Identification of linear epitopes on Semliki Forest virus E2 membrane protein and their effectiveness as a synthetic peptide vaccine

Alies Snijders, Barry J. Benaissa-Trouw, Tom A. M. Oosterlaken, Wouter C. Puijk, Willem P. A. Posthumus, Rob H. Meloen, Wil A. M. Boere, Janine D. Oosting, Cornelis A. Kraaijeveld and Harm Snippe

Eijkman-Winkler Laboratory of Medical Microbiology, Academic Hospital Utrecht, HP G04-614, Postbox 85500, 3508 GA Utrecht and Central Veterinary Institute, Postbox 65, 8200 AB Lelystad, The Netherlands

Semliki Forest virus (SFV) infection of mice was used as a model to study the applicability of synthetic peptides containing only linear epitopes as viral vaccines. The identification of linear epitopes with vaccine potential on the E2 membrane protein of SFV was based on the binding of SFV-specific antibodies to a set of overlapping synthetic hexapeptides (Pepscan) representing the whole E2 amino acid sequence. The 14 available E2-specific monoclonal antibodies which were protective in vivo proved to be unsuitable for the identification of linear epitopes because they recognized only conformational epitopes, as indicated by their lack of reactivity with unfolded, reduced E2 protein on immunoblots. Three epitopes were detected with polyclonal anti-SFV serum at amino acid positions 135 to 141, 177 to 185 and 240 to 246 of the E2 protein. Synthetic peptides containing these epitopes were coupled to a carrier protein and tested as a vaccine. Mice immunized with the peptide containing amino acids 240 to 255 of protein E2 were protected against a challenge with virulent SFV but protection of mice immunized with the peptides containing amino acids 126 to 141 or 178 to 186 was only marginally better than that of controls. The prechallenge sera of most peptide-immunized mice reacted with SFV-infected cells but none of these sera neutralized the virus in vitro. However, protection of mice correlated well with SFV-specific antibody titre, suggesting antibody-mediated protection.

Introduction

Chemically synthesized oligopeptides have become popular tools for the characterization of the interaction between protein antigens and the immune system. They allow the fine mapping, on proteins of infectious agents, of areas that are critical for induction of protective humoral and cellular immune responses (B and T cell epitopes). This technique led to the concept of synthetic immunogens for vaccination against infectious diseases (Arnon, 1972; Lerner, 1982). The advantages of such vaccines would be enormous in terms of safety, stability, and ease and cost of production. Although synthetic peptides have the disadvantage of containing only linear epitopes, the feasibility of synthetic viral vaccines is suggested by the great number of studies reporting successful in vitro neutralization of virus infectivity by antipeptide antibodies for, among others, tobacco mosaic virus (Anderer & Schlumberger, 1965), poliovirus (Emini et al., 1984), foot-and-mouth disease virus (FMDV) (Bittle et al., 1982; Fläff et al., 1982; Geysen et al., 1985), rhinovirus (Francis et al., 1987; McCray & Werner, 1987), hepatitis B virus (HBV) (Neurath et al., 1986) and human immunodeficiency virus (Chanh et al., 1986; Ho et al., 1988). In contrast to the in vitro results, there are only a few reports of full protection of test animals by peptide vaccines, i.e. for FMDV (Bittle et al., 1982; Dimarchi et al., 1986) and HBV (Itoh et al., 1986; Thornton et al., 1987). For other viruses, like murine hepatitis virus (Talbot et al., 1988), Friend murine leukaemia virus (Bayer & Hunsmann, 1987), influenza virus (Müller et al., 1982) and herpes simplex virus (Eisenberg et al., 1985; Weijer et al., 1988), the neutralizing and protective properties of antipeptide antibodies have been weak or absent. Furthermore, a problem in the development of peptide vaccines is that for many viral diseases it is not possible to measure protection, owing to the lack of a suitable animal model.

