Construction of vaccinia virus recombinants expressing several measles virus proteins and analysis of their efficacy in vaccination of mice

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Measles virus genes encoding the haemagglutinin (HA), fusion protein (F) or nucleoprotein (NP) have been inserted into the vaccinia virus genome either alone or in various combinations. In each case the measles virus genes were expressed from the 7.5K promoter and were incorporated into the thymidine kinase (tk) or K1L loci of the Copenhagen strain of vaccinia virus. Cells infected by the recombinants synthesized measles virus proteins indistinguishable from those induced in measles virus-infected cells. However, in some instances the level of expression in cells infected by recombinants expressing more than one measles virus gene was reduced when compared to those encoding a single gene. The sera from mice immunized with recombinants containing either HA, HA.F, HA.NP or HA.F.NP had similar levels of measles virus neutralizing antibodies which remained constant throughout a 7 month period. Analysis of these sera by immunoprecipitation of radiolabelled measles virus confirmed the presence of specific antibody to each of the antigens where appropriate.

The introduction of the measles virus genes into the K1L and the tk sites despite attenuating the virus for mice by 10-fold and 1000-fold respectively did not affect the vaccination efficiency, i.e. ability to induce measles virus antibody and protect mice. Vaccination of BALB/c (H2d) mice with HA and F, but not NP, recombinants completely protected the animals against a lethal measles virus challenge. In contrast, although the HA recombinant protected CBA (H2k) mice, the F recombinant did so poorly. However, by immunizing CBA mice with a recombinant expressing both F and NP, protection was increased to more than 75%. Our findings demonstrate the ability of three measles virus antigens expressed from the vaccinia virus genome alone or in combination to contribute to protective immunity against measles virus infection of mice. They also suggest that the association of measles virus antigens in a single recombinant DNA vaccine could be beneficial to overcome host-related restriction of the immune response to particular antigens.

Introduction

Eradication of smallpox using vaccinia virus (VV) as a live attenuated vaccine still remains the only case in which a human viral disease has been completely eliminated. The observations that this large DNA virus contains a number of regions of non-essential genes (for a recent list see Goebel et al., 1990; Perkus et al., 1991) which can be replaced with foreign genes has led to studies of the expression of the latter at both a fundamental level and as immunogens (Bennink & Yewdell, 1990; Piccini & Paoletti, 1988). Further studies have shown that antigens for several viruses could be expressed from a single VV recombinant and induce the appropriate immunological response in animals (Perkus et al., 1985).

Measles is an acute infection in children and despite the use of an attenuated vaccine for 30 years, there are still more than 2 million child deaths/year. The inability to control the disease in developing countries has been attributed to a number of factors including the early age of infection, maternal antibody and the instability of the vaccine in field conditions. Thus, a VV–measles recombinant virus vaccine could resolve some of these problems. Recently, we have shown that VV recombinants encoding either the haemagglutinin (HA) or fusion (F) measles virus proteins protected mice from a lethal challenge (Drillien et al., 1988). Of the two measles virus glycoproteins, HA is responsible for attachment to the host cell and is the

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immunodominant antigen inducing high levels of neutralizing antibody. The F protein catalyses the fusion of the viral and cell membranes releasing the viral components into the cytoplasm. In the late stages of infection, the accumulation of the F protein at the membrane leads to cell-to-cell fusion aiding the transmission of virus. Thus, anti-F antibodies would be important at both the initial and late stages of infection. Early studies on human vaccination with formaldehyde-inactivated measles virus failed to induce antibodies with the correct activity against the F antigen (Norrby et al., 1975). This led to an atypical disease when the children were confronted with a measles virus infection (Fulginiti et al., 1967). In contrast sera from naturally infected children contain an anti-F antibody with the desired properties (Sato et al., 1989). Murine monoclonal antibodies (MAbs) with similar properties, i.e. neutralization and anti-fusion activity, could be obtained by vaccinating mice with the VV recombinant encoding the F protein, but not with purified measles virus (Malvoisin & Wild, 1990). This suggests that the form of the presentation of the antigen is important in inducing the correct response.

