Neutralization of measles virus wild-type isolates after immunization with a synthetic peptide vaccine which is not recognized by neutralizing passive antibodies

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The sequence H379–410 of the measles virus haemagglutinin (MV-H) protein forms a surface-exposed loop and contains three cysteine residues (Cys-381, Cys-386 and Cys-394) which are conserved among all measles isolates. It comprises the minimal sequential B cell epitope (BCE) (H386–400) of the neutralizing and protective MAb BH6 that neutralizes all wild-type viruses tested. The aim of this study was to design synthetic peptides which induce neutralizing antibodies against MV wild-type isolates. Peptides containing one or two copies of T cell epitopes (TCE) and BCEs of different lengths (H386–400, BCC; H379–400, BCCC), in different combinations and orientations were produced and iteratively optimized for inducing neutralizing antibodies. Peptides with the shorter BCE induced sera that cross-reacted with MV but did not neutralize. The longer BCE containing the three cysteines (BCCC) and two homologous TCE were required for neutralization activity. These sera neutralized wild-type strains of different clades and geographic origins. Neutralizing serum was also obtained after immunization with human promiscuous TCEs. Furthermore BCCC-based peptides were fully immunogenic even in the presence of pre-existing MV-specific antibodies. The results suggest that subunit vaccines based on such peptides could potentially be used to actively protect infants against wild-type viruses irrespective of persisting maternal antibodies.

Introduction

Although current measles virus (MV) vaccines are effective both in industrialized and developing countries (Sabin, 1992), measles continues to be a major health problem in the Third World (World Health Report, 1996). The requirement of a cold chain and the possible neutralization by transplacentally acquired maternal antibodies are major drawbacks of the live-attenuated vaccines. A peptide-based vaccine, however, would be thermostable and could potentially be designed to avoid recognition by maternal antibodies (El Kasmi et al., 1999). Such a subunit vaccine should be immunogenic in a large part of the population [i.e. contain promiscuous T cell epitopes (TCEs)] and must be able to induce antibodies that neutralize wild-type viruses.

Antibodies have been shown to be sufficient for protection against MV infection even in the absence of a T cell response (Albrecht et al., 1977; Giraudon & Wild, 1985). The major target of neutralizing and protective antibodies in humans and in animal models is the MV haemagglutinin (MV-H) protein (Norrby & Hammarstjold, 1972; Varsanyi et al., 1984; Fournier et al., 1997). Such antibodies are mostly directed against conformational epitopes (Benjamin et al., 1984; Fournier et al., 1997) which are difficult to mimic with synthetic peptides. Therefore, the design of peptide-based vaccines is essentially limited to sequential B cell epitopes (BCEs).

Using a panel of neutralizing and protective MV-H-specific MAbs, we have identified two sequential BCEs: (i) H386–400 (HNE, haemagglutinin noose epitope; Ziegler et al., 1996); and (ii) H236–256 (NE, neutralizing epitope; Ziegler et al., 1996). We have recently shown that synthetic peptides based on the NE sequence induce protective and neutralizing antibodies in an animal model (El Kasmi et al., 1998, 1999). However, this epitope is only poorly conserved among different wild-type
isolates (El Kasmi et al., 1999). The HNE sequence represents a cystine loop domain of the MV-H. Its three cysteines (Cys-381, Cys-386 and Cys-394) are conserved among all MV isolates. In addition, this epitope is not recognized by maternal antibodies (Ziegler et al., 1996).

Peptide vaccines against MV have mostly been evaluated in experiments using vaccine-related clade A viruses. Although in these studies anti-peptide sera neutralized MV in vitro and protected in vivo, neutralization of wild-type viruses was never demonstrated (Obeid et al., 1995; Partidos et al., 1997; El Kasmi et al., 1999).

Here, we describe the optimization of synthetic peptides, combining the HNE domain with a promiscuous human TCE, that mimic the HNE loop and induce neutralizing serum against early passage wild-type strains, even in the presence of passively acquired antibodies.