To gain more insight into the applicability of peptide vaccines for viral diseases, we used Semliki Forest virus (SFV) infection of mice as a model. This system enabled us to test the effectiveness of peptide vaccines in a host that is highly susceptible to the virus. SFV is an alphavirus in the family Togaviridae. The SFV particle
consists of an RNA-containing capsid surrounded by an envelope in which the viral spikes, composed of trimers consisting of the three membrane proteins, E1, E2 and E3 (Vogel et al., 1986), are anchored. The nucleotide sequence of the genes encoding the structural proteins of SFV (capsid and membrane proteins) is known for the (avirulent) prototype strain of SFV (Garoff et al., 1980), making it possible to synthesize peptides representing regions of these SFV proteins. Virulent strains of SFV cause lethal encephalitis in mice. An important role for antibodies in protection against SFV is indicated by the results of previous studies (Boere et al., 1983, 1984) which demonstrated that both E1- and E2-specific monoclonal antibodies (MAbs) can protect mice against an otherwise lethal infection with SFV, and that protection can even be conferred by non-neutralizing antibodies.

Selection of peptides with vaccine potential was based on the identification of linear antibody-reactive epitopes on the E2 membrane protein. The epitopes recognized by protective, SFV-specific MAbs would be the most attractive candidates for peptide vaccines but only applicable if they are linear epitopes. To determine the ability of the available E2-specific antibodies to interact with linear structures, these antibodies were tested for their reaction with unfolded E2 (produced by treatment with SDS and 2-mercaptoethanol). Effective unfolding of the SFV E1 and E2 proteins by reduction with 2-mercaptoethanol would be expected owing to the large number of cysteine residues in the external domains of E1 and E2 (16 and 12 respectively) at conserved positions (Dalgarno et al., 1983), suggesting many disulphide bonds. To map linear epitopes, a complete set of overlapping hexapeptides (Pepscan) representing the E2 membrane protein sequence was synthesized simultaneously and tested in a subsequent ELISA with SFV-specific antibodies; peptides containing linear epitopes were tested as vaccines. The possibility of finding protective, linear epitopes on the SFV E2 membrane protein had been suggested by the results of Grosfled et al. (1989), which showed that certain cloned fragments of E2 can confer partial to complete protection against lethal SFV challenge.

Methods

Virus strains. The prototype strain of SFV (Henderson et al., 1970), obtained from Dr H. Garoff (Garoff et al., 1980), and the virulent strain of SFV, SF/LS 10 C1/A (Bradish et al., 1971), were grown and purified as described previously (Snijders et al., 1989; Kraaijeveld et al., 1979).

Preparation of anti-SFV immune sera. The immune mouse serum used in the Pepscan was prepared against prototype strain SFV passaged once in L cells (resulting in an SFV titre of 10⁹ p.f.u./ml). Male BALB/c mice were injected intraperitoneally (i.p.) with 10¹⁰ p.f.u. SFV in 0.5 ml phosphate-buffered saline (PBS) and boosted twice (2 and 6 weeks later) with 5 x 10⁶ p.f.u. SFV by i.p. injection. Sera were obtained 1 week after the last booster. This anti-SFV serum also contained antibodies to calf serum, due to its presence in the culture medium. Therefore, a second anti-SFV mouse serum was prepared, using a brain suspension from BALB/c mice containing the prototype strain of SFV, as described (Snijders et al., 1989). The latter serum was used for ELISA and immunoblot assays.

SFV-specific MAbs. Mouse MAbs, UM 4.2, UM 8.22, UM 8.77, UM 8.73, UM 8.48, UM 5.1, UM 8.62, UM 8.107, UM 8.20, UM 8.47, UM 8.64, UM 8.115 and UM 8.139, prepared against the avirulent strain of SFV, MRS MP 192/7, are described by Boere et al. (1984, 1986). Mouse MAbs UM 1.1, UM 1.2, UM 1.3, UM 1.4, UM 1.10, UM 1.13, UM 1.26 and UM 1.27 were prepared by T. A. M. Oosterlaken against the prototype strain of SFV. The procedure was similar to that for the other MAbs except that SP2/0 cells were used for fusion and hybridomas were selected for production of SFV-neutralizing, instead of SFV-binding, antibodies.

