Humoral and cellular immune responses in sheep immunized with a 22 kilodalton exported protein of *Mycobacterium avium* subspecies *paratuberculosis*

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An immunogenic 22 kilodalton exported *Mycobacterium avium* subspecies *paratuberculosis* (MAP) lipoprotein (P22) was previously identified, and found to belong to the LppX/LprAFG family of mycobacterial lipoproteins. N-terminal polyhistidine-tagged P22 was produced and purified from *Escherichia coli*. Antibody recognition of P22, and interferon-gamma (IFN- γ) responses *in vitro* using blood from a sheep vaccinated with Neoparasec, confirmed its immunogenicity. To evaluate the immunogenicity of P22 *in vivo*, five sheep were immunized with a single dose containing 0.8 mg recombinant P22 protein in adjuvant. Blood was collected at 4, 13 and 29 weeks post-immunization (p.i.) and tested for anti-P22 antibodies and P22-specific IFN- γ production. P22-specific antibodies were detected by Western blot analysis in all five Neoparasec-immunized sheep at the three time points. Three out of five P22-immunized sheep produced P22-specific antibodies for up to 13 weeks p.i., and two gave a response at 29 weeks p.i. Recombinant P22 was able to stimulate significant IFN- γ production in blood of P22-immunized sheep at 13 and 29 weeks p.i. Recombinant P22 also elicited an IFN- γ response in blood of sheep immunized with Neoparasec.

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INTRODUCTION

Ruminant animals are susceptible to Johne's disease (JD), which is a chronic inflammatory condition of the gut caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP) (Kurade *et al.*, 2004; Sigurethardottir *et al.*, 2004). The disease has a global distribution and reduces food production in both developed and developing countries. For most animals the disease is restricted to a subclinical infection; however, animals that progress to clinical disease rapidly lose condition and die due to a lack of nutrients being absorbed from the gut (Collins, 2003). Infected animals

shed MAP in the faeces, which contaminates the environment and increases the risk of other animals becoming infected.

Control of JD can be assisted by vaccination of animals with either whole killed (Gudiar, CSL) or attenuated (Neoparasec, Merial) MAP (Emery & Whittington, 2004; Harris & Barletta, 2001). Although these vaccines have been shown to be very useful tools in JD-control programmes, they are inadequate, because they induce a severe local immune reaction at the site of injection (Collett & West, 2001) and more importantly, they do not prevent infection or shedding of MAP in the faeces (Emery & Whittington, 2004; Kalis *et al.*, 2001). As a result, progressive increases in the incidence of clinical disease would inevitably follow the cessation of vaccination programmes. Additionally, the immune responses generated by these whole-cell vaccines interfere with tests to identify MAP- and *Mycobacterium tuberculosis* (MTB)-infected animals (Klawonn *et al.*, 2002; Kohler *et al.*, 2001; Muskens *et al.*, 2002). These limitations have highlighted the need for the development of an improved JD vaccine.

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Abbreviations: Ab, antibody; Ag, antigen; dH₂O, deionized water; HRP, horseradish peroxidase; IFN- γ , interferon-gamma; JD, Johne's disease; MAP, *Mycobacterium avium* subspecies *paratuberculosis*; MTB, *Mycobacterium tuberculosis*; P22, 22 kDa exported MAP protein; p.i., post-immunization; PPDA, avian purified protein derivative; RT, room temperature.

Secreted and cell-surface proteins of MAP are major immune targets. For example, the 19 kilodalton (kDa) lipoprotein (Huntley *et al.*, 2005), the 16·7 kDa protein (Mullerad *et al.*, 2003), the 85B antigen (Mullerad *et al.*, 2002b), superoxide dismutase (Mullerad *et al.*, 2002a) and MPP14 (Olsen & Storset, 2001) have been investigated to assess their diagnostic and vaccine potential. Recently, we have identified a 22 kDa exported MAP protein (P22) belonging to the LppX/LprAFG family of mycobacterial lipoproteins. Recombinant P22 protein expressed under the control of the mycobacterial β -lactamase promoter in *Mycobacterium smegmatis* was recognized by antibodies (Abs) from blood of Neoparasec-vaccinated sheep and induced interferon-gamma (IFN- γ) secretion (Dupont *et al.*, 2005).

