A chimaeric plant virus vaccine protects mice against a bacterial infection

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The plant virus cowpea mosaic virus (CPMV) is an efficient carrier of foreign peptides for the generation of strong humoral immune responses. Peptides derived from both viruses and bacteria are strongly immunogenic when displayed on the surface of CPMV and elicit high titres of peptide-specific antibody. However, the protective effects of antibodies generated using bacterial epitopes in this system have yet to be demonstrated. In this study the ability of chimaeric virus particles (CVPs) to afford protection against bacterial infection was assessed. Immunization of outbred mice with CPMV expressing a peptide derived from outer-membrane protein F of Pseudomonas aeruginosa (CPMV-PAE5) generated high titres of P. aeruginosa-specific IgG that opsonized the bacteria for phagocytosis by human neutrophils and afforded protection upon challenge with two different immunotypes of P. aeruginosa in a model of chronic pulmonary infection. When examined 8 d after challenge, CVP-immunized mice had fewer severe lung lesions and fewer bacteria in their lungs compared to mice immunized with wild-type virus. Different levels of protection were seen with CPMV-PAE5 when Freund's or alum adjuvants were used. These studies highlight the ability of CVPs to generate protective immunity against infectious disease agents.

Keywords: cowpea mosaic virus, chimaeric virus particle, Pseudomonas aeruginosa, outer-membrane protein F, vaccine

INTRODUCTION

Although live attenuated bacterial (Everest et al., 1995) and viral (Resnick et al., 1995; Rose & Evans, 1991; Sutter & Moss, 1992) vaccines as well as naked DNA vaccines (Montgomery et al., 1994) induce stronger and longer-lasting immune responses than conventional killed/subunit vaccines, even in the absence of adjuvant, there are safety concerns over the use of these vaccines (Vlacha et al., 1996; Klinman et al., 1997; WHO, 1990). In particular, persistence or reversion to virulence of the live vaccine strains and integration of, *che naked DNA vaccine into the host chromosome are major concerns. To circumvent these potential problems, more effective inactive vaccines are being developed through modification of proteins or peptides by polymerization (Reed et al., 1997), lipidation (Deprez et al., 1996), oxidation/reduction (Apostolopoulos et al., 1995) and encapsulation (Lovgren et al., 1990). The immunogenicity of proteins can also be enhanced by coupling them to carrier molecules or bacterial proteins (Zhang et al., 1996; Jahn-Schmid et al., 1997), or by producing them in the form of particulate virus-like particles (Adams et al., 1987; Ball et al., 1998). Furthermore, the use of more effective adjuvants for both mucosal and systemic delivery has resulted in increased immunogenicity of non-replicating vaccines (Gupka & Siber, 1995; Elson & Dertzbaugh, 1994).

In an effort to produce effective non-replicating vaccines, we have been examining the vaccine potential of a plant virus, cowpea mosaic virus (CPMV), genetically engineered (Usha et al., 1993; Porta et al., 1994) to express and display foreign peptides on its surface. In this presentation system, termed EPICOAT, peptides of up
to ~40 amino acids are incorporated into specific locations in either of the two virus coat proteins. This results in the presentation of 60 copies of the foreign peptide on the surface of each virus particle, circumventing the need to couple the peptides to carrier molecules. The resultant chimeric virus particles (CPVs) are easily propagated in plants and large amounts of the CPVs (up to ~1g per kg fresh weight of leaves) can be readily extracted. They have the potential for cost-effective manufacture and are not known to infect mammalian cells, thereby circumventing the safety concerns associated with live attenuated bacteria or viruses. A peptide derived from human immunodeficiency virus (HIV) gp120 was shown to be highly immunogenic when displayed on CPMV, inducing HIV-neutralizing antibodies in mice (McLain et al., 1995, 1996; Buratti et al., 1998). Also, another EPICOAT vaccine, presenting a peptide from the VP2 protein of canine parvovirus, was shown to protect against lethal challenge with the canine-parvovirus-related mink enteritis virus (Dalsgaard et al., 1997). This was the first demonstration of an experimental vaccine produced exclusively in plants conferring protection in the target animal against a viral disease.

More recently, we have shown that peptides from bacterial proteins are also highly immunogenic when displayed on CPMV. A CPMV-based CVP expressing a peptide derived from the fibronectin-binding protein of Staphylococcus aureus was shown to elicit high titres of peptide-specific antibody that completely inhibited fibronectin binding (Brennan et al., 1999a). Furthermore, a peptide derived from the outer membrane (OM) protein F of Pseudomonas aeruginosa elicited high titres of peptide-specific antibody which recognized all seven immunotypes of P. aeruginosa and enhanced their phagocytosis by human neutrophils (Brennan et al., 1999b). Despite the immunogenicity of CPMV-expressed bacterial proteins, their ability to confer protective immunity has not been demonstrated.