Methods

- **Peptide synthesis.** All peptides were synthesized by automated solid phase Fmoc chemistry on a SYRO peptide synthesizer (Multi-syntech) (Wiesmüller et al., 1992). Peptides containing combinations of TCE and BCE were collinearly synthesized and were designated TB, BT, TTB or BTT. The following three TCE were used: (a) F421–435 of the MV fusion (MV-F) protein, PVVEVNGVTIQVGSR (Tc, H2a restricted; Muller et al., 1995a); (b) F288–302 of the MV-F protein, LSEIKGVIVHRLEGV; (Tc, promiscuous for most MHC class II including the mouse H2a haplotype; Partidos & Steward, 1990); (c) t830–844 of tetanus toxin, QYIKANSKFIGITEL (Tc, human promiscuous; Demolz et al., 1989). The two BCE corresponded to: (a) H380–400 of the MV-H protein, CKGKIQALCNPWEWA (Bc), and (b) H379–400 of the MV-H protein, ETCFQQACKGKIALCNPWEWA (Bc). The MV sequence corresponds to the Edmonston strain (Alkhatib & Briedis, 1986).

- **Active and passive immunizations.** Groups of 3–5 male and female specific-pathogen-free BALB/c mice (H2b) (8–15 weeks old) were primed intraperitoneally with 100 µg (TB, BT, B and BB) or 150 µg (TTB and BTT) of peptide dissolved in water and emulsified (1:1) in complete Freund’s adjuvant (Sigma). Mice were boosted on day 14 and 45 using incomplete Freund’s adjuvant (Sigma). Serum was prepared 7–10 days after the second boost. Protective titres of anti-MV mouse serum (800 µl) were injected intraperitoneally on the day of peptide immunization and served as a model for passively acquired maternal antibodies.

- **ELISA and flow cytometry.** ELISAs were performed in microtitre plates coated with the biotinylated peptide H386–400 as previously described (Fournier et al., 1997). This peptide served as a reporter antigen to test reactivity with this BCE. To determine antibody titres of BH6 or of antiserum against the homologous peptide or the BCE, microtitre plates were coated overnight at 4 °C with free peptides (0.7 µg per well in 50 µl water). Antibody titres were defined as the highest serum dilution that yielded a signal corresponding to four times the background which was obtained with a naive control serum at a dilution of 1:500 (range of optical density background values 0.15–0.2). This serum was obtained from pre-immunization bleedings of individual mice or pooled from the pre-immunization sera. These mice were naive for MV by ELISA (Enzygnost) and by FACS on MV-infected cells and control cells. The reactivity of immune sera (1:100) with virus was tested by flow cytometry using an MV-superinfected EBV-transformed human B cell line (WMPT; gift of B. Chain, London, UK) as described previously (Muller et al., 1995b). The flow cytometry data correspond to a serum dilution of 1:100, since this concentration reflected most appropriately differences between sera. FITC-conjugate (Sigma) alone, naive serum on MV-positive and MV-negative cells or test serum on MV-negative cells served as negative controls. Data are expressed as arbitrary fluorescence units (AFU). In the rare samples which had a background above 1 AFU, the serum was discarded from the analysis. MAb BH6 (diluted 1:1000; Ziegler et al., 1996) was used to standardize flow cytometry. A neutralizing anti-MV-H serum and an anti-peptide serum served as positive controls. Data are expressed as AFU. Most sera were tested individually and pooled sera (three animals per group) accurately matched the results obtained with single sera.

- **Neutralization assay (NT).** Triplicates of two-fold serial dilutions of immune and pre-immune serum (75 µl per well; starting concentration 1/48) were pre-incubated for 3 h at 4 °C with 100 TCID₅₀ (75 µl per well) of different viruses (Table 2) in 96-well microtitre plates (Nunc) as described previously (Huiss et al., 1997). Wild-type viruses were passaged not more than eight times on B95a cells. After adding B95a cells (3.5 × 10⁶ cells in 75 µl per well), the plates were incubated under standard tissue culture conditions. On day 5, cytopathic effects were evaluated. The serum dilution that prevented cytopathic effects in at least two out of three wells corresponded to the neutralization titre (NT). All peptides were tested in two to four independent immunizations and neutralization experiments. Unless otherwise stated, neutralization was tested against the rodent-adapted MV (Table 2). The sequence of the BCE H379–400 of all viruses was confirmed by sequencing and corresponds to that of the Edmonston strain (Alkhatib & Briedis, 1986).

