antiviral immunity are rare (Andrews & Coupar, 1988).
Therefore, it was the aim of the present study to analyse
protein-specific immune responses to MV structural
components in detail.

The MV structural proteins N, P and M were
successfully cloned into VV, and all recombinant VVs,
including the VVF and VVH recombinants kindly
obtained from R. Drillien and coworkers, were shown by
different methods to express the respective MV proteins.

By immunization of Lewis rats with these VV recombi-
nants, humoral and cellular immune reactions specific
for the expressed MV protein could be induced. A
specific T cell response could be demonstrated as early as
5 days p.i. This response increased 8 and 12 days p.i.
Repeated injections with the various VV recombinants
did not result in a significant increase in the MV-specific
cellular immune response.

The efficacy of the recombinant VV-induced MV-
specific immune response was tested in our rat model for
MV encephalitis. Lewis rats were lethally infected with
the rat brain-adapted MV isolate CAM/RBH (Liebert &
ter Meulen, 1987). Prior to the challenge infection, all
animals had been immunized with recombinant VV
expressing the MV structural proteins N, P, M, F or H.
Control rats invariably died from an acute measles
encephalitis with severe disseminated inflammatory
involvement of grey and white matter of the brain. MV
was easily isolated from the brain tissue of such animals
by co-cultivation with Vero cells. Three of the MV
structural proteins, namely N, F and H, induced
protective immunity, but M and P were only partially
effective.

These data confirm the results of the protection
experiments carried out in mice with VVF and VVH
(Drillien et al., 1988) and VVN in rats (Bankamp et al.,
1991). The data presented in this paper extend our
previous knowledge of the effect of the immune response
against the M and P proteins of MV. The observations
suggest that MV infection was cleared from the CNS of

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**Table 5. Antiviral immune response following VV recombinant infection and MV challenge**

<table>
<thead>
<tr>
<th>VV recombinant used for immunization (single-shot)</th>
<th>Neutralization test</th>
<th>ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before challenge</td>
<td>After challenge</td>
</tr>
<tr>
<td>VV-wt</td>
<td>&lt;20*</td>
<td>&lt;20</td>
</tr>
<tr>
<td>VVN</td>
<td>&lt;20</td>
<td>&lt;20</td>
</tr>
<tr>
<td>VVP</td>
<td>&lt;20</td>
<td>&lt;20</td>
</tr>
<tr>
<td>VVM</td>
<td>&lt;20</td>
<td>≤20</td>
</tr>
<tr>
<td>VVF</td>
<td>≤20</td>
<td>80</td>
</tr>
<tr>
<td>VVH</td>
<td>20</td>
<td>640</td>
</tr>
</tbody>
</table>

* Antibody titres are shown as the mean reciprocal values for sera obtained from three to five different rats. The time-points of antibody
determination were the day of neurotropic CAM/RBH challenge infection and 5 to 6 days thereafter.
the N-, F- or H-vaccinated rats whereas the majority of VVP- and VVM-immunized rats were not able to clear the virus from the CNS.

This interpretation is supported by neuropathological investigations. All unprotected rats succumbed to widespread destructive brain damage, but those protected from the disease by immunization with H, F and N revealed no or only minor neuropathological lesions. Those rats immunized with VVM or VVP which remained free of clinical disease also had only mild focal encephalitic changes in their brains.

That the immunization with VV recombinants expressing the P or M proteins does not protect rats from measles encephalitis, whereas immunization with the VV recombinant expressing N does, is surprising considering that these three proteins are internal components of MV. Although it appears that the MV-specific CMI response induced by the different VV recombinants is variable, this is obviously not sufficient to explain why immunization with recombinant P and M proteins protects poorly compared to immunization with the N protein. Maybe the efficiency with which the N, P and M proteins are processed for T cell recognition in MV-infected brains is different from the processing of these proteins in tissue cultures infected with the VV recombinants. Furthermore, the N protein is the major virus protein and the P and M proteins are minor structural components of MV virions. It is conceivable that the amount of virus protein synthesized would influence its major histocompatibility complex-associated expression on the cellular surface and hence T cell recognition.

Experiments published previously suggested that recombinant VVs expressing the H and F glycoproteins of MV confer protective immunity in mice via induction of high levels of virus-neutralizing and/or fusion-inhibiting antibodies (Drillien et al., 1988). It was claimed that the humoral immune response is essential for the observed protection. Moreover, passive transfer of neutralizing antibodies directed against the haemagglutinin of MV modifies the experimental encephalitis in newborn rats; no such effect could be induced by antibodies with specificity for the MV internal proteins (Liebert et al., 1990).

