***Pseudomonas aeruginosa* outer-membrane protein F epitopes are highly immunogenic in mice when expressed on a plant virus**

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A synthetic peptide (peptide 10) representing a surface-exposed, linear B cell epitope from outer-membrane (OM) protein F of *Pseudomonas aeruginosa* was shown previously to afford protection in mice from *P. aeruginosa* infection. This peptide was expressed in tandem with the protein F peptide 18 on each of the two coat proteins of cowpea mosaic virus (CPMV). The chimaeric virus particles (CVPs) expressing the peptides on the S (small) coat protein (CPMV-PAE4) and L (large) coat protein (CPMV-PAE5) were used to immunize mice. Following subcutaneous immunization in Freund's and QuilA adjuvants, CPMV-PAE4 induced antibodies predominantly against peptide 18, whereas CPMV-PAE5 produced antibodies exclusively against peptide 10, indicating that the site of peptide expression on CPMV influences its immune recognition. The anti-peptide antibodies elicited by CPMV-PAE5 were predominantly of the IgG_{2a} isotype, indicating a highly polarized TH1-type response. The peptide-specific IgG_{2a} strongly recognized the whole F protein, but more importantly, recognized protein F in all seven Fisher-Devlin immunotypes of *P. aeruginosa*. Furthermore, the peptide-specific IgG_{2a} in CVP/QS-21 adjuvant-immunized mice was shown to bind complement and to augment phagocytosis of *P. aeruginosa* by human neutrophils *in vitro*. The ability of CPMV-PAE5 to induce *P. aeruginosa*-specific opsonic IgG_{2a} gives it potential for further development as a protective vaccine against *P. aeruginosa*.

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

INTRODUCTION

Pseudomonas aeruginosa is an opportunistic pathogen causing infection in burn patients, the immuno-suppressed and in children with cystic fibrosis, in whom it causes chronic pulmonary infections. We have previously shown that active immunization with outer-membrane (OM) protein F of *P. aeruginosa* affords protection from *P. aeruginosa* in a number of animal models, including a rat model of chronic pulmonary

infection (Gilleland *et al.*, 1988, 1992), a murine acute infection model (Gilleland *et al.*, 1984) and a murine burn wound sepsis model (Matthews-Greer & Gilleland, 1987; Matthews-Greer *et al.*, 1992). Since protein F purified from bacteria is not suitable for human vaccination due to its contamination with LPS and its requirement for detergent to prevent its denaturation into an insoluble precipitate, peptides of protein F, representing surface-exposed linear B cell epitopes, were identified and synthesized (Hughes *et al.*, 1992; Gilleland *et al.*, 1995). Three synthetic peptides, termed 9, 10 and 18, when coupled to the carrier keyhole limpet haemocyanin (KLH) elicited peptide-specific antibody that recognized whole cells of all seven Fisher-Devlin (FD) immunotypes of *P. aeruginosa* (Hughes *et al.*, 1992; Gilleland *et al.*, 1995). Subsequent studies showed that

Abbreviations: CPMV, cowpea mosaic virus; CVP, chimaeric virus particle; FCA, Freund's complete adjuvant; FD, Fisher-Devlin; IFA, incomplete Freund's adjuvant; KLH, keyhole limpet haemocyanin; NMS, normal mouse serum; OM, outer-membrane; PMN, polymorphonuclear leucocyte; wt, wild-type.

peptide 9- and 10-KLH conjugates provided protection from *P. aeruginosa* infection in a rat model of chronic pulmonary infection (Gilleland & Gilleland, 1995) and a murine acute pneumonia model (Hughes & Gilleland, 1995).

Cowpea mosaic virus (CPMV) can be genetically modified to express foreign peptides on its surface (Usha *et al.*, 1993; Porta *et al.*, 1994). Recently, we have been examining CPMV expressing OM protein F peptides on its surface as potential vaccines. These chimaeric virus particles (CVPs) 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. CPMV virions contain 60 copies of each of the L (large) and S (small) coat protein subunits. Therefore 60 copies of foreign peptide can be expressed and displayed on the surface of each virus particle (for a review see Lomonosoff & Johnson, 1991), thus obviating the need for carrier molecules such as KLH. When expressed on CPMV, a peptide derived from the envelope glycoprotein (gp41) of human immunodeficiency virus (HIV) was shown to be highly immunogenic, inducing HIV-neutralizing antibodies in mice (McLain *et al.*, 1995, 1996). Furthermore, a CPMV-based vaccine expressing a peptide from the VP2 protein of canine parvovirus was shown to protect mink from a lethal challenge with the canine-parvovirus-related mink enteritis virus (Dalsgaard *et al.*, 1997).

