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Small protective fragments of the *Yersinia pestis* V antigen

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ABSTRACT

Yersinia pestis is the causative agent of plague. Naturally occurring cases of the disease and the potential use of *Y. pestis* as a bioweapon fuel the need for efficacious vaccines. The most recent plague vaccine is a killed whole cell preparation that is expensive to manufacture and its side effects are common. The protective antigens F1 and V have been identified and are currently being developed as a combined subunit vaccine. Protective epitopes of the V antigen have previously been shown to reside in the central part of the protein. In order to identify the minimum protective fragment of the V antigen that can provide protection against plague, the structures of several small fragments of the antigen were modelled *in silico* and recombinant proteins were produced. These fragments were probed for the retention of a protective epitope using a protective monoclonal antibody and protection against *Y. pestis* in mice was determined. The smallest protective fragment of V antigen identified comprised amino acids 135–262. Finally the ability of this fragment to confer protection when given in the context of a DNA vaccine was confirmed.

1. Introduction

Yersinia pestis is the causative agent of plague, a disease which can be transmitted from rodents via flea bites to humans, giving rise to the bubonic form of the disease. Untreated, the disease can progress into septicaemic or pneumonic forms, the latter being highly transmissible between humans via the aerosol route and often proving fatal, even where antibiotics are administered [1]. Naturally occurring cases of the disease in endemic areas and the potential use of *Y. pestis* as a bioweapon fuel the need for the development of efficacious vaccines.

The most recently used plague vaccine United States Pharmacopoeia (USP) is a formaldehyde-killed whole cell formulation preserved in phenol [2]. The production of killed vaccines from highly pathogenic bacteria requires high-containment facilities, making this type of vaccine very expensive and hazardous to make. In addition, side effects associated with the plague vaccine (USP) are common and increase with the number of booster doses of vaccine given [3]. Furthermore, this vaccine failed to provide high levels of protection against aerosol challenge in animal models [4]. Another plague vaccine developed is the live attenuated EV76 strain, which is neither available nor licensed for use in humans outside of the Former Soviet Union. EV76 is Pgm$^-$ (deleted in the pigmentation genes) rendering it non-pathogenic. However, vaccination with EV76 gave rise to serious side effects and there was a danger of reversion to virulence. The efficacy of the vaccine was also questionable [2]. More recent advances in plague vaccine development have focused upon the delivery of protein subunits in the hope that the problems associated with killed whole cell vaccines can be overcome. Two major proteins have been investigated. The F1 capsular antigen is thought to be the major immunostimulatory component of killed whole cell vaccines [5] and has been found to be protective against plague when administered to mice as a recombinant protein [6]. However, acapsular strains of *Y. pestis* exist to which protection would not be afforded by a vaccine comprising the F1 antigen alone [7]. A further antigenic protein, the V antigen (LcrV), has also been shown to afford protection against plague in mice [8]. Thus, the combination of the F1 and V antigens as separate proteins co-delivered in a single vaccine or as a fusion protein is now in clinical trials in the UK and USA, respectively [9,10].

The V antigen is a 37 kDa protein (326 amino acids) encoded by the *lcrV* gene. It is a component of the *Y. pestis* type III secretion system and acts intracellularly as a regulator of secretion via binding of the LcrG protein [11] and is a component of a needle-like injectisome on the surface of *Yersinia enterocolitica* [12]. Recent investigations of the injectisome components by scanning transmission electron microscopy (STEM) imaging and from data obtained with hybrids of LcrV, PcrV (of *Pseudomonas aeruginosa*) and AcrV (of *Aeromonas salmonicida*) suggests that LcrV exists as a pentamer at the injectisome tip [13]. V antigen also possesses anti-inflammatory activity via induction of IL-10 [14] which is currently thought to be achieved by signalling through a TLR2/TLR6/CD14

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complex [15]. Antibody responses to V antigen are known to correlate with protection against plague [16] and polyclonal serum or monoclonal antibodies to V antigen also offer protection [17–19]. The V antigen contains a number of protective epitopes that map to a central region of the protein between amino acids (a.a.) 135 and 275 [19]. The publication of the crystal structure of V antigen [20] has allowed further insights into the binding of protective monoclonal antibodies, and initial observations suggest that a smaller protective fragment than a.a. 135–275 is possible.