Results

**MV cross-reactivity induced by TB⁺Cc and TTBCc peptides**

MAb BH6 recognized specifically cystine loops formed by the two 15-mer peptides H381–395 and H386–400 (Ziegler et al., 1996) both of which shared the two cysteines Cys-386 and Cys-394. Single copies or tandem repeats of the TCEs Tₐ, Tₐ or Tc were combined with H386–400 (Bc) (Fig. 1a). These peptides were recognized by BH6 (titres 1:10³–5) indicating that they mimicked the viral loop conformation. All anti-peptide sera reacted strongly with the BCE peptide (1:10⁶), the homologous peptide (1:10⁸–⁸) and the BCE moiety (1:10⁸–⁸⁵) (Fig. 1a). The sera also cross-reacted with the virus (mean AFU of 6–32). The strongest anti-MV response was obtained after immunization with Tc (mean AFU of 25–32) (Fig. 1a). Adding an additional TCE to the TB peptides did not critically improve the anti-peptide or the anti-MV response (Fig. 1a). Neither TB nor TTB peptides induced MV-neutralizing serum (data not shown) suggesting that the loop formed between Cys-386 and Cys-394 in the BcCc peptide may have been suboptimal for the induction of functional antibodies.

**Iterative optimization of the BCE and the role of the cysteines**

To improve the anti-MV response, peptides were modified in several ways: (i) The BcCc peptide was N-terminally elongated to include Cys-381 as a third cysteine (BcCc,
Neutralization of measles virus

H379–400). This peptide generated a strong antibody response against the reporter peptides as well as against the virus (Fig. 1b). (ii) After conjugation of B_CCC with the TCEs T_ac and T_ce (the best TCE in Fig. 1a) antibody titres against the C-terminal BCE reporter peptide and against whole virus further increased in the serum of animals which were immunized with T_aB_CCC, but the immune response did not improve in animals injected with T_cB_CCC. (iii) When the orientation of the epitopes was changed from T_aB_CCC to B_CCC T_a most of the antibodies were again directed against the C-terminal moiety. Since in this case the TCE was C-terminal, the cross-reactivity with the BCE reporter peptides and the MV was considerably reduced (Fig. 1b). Peptides used in the above experiments (i) to (iii) were all recognized by the neutralizing MAb BH6 (titres, 1:10^6–6.2; data not shown) but none of them induced neutralizing serum. (iv) The three cysteines of B_CCC could result in several different conformations of the epitope of which only some may be able to induce functional antibodies. To analyse the role of these residues, the cysteines were consecutively replaced by alanines (Table 1). Antigenicity and cross-reactive immunogenicity was first tested with T_B peptides containing only one or no cysteine. Table 1 shows that, except for B_AAC, these peptides were not recognized by BH6 and none of them induced MV cross-reactive serum.

Loop conformation was also evaluated with peptides containing two cysteines. Although BH6 did not react with B_CCA, it did react with the peptides B_AAC and B_CAC. The latter two peptides were also the only substitution analogues that induced anti-MV serum (Table 1); however, they did not have neutralizing activity. These results suggest that the loops Cys-386/Cys-394 (in B_CC and B_CCC) and Cys-381/Cys-394 (in B_CAC) may both be suboptimal to induce functional antibodies. Since all three cysteines are highly conserved in all MV strains, the presence of a third cysteine, not participating in loop formation, might be critical for the induction of high-affinity antibodies. Therefore, only peptides containing the three cysteines (B_CCC) were further optimized.