We have expressed peptides 10 and 18 from OM protein F of *P. aeruginosa* in tandem on both the S and L coat proteins of CPMV and have examined the immunogenicity of these protein F-based CVPs following subcutaneous immunization in mice. The protein F-based CVPs induced high titres of *P. aeruginosa*-specific IgG_{2a} that recognized all seven immunotypes of *P. aeruginosa* and enhanced the phagocytosis of the bacterium by human neutrophils *in vitro*.

METHODS

Bacterial strains and culture conditions. The strains of *P. aeruginosa* used included the following: PAO1 (serotype 0-5 by Difco typing sera), which corresponds to the Fisher-Devlin (FD) immunotype 7, ATCC 27584 (Difco 0-6; FD immunotype 1), ATCC 27313 (Difco 0-11; FD immunotype 2), ATCC 27314 (Difco 0-2; FD immunotype 3), ATCC 27315 (Difco 0-1; FD immunotype 4), ATCC 27585 (Difco 0-10; FD immunotype 5), ATCC 27579 (Difco 0-7,8; FD immunotype 6) and KG1077, which is a protein F-deficient mutant of PAO1 (Gotoh *et al.*, 1989) obtained from N. Gotoh (Kyoto, Japan). All 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-PAE4 and CPMV-PAE5 virions. The genome of CPMV consists of two molecules of single-stranded, plus-sense RNA, both of which have been cloned on separate plasmids as full-length cDNAs termed pCP1 and pCP2 (Dessens & Lomonosoff, 1993). The construction of vector pCP2-0.51 has been previously described (Dalsgaard *et al.*, 1997). To make pCP2-

PAE4, the human rhinovirus (HRV) sequences were removed from pCP2-0.51 by digestion with *NheI* and *AatII* and replaced by oligonucleotides encoding the excised wild-type (wt) CPMV sequences together with OM protein F peptide 18 linked to OM protein F peptide 10 by a short linker sequence (SG; see below) and additional OM protein F amino acids (D and AEV; see below) either side of peptide 10. The latter were included to make the peptide more amenable to presentation on the surface of CPMV. The amino acid sequence of the insert, with peptides 18 and 10 emboldened, was therefore: **NEYGVEGGRVNAVGS**GDNATA**EGR**AINRR**VEAEV**. A potential insertion site on the L coat protein subunit of CPMV was identified by superposition of the crystal structure of CPMV upon that of HRV14. There is a high degree of structural similarity between VP2 of HRV14 and the C domain of the L coat protein subunit of CPMV; however, there is a prominent difference where an extended loop on HRV14, which makes up the NIM II antigenic site (Sherry *et al.*, 1986), is much smaller on CPMV. Therefore larger loops, made by epitope insertions between amino acids 98 and 99 of the L coat protein subunit (in the β E- α B loop), should be stably accommodated by CPMV. To make pCP3-PAE5, the cauliflower mosaic virus 35S promoter-CPMV RNA2 cDNA cassette was excised from pCP2-0.51 by digestion with *SacII* and *EcoRI* and recloned into similarly digested pBluescript II SK(+) (Stratagene) to make pCP7-HRV. The HRV sequences were removed from this vector by digestion with *AatII* and *NheI* and replaced by oligonucleotides encoding the wt CPMV sequences to make pCP7. Vector pCP3 was created by oligonucleotide-directed mutagenesis of pCP7 (Kunkel, 1985) such that RNA2 nucleotides 1829, 1830, 1838 and 1843 were changed from G, G, A and T to C, C, G and C, respectively, in order to create two new unique restriction sites, *StuI* and *KpnI* (the *KpnI* site in the pBluescript II polylinker having first been removed). To make pCP3-PAE5, pCP3 was digested with *StuI* and *KpnI* and the excised fragment replaced with oligonucleotides encoding the excised wt CPMV sequences together with the OM protein F sequences described above.

Plasmids pCP2-PAE4 and pCP3-PAE5 were linearized and individually mixed with linearized pCP1 and inoculated onto young cowpea plants as described by Dessens & Lomonosoff (1993). CVP master stocks were purified from the first inoculation and passaged a second time to produce virus working stocks as described previously (Dalsgaard *et al.*, 1997). Master and working stocks were characterized by RT-PCR, sequencing and SDS-PAGE, and quantified also as described by Dalsgaard *et al.* (1997). Expression of OM protein F peptides on the surface of the CVPs was determined by ELISA, using hyperimmune sera from mice immunized with KLH-conjugated peptides (Hughes & Gilleland, 1995). Each virion expresses 60 copies of the OM protein F 18/10 peptide (Usha *et al.*, 1993), and 1 µg CVP contains approximately 40 ng of peptide. Chimaeric virus particles were purified and characterized as described previously (Dalsgaard *et al.*, 1997) and were designated CPMV-PAE4 or CPMV-PAE5 as appropriate.

Immunization of mice. Mice (aged 6–8 weeks) were obtained from Harlan-Olac and housed at the Department of Pathology, University of Cambridge. All procedures were performed according to the Home Office guidelines for animals in medical research.