From our results and those of others, a minimum requirement for a VV recombinant vaccine against measles infection would include one of the measles virus glycoproteins. Recent studies of other viruses such as influenza, rabies and respiratory syncytial viruses have shown that internal structural proteins, especially the nucleoprotein (NP), play an important role in the immunization against disease. The NP of influenza and respiratory syncytial viruses have been implicated in cell-mediated immunity at both the T helper and T cytotoxic levels (Bangham et al., 1986; Gao et al., 1989; McMichael et al., 1986; Openshaw et al., 1988; Townsend et al., 1984). Although the NP does not induce virus-neutralizing antibodies directly, immunization with this antigen and subsequent challenge with sub-immunizing levels of virus does (Ertl et al., 1989; Tite et al., 1988). Further studies have shown that this phenomenon can be attributed to the role of NP T helper cells (Ertl et al., 1989). At the cytotoxic T cell level, the NP of a number of negative strand viruses has been shown to be the major antigen concerned (McMichael et al., 1986; Puddington et al., 1986; Townsend et al., 1984). If, as for many other viruses, measles virus NP also induces cell-mediated immune responses, it could be beneficial to include a gene encoding this antigen in a VV recombinant vaccine.

In the present study, we have constructed a number of VV–measles recombinant viruses containing different combinations of the genes coding for the HA, F and NP measles virus proteins to examine the feasibility of a multigene recombinant for immunization against measles. We show that such recombinants direct the synthesis of measles virus proteins and that they induce an immune response in mice. Further, a haplotype restriction on the immune response of the F protein can be partially overcome by the incorporation of the NP gene in the recombinant. We propose that a VV–measles recombinant virus expressing the HA, F and NP measles virus proteins would be potentially more appropriate as a recombinant DNA vaccine than one expressing only a single measles virus antigen.

**Methods**

**Construction of plasmids**

(i) A plasmid vector for insertion of the measles virus HA and F genes (pTG3115). A plasmid designated pTG1169 was previously constructed so as to contain the measles virus HA gene (Gerald et al., 1986) under the control of the VV p7.5K promoter flanked on both sides by the VV thymidine kinase (tk) gene (Drillien et al., 1988). In order to add a second expression unit to this plasmid, pTG1169 was cut with BglII at a unique restriction site at the 3' end of the HA gene, and another p7.5K promoter element (297 bp BglII–BamHI fragment) was inserted to run in the opposite direction to the HA gene. The plasmid thus generated now contained a unique BamHI site at the 3' end of the newly added p7.5K promoter. This BamHI site was cut and 5' end of the measles virus F gene (Buckland et al., 1987), coding only for the N-terminal portion of the F protein, was added as a BglII–BamHI fragment thus creating the plasmid pTG2133. In previous work we had noticed that a bacterial plasmid with two poly(A) tails in a tail-to-tail configuration was unstable in *Escherichia coli*. Therefore before adding on the 3' end of the measles virus F gene encoding the C-terminal portion of the F protein, we modified the 3' end of the F gene by site-directed mutagenesis using the oligonucleotide 5' AAT-TATCTCCTGCTATTAGTCGGCCGAACAAATCGG 3'. This process created a BglII site 246 nucleotides downstream of the unique BamHI site within the F gene and upstream of the poly(A) tail on the cDNA clone. The 246 bp fragment was then isolated by cutting with BamHI and BglII and was inserted into the unique BamHI site of pTG2133. The overall result was a plasmid, pTG3115, which contained the measles virus HA and F genes in a tail-to-tail configuration under the transcriptional control of two identical p7.5K promoter elements and flanked by the VV tk gene.