In this study, we overexpressed P22 in an *Escherichia coli* expression system and demonstrated that purified protein retained immunogenicity when used in *in vitro* assays. In addition, we demonstrated that P22 can elicit humoral and cell-mediated (IFN- γ) responses when injected into sheep.

METHODS

Bacterial strains and plasmids. The strains and plasmids used in this study are shown in Table 1.

Culture media and reagents. *E. coli* strains were grown in Luria broth (LB) or on LB agar plates. When used, antibiotics were added to concentrations of 100 μ g ampicillin ml⁻¹, 50 μ g chloramphenicol ml⁻¹ and 30 μ g kanamycin ml⁻¹.

PCR and cloning methodology. Platinum *PfuTurbo* DNA polymerase (Stratagene) was used for all PCR reactions with the following conditions: 1 cycle at 95 °C for 10 min followed by 30 cycles consisting of 1 min at 95 °C, 1 min at 60 °C and 1 min at 72 °C. The primers p22-SF (ggg aat tcc ata tgt tga tgc ccg gct gct cgt cgg gc) and p22-SR (ggg aat tcc ata tgt cac gag ctc acc ggg ggc ttg gtg) were used to amplify the gene encoding P22 (minus the 57 bp signal sequence) from pMIP-p22. The PCR product was cloned as an *NdeI* fragment into pET14b using standard cloning techniques. The two primers introduced *NdeI* sites to the N-terminal- and C-terminal-encoding portions, respectively, of p22. The recombinant protein was expressed in pET14b with six histidines at the N terminus. The plasmid pAM1 was cloned and maintained in *E. coli* XL-1 Blue.

pAM1 was sequenced to verify that no mutations had occurred during PCR amplification.

Expression of P22 from pAM1. For recombinant protein expression, pAM1 was transformed into *E. coli* BL-21 CodonPlus (DE3) RP. A single colony was picked from an LB agar plate containing ampicillin and chloramphenicol and grown overnight at 37 °C with shaking at 250 r.p.m. LB broth (500 ml) containing ampicillin was inoculated with 1% (v/v) of the overnight culture. This culture was grown at 37 °C with shaking at 250 r.p.m. for 2 h, and then IPTG was added to a final concentration of 1 mM. Following induction for 3 h at 37 °C with shaking at 250 r.p.m., the cell pellet was harvested by centrifugation.

Purification of P22. The pelleted cells were resuspended in 100 ml lysis buffer containing 6 M guanidine hydrochloride, 20 mM sodium phosphate and 500 mM sodium chloride at a final pH of 7·8. The mixture was rocked at room temperature (RT) for 5–10 min and then sonicated on ice with three 5 s pulses. Insoluble debris was removed by centrifugation at 3000 g for 15 min. The supernatant was added to pre-equilibrated ProBond nickel-chelating resin (Invitrogen) and rocked for 10 min at RT. The mixture was then centrifuged at 800 g, and the supernatant removed. All buffers [binding (pH 7·8), washing (pH 6·0 and pH 5·3) and elution (pH 4·0)] contained 8 M urea, 20 mM sodium phosphate and 500 mM sodium chloride. Binding buffer (pH 7·8) was added at a ratio of 4 ml for every 5 ml of lysate added to the resin. The mixture was incubated at RT with gentle rocking for 2 min, followed by centrifugation at 800 g and removal of the supernatant. This wash procedure was repeated four times, using wash buffer at pH 6·0 for the first two washes and wash buffer at pH 5·3 for the final two washes. Recombinant P22 was then eluted from the resin by adding 5 ml elution buffer (binding buffer at pH 4·0) for every 5 ml of lysate. Resin-free fractions were obtained by adding the mixture to a gravity-flow column, or by centrifugation at 800 g. Eluted fractions and wash samples were electrophoresed in 12% SDS-PAGE gels and stained with Coomassie blue (Bio-Rad). Those fractions considered to be free of any significant amount of *E. coli* were pooled together. To remove the urea and obtain soluble P22, the pooled fractions were dialysed against six changes of PBS containing decreasing amounts of urea (from 8 to 0 M).