In order to investigate further the role of VVH- and VVF-induced virus-neutralizing antibodies, their occurrence after immunization and MV challenge infection was analysed. Since in the case of VVH immunization virus-neutralizing antibodies, although of low titre, are already present at the time of MV infection, their contribution to the observed protection cannot be denied. Following immunization with VVF however, the time-lag of at least 3 days before antibodies start to rise makes it unlikely that these antibodies contribute to protection from encephalitis. A similar observation was made by Wild et al. (1990). This interpretation is supported by passive transfer experiments using mixtures of MV-neutralizing monoclonal antibodies. Even when a titre of 1 in 80 was reached within 72 h of infection by repeated i.p. injections, the development of MV encephalitis was not prevented. Since in animals immunized with VVF it lasted a further 3 to 4 days after MV challenge infection before a comparable average titre was reached, the neutralizing antibodies apparently appear too late to contribute to the VVF-induced protective immunity against MV encephalitis. In the experiments where VVN was used the cellular immune reaction could have played an essential role since neutralizing antibodies were not induced in VVN-immunized rats (Tables 1 and 5). These results suggest that in vivo activated functional N or F protein-specific T lymphocytes can effectively move to MV-infected brain tissue and eliminate virus-infected cells. Whether or not H-specific T cells alone can also effectively eliminate virus-infected cells from the brain in the absence of virus-neutralizing antibodies is uncertain.

CMI responses have been shown clinically to be essential for overcoming acute measles in man (Cherry, 1987). Most observations point to CD8+ cytotoxic T lymphocytes as the essential component. In an attempt to find out whether activated CD8+ lymphocytes constitute the effector cell population which restricts virus spread in brain tissue, CD8+ T cells were eliminated from VVN-, VVF- and VVH-immunized rats by injection of monoclonal antibodies directed against the CD8 molecule. This treatment results in the prolonged (at least 35 days) and complete depletion of CD8+ T cells (Sedgwick, 1988; Bankamp et al., 1991). Except for one VVN-immunized rat, none of the CD8-depleted rats developed clinical signs after challenge infection and MV could not be isolated from the brain tissue of CD8-depleted VVN-, VVF- or VVH-immunized rats between 13 and 16 days p.i. Histopathological examination revealed residual non-inflammatory lesions or focal encephalitic lesions in about 25% of the rats but no lesions in the remainder (Table 4). The changes were no different from non-depleted, protected rats. These results show that CD8+ MV-specific lymphocytes are not a prerequisite for protection from measles encephalitis in rats. At least in the VVN- and VVF-immunized rats, CD4+ T lymphocytes specific for the N or F proteins are obviously sufficient to protect rats from measles encephalitis in the absence of neutralizing antibodies and CD8+ cytotoxic T lymphocytes.

It has been reported previously that the persistence of measles virus in rat brain neurons may be promoted by depletion of CD8+ T cells (Maehlen et al., 1989). In the light of the data presented in this paper it is possible that...
both CD8+ and CD4+ T cells may cooperate and be capable of protecting rats from measles encephalitis as in the case of murine cytomegalovirus infection (Jonjic et al., 1990).

Two possible mechanisms for the protective CMI response can be discussed. Firstly, the CD4+ MV-specific T cells may be cytotoxic effectors themselves. The presence in human peripheral blood lymphocytes of MV-specific CD4+ T cells with cytotoxic effects in addition to CD8+ cytotoxic T cells would support this hypothesis (Jacobson et al., 1984; van Binnendijk et al., 1990). However, it has not yet been possible to demonstrate the specific lysis of MV-infected target cells by our MV-specific rat T cell lines in vitro (S. Niewiesk & U. G. Liebert, unpublished observations). Second, cytokines secreted from T cells invading the CNS may attract phagocytes into the brain. Such an observation was made in experimental allergic encephalomyelitis where CD4+ T cells were shown to be inducing and transferring the disease (Sedwick et al., 1987). However, blocking macrophage invasion into the CNS across the blood–brain barrier prevented clinical symptoms and reduced histopathological lesions (Huitinga et al., 1990).

Antiviral neutralizing antibodies are apparently instrumental in overcoming CNS infection after immunization with VVH (Drillien et al., 1988; Malvoisin & Wild, 1990). The effect of T cells induced by VVH immunization in the absence of virus-neutralizing antibodies is uncertain. At present no definite answer can be given as to which immune mechanism confers protection against MV encephalitis in the rat model.

The observation that immune responses to individual MV proteins control infection of the CNS in the rat model raises the question why in SSPE the immune system fails to eliminate MV despite the fact that infected brain cells always contain the N protein (Liebert et al., 1986). So far no specific immunological defects have been seen in SSPE patients which would explain the failure of the host defence mechanism in controlling the MV infection. Perhaps mutations of MV genes arising as a consequence of virus persistence (reviewed by Billeter et al., 1989) or an impairment of antigen processing and presentation (Del Val et al., 1989; David-Watine et al., 1990) allow the CNS disease process to escape immune surveillance. Such effects would ultimately result in the inaccessibility of the major target cells to the T cell-mediated immune reaction, and enable MV persistence in the human CNS.

The animal model will help to define the parameters by which CMI reactions block MV infection in brain tissues. With this information it may be possible to gain more insight into the patho-genetic mechanisms involved in SSPE and other persistent viral infections of the CNS.

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References


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