(ii) A plasmid vector for insertion of the measles virus NP gene (pTG3109). The full-length cDNA coding for the measles virus NP has been previously isolated (Buckland et al., 1988). The 5' end of this cDNA was inserted as a Psrl–XbaI fragment into the single-stranded phage vector M13 TG131 and modified by site-directed mutagenesis with the oligonucleotide 5' CCTTAAAAAGGTTGGCCCATCTTATTCCTTATACCCATTACGTC 3'. This created a BglII site three nucleotides upstream of the NP translation initiation codon. This phage vector designated M13 TG2126 was then cut with Xbal and EcoRI at a restriction site within the coding region of the NP gene, and Smal, located within the polylinker region of the M13 phage vector. The 3' end of the NP gene was then added as an XbaI–HpaI fragment. Finally the entire coding sequence of the NP gene thus reconstructed was cut out with BglII and EcoRI and ligated into the VV transplacement vector pTG186 (Kieny et al., 1984) then cut open with BamHI and EcoRI which have two restriction sites downstream of the p7.5K promoter element. This procedure generated a plasmid designated pTG3109.
(iii) A plasmid vector for insertion of the E. coli lacZ gene at the locus of the VV K1L gene. The 5-2 kbp EcoRI K fragment from the left end of the VV Copenhagen genome (Gillard et al., 1985) was cloned into the EcoRI site of pUC8. This plasmid was then subjected to partial digestion with BglII followed by religation so as to delete an 855 bp fragment encoding a host range gene (Gillard et al., 1986), also designated K1L (Perkus et al., 1989), thus generating a plasmid named pUC8Khr. A 3-4 kbp BglII fragment containing the VV sequences surrounding the K1L gene was then removed from pUC8Khr and ligated into the BamHI site of pUC7. This plasmid, designated pBAC1, contained a unique BglII site at the position of the deleted K1L gene. Plasmid pBAC1 was cut with BglII, and a lacZ fusion gene encoding β-galactosidase (Hall et al., 1983) was inserted as a 3-8 kbp fragment with a flanking BglII site at its 5' end and a BamHI site at its 3' end. The VV p7-SK promoter contained within a BglII fragment was then ligated upstream and in the same orientation as the lacZ gene at the BglII site. This generated a plasmid designated pTG2131 in which the orientation of the lacZ gene was from left to right with respect to the VV sequences.

(iv) A plasmid vector for insertion of the measles virus F gene and the NP gene at the locus of the VV K1L gene. The plasmid pBAC1 was modified by addition of a double-stranded oligonucleotide linker so as to contain a unique XhoI site to the left of the unique BglII site with respect to the VV genome. The plasmid thus created was cut with XhoI and BglII and the VV p7-SK promoter was inserted as a 3' SalI-3' BglII fragment in such a manner that the promoter ran from left to right with respect to the virus genome. The two EcoRI sites flanking the pUC7 portion of this plasmid were then removed by two successive rounds of EcoRI digestion, filled in with the Klenow fragment of E. coli DNA polymerase and religated. A polylinker segment flanked by a BglII site and a BamHI site (Lathe et al., 1987) was then inserted at the unique BglII site to generate pTG2147. This plasmid, which contained the VV p7-SK promoter upstream of a polylinker segment and surrounded by VV sequences flanking the K1L gene, could be used for convenient introduction of any foreign gene. In this study the measles virus gene encoding the F protein was excised from pTG1173 with BglII and EcoRI and then inserted downstream of the p7-SK promoter in the correct orientation by ligation into the BglII and EcoRI sites of pTG2147. The plasmid containing the measles virus F gene was designated pTG2148. In another plasmid construction designated pTG3128 the measles virus NP gene was recovered from the plasmid pTG1109 by digestion with PstI and EcoRI then inserted into the PstI and EcoRI sites of pTG2147. Schematic representations of the procedures followed for the construction of the plasmids are available upon request.

Construction of VV recombinants

Insertion into the tk gene. Primary chicken embryo fibroblasts (CEF) were infected with the VV ts7 mutant and cotransfected with wild-type (wt) DNA and one of the plasmids containing insertions in the tk locus. Transfection and selection of tk- virus on Ltk- cells were carried out as previously reported (Kiény et al., 1984; Mackett et al., 1982).