The feasibility of conferring protection against plague using a DNA vaccine encoding full-length V antigen has been reported by others and us [21,22]. Indeed, it has been shown that a DNA-based plasmid cocktail approach can stimulate protective immune responses against a range of biodefence agents such Ebola, plague and anthrax [21,23]. While these results are encouraging the approach suffers from the fact that the two antigens are located on separate plasmids making the vaccine difficult to formulate. To simplify this approach we are seeking to identify the regions of each antigen which are key to protection so that they may be incorporated into a single plasmid construct. This work seeks to identify the minimum fragment of a.a. 135–275 that may be used to provide protection against *Y. pestis*.

2. Materials and methods

2.1. In silico analysis

In order to generate small protective regions of the V antigen this work aimed to rationally reduce the size of the a.a. 135–275 fragment previously shown to be protective [19] by removing parts of the structure thought not to be involved in binding a protective monoclonal antibody. The crystal structure of the *Y. pestis* V antigen [20] was used to generate three-dimensional computer models that could be manipulated in order to model a minimal fragment of V antigen that would bind a protective monoclonal antibody. All modelling and visualization was carried out using SYBYL 7.1 (Tripos Ltd., Milton Keynes, UK). Molecular surface representation and property mapping was carried out with the MOLCAD module of SYBYL. Energy calculation was carried out with Maximin2 using the Tripos force field parameters with a 1000 iteration limited minimization to allow a qualitative analysis of localized energetic strain. All models were based on the rationale that the core fold of remaining secondary structures would be maintained for effective antibody binding and immunogenicity, and the interpretation of resulting models would elucidate structures likely to be stable.

The file 1R6F containing the V antigen crystal structure was extracted from the Protein Databank (http://www.rcsb.org) then visualized and checked for regions of poor geometry and missing structure. Missing or incomplete sidechains were reconstructed from the standard sidechain conformation database. Residues prior to residue 28 and following residue 322 were not present in the crystal structure and no attempt was made to model the N- or C-termini. Loop regions Tyr50-Ala60 and Asn263-Cys273 of the structure data included undefined residues and these were modelled using SYBYL's loop search procedure. Modelled structures of previously produced experimental fragments a.a. 135–275 [19], 168–325 [24], 135–245 [19] and 135–275 Δ218–234 [25] were generated computationally for comparative purposes. Fragments 135–275, 168–325 and 135–245 were created by deletion of unwanted residues and limited optimisation of remaining structure. Fragment 135–275 Δ218–234 was generated by deletion and application of the SYBYL loop search process followed by limited optimisation. Based on analysis, models of a.a.168–275, 175–275, 135–268, 135–262 and 135–275 Δ218–234 (Table 1, Fig. 1) were chosen for experimental production and evaluation of protective antibody binding.

2.2. Bacterial strains and plasmids

Oliognucleotide primers incorporating appropriate restriction sites were used to amplify *lcrV* fragments from *Y. pestis* GB DNA by PCR under standard PCR conditions. (Primers: 135F 5′ CGGATCCATTTTGAAAGTGATTGTTG 3′, 168F 5′ CGGATCCATAGTTATTCAAGCCGAAA 3′ 175F 3′ CGGATCCATTAAGCATCTGTCTAGTA 5′ 262R 5′ CGTCGACTCAATCTTTATTATAAGAG 3′, 268R 5′ CGTCGACTCAGTGAGATAATTCATTA 3′, 275R 5′ CGTCGACTCAATCCGAGCAGGTGGTG 3′ restriction sites underlined.) Internal deletions within *lcrV* fragments were created using overlap extension PCR. (Primers: IntF 5′ AAAATTCTCGAGAAAGTCTCGATAAAGGAC 3′, IntR 5′ GTCCTTTATCGAGACTTTCTCGAGAATTTT 3′.) The amplicons were cloned in frame with a glutathione *S*-transferase (GST) tag in plasmid pGEX6P-1 (GE Healthcare, Amersham, UK). For expression of recombinant proteins, plasmids were transformed into chemically competent *Escherichia coli* BL21 (DE3) pLysS (Invitrogen). DNA vaccine plasmids were constructed incorporating *lcrV* fragment DNA sequences codon-optimised for expression in the mouse, synthesised *de novo* by Geneart AG (Regensburg, Germany). An in-frame GST tag was included within the DNA vaccine plasmids to allow direct comparison with the GST-tagged proteins. Synthesised DNA was cloned into the vector pSTU2 [26].