Tandem repeats induce neutralizing serum

In previous studies, we and others have observed that tandem TCEs may be more efficient in inducing functional antibodies (El Kasmi et al., 1999; Obeid et al., 1995). Therefore, B_CCC was coupled in two different orientations to a tandem TCE. Since the results shown in Fig. 1(b) indicated that the B_CCC contained a TCE, this sequence was also tested as a free tandem repeat (BCCCCCCCCCCCCCCCCCC). All of these peptides cross-reacted with BH6 (1:10^6–6.6) and induced peptide cross-reactive (e.g. anti-BCE, 1:10^5–6.8) and MV cross-reactive antisera (mean AFU of 5–35) (Fig. 1c). The best reactivity against MV was obtained with anti-B_CCCB_CCC serum. More importantly, however, antibodies generated with T_aT_cB_CCC and with B_CCB_CCC neutralized the neuro-adapted MV in vitro (Fig. 1c). The consecutive replacement of cysteines by alanines in T_aT_cB_CCC peptides confirmed the results obtained with the alanine-substituted T/B peptides (Table 1), inasmuch as all three cysteines were required to induce neutralizing serum (Table 2).

Anti-peptide serum neutralizes wild-type strains

As part of a subunit vaccine, peptides must induce antibodies that are able to neutralize not only laboratory strains but also wild-type viruses. For this purpose, a pool of anti-B_CCB_CCC serum was produced and tested against representative viruses of clades A, B, C and D. These viruses represent early passage isolates from different geographic
Table 1. Antigenicity of alanine-substituted TTB and T_a TTB peptides with respect to BH6 and cross-reaction of anti-peptide sera with MV

<table>
<thead>
<tr>
<th>BCE</th>
<th>Sequence*</th>
<th>TTB</th>
<th>BH6†</th>
<th>MV†</th>
<th>T_a TTB</th>
<th>BH6†</th>
<th>MV†</th>
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<tr>
<td>B_{CCC}</td>
<td>ETCTFQQACKGKIQALCENPEWA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B_{CAA}</td>
<td>--C-----A-------A------</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B_{ACA}</td>
<td>--A-----C-------A------</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B_{AAC}</td>
<td>--A-----A-------C------</td>
<td>4:7</td>
<td></td>
<td></td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B_{CCA}</td>
<td>--C-----C-------A------</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B_{ACC}</td>
<td>--A-----C-------C------</td>
<td>4:7</td>
<td>/NT−</td>
<td></td>
<td>6:5</td>
<td>/NT−</td>
<td></td>
</tr>
<tr>
<td>B_{CAC}</td>
<td>--C-----C-------A------</td>
<td></td>
<td>4</td>
<td>/NT−</td>
<td>4</td>
<td>/NT−</td>
<td></td>
</tr>
<tr>
<td>B_{AAA}</td>
<td>--A-----A-------A------</td>
<td></td>
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</tbody>
</table>

* Sequence of the BCE within the TTB or T_a TTB peptide.
† Titres are given as $\log_{10}$; --, titre $< 2.0$.
‡ +/NT−, MV-cross-reactivity $> 4$ times background but no neutralizing activity; --, no MV cross-reactivity.

Table 2. Neutralization titres of anti-peptide B_{CCC}B_{CCC} serum against different MV strains

<table>
<thead>
<tr>
<th>Laboratory name</th>
<th>WHO designation</th>
<th>Clade</th>
<th>Titre</th>
</tr>
</thead>
<tbody>
<tr>
<td>MV310</td>
<td>Edmonston wt/USA/1954*</td>
<td>A</td>
<td>1:72</td>
</tr>
<tr>
<td>MV503</td>
<td>CAM-70 mouse-brain-adapted†</td>
<td>A</td>
<td>1:72</td>
</tr>
<tr>
<td>MV126</td>
<td>MV/Ilbadan.NIE/9986#</td>
<td>B3</td>
<td>1:72</td>
</tr>
<tr>
<td>MV201</td>
<td>MV/Reuler.LUX/1396#</td>
<td>C2</td>
<td>1:48</td>
</tr>
<tr>
<td>MV224</td>
<td>MV/Luxembourg.LUX/2397#</td>
<td>C2</td>
<td>1:72</td>
</tr>
<tr>
<td>MV312</td>
<td>wt/Chicago-1, USA/1989*</td>
<td>D3</td>
<td>1:144</td>
</tr>
<tr>
<td>MV227</td>
<td>MV/Luxembourg.LUX/3197#</td>
<td>D5</td>
<td>1:48</td>
</tr>
</tbody>
</table>

* Viruses kindly provided by P. Rota, CDC, Atlanta, GA, USA; wt, wild-type isolate.
† Derived from the CAM/RB strain; a gift from U. G. Liebert, Leipzig, Germany.
‡ Viruses isolated at the Laboratoire National de Santé, Luxembourg; submitted to GenBank.