(ii) Insertion into the K1L gene. In a first step, we selected VV recombinants containing the lacZ gene at the K1L locus. CEFs were infected with the VV ts7 mutant and cotransfected with VV wt DNA and pTG2131. Viral progeny from the transfection was plated on CEFs and stained with X-Gal (300 μg/ml) to detect β-galactosidase activity (Chakrabarti et al., 1985; Panicali et al., 1986). A single blue plaque was picked and was shown by DNA restriction digestion to have the lacZ gene at the position of the K1L gene. This virus, designated VV hrlacZ, had an altered host range since it was unable to multiply in rabbit RK13 cells. Furthermore, it displayed a characteristic plaque morphology on CEFs, in that it failed to produce extensive cell aggregation typical of VV wt plaques. In a second step a virus recombinant containing the lacZ insert as well as the ts7 mutation was isolated. CEFs were co-infected with the VV ts7 mutant and VV hr lacZ each at about 5 p.f.u./cell. After complete cell necrosis, 1 day later, the viral progeny were plated on fresh CEFs at 33°C. The plaque enlargement technique was then used to identify temperature-sensitive (ts) virus (Drillien et al., 1982) and X-Gal staining was employed to detect virus containing the lacZ gene. A viral recombinant displaying both phenotypes was isolated, plaque-purified once again and designated VV ts hrlacZ.

To isolate viral recombinants containing either the measles virus NP gene or F gene within the K1L locus, CEFs were infected with VV ts hrlacZ then cotransfected with intact DNA from VV hrlacZ and either pTG3128 or pTG2148 respectively. Viral progeny was plated on chick cells and plaques were stained by the addition of X-Gal to the medium. Viral plaques that failed to stain blue and could therefore have the NP or F gene inserted at the position of the lacZ gene were picked and plaque-purified a second time. The phenotype of the recombinants was confirmed by studying expression of the measles virus proteins in cells infected with the VV recombinants.

(iii) Isolation of VV recombinants encoding two or three measles virus antigens. To isolate recombinants encoding two or three measles virus antigens, CEFs were co-infected with one of the recombinants containing an insert at the tk locus and another with an insert at the K1L locus. After complete necrosis of the infected cells, progeny virus was plated on Ltk- cells in the presence of 100 μg/ml 5’ bromodeoxyuridine. Individual plaques were picked and inoculated on CEF monolayers for examination of their morphology. Viral recombinants that displayed a plaque morphology typical of virus deleted in the K1L gene were recovered and cloned once again. Further analysis revealed that they contained inserts both at the tk and K1L loci.

(iv) Designation of recombinant viruses. VV recombinants were designated according to the plasmid that had been used to generate them and according to the measles virus genes they encode. For instance VV TG 1173 HA encodes the measles virus HA. In cases where the virus was isolated by in vivo recombination between two other viruses the locus of insertion of the measles virus genes is indicated by a subscript in the virus name. For instance VV- NP K1L F tk is a VV recombinant containing the NP gene inserted at the K1L locus and the F gene inserted at the tk locus. For simplicity and where no confusion is possible, viruses are designated using only the abbreviation of the measles virus gene they encode.

Expression of measles virus antigens in cells infected with VV recombinants. Measles virus antigens were visualized in mouse Ltk- cells infected with the various recombinants using standard immunofluorescence assays. Infected cells were fixed in acetone; mouse MAb directed against one of the measles virus antigens and fluorescein isothiocyanate-labelled goat anti-mouse antibody were employed for detection. Immunoprecipitation coupled with PAGE were used to determine the apparent Mr values of the antigens. Cells infected for 15 h were labelled with [35S]methionine (50 μCi/ml) in a methionine-free medium. Immune complexes were then formed with mouse MAbs directed against one of the measles virus antigens and isothiocyanate-labelled goat anti-mouse antibody were employed for detection. Immunoprecipitation coupled with PAGE were used to determine the apparent M r values of the antigens. Cells infected for 15 h were labelled with [35S]methionine (50 μCi/ml) in a methionine-free medium. Immune complexes were then formed with mouse MAbs directed against each of the measles virus structural proteins. The MAbs directed against HA, NP and F were respectively CI. 55, CI. 105 and 19F11VII. Immune complexes were purified using Protein A-coupled Sepharose beads and the proteins separated on SDS-polyacrylamide gels. The gels were treated for fluorography, dried and subjected to autoradiography.