Preparation of antipeptide immune sera. Peptides containing amino acid sequences corresponding to regions of the membrane protein E2, with a cysteine residue added to either the N or C terminus were synthesized by Dr J. van Zoelen (Hubrecht Laboratory, Utrecht, The Netherlands) on a Biosearch SAM-2 synthesizer by the solid-phase method of Barany & Merrifield (1980). The amino acid composition of the peptides was confirmed by analysis of the total hydrolysate. Peptides were treated with 2-mercaptoethanol, purified by gel filtration and coupled to the carrier protein, keyhole limpet haemocyanin (KLH), by the method of Lee et al. (1980), using γ-maleimidobutyric acid N-hydroxysuccinimide ester (GMBS; Calbiochem) as the coupling agent. After treatment of KLH with GMBS, which resulted in between eight and 20 maleimide groups per 100K of KLH, a 1-2-fold molar excess of reduced peptide was added to the pretreated KLH. Each peptide–KLH conjugate (1 mg containing about 0.1 to 0.3 mg peptide) was emulsified in Freund's complete adjuvant (FCA) and injected intramuscularly into a New Zealand White rabbit. A booster injection with 1 mg peptide–KLH conjugate emulsified in Freund's incomplete adjuvant (FIA) was given 3 weeks later. Immune serum was obtained 2 weeks after the booster. Control sera were obtained by preimmunization bleeding of the same rabbits.

Groups of inbred male BALB/c mice were injected i.p. with 50 μg peptide–KLH emulsified in FCA. Booster injections were given after 2 and 4 weeks with the same dose of peptide–KLH in FIA (protocol 1). Alternatively, the groups of BALB/c mice were injected subcutaneously (s.c.) with a mixture of 50 μg peptide–KLH and 50 μg adjuvant Quil A (Superfos Biosector) and received one s.c. booster injection with the same dose of peptide–KLH and Quil A 5 weeks later (protocol 2). Immune sera were obtained about 2 weeks after the last booster; sera from the mice of each group were pooled.

Solid-phase ELISA. Peptides (1 μg/well) were coated in high-binding microtiter plates (Nunc; Maxisorb) by drying peptide solution overnight at 37 °C. An SFV prototype preparation (obtained by infecting L cells at an m.o.i. of 0.5 and harvesting the supernatant after an infection period of 18 to 20 h) was similarly coated by drying. A coat of SFV-infected L cell monolayers was obtained by virus inoculation at an m.o.i. of 40 and subsequent incubation for 6.5 h at 37 °C (Boere et al., 1986). The cells were fixed with 0.05% glutaraldehyde and washed. Sera were diluted in PBS, 0.5% Tween, supplemented where necessary with 3% dried milk proteins (Protifar; Nutricia) or 5% calf serum. Horseradish peroxidase (HRPO)-coupled goat anti-rabbit Ig and goat anti-mouse Ig were obtained from Nordic.

SDS–PAGE and immunoblot analysis. SDS–PAGE and blotting (40 mA, 2 to 3 h) were performed by standard techniques. Blots were preincubated overnight in PBS containing 3% bovine serum albumin.
Table 1. Characteristics and reactivities of E2-specific MAbs

<table>
<thead>
<tr>
<th>MAb*</th>
<th>Antibody isotype</th>
<th>Neutralization†</th>
<th>In vitro protection‡</th>
<th>Binding to E2 on immunoblot§</th>
<th>Epitope location</th>
</tr>
</thead>
<tbody>
<tr>
<td>UM 4.2</td>
<td>IgG2a</td>
<td>0</td>
<td>+</td>
<td>Non-reduced</td>
<td>169 to 231</td>
</tr>
<tr>
<td>UM 8.22</td>
<td>IgG2a</td>
<td>0</td>
<td>+</td>
<td>reduced</td>
<td></td>
</tr>
<tr>
<td>UM 8.77</td>
<td>IgG2b</td>
<td>0</td>
<td>+</td>
<td>Cloned</td>
<td></td>
</tr>
<tr>
<td>UM 8.73</td>
<td>IgG2a</td>
<td>1.5</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UM 5.1</td>
<td>IgG2a</td>
<td>5.3</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UM 8.62</td>
<td>IgG2a</td>
<td>6.0</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UM 8.107</td>
<td>IgG1</td>
<td>0</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UM 1-1</td>
<td>IgG2a</td>
<td>5.6 (5.9)</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UM 1-3</td>
<td>IgG2a</td>
<td>1.7 (0.7)</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UM 1-10</td>
<td>IgG2a</td>
<td>1.4 (1.5)</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UM 1-4</td>
<td>IgG2a</td>
<td>4.6 (4.2)</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UM 1-27</td>
<td>IgG2a</td>
<td>2.5 (2.6)</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UM 1-13</td>
<td>IgG2a</td>
<td>5.8 (5.2)</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UM 1-26</td>
<td>IgG2a</td>
<td>4.7 (5.2)</td>
<td>+</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* MAbs marked with the same number are mutually competing.
† Log_{10} of the reciprocal ascites dilution giving 50% plaque reduction of the virulent (or the prototype) strain of SFV.
‡ + +, Full protection after i.v. injection of 1 μg purified MAb against i.p. challenge with virulent SFV; +, full protection with 100 μg MAb; +, partial protection with 100 μg MAb; -, no protection.
§ Immunoblot assays as described in Fig. 1. Tested cloned fragments of E2 are described in the text.