P. aeruginosa is an important opportunistic bacterial pathogen that causes severe infections in immuno-compromised humans, including burn patients, the immunosuppressed, and in children with cystic fibrosis (CF). In CF patients, P. aeruginosa remains the leading cause of morbidity and mortality (Buret, 1994) due to chronic colonization of the CF lung. No means are currently available to block the colonization of the CF lung by P. aeruginosa. The development of a vaccine that could successfully accomplish this remains a highly sought-after goal. Among the most promising vaccine candidates for use in this clinical situation are those based on OM protein F of P. aeruginosa. In this study, we sought to determine if vaccination using a CVP displaying a peptide derived from the OM protein F could provide protection from a bacterial infection. Outbred mice were immunized with the CVP in either Freund’s or alum adjuvants and the levels of protection assessed in a clinically relevant model of P. aeruginosa infection.

**METHODS**

**Bacterial strains and culture conditions.** The strains of P. aeruginosa used in this study were ATCC 27313 (Difco 0-11; FD immunotype 2) and ATCC 27315 (Difco 0-1; FD immunotype 4). Both strains were grown at 30°C with shaking in BBL nutrient broth (Becton-Dickinson Microbiology Systems), or on nutrient agar (Difco) plates.

**Construction, propagation and purification of CPMV-PAe5 virions.** The construction, propagation and purification of the chimeric CPMV particles (CPMV-PAe5) have been described previously (Brennan et al., 1999b). CPMV-PAe5 expresses a 34-amino acid peptide (NEYVEGGRNVAGSDGNATAGRAIMVRV8) inserted between amino acids 98 and 99 of the L coat protein subunit (in the βE→βB loop) of CPMV. This sequence comprises OM protein F peptide 18 linked to OM protein F peptide 10 (peptides 18 and 10 in bold) by a short linker sequence and additional OM protein F amino acids either side of peptide 10. The latter were included to make the peptide more amenable to presentation on the surface of CPMV. Although peptide 10, unlike peptide 10, has not been shown to provide protection from P. aeruginosa challenge (Hughes & Gillett, 1995), it was found that higher rates of plant infection and virus yield were achieved if peptide 18 was coexpressed with peptide 10 than if peptide 10 was expressed alone. Each virion expresses 60 copies of the OM protein F 18/10 peptide, and 1μg CVP contains approximately 40 ng tandem peptide. CPMV-PAe5 was shown previously to elicit antibodies only to peptide 10 (Brennan et al., 1999b).

**Immunization of mice.** Mice (5-week-old female specific-pathogen-free outbred ICR mice from Sprague–Dawley) (45–50 per group) were subcutaneously immunized with 100μg of either CPMV-PAe5 or wild-type CPMV (wtCPMV) in either Freund’s complete adjuvant (FCA) (1:1, Sigma) or alum (25 mg ml−1, aluminium hydroxide concentrate, Pharmaceutical Associates). Booster injections of 10μg virus were administered in the same adjuvant (except that Freund’s incomplete adjuvant (FIA) replaced FCA) on days 14, 28 and 42. Five mice from each group were culled on each of days 0, 27, 41 and 53 and the sera from mice of each group pooled and stored at −20°C for future analysis. Fourteen days after the last immunization (day 56), all remaining immunized mice (21–28 per group) were challenged with agar beads containing approximately 5×106 live P. aeruginosa of the Fisher–Devlin (FD) immunotype 2 (FD2) strain (described below). In a second study using the wtCPMV in alum, CPMV-PAe5 in alum and CPMV-PAe5 in FCA/FIA groups, and an identical immunization regimen, mice were challenged with the FD4 strain. In a follow-up experiment, a wtCPMV in FCA/FIA and a CPMV-PAe5 in FCA/FIA group were challenged with the FD4 strain, and the results combined with those of the initial experiment.