Protein concentration was determined using the Bio-Rad protein assay. Protein preparations were further concentrated using Centriplus (10 000 Da molecular mass cut-off) centrifugal filter devices (Amicon, Millipore).

Animals and immunization. Sixteen 8-month-old New Romney (castrated males) sheep were grazed on MAP-free pasture throughout

Table 1. Strains and plasmids used in the study

Strains and plasmids	Description/genotype	Source/reference
Strains		
MAP strain 316F	Attenuated vaccine strain (Neoparasec)	Meriel
<i>E. coli</i> XL-1 Blue	<i>RecA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac[F' proAB lacI^qΔM15Tn10(Tet^r)]</i>	Stratagene
<i>E. coli</i> BL-21 CodonPlus (DE3) RP	<i>E. coli</i> B F ⁻ <i>ompT hsdS(r_B⁻ m_B⁻) dcm⁺ Tet^r gal^l (DE3) endA Hte [argU proL Cam^r]</i>	Stratagene
Plasmids		
pET14b	Amp ^R ; <i>E. coli</i> expression vector	Novagen
pAM1	pET14b containing the <i>p22 NdeI</i> fragment	This work
pMIP-p22	pMIP12 containing the <i>p22</i> ORF	Dupont <i>et al.</i> (2005)

the trial. The sheep were randomly separated into three groups and numbered as follows: Neoparasac immunized (811, 813, 817, 819, 821), non-immunized (control) (808, 823, 830, 834, 863, 869) and P22 immunized (820, 825, 828, 829, 848). Each sheep was injected subcutaneously in the neck with one dose of one of the following: 1 ml commercial vaccine (Neoparasac, Merial) containing live attenuated MAP formulated in water-in-oil emulsion adjuvant, or 2 ml recombinant P22 containing 0.8 mg protein mixed 1:1 (v/v) with the same adjuvant.

After immunization, the sheep were monitored for any signs of adverse reactions at the site of the injection. They were also weighed 1 month prior to immunization, and 5 weeks and 8 months post-immunization (p.i.). To obtain a baseline reference of the animals' immune response towards P22, heparinized and non-heparinized blood samples were collected 1 month prior to immunization (pre-bleed) and tested for IFN- γ production and the presence of anti-P22 Abs (see Methods, IFN- γ assay, and Methods, Western immunoblotting, below).

IFN- γ assay. IFN- γ assays were performed using the whole blood Bovigam EIA bovine interferon test (Commonwealth Serum Laboratories), as previously described (Dupont *et al.*, 2005). The following antigens (Ags) pre-diluted in sterile deionized water (dH₂O) were used: avian purified protein derivative (PPDA; CSL, 12.56 $\mu\text{g ml}^{-1}$, positive control), PBS (negative control), P22 (10 $\mu\text{g ml}^{-1}$) and concavalin A (Con A; Sigma, 20 $\mu\text{g ml}^{-1}$, non-specific T-cell mitogen to verify cell viability). The results were expressed as corrected individual mean A_{450} units of the duplicate samples after the mean negative-control A_{450} units were subtracted from the mean sample A_{450} units.

Statistical analysis. IFN- γ results of wells treated with PPDA Ag and P22 Ag were analysed using the MIXED procedure of the Statistical Analysis system package, version 9.1 (SAS Institute). The linear model included the fixed effects of treatment (Neoparasac immunized, non-immunized and P22 immunized), week and their interaction, and the random effect of animal within treatment (Littell *et al.*, 1998). Using the Akaike's information criterion, a compound symmetry error structure was determined as the most appropriate residual covariance structure for repeated measures over the week within animals. Least square means and their standard errors were obtained for each treatment for weeks 4, 13 and 29, and used for multiple comparisons between treatment effects.