The subsequent ELISA was performed in PBS with 0.5% Tween and 3% dried milk proteins.

Antibody-dependent complement-mediated cytotoxicity (ADCMC). ADCMC of SFV-infected L cells by the concerted action of antibodies and 10% guinea-pig complement was performed essentially as described (Boere et al., 1985) except that antiserum and complement dilutions were added 4 h after infection of the ^51Cr-labelled L cells. The supernatant was harvested 4 h later.

Neutralization and protection studies. The 50% plaque reduction assay for both strains of SFV was performed as described previously (Boere et al., 1984).

Passive protection studies were performed by injecting groups of five or six male BALB/c mice intravenously (i. v.) with immune serum or purified MAb (diluted in 0.5 ml PBS), followed 3 h (MAb) or 24 h (serum) later by an i.p. challenge with 16 p.f.u. virulent SFV. Control mice received the same dose of a corresponding control serum or a control MAb before challenge. To quantify protection, mice were observed for 21 days.

Protection by vaccination was studied in groups of between five and 10 male BALB/c mice immunized with peptide-KLH conjugate (as described for antipeptide sera). Control mice received only KLH (or PBS) and adjuvant. All mice were challenged i.p. 2 or 3 weeks after the last booster with 400 p.f.u. (mice immunized by protocol 1) or 16 p.f.u. (mice immunized by protocol 2) virulent SFV.

Results

Reaction of SFV-specific antibodies with reduced and non-reduced E2 protein

To determine the capacity of E2-specific MAbs to react with linear epitopes, the effect of unfolding and reduction of the E2 protein on the binding of these antibodies in immunoblot assays was investigated. Fifteen different MAbs from 10 different competition groups were tested (Table 1; Fig. 1). All 14 protective
MAbs bound to SDS-treated, non-reduced E2 on immunoblots, but weakly (MAb UM 5-1) or undetectably (the other 13 MAbs) to SDS-treated, reduced E2. In contrast, MAb UM 8-107 bound the non-reduced and reduced forms of the E2 protein equally well; however, this MAb does not neutralize the virus in vitro and is non-protective in vivo. Similar results were obtained with five different E1-specific MAbs (not shown). These results indicate that protective and, especially, neutralizing SFV-specific MAbs generally recognize conformational epitopes that are destroyed by reduction of the disulphide bonds.

non-reduced hybrid protein expressed from plasmid pSH3-2 in *Escherichia coli*, which contains amino acids 1 to 350 of E2 (Snijders et al., 1989), was bound only by the non-protective MAb UM 8-107. When tested with smaller cloned fragments of E2, the epitope recognized by MAb UM 8-107 could be localized to amino acids 169 to 231 (results not shown).

**Identification of epitopes on the E2 membrane protein using polyclonal anti-SFV serum**

Although the tested panel of protective, SFV-specific antibodies recognized only conformational epitopes, there might still be other protective antibodies present in anti-SFV serum that recognize linear epitopes. Using the published nucleotide and amino acid sequences for the structural proteins of the prototype strain of SFV (Garoff et al., 1980), a complete set of overlapping hexapeptides, corresponding to amino acids 1 to 6, 2 to 7, 3 to 8 etc. of the E2 membrane protein of SFV, was synthesized on a solid support as described (Geysen et al., 1984). The support-coupled peptides were subjected to an indirect ELISA with hyperimmune serum raised in mice against L cell-derived SFV of the prototype strain (Fig. 2a), or with the same serum after absorption with a concentrated suspension of purified intact prototype strain SFV (Fig. 2b). This resulted in the identification of three major continuous regions (linear epitopes) on the E2 membrane protein recognized by antibodies in polyclonal anti-SFV serum. These continuous epitopes were located at amino acids 135 to 141, 177 to 185 and 240 to 246.