In an initial experiment, groups of eight BALB/c (H-2^d) mice were immunized subcutaneously on day 0 with 100 µg CPMV-PAE4, CPMV-PAE5 or wt CPMV in Freund's complete adjuvant (FCA), with booster injections of 25 µg virus in

incomplete Freund's adjuvant (IFA) on days 14 and 28. Since titres were relatively low (< 5000) on day 42, booster injections of 25 μ g virus in QuilA were given on days 49 and 70. Three separate subcutaneous immunization experiments were performed with CPMV-PAE5 in C57BL/6 (H-2^b) mice. In a first experiment, mice (eight per group) were immunized subcutaneously on day 0 with 100 μ g CPMV-PAE5 with 20 μ g of either QS-21 (Aquila Biopharmaceuticals) or QuilA (Superfos), with booster injections of 25 μ g on days 14 and 28 in the same adjuvant as used for the initial immunization. In a second experiment, six mice received three injections of 50 μ g CPMV-PAE5 in QS-21 on days 0, 14 and 28. In a third experiment, mice (five per group) were immunized with either 25 μ g CPMV-PAE5 or 25 μ g KLH-conjugated OM protein F peptide 10 in QS-21 on days 0 and 21. In all experiments, blood was collected 2 weeks after the final booster dose by tail-bleeding or following exsanguination, and sera were collected and stored at -20°C .

ELISA for detection of serum antibody. Antibodies in sera were assayed by ELISA in microtitre plates (Dynatech; Immulon 4) coated with either 0.1 μ g CPMV per well, or with 0.5 μ g OM protein F peptides 10 or 18 (Genosys Biotechnologies) per well, or a mixture of the two peptides as described previously (Matthews-Greer & Gilleland, 1987). In some assays, plates were also coated with 0.5 μ g OM peptide 9 (Hughes *et al.*, 1992) per well as a control. ELISA plates were also coated with protein F purified from the cell envelopes of the PAO1 strain of *P. aeruginosa* as previously described (Gilleland *et al.*, 1984) or plates (Dynatech; Immulon 1) were coated with a suspension of whole *P. aeruginosa* (protein F-deficient and FD immunotypes 1–7) cells prepared according to the method of Abdillahi & Poolman (1987). Serum dilutions were incubated on the antigen-coated plates for 1 h at 37°C . Bound antibody was detected with either an alkaline-phosphatase-conjugated goat anti-mouse IgG₁ and IgG_{2a} (as a 1:1 mix or used separately) or with alkaline-phosphatase-conjugated goat anti-mouse IgG (all Southern Biotechnologies) with *p*-nitrophenyl phosphate (Sigma) as the substrate. The products were quantified with an automated ELISA reader at 405 nm. For CPMV-specific titres, the results are expressed as the end-point titre, calculated as the inverse of the dilution that gave a mean OD₄₀₅ higher than the OD₄₀₅ obtained with a 1:50 dilution of pooled serum from unimmunized mice (normal mouse serum; NMS). For OM protein F-specific titres, end-point titres were calculated as the inverse of the dilution that gave a mean OD₄₀₅ higher than the OD₄₀₅ obtained with a 1:50 dilution of pooled serum from wt CPMV-immunized mice.

Western blot analysis of sera for recognition of *P. aeruginosa* OM protein F. Western immunoblotting was performed using a 1:200 dilution of pooled sera from mice immunized with wt CPMV, CPMV-PAE4 or CPMV-PAE5. Proteins extracted from cell envelopes of strains of *P. aeruginosa* representing each of the seven FD immunotypes and from the protein F-deficient KG1077 strain, as well as purified OM protein F, were separated by SDS-PAGE, transferred to nylon membranes and the membranes developed as described by Matthews-Greer & Gilleland (1987).

C1q and C3 binding assay. C1q binding was assayed as described by Eckhardt *et al.* (1991). Briefly, microtitre plates were coated with 0.5 μ g OM protein F peptide 10 per well. After blocking non-specific binding sites, 50 μ l twofold dilutions of heat-inactivated serum from either CPMV-PAE5-, wt CPMV- or KLH-10-immunized mice or NMS were added together with 50 μ l rabbit complement (diluted 1:50; Harlan Sera-Lab). After incubation for 1 h at 37°C , the plates were

washed and C1q and C3 binding were detected using a primary goat anti-rabbit C1q or goat anti-rabbit C3 polyclonal serum (Harlan Sera-Lab), respectively, followed by secondary alkaline-phosphatase-labelled rabbit anti-goat antibody (Southern Biotechnologies) with *p*-nitrophenyl phosphate as the substrate. The products were quantified with an automated ELISA reader at 405 nm.