Western immunoblotting. Purified P22 (6.12 μg per gel) was denatured in loading buffer containing β -mercaptoethanol and electrophoresed on 12% SDS-PAGE gels. Following separation, the protein was blotted onto PVDF membranes (Pall) using a Trans-Blot Semi-Dry Transfer Cell (Bio-Rad) and stained with Ponceau S to verify that protein transfer had taken place. After destaining with dH₂O, the membranes were cut into strips and blocked overnight at 4 °C in blocking buffer containing 0.1% (v/v) Tween 20, 10 mM Tris (pH 7.4), 150 mM NaCl and 5% (w/v) skimmed milk powder. Fresh blood obtained from P22-, Neoparasac- and non-immunized sheep was allowed to coagulate, and the serum was collected after centrifugation at 3000 *g* for 15 min at RT. Serum samples, diluted 1:500 in blocking buffer, were added separately to membrane strips and incubated for 1 h at RT with continuous agitation. The strips were then washed three times for 10 min with wash buffer [0.1% (v/v) Tween 20, 10 mM Tris (pH 7.4), 150 mM NaCl] and incubated in goat anti-sheep IgG horseradish peroxidase (HRP)-conjugated Ab (Sigma, diluted 1:40 000) for 1 h at RT. For the detection of histidine-tagged P22, anti-hexahistidine HRP-conjugated Ab (Roche Molecular Biochemicals) was added at 1:500, incubated for 1 h at RT and washed as previously described. Development by chemiluminescence was performed using SuperSignal West Femto

Maximum Sensitivity Substrate (Pierce Scientific) according to the manufacturer's instructions, prior to exposure to radiographic film (BioMax MR, Kodak) for 10 s.

RESULTS AND DISCUSSION

We have previously identified P22 as an immunogenic exported lipoprotein of MAP (Dupont *et al.*, 2005). Purified P22 produced in the heterologous host *M. smegmatis* elicited IFN- γ secretion in blood from sheep vaccinated with the live attenuated MAP strain 316F, and vaccinated animals also produced Ab that recognized P22 in Western blot assays. In addition, Abs were detected in cattle naturally infected with MAP. This work indicated that the P22 protein was an immunogenic component of the vaccine strain and wild-type field strains. To extend this work, we have cloned the gene encoding P22 into an *E. coli* expression system in order to produce sufficient quantities to undertake immunization studies in sheep. Overexpression of P22 in *E. coli* was achieved after 3 h induction with IPTG (Fig. 1). The recombinant P22 was found to be in the insoluble fraction of the cell pellet. Denaturing purification was performed using Probond resin in the presence of guanidine-HCl and urea. Protein was obtained in soluble form by gradually reducing the amount of urea from 8 to 0 M, by dialysis in PBS over a 42 h period.

Fig. 1. Expression of recombinant P22 from *E. coli*. One colony of transformed BL-21 CodonPlus (DE3) RP cells harbouring pAM1 was inoculated in 5 ml LB containing chloramphenicol and ampicillin. After overnight incubation at 37 °C with shaking at 250 r.p.m., 50 μl culture was added to 1 ml fresh LB containing ampicillin alone and incubated under the same conditions for 2 h. An 80 μl sample was removed (uninduced, UI) and stored at 4 °C for further analysis. IPTG (1 mM final concentration) was added to the remaining cells and incubated for a further 3 h. An 880 μl portion of sample was then removed (induced, I). Both samples were then centrifuged, and pellets were resuspended in 20 μl loading buffer containing β -mercaptoethanol and heated at 95 °C for 5 min before electrophoresing on a 12% SDS-PAGE gel. The numbers to the left of lane M (broad range molecular weight marker, Biolabs) indicate the protein size in kDa.