**Recognition of hexapeptides by SFV-specific MAbs**

The support-coupled peptides were also subjected to an indirect ELISA with eight different E2-specific MAbs, UM 4-2, UM 8-22, UM 8-77, UM 8-73, UM 8-48, UM 5-1, UM 8-62 and UM 8-107 (Table 1), which recognized at least five different epitopes (Boere et al., 1984, 1986). The result of the Pepscan of E2 with MAb UM 5-1 is shown in Fig. 2(c). The results obtained with these MAbs were negative, as would be expected from their lack of binding to reduced E2, but even the non-protective UM 8-107, which did recognize reduced E2 and cloned fragments of E2 on immunoblots, did not bind any of the hexapeptides in the Pepscan.

**Reactivities of antipeptide antibodies**

Peptides containing the epitopes identified with polyclonal anti-SFV serum were synthesized, coupled to the carrier protein KLH and injected with adjuvant (FCA or Quil A) into mice and also, to obtain large quantities of serum, into rabbits. The resulting antipeptide sera were tested for their binding and neutralizing capacities. Measured by indirect ELISA both rabbit and mouse antipeptide antibodies bound very well and specifically to homologous peptide (Table 2). The antibody titre varied between peptides but mouse and rabbit sera against the same peptide had comparable antibody titres, although on the basis of body weight the mice received a five- to 10-fold higher dose of peptide–KLH conjugate. Rabbit and mouse antibodies to peptide 240 to 255 were clearly cross-reactive with SFV because they reacted quite well with SFV-infected cells (Table 2; Fig. 3) but the cross-reactivity with SFV of the antibodies raised to the other peptides was rather weak. However, for three of the rabbit antipeptide sera, the E2 specificity could be confirmed by their binding to the E2 membrane protein of SFV on immunoblots (Table 2). The antibody titres of individual sera of mice immunized with peptide 126 to 141 or 178 to 186 (measured by ELISA on SFV-infected L cells) varied from <2 to 3, and for peptide 240 to 255 from 3 to >3⋅5. We chose to measure antibody...
Table 2. Reactivities of antipeptide sera

<table>
<thead>
<tr>
<th>Antiserum against peptide*</th>
<th>Immunized animal†</th>
<th>Antibody titre‡</th>
<th>Binding to E2 on immunoblot§</th>
<th>Lysis in ADCMC (%)¶</th>
<th>Neutralization titre¶</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Homologous peptide</td>
<td>SFV</td>
<td>SFV-infected L cells</td>
<td></td>
</tr>
<tr>
<td>126 to 141</td>
<td>m</td>
<td>4.5</td>
<td>&lt;2</td>
<td>&lt;2</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>r</td>
<td>4.5</td>
<td>&lt;2</td>
<td>&lt;2</td>
<td>+</td>
</tr>
<tr>
<td>134 to 141</td>
<td>r</td>
<td>&gt;5.5</td>
<td>2-5</td>
<td>2.5</td>
<td>+ +</td>
</tr>
<tr>
<td>178 to 186</td>
<td>m</td>
<td>4.0</td>
<td>2</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>r</td>
<td>3.5</td>
<td>&lt;2</td>
<td>&lt;2</td>
<td>-</td>
</tr>
<tr>
<td>240 to 255</td>
<td>m</td>
<td>&gt;5.5</td>
<td>3</td>
<td>3.5</td>
<td>+ +</td>
</tr>
<tr>
<td></td>
<td>r</td>
<td>5.0</td>
<td>3</td>
<td>3.5</td>
<td>+ +</td>
</tr>
</tbody>
</table>

* Numbers indicate the amino acid positions of the region of E2 present in the peptide.
† The antisera were either pooled prechallenge sera of six mice immunized by protocol 1 (m), or individual rabbit sera (r).
‡ = Log10 of the highest serum dilution that resulted in a ratio of > 1.8 between the extinction values obtained for immune and control serum in an indirect ELISA on the indicated specific coat and, concomitantly, in a difference > 0.10 between the extinction value obtained with immune serum at that dilution on the indicated specific coat and the extinction value on the corresponding control coat (heterologous peptide, calf serum or uninfected L cells).
§ Binding of 100-fold dilutions of rabbit antipeptide sera to E2 on immunoblots in an indirect ELISA performed as described in Fig. 1.
¶ ADCMC of SFV-infected L cells using 30-fold diluted rabbit antipeptide serum. Less than 5% lysis was measured in controls of infected cells with antiserum and inactivated complement and 75% with control serum and active complement.
¶ Log10 of the reciprocal serum dilution that gave a 50% plaque reduction of prototype SFV.