Assay for complement-mediated bacteriolysis. Complement-mediated antibody-dependent bacteriolysis of *P. aeruginosa* cells was performed as described by Gilleland *et al.* (1992) using *P. aeruginosa* strains FD2 and FD4. Briefly, the bacterial cells and undiluted test sera (heat-inactivated at 56°C for 30 min) were mixed and incubated for 30 min at 37°C with shaking. Next, guinea pig complement (diluted 1:32) was added and incubation at 37°C with shaking was continued for a further 1 h. Serial tenfold dilutions were plated from samples taken at time 0 and at the end of the assay to determine the number of viable bacteria present.

Assay for opsonic activity of antisera. The ability of antisera from CPMV-PAE4- and CPMV-PAE5-immunized mice to mediate the uptake of *P. aeruginosa* cells by human polymorphonuclear leucocytes (PMNs) was compared with the ability of NMS and wt CPMV-immunized mouse sera to do likewise. The assay was performed as described previously (Gilleland *et al.*, 1992). The FD immunotype 2 and 4 strains were used in the assays. Briefly, bacterial cells were mixed with undiluted heat-inactivated sera and incubated with gentle shaking at 37°C for 30 min. Human whole blood was added to the mixture and incubated for a further 30 min at 37°C . After incubation of the blood with the bacteria and antisera, slides were prepared of the mixture and treated with Giemsa's stain. Each slide was examined microscopically and the number of bacterial cells contained within the first 75 isolated, intact PMNs encountered was determined for each reaction mixture. The mean number ($\pm$ SD) of bacterial cells per PMN was calculated. The assay was performed at least twice for each antiserum. The statistical significance of the differences noted between groups was evaluated using the unpaired Student's *t*-test with $P \leq 0.05$ considered statistically significant.

RESULTS

Assembly and production of CPMV-PAE4 and CPMV-PAE5 CVPs

Initially, attempts were made to express peptides 10 and 18 individually on CPMV; however 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. Inoculation of cowpea plants with pCP1 and either pCP2-PAE4 or pCP3-PAE5 led to the establishment of infections in four out of five cowpea plants in both cases. Subsequent infection rates using virus master stocks were 100% and working stock yields were 1.2 mg (g leaf tissue)⁻¹ for CPMV-PAE4 and 1.0 mg (g leaf tissue)⁻¹ for CPMV-PAE5. These yields were equivalent to those routinely achieved with wt virus. The SDS-PAGE profiles for both CVPs showed coat protein bands (either L or S) migrating slower than the corresponding wt coat protein bands, as expected (data not shown). In addition, strand breakage on the C-terminal side of the peptide was observed for both CVPs, as has been seen with most

Fig. 1. Subcutaneous immunization with CPMV-PAE4 and CPMV-PAE5 in FCA/IFA/QuilA induces protein F peptide 10/18- and CPMV-specific serum IgG. BALB/c mice (eight per group) were immunized subcutaneously with 100 μg CPMV-PAE4 (a), CPMV-PAE5 (b) or wild-type CPMV (c) in FCA and boosted with 25 μg on days 14 and 28 using IFA, and on days 49 and 70 using QuilA. Sera were collected on day 82 and tested for peptide 10/18-specific (▨) and CPMV-specific (□) IgG_1 and IgG_{2a} by ELISA. Data are expressed as end-point titres obtained with sera from individual mice.

other CVPs made to date (Dalsgaard *et al.*, 1997; McLain *et al.*, 1995). Therefore, constructs containing inserted epitopes in the L coat protein subunit insertion site are infectious on plants and appear to have similar characteristics to those containing epitopes in the S coat protein insertion site.

Subcutaneous immunization with CPMV-PAE4 and CPMV-PAE5 induces OM protein F-specific antibody in serum which recognizes all seven major immunotypes of *P. aeruginosa*

BALB/c mice immunized with either CPMV-PAE4, CPMV-PAE5 or wt CPMV in FCA/IFA/QuilA produced very high end-point titres of CPMV-specific antibody (titres >400000 in all mice; Fig. 1a–c).

Fig. 2. Relative titres of anti-peptide 10 and anti-peptide 18 serum IgG in mice immunized with CPMV-PAE4 and CPMV-PAE5. BALB/c mice were immunized subcutaneously with 100 μg CPMV-PAE4 (a) or CPMV-PAE5 (b) in FCA and boosted with 25 μg on days 14 and 28 using IFA, and on days 49 and 70 using QuilA. Sera were collected on day 82 and tested for peptide 10- (□) or peptide 18- (▨) specific serum IgG_1 and IgG_{2a} by ELISA. Data are expressed as end-point titres obtained with sera from individual mice.