**ELISA for detection of P. aeruginosa-specific serum antibody.** Pooled sera from each of the groups were examined for titres of IgG antibodies against P. aeruginosa FD2 and FD4 by ELISA as described previously (Hughes et al., 1992; Staczek et al., 1998). Briefly, plates (Dynatech; Immunlon 1) were coated with a suspension of whole P. aeruginosa (FD2 or FD4) cells prepared according to the method of Abdullahi & Poolman (1987). Dilutions of sera were incubated on the bacteria-coated plates and bound antibody was detected with alkaline-phosphatase-conjugated goat anti-mouse IgG (Southern Biotechnology Associates) with p-nitrophenyl phosphate (Sigma) as the substrate. The absorbance was measured with an
Plant virus vaccine against P. aeruginosa

RESULTS

CPMV-PAE5 elicits P. aeruginosa-specific opsonizing antibody

Mice immunized with CPMV-PAE5 in either FCA/FIA or alum produced both P. aeruginosa FD2- and FD4-specific IgG (Fig. 1a and b, respectively), which was absent in mice immunized with wtCPMV in either alum or FCA/FIA. Immunization with CPMV-PAE5 in Freund’s adjuvants elicited higher titres of both FD2 and FD4-specific antibodies than did immunization with CPMV-PAES in alum at all time-points examined. CPMV-PAE5 in Freund’s adjuvants elicited specific antibody as early as day 27 (after two immunizations) whereas antibody was not detected in mice vaccinated with CPMV-PAES in alum until after four immunizations, on day 53 (Fig. 1).

The CPMV-PAE5-immunized mice produced antisera by day 53 that were highly opsonic for P. aeruginosa (Table 1). Sera from mice immunized with CPMV-PAE5 in either FCA/FIA or alum were significantly more opsonic for FD2 than sera from wtCPMV-immunized groups. The degree of opsonization (FCA/FIA > alum) paralleled the titres of FD2-specific IgG present in the sera (Fig. 1a). The level of opsonization of FD2 cells observed with sera from mice immunized with purified protein F in alum was significantly higher (ratio of 1:76) than with sera from CPMV-PAE5-immunized mice when alum was used (ratio of 1:35) but not when


**Table 1.** Phagocytic uptake by PMNs of *P. aeruginosa* exposed to the various antisera

<table>
<thead>
<tr>
<th>Strain</th>
<th>Test serum</th>
<th>Mean no. of bacteria per PMN ± SD</th>
<th>Ratio*</th>
<th>P value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>FD2</td>
<td>wtCPMV in alum</td>
<td>9.68 ± 2.23</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>CPMV-PAE5 in alum</td>
<td>13.09 ± 3.49</td>
<td>1.35</td>
<td>0.003</td>
<td></td>
</tr>
<tr>
<td>wtCPMV in FCA/FIA</td>
<td>9.83 ± 1.22</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>CPMV-PAE5 in FCA/FIA</td>
<td>18.96 ± 2.36</td>
<td>1.93</td>
<td>&lt;0.000001</td>
<td></td>
</tr>
<tr>
<td>NMS</td>
<td>11.10 ± 1.43</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Protein F in alum</td>
<td>19.58 ± 6.22</td>
<td>1.76</td>
<td>&lt;0.000001</td>
<td></td>
</tr>
<tr>
<td>FD4</td>
<td>wtCPMV in alum</td>
<td>12.97 ± 1.76</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>CPMV-PAE5 in alum</td>
<td>17.28 ± 7.48</td>
<td>1.33</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>wtCPMV in FCA/FIA</td>
<td>13.03 ± 1.043</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>CPMV-PAE5 in FCA/FIA</td>
<td>20.87 ± 1.460</td>
<td>1.6</td>
<td>&lt;0.000001</td>
<td></td>
</tr>
</tbody>
</table>

*Ratio represents the mean number of bacteria associated per PMN for the test serum divided by the mean number per PMN for its control.† P values determined by the unpaired two-tail Student's t-test.

**Table 2.** Scoring of lung lesions in mice after challenge with the FD2 or FD4 immunotypes of *P. aeruginosa*

<table>
<thead>
<tr>
<th>Strain</th>
<th>Immunization group</th>
<th>No. of mice with lesions scored as*:</th>
<th>Proportion with lesions ≥2+</th>
<th>P value†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0  1+  2+  3+  4+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FD2</td>
<td>wtCPMV in alum</td>
<td>3  0  2  9  13</td>
<td>24/27 (88.9%)</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>CPMV-PAE5 in alum</td>
<td>10 0 3 9 6</td>
<td>18/28 (64.3%)</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>wtCPMV in FCA/FIA</td>
<td>3 2 1 6 15</td>
<td>22/27 (81.5%)</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>CPMV-PAE5 in FCA/FIA</td>
<td>15 3 2 6 1</td>
<td>9/27 (33.3%)</td>
<td>0.0004</td>
</tr>
<tr>
<td>FD4</td>
<td>wtCPMV in alum</td>
<td>3 1 0 9 14</td>
<td>23/27 (85.2%)</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>CPMV-PAE5 in alum</td>
<td>7 2 3 7 2</td>
<td>12/21 (57.1%)</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>wtCPMV in FCA/FIA</td>
<td>3 0 1 11 6</td>
<td>18/21 (85.7%)</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>CPMV-PAE5 in FCA/FIA</td>
<td>21 5 3 14 4</td>
<td>21/47 (44.7%)</td>
<td>0.0014</td>
</tr>
</tbody>
</table>