Fig. 3. Antipeptide and antiviral reactivity of rabbit serum against peptide 240 to 255 measured by indirect ELISA. The extinction value obtained in ELISA on a coat of peptide 240 to 255 (a) or on a coat of SFV-infected L cells (b) was plotted against the log10 of the reciprocal serum dilution, using rabbit serum against peptide 240 to 255 (●), preimmune serum from the same rabbit (○), mouse serum against SFV (prototype strain) grown in L cells (▲), mouse serum against L cells (△), mouse serum against SFV obtained from mouse brain (▽) or normal mouse serum (◇) and HRPO-coupled goat anti-rabbit or goat anti-mouse Ig.

titres to SFV mainly on SFV-infected L cells because much smaller amounts of SFV were needed for that method and the results on SFV or SFV-infected cells seemed comparable (Table 2). Furthermore, the two rabbit antipeptide sera with the highest antibody titre on (fixed) SFV-infected cells proved able to cause complement-mediated lysis (ADCMC) of SFV-infected cells (Table 2) in vitro in the presence of 10% guinea-pig complement, showing that the antipeptide antibodies also reacted with unfixed SFV-infected cells. However, in spite of the cross-reactivity with SFV, none of the antipeptide sera (Table 2) showed any detectable capacity to neutralize SFV in vitro, whereas the polyclonal mouse anti-SFV sera had neutralization titres of 4.5 (for the serum against L cell-grown SFV) and 3.6 (for the serum against brain-passaged SFV) in the same test.

Protection of peptide-immunized mice

The same antibody-selected peptides that were used to raise antipeptide sera were (after conjugation to KLH) tested as a vaccine against lethal SFV infection in male BALB/c mice. Of 21 mice immunized with the KLH-coupled peptide containing amino acids 240 to 255 of E2, 20 were protected against a challenge with otherwise lethal doses of virulent SFV (Table 3), as were some of the mice immunized with the KLH-coupled peptide 126 to 141 or 178 to 186. A high dose of virulent SFV was needed to study protection of mice immunized by protocol 1 using FCA as the adjuvant, otherwise substantial numbers of control mice (which received only FCA, or KLH and FCA) survived after challenge. Even after challenge with as high a dose as 400 p.f.u. non-specific survival sometimes occurred (results not shown). Using immunization protocol 2 with Quil A as the adjuvant, no non-specific survival was observed after challenge with 16 p.f.u. SFV, the 10 LD50 dose for naive male BALB/c mice, as determined previously (Kraaijeveld et al., 1979).

All surviving mice immunized by protocol 1 had neutralizing antibodies in their serum 3 weeks after the
Table 3. Survival of peptide-immunized mice

<table>
<thead>
<tr>
<th>KLH-conjugated peptide†</th>
<th>Adjuvant used</th>
<th>Route</th>
<th>Booster (day)</th>
<th>Antibodies to SFV‡</th>
<th>Infection of mice</th>
<th>Mean survival time (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Serum obtained (day)</td>
<td>Antibody titre§</td>
<td>Challenge i.p. (day)</td>
</tr>
<tr>
<td>PBS</td>
<td>FCA/FIA</td>
<td>i.p.</td>
<td>14,28</td>
<td>41</td>
<td>&lt; 2.0</td>
<td>42</td>
</tr>
<tr>
<td>KLH</td>
<td>FCA/FIA</td>
<td>i.p.</td>
<td>14,28</td>
<td>41</td>
<td>&lt; 2.0</td>
<td>42</td>
</tr>
<tr>
<td>126 to 141</td>
<td>FCA/FIA</td>
<td>i.p.</td>
<td>14,28</td>
<td>41</td>
<td>&gt; 2.0</td>
<td>42</td>
</tr>
<tr>
<td>178 to 186</td>
<td>FCA/FIA</td>
<td>i.p.</td>
<td>14,28</td>
<td>41</td>
<td>3.5</td>
<td>42</td>
</tr>
<tr>
<td>240 to 255</td>
<td>FCA/FIA</td>
<td>i.p.</td>
<td>14,28</td>
<td>41</td>
<td>3.5</td>
<td>42</td>
</tr>
<tr>
<td>KLH</td>
<td>Quil A</td>
<td>s.c.</td>
<td>36</td>
<td>48</td>
<td>&lt; 2.0</td>
<td>72</td>
</tr>
<tr>
<td>126 to 141</td>
<td>Quil A</td>
<td>s.c.</td>
<td>36</td>
<td>48</td>
<td>&lt; 2.0</td>
<td>72</td>
</tr>
<tr>
<td>178 to 186</td>
<td>Quil A</td>
<td>s.c.</td>
<td>36</td>
<td>48</td>
<td>2.5</td>
<td>72</td>
</tr>
<tr>
<td>240 to 255</td>
<td>Quil A</td>
<td>s.c.</td>
<td>36</td>
<td>48</td>
<td>&gt; 3.5</td>
<td>72</td>
</tr>
<tr>
<td>KLH</td>
<td>Quil A</td>
<td>s.c.</td>
<td>34</td>
<td>49</td>
<td>&lt; 2.0</td>
<td>64</td>
</tr>
<tr>
<td>240 to 255</td>
<td>Quil A</td>
<td>s.c.</td>
<td>34</td>
<td>49</td>
<td>4.3</td>
<td>64</td>
</tr>
</tbody>
</table>