However, only the CVP-immunized mice produced antibodies specific for protein F peptides 10 and 18 (ELISA plates coated with a mixture of peptides 10 and 18). Titres generated by CPMV-PAE5 (geometric mean 11738 ± 4698) were generally higher and less variable than those generated by CPMV-PAE4 (geometric mean 1467 ± 1028). When the responses to the individual peptides were examined, CPMV-PAE4-immunized mice produced antibodies predominantly (approx. 85%) to peptide 18 with anti-peptide 10 levels much lower (Fig. 2a). In contrast, CPMV-PAE5-immunized mice produced exclusively anti-peptide 10 antibodies with no or undetectable levels of anti-peptide 18 antibody (Fig. 2b). Neither of the CVPs elicited antibody that recognized the irrelevant control peptide 9 from protein F (data not shown). The anti-peptide antibodies elicited by both CVPs also recognized the protein F whole protein (Fig. 3). Furthermore, the sera were highly reactive with all seven FD immunotypes of *P. aeruginosa*, but not with a protein F-deficient strain when examined by both whole-cell ELISA (Fig. 3) and by Western immunoblot analysis (Fig. 4). In immunoblots, the sera from the wt-CPMV-immunized mice did not react with any proteins extracted from the various immunotype strains of *P. aeruginosa* or with purified protein F (data not shown). Sera from both the CPMV-PAE4-immunized mice (Fig. 4a) and the CPMV-PAE5-immunized mice (Fig. 4b) reacted with purified protein F (lane 1) and with the protein F band in all seven immunotype strains (lanes

Fig. 3. Pooled sera from mice immunized with CPMV-PAE4 or CPMV-PAE5 specifically binds to all seven immunotypes of *P. aeruginosa* by ELISA. BALB/c mice were immunized subcutaneously with 100 µg CPMV-PAE4 (▨) or CPMV-PAE5 (▩) in FCA and boosted with 25 µg on days 14 and 28 using IFA, and on days 49 and 70 using QuilA. Sera were collected on day 82, pooled and tested for specific IgG against the OM protein F whole protein and against whole cells of all seven FD immunotypes of *P. aeruginosa*. Whole cells of a protein F-deficient (F⁻) strain were used as a control. NMS (■) and pooled wt CPMV-immunized mouse serum (□) were tested in parallel as negative controls for specific reactivity of sera. Data are expressed as end-point titres obtained with pooled sera from individual mice.

Fig. 4. Western immunoblots of pooled antisera reacted with cell envelope proteins of seven immunotype strains of *P. aeruginosa*. Lanes 1–9 contain, respectively, purified OM protein F from the PAO1 strain (lane 1), proteins extracted from FD immunotype 1–7 strains (lanes 2–8), and proteins from the KG1077 protein F-deficient strain derived from the PAO1 strain (lane 9). (a) Reaction with sera from BALB/c mice immunized with CPMV-PAE4; (b) reaction with sera from BALB/c mice immunized with CPMV-PAE5. A protein F-specific reaction is noted similar to that of the PAE4-sera.

2–8). Neither sera reacted with the proteins from the protein F-deficient KG1077 strain (lane 9), further indicating that the reactive band seen was indeed protein

F. The very faint bands seen in Fig. 4 represent non-specific background bands that have become visible due to overdevelopment of the blot to allow maximum enhancement of the protein F band.

Subcutaneous immunization with CPMV-PAE5 induces a highly polarized TH1/IgG_{2a} antibody response to OM protein F peptide 10

The CPMV-PAE5 construct elicited antibodies to the protective peptide 10 when administered with FCA/IFA/QuilA, therefore further studies were undertaken with this CVP using the saponin-based adjuvants QS-21, which has been used in human clinical trials (Kensil *et al.*, 1991, 1995), and QuilA. In addition, the isotype of the peptide-specific IgG was examined to identify any TH1/TH2 bias in the responses.

C57BL/6 mice immunized with three doses of 100, 25 and 25 µg, respectively, CPMV-PAE5 in QS-21 or QuilA (experiment 1) had high titres of protein F peptide 10-specific (Fig. 5a) and *P. aeruginosa* FD4-specific (Fig. 5b) IgG_{2a}, with low or undetectable levels of specific IgG₁. The response to the CPMV carrier was also predominantly of the IgG_{2a} isotype, although significant levels of IgG₁ were also detected (data not shown). Similar titres were achieved with three doses of 50 µg CPMV-PAE5 and QS-21 in a second experiment (Fig. 5a, b). In a third experiment, mice immunized only twice with 25 µg CPMV-PAE5 in QS-21 produced the same bias towards the IgG_{2a} isotype (Fig. 5a, b), although the titres were significantly lower ($P < 0.05$) than when three doses were used (experiments 1 and 2). Mice immunized with two doses of 10 µg peptide 10 coupled to KLH in QS-21 produced equivalent levels of peptide-specific IgG₁ and IgG_{2a} (Fig. 5a, b). The TH1-bias of the response to peptide 10 when expressed on CPMV was confirmed when the sera from the BALB/c mice immunized with CPMV-PAE5 in FCA/IFA/QuilA were also found to contain predominantly peptide-specific IgG_{2a} (data not shown).