Table 2 shows that the antisera neutralized all viruses tested. It can be concluded that the neutralizing antibodies are solely directed against the HNE domain, since no additional TCE was used to generate these antisera. Anti-T_a T_{a} B_{CCC} serum also neutralized wild-type viruses with similar titres (data not shown).

HNE peptides are not recognized by passively acquired antibodies

To demonstrate immunogenicity of the BCE in the presence of maternal antibodies, the MV Edmonston strain was used to generate a large pool of neutralizing (titre 1:1024) and protective serum. Adult mice were injected intra-peritoneally (i.p.) with protective levels of this serum (800 µl, giving at least 80% protection). Within 1 h after the injection, mice were actively immunized i.p. with the T_a T_{a} B_{CCC} peptide. Serum from each mouse was tested by ELISA against the reporter BCE and against the peptide used for immunization (Fig. 2a). Neither the pre-immune sera nor the anti-MV serum pool reacted with the reporter peptide H386–400 (Fig. 2a).
peptide induced BCE-reactive antibody titres in all infused mice that were similar to those observed in the absence of passive MV serum (P value not significant). These results demonstrate that such a peptide can induce virus-specific antibodies in the presence of pre-existing passive protective antibodies.

Neutralization with TTB_{ccc} peptides and human promiscuous TCEs

To design peptides that would be immunogenic in a large part of the population, the B_{ccc} was tested in combination with promiscuous TCEs. Tandem repeats of T_1 and T_5 were combined with B_{ccc} (Fig. 2h). These constructs were also recognized by BH6 (1:10^{5.5-5.6}). The corresponding antisera reacted with the reporter peptides (e.g. anti-BCE, 1:10^{6-8}) and MV (mean AFU of 4–10) (Fig. 2o). Furthermore, both peptides induced neutralizing serum against the rodent-adapted MV as well as against wild-type isolates (titres of 1:48).

Discussion

Protective immunity against murine measles encephalitis has been obtained after passive transfer of antibodies induced with linear epitopes of the MV-H (El Kasmi et al., 1999) and MV-F proteins (Obeid et al., 1995). In these experiments, mice were challenged with a rodent-adapted neurotropic virus (CAM/RB), a virus that is highly susceptible to neutralization by MAbs directed against the Edmonston strain (unpublished data). Protective anti-peptide serum against the MV-F protein, however, did not neutralize a laboratory strain of MV (Partidos et al., 1997). The authors attributed this to a putative difference in the presentation of the epitope within the laboratory strain compared to the MV strain used for protection. Recently, we have also shown that wild-type viruses are much more resistant to neutralization by protective anti-peptide serum directed against a BCE of the MV-H protein. This was partially due to differences in the sequence of the sequential epitopes, but even wild-type viruses with no mutation in the BCE were not neutralized by sera that otherwise inactivated the CAM or a clade A laboratory strain (El Kasmi et al., 1999).

This problem, therefore, cannot necessarily be circumvented by designing peptides that contain mutations of the wild-type viruses or by targeting a highly conserved BCE. We have evidence that antibodies binding to the same sequence of a vaccine-like virus and wild-type viruses, only neutralize the former (unpublished data). Neutralization of wild-type viruses after immunization with peptide-based immunogens is therefore critical and has not been demonstrated in previous studies with MV peptides (El Kasmi et al., 1999; Obeid et al., 1995). Protection against MV wild-type strains can only be inferred from in vitro neutralization assays since small animal models using wild-type virus are only just being developed (Niewiak, 1999). In this study, we induced antibodies to a sequential epitope that mediates neutralization of both vaccine-like viruses and wild-type virus. After iterative optimization, peptides based on the sequence of the HNE domain neutralized early passage isolates from different geographic origins and clades.