* Results of three separate experiments are given.
† Numbers indicate the amino acid position of the E2 region present in the peptide.
‡ Results of pooled sera are given. Antibody titres were measured by indirect ELISA against SFV-infected L cells.
§ Titre as defined in Table 2.

challenge (results not shown), whereas their prechallenge sera had no neutralizing activity, indicating that some virus replication probably occurred to create an immunogenic dose. However, only one of the five surviving mice immunized with peptide 240 to 255 by protocol 2 had neutralizing activity in its serum after challenge (results not shown). The antibody titre on SFV-infected cells of the individual prechallenge sera of the peptide-immunized mice (protocol 2) were also determined. The titres were < 2 for peptide 126 to 141-immunized mice and > 3-5 and 4.3 for peptide 240 to 255-immunized mice, and varied from < 2 to 3 for mice immunized with peptide 178 to 186. The prechallenge sera of individual surviving mice had SFV-specific antibody titres at least equal to, but usually higher than, those of non-surviving mice in the same group (results not shown). Immunized mice which did not survive infection had a longer mean survival time than control mice that did not survive (Table 3).

**Protection by antipeptide serum**

To test whether antipeptide immune serum was able to produce protective immunity against SFV in naive mice, a passive transfer experiment was performed: a group of 16 donor mice was immunized s.c. with KLH-conjugated peptide 240 to 255 and Quil A (by protocol 2). Sera were collected 2 weeks after the last booster injection and pooled; control serum was obtained from mice immunized similarly with KLH or Quil A alone. The undiluted pooled sera were injected i.v. into naive male BALB/c mice (0.5 ml per mouse). Blood was taken from two mice from each group of recipients 24 hours later. The SFV-specific antibody titre of serum in immune serum recipients was 3.1 compared to 3.8 for the donor serum. Subsequently, all recipient mice were challenged i.p. with 16 p.f.u. SFV. The presence of viraemia was determined 24 h after infection in four mice from each group. Recipients of immune serum showed no detectable viraemia in contrast to that observed in all control animals tested (Table 4). However, of six recipients of immune serum, three died having lived longer (9-0 days compared to 7-0 days) than control mice. The combined results suggest that non-neutralizing antibodies to SFV induced by peptide 240 to 255 mediate protection against virulent SFV in vivo.

**Discussion**

In this study, we used SFV infection of mice as a model to analyse the applicability of viral synthetic peptide vaccines. The choice of SFV infection of mice as a model for these vaccines was based on the fact that virulent strains of SFV cause a lethal encephalitis in mice which can be completely prevented by administration of antibodies (Boere et al., 1984, 1985). Peptides to be tested as a vaccine were selected on the basis of their reactivity with antiviral antibodies. Of the selected peptides (coupled to KLH), one proved able to protect mice against a challenge with virulent SFV.