Fig. 5. Subcutaneous immunization with CPMV-PAE5 induces OM protein F peptide 10-specific IgG_{2a} with little or no specific IgG₁. C57BL/6 mice were immunized with CPMV-PAE5 in three separate experiments. Experiment 1, 100 µg CPMV-PAE5 in QS-21 or QuilA ($n = 8$ per group) on day 0 and boosting with 25 µg on days 14 and 28; experiment 2, 50 µg CPMV-PAE5 in QS-21 on days 0, 14 and 28 ($n = 6$); experiment 3, 25 µg of either CPMV-PAE5 or peptide 10–KLH conjugate in QS-21 on days 0 and 21 ($n = 5$ per group). Sera were collected on day 42 and tested for peptide 10-specific (a) and *P. aeruginosa* FD4 whole-cell-specific (b) IgG₁ (□) and IgG_{2a} (▨) by ELISA. Data are expressed as geometric mean end-point titres $\pm$ SD obtained with the sera from mice within each group.

OM protein F-specific antibody in CPMV-PAE5-immunized mice fixes complement C1q and C3 but does not induce complement-mediated bacteriolysis

The peptide 10-specific antibody in sera from both CPMV-PAE5-immunized C57BL/6 and BALB/c mice was able to bind both complement components C1q and C3 when examined by ELISA (Fig. 6; C57BL/6 results shown for the three individual immunization experiments). However, these sera failed to induce complement-mediated bacteriolysis (data not shown). In none of the various complement-mediated bacteriolysis assays performed was a noteworthy decrease in the number of bacterial cells surviving exposure to the sera-complement mixture observed with the CPMV-PAE4 or CPMV-PAE5 sera compared to the negative control sera.

CPMV-PAE5-immunized mouse sera augments phagocytosis of *P. aeruginosa*

The CPMV-PAE5-immunized mice produced sera which were significantly more opsonic than the control sera in BALB/c mice, as well as in C57BL/6 mice when QS-21 was used as the adjuvant (more opsonic in three of four

Fig. 6. CPMV-PAE5-immunized mouse sera fix complement components C1q and C3. C57BL/6 mice were immunized subcutaneously with CPMV-PAE5 in three separate experiments. Experiment 1, 100 µg CPMV-PAE5 in QS-21 or QuilA ($n = 8$ per group) on day 0 and boosting with 25 µg on days 14 and 28; experiment 2, 50 µg CPMV-PAE5 in QS-21 on days 0, 14 and 28 ($n = 6$); experiment 3, 25 µg of either CPMV-PAE5 or peptide 10–KLH conjugate in QS-21 on days 0 and 21 ($n = 5$ per group). Sera were collected on day 42 and tested for peptide 10-specific-antibody that could bind complement components C1q (□) and C3 (▨) by ELISA. NMS ($n = 4$) and wt CPMV-immunized sera ($n = 6$) were tested in parallel. Complement binding is expressed as geometric mean OD₄₀₅ $\pm$ SD obtained with a 1:20 dilution of serum from the mice in each group.

assays), but not in C57BL/6 mice when QuilA was used as the adjuvant (more opsonic in none of four assays) (Table 1). The CPMV-PAE5-sera were opsonic for both FD2 and FD4 cells. The importance of the adjuvant used in eliciting functional antibodies is indicated by the relative opsonic activities of the CPMV-PAE5-elicited antibodies in the C57BL/6 mice upon use of QS-21 versus QuilA as adjuvant (Table 1). Sera from CPMV-PAE5-immunized BALB/c mice gave statistically significant opsonic activity in 11 of 11 assays compared to sera from wt CPMV-immunized mice.

DISCUSSION

In this study we have demonstrated that the plant virus CPMV is a highly effective carrier of peptides from OM protein F of *P. aeruginosa* for the generation of *P. aeruginosa*-specific IgG in mice. Two peptides (10 and 18) representing linear B cell epitopes from protein F of *P. aeruginosa* were expressed in tandem on either the S or L coat proteins of CPMV. Five doses totalling 200 µg of the two chimaeric viruses in FCA/IFA/QuilA, constituting ~ 8 µg of the tandem peptide, elicited high titres of protein F-specific IgG which recognized all seven FD immunotypes of *P. aeruginosa*. Although the display of peptides on the S subunit of CPMV has been described (McLain *et al.*, 1995, 1996; Dalsgaard *et al.*, 1997), this is the first report of the expression of an immunogenic peptide on the L subunit of CPMV. Interestingly, the expression of the peptide on the L subunit elicited antibodies predominantly to peptide 10, whereas expression on the S subunit induced a response predominantly directed to peptide 18. The reasons for

Table 1. Quantification of phagocytic uptake of *P. aeruginosa* by PMNs exposed to antisera from BALB/c and C57BL/6 mice