Several other features of these peptides make them attractive candidates as a component of a subunit vaccine. (i) In infants, current live-attenuated vaccines are neutralized by transplacentally acquired maternal antibodies. HNE-based peptides are not recognized by passively transferred anti-MV immunoglobulins in the mouse. This explains why even protective levels of anti-whole virus antibodies do not suppress active seroconversion induced by the peptide antigen. We have previously shown that humans, particularly women of a child-bearing age, do not produce antibodies against the HNE domain (Ziegler et al., 1996). The results therefore suggest that vaccine formulations based on such peptides could be administered to infants, irrespective of the level of persisting maternal antibodies.

(ii) Another problem that may limit the use of peptide-vaccines is MHC restriction. This is further complicated by the fact that the choice of the TCE influences the conformation of the BCE, and its ability to induce functional antibodies (El Kasmi et al., 1999). HNE peptides combined with promiscuous human TCEs induced neutralizing antibodies. Such peptides could also be immunogenic in large parts of the human population. The TCE derived from the MV-F protein may induce memory T cells that could become activated when challenged with MV (Partidos & Steward, 1990).

(iii) Finally, an increasing number of immune-suppressed children, who might develop complications with a live vaccine, may benefit from a subunit vaccine.

The loop between Cys-381 and Cys-394 is thought to account for the principal tertiary structure of the HNE domain (Hu & Norrby, 1994). The iterative optimization of the peptide demonstrated that the induction of MV cross-reactive serum required one of the loops formed by Cys-386 or Cys-381 with Cys-394. However, neutralizing antibodies were solely induced with peptides containing all three cysteines (B_{ccc}). Either one of the two loops is also present in the virus, since MAb BH6, specific for the sequences H381–394 and H386–400, does not recognize the virus in its reduced form. This MAb also reacts only with oxidized peptides that contain either the Cys-386/Cys-394 or the Cys-381/Cys-394 loop (Ziegler et al., 1996). In keeping with previous studies, we could also attribute an important role to Cys-394 in the binding of neutralizing antibodies (Ziegler et al., 1996).

Whereas Hu & Norrby (1994) suggest that Cys-386 may not participate into the loop formation, our data are ambiguous as to the role of Cys-381 and Cys-386. The present results, however, do not allow any conclusion as to which loop is actually required to induce neutralizing antibodies. Consistent with previous observations (El Kasmi et al., 1999; Obeid et al., 1995) regarding other sequential epitopes, tandem repeats of TCEs considerably improved the functional activity of the
antibodies. Since the BCE used here also contained a TCE, the B$_{TCE}$ dimer may have benefited from the same enhanced T cell help; moreover, the B$_{TCE}$ dimer may enhance B cell activation via surface Ig cross-linking (Rehe et al., 1990; Wortis et al., 1995). The TCEs as flanking sequences have profound influences on the structural presentation of the BCE but all TCEs used here appeared to preserve the loop conformation of the BCE. Conversely, the orientation of the BCEs as flanking sequences can influence the immunogenicity of the TCE due to modulation of processing and presentation (Levely et al., 1990; Vacchio et al., 1989; Martineau et al., 1996; Partidos & Steward, 1992). However, all TCEs in TB orientation supported the induction of neutralizing serum.

Although protection against MV encephalitis has been demonstrated after immunization with MV-derived peptides (El Kasmi et al., 1999; Obeid et al., 1995; Partidos et al., 1997), this is the first report showing that neutralization of wild-type MV strains after immunization with a peptide is possible.

A peptide-based vaccine with the above features could potentially be given at an early age irrespective of maternal antibody titres and it would also protect against infection with wild-type MV as demonstrated here. If such a vaccine could close the window of vulnerability that exists when maternal antibodies have waned until the child is protected by active immunization at the age of 9–15 months, considerable progress towards measles control in infants could be achieved.

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References


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