In vitro evaluation of P22 immunogenicity

To establish if P22 retained immunogenicity when produced in *E. coli*, the recombinant protein was tested in Western blot analysis and IFN- γ assays using sera and cells obtained from a sheep vaccinated with Neoparasac and a control non-immunized animal. A strong band corresponding to a molecular mass of 22 kDa was seen using the serum from the vaccinated but not from the control animal (data not shown). P22 also stimulated IFN- γ production in peripheral blood mononuclear cells from the Neoparasac-vaccinated sheep but not from the control animal (data not shown). These results indicated that recombinant P22 produced in *E. coli* retained immunogenicity and was capable of stimulating P22-specific IFN- γ production from blood, and that it was recognized by Abs from a sheep sensitized to MAP antigens.

In vivo evaluation of P22 immunogenicity

IFN- γ production. The group of sheep used to investigate the immunogenicity of P22 *in vivo* were initially tested to ensure that they did not have any reactivity against mycobacterial antigens by testing the responses of peripheral blood to PPDA in IFN- γ assays. Testing of sheep showed no significant reactivity to PPDA prior to vaccination (data not shown), and there was no significant difference between the animal groups in weight gain during the trial (data not shown). All the Neoparasac-immunized animals developed large lumps approximately 3 cm wide at the injection site which lasted for over 6 months. In contrast, two out of the five P22-immunized animals showed a swelling at the injection site that disappeared within 2 months.

The IFN- γ responses of blood from the sheep groups were tested against PPDA and P22 at 4, 13 and 29 weeks p.i. The group means and individual animal responses are shown in Fig. 2. As expected, the animals immunized with Neoparasac gave a significant response ($P < 0.05$) to PPDA at the three time points (Fig. 2a–c). Notably, these animals also made a response to P22 at the same time points. Following P22 stimulation, the mean difference in IFN- γ production between P22-immunized animals and controls was significant ($P < 0.05$) at 13 and 29 weeks p.i. (Fig. 2b, c).

Antibody reactivity

At 4 weeks p.i., visible bands of reactivity against P22 could be observed with sera from three out of five P22-immunized animals (Fig. 3a). These reactions were seen with sera from animals 820, 825 and 828. By 13 weeks p.i., only animals 820 and 828 had a detectable Ab reactivity (Fig. 3b) that was maintained at 29 weeks p.i. (Fig. 3c). Neoparasac-vaccinated animals all showed detectable Ab recognition of P22 at all three time points (Fig. 3a–c). Animal 817 gave the strongest band of Ab reactivity towards P22 Ag in the Neoparasac group, and this was maintained at both 13 and 29 weeks p.i. The intensity of the bands for all the animals in this group was consistent over the three time points. No

Fig. 2. IFN- γ induction of blood from sheep immunized with Neoparasac or recombinant P22. Whole blood from sheep immunized with Neoparasac (squares), P22 (triangles) or non-immunized negative control sheep (circles) at (a) 4 weeks, (b) 13 weeks and (c) 29 weeks p.i. was incubated with either PPDA (12.56 $\mu\text{g ml}^{-1}$, open symbols) or P22 (10 $\mu\text{g ml}^{-1}$, filled symbols) in duplicate wells. IFN- γ assays were performed as described in Methods. The corrected mean A_{450} was calculated using the formula (mean sample A_{450} value – mean PBS control A_{450} value). The bars represent the group mean. Note that at 4 weeks p.i., the blood from two sheep only was stimulated with P22 antigen in the Neoparasac and non-immunized groups.

Fig. 3. Detection of Ab against Ni^{2+} affinity-enriched P22 in sheep immunized with either Neoparasec or recombinant P22. Western blots of 6–12 μg recombinant P22 were incubated with a 1 : 500 dilution of serum from sheep either immunized with Neoparasec or non-immunized, at (a) 4 weeks, (b) 13 weeks and (c) 29 weeks p.i. Reactivity was detected using a 1 : 40 000 dilution of goat anti-sheep IgG HRP-conjugated Ab. The number on the left indicates the protein size in kDa.

P22-specific Ab recognition was detected in the non-immunized control animals at 4, 13 and 29 weeks p.i.