Immunization of mice and challenge with measles virus. Four-week-old BALB/c female mice (IFCA/CREDO, France) were immunized by tail scarification with the different recombinants. Three weeks later the mice were challenged by intracerebral (i.c.) inoculation of a mouse-adapted measles virus strain (Wild et al., 1979). In non-vaccinated mice, virus-induced mortality occurred 7 to 15 days after challenge.
Normally, mice were observed during a 28 day period after measles virus infection. Deaths in the first 3 days were regarded as non-specific.

**Measles virus antibody levels.** Measles virus neutralizing antibody in mouse serum was measured by a microplate method using 50 p.f.u. of virus per well with twofold dilutions of serum as previously described (Wild et al., 1979).

Immunoprecipitation and SDS-PAGE with serum from vaccinated mice were carried out as described above, except the 35S-labelled antigen used was partially purified measles virus.

**Results**

**Construction of VV recombinants encoding measles virus antigens**

VV recombinants encoding measles virus antigens were constructed by inserting the foreign genes either at the tk locus (Hruby et al., 1983; Weir & Moss, 1983) or at a host range locus (Gillard et al., 1986) also designated K1L (Perkus et al., 1989). Construction and selection of recombinants containing foreign genes at the tk locus followed previously described procedures (Kieny et al., 1984; Mackett et al., 1982), whereas a different method was devised for insertion at the K1L locus (see Methods).

We noticed that inactivation of the K1L gene resulted in two distinct phenotypes. On the one hand virus deleted in K1L failed to produce plaques on rabbit RK13 cells as reported previously by Perkus et al. (1990) and on the other hand such virus displayed an atypical plaque morphology on CEFs, characterized by failure to aggregate infected cells as effectively as the wt virus.

Two different strategies were used to isolate VV recombinants containing more than one measles virus gene. In one method, we inserted both genes at the tk locus under the control of two p7.5K promoter elements. To ensure stability of the recombinants, despite the occurrence of repetitive sequences, the two promoter elements as well as the two genes were positioned in opposite directions as previously described for fowlpox virus recombinants (Spehner et al., 1990). In a second method, cells were co-infected with two different recombinant viruses containing inserts either at the tk locus or at the K1L locus. Progeny virus from the mixed infection that simultaneously retained the tk− phenotype and the plaque morphology phenotype typical of a virus deleted in the K1L gene was isolated and shown to encode two measles virus antigens (see below). The latter method also enabled the isolation of VV recombinants encoding three antigens from mixed infections with a VV recombinant containing two genes inserted at the tk locus and a recombinant with a single gene inserted at the K1L locus. A schematic representation of the various VV recombinants constructed for this study is provided in Fig. 1.

**Expression of the measles virus antigens in infected cells**

BHK-21 cells were infected with the various recombinants and proteins synthesized between 15 and 18 h after infection were radiolabelled with [35S]methionine. Measles virus proteins were then immunoprecipitated from cell lysates using mouse MAbs directed against
each of the antigens (Fig. 2). In every instance this method enabled detection of the expected measles virus proteins. Antibodies against the HA recognized a characteristic doublet of about 80K. Immunoprecipitation with the MAb against the F protein revealed the expected precursor F₀ at 60K and one of the cleavage products, F₁, at 40K. The 20K F₂ species was detected in other experiments (not shown). Immunoprecipitation with an anti-NP MAb revealed a 60K product corresponding to the size of authentic NP. The relative amount of measles virus proteins synthesized was lower in cells infected with a recombinant virus containing inserts of both the F and HA genes, at the tk locus, as compared to a recombinant virus containing either the F or HA genes at the tk locus. This could possibly be explained by the fact that the promoters used to obtain transcription run in converging directions and may therefore interfere with one another. There was also slightly less NP synthesized in cells infected with the recombinant virus encoding three measles virus antigens. Other minor differences noticed in the amount of measles virus proteins synthesized using the various recombinants have not been substantiated by further experiments.

The cellular localization of the antigens was visualized by immunofluorescence (results not shown). As in previous experiments (Drillien et al., 1988) the HA and F antigens were detected at the cell surface whereas the NP antigen was localized both in the cytoplasm and the nucleus.