*Lesions were scored as follows: 0, absence of any macroscopic lesion; 1+, presence of one or two small lesions not exceeding 1 mm in diameter; 2+, presence of three or more small lesions not exceeding 1 mm in diameter; 3+, presence of a medium lesion 2-5 mm in diameter; 4+, presence of a large lesion exceeding 5 mm in diameter.† P value was determined by Fisher’s exact test.

FCA/FIA (ratio of 1.93) was used (Table 1). In parallel studies, sera from mice immunized with CPMV-PAE5 in either FCA/FIA or alum were also opsonic for FD4 (Table 1).

**CPMV-PAE5 immunization protects mice from *P. aeruginosa* challenge**

Mice immunized with CPMV-PAE5 in either FCA/FIA or alum were significantly protected against the development of severe (≥2+) lung lesions (Table 2). Following challenge with FD2 in mice in which FCA/FIA was used as the adjuvant, 81.5% of those immunized with wtCPMV had severe lesions, scored as ≥2+, whereas only 33.3% (*P* = 0.0004) of mice immunized with CPMV-PAE5 had severe lesions. Although protection conferred by CPMV-PAE5 was higher when FCA/FIA was used, significant protection was also seen with CPMV-PAE5 when alum was used as the adjuvant: 88.9% of mice immunized with wtCPMV had severe lesions, whereas only 64.3% (*P* = 0.03) of mice immunized with CPMV-PAE5 had severe lesions. Similar levels of protection from FD4 challenge were also seen in mice immunized with CPMV-PAE5 in FCA/FIA or alum (Table 2). A second indicator of protection afforded by the CVPs was the decrease in bacteria...
Table 3. Number of P. aeruginosa in lungs of mice after challenge with the FD2 or FD4 immunotypes of P. aeruginosa

<table>
<thead>
<tr>
<th>Strain</th>
<th>Immunization group</th>
<th>No. of mice with no growth/total no. of mice</th>
<th>P value*</th>
<th>No. of mice with &lt;5 × 10⁸ c.f.u. in lungs/total no. of mice</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>FD2</td>
<td>wtCPMV in alum</td>
<td>4/27 (14.8%)</td>
<td></td>
<td>5/27 (18.5%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CPMV-PAE5 in alum</td>
<td>8/28 (28.6%)</td>
<td>0.18</td>
<td>11/28 (39.3%)</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td>wtCPMV in FCA/FIA</td>
<td>7/26 (26.9%)</td>
<td></td>
<td>7/26 (26.9%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CPMV-PAE5 in FCA/FIA</td>
<td>17/27 (63%)</td>
<td>0.009</td>
<td>20/27 (74.1%)</td>
<td>0.0007</td>
</tr>
<tr>
<td>FD4</td>
<td>wtCPMV in FCA/FIA</td>
<td>7/21 (33.3%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CPMV-PAE5 in FCA/FIA</td>
<td>29/47 (61.7%)</td>
<td>0.028</td>
<td>37/47 (78.7%)</td>
<td>0.012</td>
</tr>
</tbody>
</table>

* P values were determined by Fisher’s exact test.