Mice	Bacterial strain	Test serum	Mean number of bacteria per PMN $\pm$ SD†	
			Expt 1	Expt 2
BALB/c	FD2	NMS	6.0 $\pm$ 6.4	5.2 $\pm$ 4.8
		wt CPMV	5.7 $\pm$ 6.2	4.8 $\pm$ 5.0
		CPMV-PAE4	7.9 $\pm$ 7.6*	4.6 $\pm$ 5.2
		CPMV-PAE5	9.6 $\pm$ 9.2**	8.5 $\pm$ 10.4**
BALB/c	FD4	NMS	11.1 $\pm$ 5.9	11.9 $\pm$ 7.0
		wt CPMV	11.8 $\pm$ 7.3	9.0 $\pm$ 6.3
		CPMV-PAE4	13.5 $\pm$ 10.1	11.1 $\pm$ 7.5
		CPMV-PAE5	17.6 $\pm$ 11.2**	17.5 $\pm$ 11.7**
C57BL/6	FD2	NMS	4.6 $\pm$ 6.4	5.2 $\pm$ 5.0
		CPMV-PAE5 (QS-21)	7.3 $\pm$ 10.4*	6.4 $\pm$ 9.2
		CPMV-PAE5 (QuilA)	5.9 $\pm$ 5.0	4.2 $\pm$ 3.8
C57BL/6	FD4	NMS	12.3 $\pm$ 8.2	7.5 $\pm$ 5.7
		CPMV-PAE5 (QS-21)	18.0 $\pm$ 11.8**	11.3 $\pm$ 8.8**
		CPMV-PAE5 (QuilA)	12.7 $\pm$ 9.5	8.8 $\pm$ 6.0

† P-value determined by unpaired Student's *t*-test, by comparison with wt CPMV-antisera for BALB/c mice and with NMS for C57BL/6 mice. *, significantly different from control at $P = 0.05$; **, significantly different from control at $P < 0.001$.

the differential recognition of the peptides on the L and S proteins are unclear. The peptides may adopt different conformations when presented on the different subunits, giving rise to differing exposures for antibody recognition and binding. The peptide in the S coat protein insertion site is highly exposed at the tip of the fivefold axis of the virus and can therefore freely interact with the solvent, whereas the peptide in the L coat protein insertion site is located in a 'valley' on the viral surface where it is likely that residues on neighbouring surfaces of the virus particle may influence its conformation. Titres in serum were substantially less to the peptide than to the CPMV (mean titres to the peptide approximately 40-fold lower), probably because the CPMV virion contains multiple epitopes, both linear and conformational, while the expressed peptide comprises two linear epitopes only. Since the CPMV-PAE5 construct elicited IgG to peptide 10, which has been shown to protect mice from *P. aeruginosa* infection (Hughes & Gilleland, 1995), further studies were performed with this CVP.

Since FCA has never been registered for use in humans (Gupka & Siber, 1995), less toxic adjuvants were sought that could also elicit high titres of peptide-10-specific antibody. The saponin-based adjuvant QS-21, believed to be a less toxic but adjuvant-active fraction of QuilA, has been used in human clinical trials (Kensil *et al.*, 1991, 1995) and may gain FDA approval for human administration in the USA.

C57BL/6 mice immunized three times with CPMV-PAE5 in either QS-21 or QuilA elicited even higher and

more consistent anti-peptide 10 IgG titres than those seen in BALB/c mice given five injections of CPMV-PAE5 in FCA/IFA/QuilA. When only two doses were used, the peptide 10-specific titres were substantially lower, implying that three immunizations may be necessary to elicit high titres. Indeed previous studies have shown that at least four immunizations of KLH-peptide 10 are required to generate high peptide-specific titres (Gilleland *et al.*, 1995).

When the isotype of the anti-peptide 10 IgG was examined in the BALB/c mice immunized with CPMV-PAE5 and in C57BL/6 mice immunized with CPMV-PAE5 in either QS-21 (varying immunization regimens) or QuilA, it was found to be exclusively IgG_{2a} with no detectable IgG₁. IgG_{2b} was also detected but at lower levels than IgG_{2a} (data not shown). This indicates that CPMV-PAE5 induces a highly polarized activation of CPMV-specific T cells of the TH1 subset that are inducing protein F-specific B cell class-switching to IgG_{2a} production. In support of this, spleen cells from CVP-immunized mice produce high levels of interferon- γ and no IL-4 when stimulated with CPMV *in vitro*, further supporting the idea that CVPs prime predominantly CPMV-specific T cells of the TH1 subset (F. R. Brennan & T. Bellaby, unpublished observations). C57BL/6 mice immunized with peptide 10 conjugated to KLH and administered in QS-21 induced equivalent amounts of peptide 10-specific IgG₁ and IgG_{2a}, implying that there is no biasing of the responses toward a particular T helper cell subset when KLH is used as a carrier of peptide 10. The theory that the type of adjuvant can skew the response towards a particular T