**Immunization**

Our previous results established that immunization of BALB/c mice with the recombinants VV-HA or VV-F induced measles virus-neutralizing antibody and the animals were protected against a lethal measles virus infection (Drillien et al., 1988). To test whether the NP played a role in protection in the mouse model, animals were immunized with the VV-NP recombinant and challenged 21 days later with measles virus (Table 1). No serum neutralizing antibodies were found after immunization (titre <40) and only a few animals survived the infection (20 to 30%). The sera of the surviving animals had neutralizing antibody when examined 1 month after the challenge infection, showing that these mice had not escaped infection.

To study the efficiency of immunization when two or three measles virus genes are expressed from the same vector, BALB/c mice were immunized with either VV-HA, VV-HA-NP, VV-HA-F or VV-HA-F-NP and the sera examined at intervals up to 7 months for measles virus-neutralizing antibodies (Table 2). Similar levels of neutralizing antibody were induced in the mice by all four recombinants and the titres were stable over the 7 month observation period. In further experiments with mice vaccinated with the VV-HA recombinant, both the VV- and measles virus-neutralizing antibody levels were stable during a 15 month period (data not shown).

To study the antibody response to the individual measles virus antigens, sera from mice, 3 months after immunization, were incubated with [35S]methionine-labelled measles virus and after immunoprecipitation examined by SDS-PAGE (Fig. 3). The presence of HA, F and NP (where appropriate) could be detected. The expression of more than one measles virus gene in the VV

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**Table 1. Vaccination of BALB/c mice with VV-NP**

<table>
<thead>
<tr>
<th>Recombinant virus</th>
<th>Protected/total*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td></td>
</tr>
<tr>
<td>VV-NP</td>
<td>6/20</td>
</tr>
<tr>
<td>VV-wt</td>
<td>0/6</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
</tr>
<tr>
<td>VV-NP</td>
<td>2/10</td>
</tr>
<tr>
<td>VV-wt</td>
<td>0/10</td>
</tr>
</tbody>
</table>

* Mice were immunized by tail scarification with 2 × 10⁷ p.f.u. of VV or the NP recombinant and challenged 21 days later by i.c. inoculation of measles virus.

**Table 2. Serum neutralizing antibody titres (measles virus) in mice immunized with VV-measles recombinant viruses**

<table>
<thead>
<tr>
<th>Time after vaccination (months)</th>
<th>VV-HA</th>
<th>VV-HA-NP</th>
<th>VV-HA-F</th>
<th>VV-HA-F-NP</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9.8 ± 0.9†</td>
<td>8.6 ± 0.7</td>
<td>8.9 ± 0.5</td>
<td>8.8 ± 1.0</td>
</tr>
<tr>
<td>3</td>
<td>10.6 ± 0.7</td>
<td>9.6 ± 1.0</td>
<td>9.0 ± 1.2</td>
<td>9.5 ± 0.8</td>
</tr>
<tr>
<td>7</td>
<td>10.1 ± 0.7</td>
<td>9.6 ± 1.3</td>
<td>9.5 ± 0.8</td>
<td>9.2 ± 1.6</td>
</tr>
</tbody>
</table>

* Four-week-old female BALB/c mice (six to seven per group) were immunized with the different viruses by tail scarification (10⁶ p.f.u./mouse).
† Results are the average from each group of mice expressed as the reciprocal of the dilution (log₂) of serum that gave complete neutralization of 50 p.f.u. of measles virus. Neutralization titres of unvaccinated mice were <4.3.
recombinants did not affect the immune response against the individual antigens.

It has been reported that the inactivation of the tk gene in the WR strain of VV reduced its neurovirulence 1000-fold (Buller et al., 1985). In the construction of our recombinants with the Copenhagen strain, we have used both the tk and K1L sites. We established that intraperitoneal inoculation of the K1L- and tk- viruses into 3- to 4-day-old mice reduced the LD50 by 10- and 1000-fold respectively (data not shown). If insertion of foreign genes into these sites attenuates the virus, it may also reduce the immunization efficiency. To examine this, mice were immunized with 10-fold dilutions of the VV-F recombinants in which the measles virus F gene was inserted in the tk or K1L site and a third group were immunized with the recombinant VV-NPtkL.HA.Fm

The mice were challenged 21 days later with measles virus (Table 3). In all three cases 104 p.f.u. of the recombinant virus protected some of the animals, whereas 103 p.f.u. protected all of the mice against measles virus infection.