Using a Pepscan of the E2 membrane protein, three linear epitopes were identified by the reactivity of antibodies in polyclonal SFV-specific serum (Fig. 2). It is likely that more linear epitopes would have been detected if the peptides in the Pepscan of E2 had been
later, as indicated by the lack of reactivity of MAbs to SFV with the hexapeptides in the Pepscan although it did recognize a linear epitope on E2 (Table 1). Recently, Ariel et al. (1990) showed that a greater number of linear determinants could be detected with 20 amino acid-long overlapping synthetic peptides of the SFV E2 protein. However, for the protective MAbs the lack of binding to the peptides of the Pepscan is fully explained by the lack of binding to unfolded E2.

These findings are in agreement with those of others that determinants recognized by neutralizing antibodies on the membrane proteins of SFV (Greiser-Wilke et al., 1989; Wust et al., 1989) and Sindbis virus (Roehrig et al., 1982; Stanley et al., 1985) are conformational. Furthermore, most epitopes on protein antigens are of a discontinuous nature (Benjamin et al., 1984) and the frequency of peptide-reactive antibodies is low after immunization with native protein (Jemmerson, 1987). We would like to emphasize the strong correlation found between the protective capacity of the antibody and the conformational nature of the epitope. This correlation indicates that the chances of identifying protective linear epitopes with antiviral antibodies are low. This will probably be true in many viral systems.

Carrier-coupled peptides incorporating the identified epitopes were tested as vaccines. One of them (peptide 240 to 255) proved effectively protective, causing mice immunized with this peptide to be protected against a lethal SFV challenge although the peptides induced only non-neutralizing antibodies; the two other peptides showed very limited protective capacity. These results are in agreement with those of Grosfeld et al. (1989) who showed that some fragments of E2 from SFV (amino acids 115 to 150, 218 to 288 and 289 to 352), selected on the basis of a hydrophilicity pattern and produced as hybrid proteins with β-galactosidase in E. coli, are recognized by anti-SFV antibodies and can confer partial to complete protection although these fragments induce non-neutralizing, SFV-specific antibodies. Our studies differ in some respects. The cloned fragments of E2 used by Grosfeld et al. (1989) are considerably larger than our synthetic peptides and could, as they suggest, still retain some of the spatial conformation of the native protein. This might explain why they identify the fragment containing amino acids 289 to 352 of E2 as being the most effective in the induction of a protective response, whereas we failed to identify a linear epitope located within the 289 to 352 amino acid region. Our approach, however, led to a more precise localization of linear epitopes.

In our study, peptide 240 to 255, which showed the strongest protective capacity, induced SFV-specific antibodies to a high titre, whereas the sera of mice immunized with peptide 126 to 141 or 178 to 186, which showed only limited protection, were hardly cross-reactive with SFV. The antibody titre of the pooled prechallenge sera of the groups of peptide-immunized mice (measured on SFV-infected cells) correlated well with the survival rate of each group (Table 3). A good correlation was also found between the SFV-specific antibody titre of individual prechallenge sera and individual survival (results not shown). These combined results indicate that the protection induced by the peptides is antibody-mediated, in agreement with the way in which they were selected. However, passive protection experiments with sera indicated that high doses of antibody are needed for protection (Table 4).

The mechanism of peptide-induced protection may involve Fc receptor-mediated uptake of opsonized SFV by non-permissive mouse macrophages and antibody-dependent lysis of SFV-infected cells by complement, as described for protection by non-neutralizing, protective SFV-specific MAbs (Boere et al., 1985, 1986).

In conclusion, we have identified three linear epitopes on E2 of SFV by the reaction of anti-SFV antibodies with a Pepscan of E2. A short synthetic peptide containing one of these linear epitopes could induce protective immunity against virulent SFV in mice by inducing high levels of non-neutralizing antibodies; peptides containing the other linear epitopes were only slightly protective. Our results show that, although protective, SFV-specific antibodies generally recognize epitopes with a
discontinuous conformational nature and, although antipeptide antibodies are generally inferior in inducing protection compared to antiviral antibodies, a peptide containing a linear epitope selected with anti-SFV antibodies proved, after coupling to a carrier and application with adjuvant, effectively protective as a synthetic peptide vaccine.

We would like to thank Hetty Vernooy, Geert Ekstijn and Martin Becker for technical assistance and Peter Capel for critically reading the manuscript. This work was supported by grant 178BT/93-138/X from the Dutch Organization for Scientific Research (NWO).

References


(Received 8 October 1990; Accepted 29 November 1990)