Overview of results

The Western blots and $\text{IFN-}\gamma$ assays confirm that P22 is an immunologically active component of the 316F attenuated strain of MAP, and that P22 produced in *E. coli* retains determinants of its B-cell and T-cell activity. It is well known that some mycobacterial proteins display reduced immunological activity when expressed in an *E. coli* vector. MTB heparin-binding haemagglutinin (HBHA) requires a complex methylation pattern of the C-terminal domain for induction of T-cell immunity. These methyl groups are absent in the recombinant form of HBHA produced by *E. coli* (Temmerman *et al.*, 2004). The demonstration that soluble immunologically active P22 can be produced in *E. coli* will facilitate studies with this protein, as large-scale production and purification can be easily achieved using standard techniques.

Vaccination of sheep with P22 induced both humoral and cell-mediated immune responses in some animals as early as 4 weeks p.i. The animals which gave the highest $\text{IFN-}\gamma$ response to P22 Ag at 4 weeks (animals 820, 825 and 828) also produced good Ab reactivity towards P22 at that time point. Whilst animals 820 and 828 maintained a high $\text{IFN-}\gamma$ and Ab response at 29 weeks, the Ab reactivity of animal 825 was not detectable at 13 weeks, and the $\text{IFN-}\gamma$ response had diminished by 29 weeks. Similarly, Neoparasec-vaccinated animal 817 gave the highest $\text{IFN-}\gamma$ response to P22 at 4, 13 and 29 weeks p.i., corresponding to the strongest Ab reactivity to this Ag at the same time points.

Protective immune responses against MAP are considered to be cell mediated, but the role (if any) of Ab is unknown. A recent study to identify immune markers of protection afforded by the Neoparasec vaccine in sheep has shown that an early and strong cell-mediated immune response ($\text{IFN-}\gamma$) is important in determining disease outcome (Begg & Griffin, 2005). However, this response is also accompanied by an early Ab response, although the contribution of this humoral response to protection has yet to be elucidated. Infection studies performed in a mouse model using MTB or *Mycobacterium bovis* preincubated with mycobacterial-specific monoclonal Abs have shown in some cases an enhanced survival of mice (Chambers *et al.*, 2004; Teitelbaum *et al.*, 1998) and reduced dissemination of bacteria (Pethe *et al.*, 2001). Although this evidence implies that anti-mycobacterial Abs could have a beneficial role in protection, this has yet to be investigated in a sheep model against MAP infection.

There is no information on the biological function of P22, so the importance and relevance of this protein in MAP pathogenesis is purely speculative. *In silico* comparison of P22 with other mycobacterial proteins produced an alignment (68 % identity) with a 27 kDa lipoprotein LprG precursor which has been shown by other workers to be biologically active. Gehring *et al.* (2004) have shown that prolonged incubation of macrophages with the 27 kDa LprG lipoprotein inhibits TLR-2-dependent major histocompatibility complex-II (MHC-II) antigen processing. This may be an immune-evasion mechanism evoked by MTB. As a consequence of the lack of MTB Ag presentation via the MHC-II, there would be a down-regulation of the activity of CD4^{+} T lymphocytes and the ensuing adaptive immune response. The 27 kDa lipoprotein may be linked to

virulence, as knocking out the *LprG-Rv1410* operon in MTB results in a 2.5-log decrease in bacterial load in mice (Bigi *et al.*, 2004). Hovav *et al.* (2003) have demonstrated that despite a good Th-1 immune profile considered important for protection, 27 kDa protein-vaccinated mice are not protected against BCG or MTB challenge. The relevance of these studies to P22 is yet to be determined, but it will be interesting to undertake similar work in appropriate model systems. The pathogenesis of MAP infection in the gut is quite different to that of MTB infection of the lungs. Thus, it is reasonable to assume that the correlates of protection in the two diseases will also be different.

The work described here contributes to the increasing knowledge of MAP antigens. Recombinant P22 generated IFN- γ levels comparable to those seen following immunization by an attenuated whole-cell vaccine, suggesting that it may be a candidate for inclusion in a subunit vaccine.

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