Immunization of CBA mice

To extend our studies to mice of a different haplotype, we immunized CBA (H2k) mice with the recombinants VV-HA or VV-F and challenged them with measles virus 21 days later (Table 4). In contrast to BALB/c mice, CBA mice were not significantly protected by VV-F. Although the amount of VV-F used in the vaccination did not affect the eventual outcome, mice receiving larger inocula of the recombinant survived longer (Fig. 4).

Table 3. Effect of insertion of measles virus genes in the K1L and tk sites of the VV genome on the efficiency of protection

<table>
<thead>
<tr>
<th>Recombinant virus</th>
<th>VV-Ftx</th>
<th>VV-FtkL</th>
<th>VV-NPtkL.F. HAtx</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dose (p.f.u./mouse)</td>
<td>10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>5/6†</td>
<td>5/6</td>
</tr>
<tr>
<td></td>
<td>10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>5/5</td>
<td>6/6</td>
</tr>
<tr>
<td></td>
<td>10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>3/6</td>
<td>3/6</td>
</tr>
<tr>
<td></td>
<td>10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>0/6</td>
<td>1/5</td>
</tr>
</tbody>
</table>

* Twenty-one days after vaccination with the viruses, the mice were challenged with measles virus and observed for a further 6 weeks. † Number of animals protected/total inoculated.

Table 4. Protection of CBA mice immunized with VV–measles recombinant viruses

<table>
<thead>
<tr>
<th>Recombinant*</th>
<th>VV wt</th>
<th>VV-HA</th>
<th>VV-F</th>
<th>VV-NP</th>
<th>VV-NP.F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dose (p.f.u./mouse)</td>
<td>Experiment 1</td>
<td>3 × 10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>0/6†</td>
<td>6/6</td>
<td>1/6</td>
</tr>
<tr>
<td></td>
<td>3 × 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>6/6</td>
<td>1/6</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3 × 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>6/6</td>
<td>1/6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Experiment 2</td>
<td>3 × 10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>2/10</td>
<td>0/10</td>
<td>7/9</td>
<td></td>
</tr>
</tbody>
</table>

* Mice were immunized with the recombinant viruses and challenged with measles virus 21 days later. † Number of animals protected/total inoculated.

To investigate whether the reduced response to the F antigen was specific to the H2k haplotype or to other 'background' factors, the experiment was repeated using H2k BALB/c mice (Table 5). This confirmed that the
failure of VV-F to protect was associated with the H2k haplotype.

Studies of influenza and rabies viruses have shown that the NP via its T helper response can aid the antibody response to the other virus proteins (Ertl et al., 1989; Tite et al., 1988). To examine whether the measles virus NP could aid or complement the anti-F response in H2k mice, we constructed a recombinant containing both the NP and F genes. When CBA mice were immunized with this recombinant more than 75% of the mice were protected (Table 4).

### Discussion

Measles virus is responsible for an acute infection in children which is followed by a life-long immunity. The nature of the latter is unknown, but in the rare disease subacute sclerosing panencephalitis the virus may become activated and establish a persistent, invariably fatal infection. Although passive administration of γ-globulin or anti-measles virus MAbs prevents disease (Mitus et al., 1985), cell-mediated immunity is of major importance in clearing virus infection (Enders et al., 1959; Good & Zak, 1956). Thus, antibody may be sufficient to block the infection, but not to eliminate it. A vaccine should, therefore, induce not only neutralizing antibodies, but also a cytotoxic T cell response. Such a response should ideally include both class 1- and class 2-restricted cells. Recently, it has been shown that the major cytolytic response in children recovering from measles is class 1-restricted (Van Binnendijk et al., 1990).

Studies on a number of viruses have implicated internal and non-structural proteins as target antigens for cytotoxic T lymphocyte activity. In several negative-strand viruses, the NP is the major antigen for T cell cytolytic activity (McMichael et al., 1986; Townsend et al., 1984). Mice infected with VV-respiratory syncytial recombinant viruses encoding the F and NP proteins generated T cell cytolytic memory cells, whereas recombinants encoding the glycoprotein, matrix and NS antigens were ineffective (Bangham et al., 1986; Pemberton et al., 1987). This has also been found for T helper cells even though different classes of the major histocompatibility complex are involved (Openshaw et al., 1988).