helper cell subset (for a review see Cooper, 1994) did not seem to hold true in these experiments. QuilA and QS-21 are thought to amplify TH2 responses (Gupka & Siber, 1995; Kensil *et al.*, 1995), and although enhanced TH1 responses have been achieved with QS-21 (Kensil *et al.*, 1995), a strong TH1 bias toward peptide 10 was observed in the studies described here. Also, BALB/c mice are known to favour TH2-type responses (Natsuume-Sakai *et al.*, 1977), and yet there was a strong peptide 10-specific TH1 response in these mice. Other peptides from HIV-1, *Staphylococcus aureus* and human chorionic gonadotrophin have also shown a strong TH1/IgG_{2a} bias when expressed on CPMV (F. R. Brennan, unpublished observations). When a purified *P. aeruginosa* protein I vaccine was evaluated recently in humans (von Specht *et al.*, 1996), there was no preferential increase in any subclass of protein I-specific human IgG. It remains to be seen if the highly polarized TH1 response to CPMV-PAE5 applies to other animal species and to man.

The TH1 response elicited by CPMV-PAE5 is of major consequence for a *P. aeruginosa* vaccine. IgG_{2a} is the major IgG isotype in mice that facilitates both antibody-dependent phagocytosis and fixes complement leading to complement-mediated phagocytosis and potentially to bacteriolysis (Huesser *et al.*, 1977; Klaus *et al.*, 1979). Hence, CPMV-PAE5-immunized sera were shown to fix complement components C1q and C3. However, the sera did not induce complement-mediated bacteriolysis. We have shown previously that mice immunized with recombinant OM protein F produce specific antibody that also failed to induce complement-mediated bacteriolysis of *P. aeruginosa* (Gilleland *et al.*, 1992). Likewise, selected monoclonal antibodies to protein F have been reported to mediate opsonic complement-independent phagocytosis but not to promote complement-mediated bactericidal killing of *P. aeruginosa* (Battershill *et al.*, 1987; Hancock *et al.*, 1985). Since lysis is dependent on the antigen density, the spacing between the antigens and their relative orientation, and the distance from the cell surface (Sandlie & Michaelsen, 1991), it is possible that not all of these conditions are optimal for OM protein F to induce bacteriolysis. The binding of C1q and C3 to peptide 10-specific IgG_{2a}, however, should result in C3b production *in vivo* which would opsonize the bacteria for phagocytosis by C3b receptor (CR1 and CR3)-bearing neutrophils. Serum from C57BL/6 mice immunized with CPMV-PAE5 in QS-21 (but not in QuilA) facilitated opsonic phagocytosis of *P. aeruginosa* by human neutrophils, implying that the choice of adjuvant will be important in the formulation of a protective vaccine.

Although it seems reasonable to assume that the generation of specific lung antibody would be required to acquire maximum protection from an organism such as *P. aeruginosa* that causes respiratory infections, the actual importance of high levels of *P. aeruginosa*-specific IgA and IgG in the lung for protection from bacterial challenge is unclear. Others have shown using an acute model of infection that anti-protein F antibody did not

correlate with protection (Cripps *et al.*, 1994). However, the clearance of *P. aeruginosa* from the lung correlates with levels of protein F-specific antibody in alveolar secretions (Cripps *et al.*, 1995). Furthermore, it has previously been demonstrated that combined intranasal/parenteral vaccination with KLH-conjugated protein F peptides induced high titres of *P. aeruginosa*-specific serum antibody and, despite there being little or no *P. aeruginosa*-specific IgA or IgG in the lungs of these mice prior to challenge, they were still protected from a lethal *P. aeruginosa* infection (Hughes & Gilleland, 1995). Hence, it is most likely that the opsonic serum IgG_{2a} 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). Vaccination against whooping cough and influenza are routinely performed via the intramuscular route and high level protection is achieved to these organisms that infect the respiratory tract. Hence, the preferred route of delivery for a *Pseudomonas* vaccine may be the one that elicits maximum level of specific antibody, either in serum or mucosa. Preliminary studies have shown that intranasal immunization of mice with CPMV-PAE5, with or without cholera toxin, fails to elicit OM protein F-specific lung IgG or IgA and levels of specific serum antibody were lower than those achieved following subcutaneous immunization (F. R. Brennan & H. E. Gilleland, unpublished observations). Therefore parenteral immunization may be the preferred route of delivery for CPMV-PAE5.

In conclusion, we have shown that *P. aeruginosa* OM protein F peptides are highly immunogenic when expressed on CPMV. Since these plant-virus-derived vaccines have the potential for cost-effective manufacture and are not known to infect mammalian cells, they have the potential for further development as a protective vaccine against human *P. aeruginosa* infections. Studies to determine the protective capacity of CPMV-PAE5 in the mouse models of chronic (Gilleland & Gilleland, 1995) and acute (Hughes & Gilleland, 1995) pneumonia are planned. In addition, further constructs containing peptides derived from other *P. aeruginosa* surface proteins are being considered for display on CPMV together with protein F peptide 10.

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