DISCUSSION

We have demonstrated here that a chimaeric plant virus particle expressing a tandem peptide containing two linear B cell epitopes from OM protein F of P. aeruginosa (Gilleland et al., 1995) confers protection upon outbred mice when challenged with P. aeruginosa in a chronic model of pulmonary infection. The protection observed is mediated by the peptide 10 epitope, since immunization with CPMV-PAE5 fails to elicit antibodies to peptide 18 (Brennan et al., 1999b). The peptide 10 epitope of OM protein F is conserved among the various strains of P. aeruginosa as evidenced by antibodies directed towards peptide 10 reacting at high titre to all strains of P. aeruginosa tested, including strains representing each of the seven Fisher-Devlin immunotypes and over 50 clinical strains (Gilleland & Gilleland, 1995). The four doses administered represent ~ 5-2 µg of the tandem peptide and thus only ~ 2.6 µg of the protective peptide 10 epitope. Very low doses of OM protein F peptide, when expressed on CPMV, elicited high titres of P. aeruginosa-specific antibody in serum, but not in the whole P. aeruginosa (Cripps et al., 1994) can elicit P. aeruginosa-specific antibody in serum, but not in the lung, and can provide protection from P. aeruginosa challenge. CVP-immunized mice had fewer severe lesions and lower numbers of bacteria in their lungs compared to mice immunized with the wild-type virus following challenge with P. aeruginosa. It has previously been demonstrated that KLH-conjugated protein F peptides (Hughes & Gilleland, 1995) as well as killed whole P. aeruginosa (Cripps et al., 1994) can elicit P. aeruginosa-specific antibody in serum, but not in the lung, and can provide protection from P. aeruginosa challenge. It is most likely therefore that opsonic serum IgG is entering the lung and conferring protection. Indeed, clearance of P. aeruginosa infection from the lung was enhanced by the intravenous infusion of immune serum (Dunkley et al., 1995).

In this study, mice were immunized with CPMV-PAE5 in Freund’s adjuvants since previous studies (Brennan et al., 1999b) demonstrated that immunization of mice present in the lungs of immunized mice 8 d after challenge (Table 3). When FCA/FIA was used as the adjuvant and mice were challenged with the FD2 strain of P. aeruginosa, the proportion of mice whose lungs yielded no bacterial growth was increased from 26.9% in mice immunized with wtCPMV to 63% (P = 0.009) in mice immunized with CPMV-PAE5. When CPMV-PAE5 in alum was used, a higher (approx. double) proportion of the lungs from immunized mice also had no growth compared to mice immunized with wtCPMV in alum, but these differences were not significant (Table 3). A significantly (P = 0.028) higher number (61.7%) of mice immunized with CPMV-PAE5 in FCA/FIA and challenged with the FD4 immunotype strain of P. aeruginosa (Table 3) had no bacterial growth in their lungs when compared to mice immunized with wtCPMV in FCA/FIA (33.3%).

In mice challenged with the FD2 strain, there was a significantly (P = 0.0007) higher number immunized with CPMV-PAE5 in FCA/FIA (74.1%) that had <5 × 10⁸ c.f.u. in their lungs compared to mice immunized with wtCPMV in FCA/FIA (26.9%). When alum was used, although a higher number of CPMV-PAE5-immunized mice (39.3%) had <5 × 10⁸ c.f.u. in their lungs compared to wtCPMV-immunized mice (18.5%), this difference was not significant (P = 0.08). In mice challenged with FD4 (Table 3) a significantly (P = 0.0012) higher number (78.7%) of mice immunized with CPMV-PAE5 in FCA/FIA had <5 × 10⁸ c.f.u. in their lungs compared to mice immunized with wtCPMV in FCA/FIA (47.6%).

The 5 × 10⁸ c.f.u. cut-off has been shown previously to represent a 95% decline from the mean number of bacteria found in the lungs of challenged, control mice on day 8 after challenge in this model (Staczek et al., 1998). Since approximately 5 × 10⁸ c.f.u. of P. aeruginosa were inoculated into the lungs at the time of challenge, this cut-off also indicates whether the inoculated bacteria were controlled or were capable of establishing an infection that resulted in a substantial increase in bacterial number within the lung.
with CPMV-PAE5 in Freund's adjuvants elicited the production of high titres of both peptide- and whole P. aeruginosaspécific IgG. The more clinically applicable alum adjuvant was also tested with CPMV-PAE5 as alum is the only adjuvant currently accepted for human use (Gupka & Siber, 1995). The titres of P. aeruginosaspécific IgG elicited by CPMV-PAE5, and consequently the levels of P. aeruginosaprotection following challenge, were substantially higher with Freund's adjuvants than with alum. This implies that more effective adjuvants must be sought for use in humans to enhance the immunogenicity of CPMV-PAE5.

The commonly used carrier molecule keyhole limpet haemocyanin (KLH) has been used previously to deliver OM protein F peptides to the immune system of both mice and rats (Gilleland & Gilleland, 1995; Hughes & Gilleland, 1995). Peptide 10, when coupled to KLH and used in humans to enhance the immunogenicity of alum adjuvant was also tested with CPMV-PAE5. This work was performed under MAFF licence no. PHL 91/2275 (08/1997).

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