In our previous studies, we established that BALB/c mice immunized with VV recombinants encoding either the HA or F measles virus antigens were protected against a lethal infection. Studies in humans with formaldehyde-inactivated measles virus concluded that although high levels of anti-HA antibodies could protect against an acute infection, a correct anti-F response was required to avoid complications (Fulginiti et al., 1967; Norrby et al., 1975). Studies in both mice and humans with formaldehyde-inactivated respiratory syncytial virus came to the same conclusion (Fulginiti et al., 1969; Kim et al., 1969).

In the present study, we have examined the possibility of expressing more than one measles virus gene from the same VV vector. Among the aspects we wished to study were the relative levels of expression of the different measles virus proteins and the possibility of synergistic effects on the immune system. In vitro, the three measles virus genes could be successfully expressed from a single vector. Expression of the F and HA genes in the same orientation within the tk locus gave a recombinant which initially expressed both measles virus genes, but on subsequent passage lost the F gene (Spehner et al., 1990).

In contrast, when the genes were constructed in the opposite orientation the resulting recombinant was stable. However, the amount of the measles virus proteins were estimated to be approximately half of that synthesized by the VV-F recombinant.

Immunization of mice with recombinant viruses encoding one, two or three measles virus proteins (HA, F, NP) induced similar levels of neutralizing antibodies, and these were stable over a 7 month observation period. No synergistic effect was observed at this level. Recently, Mason et al. (1991) reported the construction of VV recombinants expressing Japanese encephalitis virus proteins which assembled into extracellular particles. Assembly of these particles was correlated to the ability to induce high levels of neutralizing antibody. In the case of measles virus, it may also be necessary to construct a recombinant that can form extracellular particles in order to boost the neutralizing antibody level to a greater order of magnitude.

In contrast to vaccination of BALB/c (H2d) mice, CBA (H2k) mice immunized with VV-F were not protected against measles, but by including the NP gene in the vaccination the protection could be increased to more than 75%. The role of the NP in this phenomenon is unknown. However, it has been shown for rabies and influenza viruses that the presence of NP-specific T

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**Table 5. Immunization of BALB/c (H2k) mice with VV-measles recombinant viruses**

<table>
<thead>
<tr>
<th>Recombinant</th>
<th>Protected/total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td></td>
</tr>
<tr>
<td>VV-NP</td>
<td>0/9</td>
</tr>
<tr>
<td>VV-F</td>
<td>2/15</td>
</tr>
<tr>
<td>VV-HA</td>
<td>10/10</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
</tr>
<tr>
<td>VV wt</td>
<td>0/4</td>
</tr>
<tr>
<td>VV-F</td>
<td>0/5</td>
</tr>
</tbody>
</table>

* Mice were immunized with the viruses and challenged with measles virus 21 days later.

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helper cells boosts the antibody response to the other viral antigens (Ertl et al., 1989; Tite et al., 1988). Bankamp et al. (1991) have shown that VV recombinants expressing NP protect rats against a measles virus challenge. Adoptive transfer of the recombinant-induced NP-specific CD4+ T lymphocytes also protected the animals.

Observations have shown that non-responsiveness to certain antigens can be overcome by increasing the amount of antigen in the immunization and so the haplotype block need not be absolute (Tite et al., 1988). The apparent haplotype restriction is unusual and needs to be confirmed by assay of cellular responses. An alternative explanation would be that CBA mice do not make antibodies against the appropriate epitopes. However, a booster immunization after 1 month induces measles virus-neutralizing antibodies (unpublished observations). It is not clear in the case of the co-expression of the measles virus F and NP whether any of the above phenomena is involved, nor is it clear why CBA mice receiving higher doses of VV-F recombinant survive longer. Thus, the mechanism by which NP interacts with F in the CBA system remains to be clarified. Nevertheless, our studies in conjunction with previous observations suggest that a recombinant DNA vaccine against measles should encode several of the measles virus antigens.

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