2.3. Production of recombinant proteins

E. coli BL21 (DE3) pLysS cultures were grown to mid-log phase and then isopropyl-beta-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM to induce expression of the recombinant proteins. Cells were harvested after 3 h and were resuspended in PBS containing DNAase (Roche) and a complete protease inhibitor cocktail tablet (Roche). The suspension was then sonicated to release intracellular protein and cell debris was removed by centrifugation followed by filtration through a 0.45 μm syringe filter. FPLC was used to purify proteins via affinity chro-

Table 1
Suggested V antigen fragments which may retain binding to protective monoclonal antibodies.

Fragment	Deletion type	Amino acids	Reasoning for selection of fragment
V1	–	135–275	Previously shown to be protective, control
V2	N-terminal	168–275	Previous experimental data
V3	N-terminal	175–275	Previous experimental data
V4	C-terminal	135–268	Removes part of the remnant of a loop structure partially destroyed in 135–275 but leaves enough structure to cover potentially lipophillic patch
V5	C-terminal	135–262	Removes the entire remnant of a loop structure partially destroyed in 135–275
V6	Internal	135–275 Δ218–234	Removes 2 β-sheets not thought to be involved in antibody binding

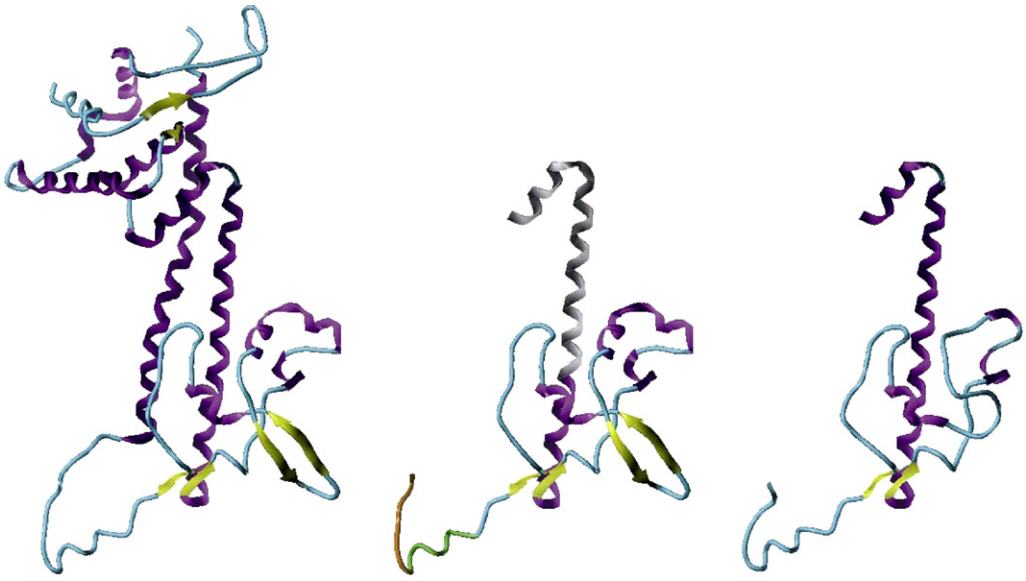

Fig. 1. Ribbon-tube representation of modelled structures coloured by secondary structure; strands, yellow ribbon; helices, purple ribbon; loops, cyan tube. Left image: V antigen structure (a.a. 28–322) including modelled undefined loop regions; middle image: a.a. 135–275 including a.a. 135–167 (deleted in V2 fragment and shown in white), a.a. 269–275 (deleted in V4 fragment and shown in orange), a.a. 263–269 (deleted in V5 fragment and shown in green). Right image: V6 (a.a.135–275Δ218–234). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

matography. Proteins were loaded onto a GSTrapFF column (GE Healthcare) and eluted with a buffer containing 50 mM Tris–HCl and 10 mM reduced glutathione. Proteins were dialysed against PBS overnight and assayed for protein content using the bicinchoninic acid (BCA) method.

2.4. Immunoassay of V antigen fragments

Protein samples were subjected to ELISA using the monoclonal anti-V antigen antibody mAb 7.3 [19]. Briefly, 96-well plates were coated with 50 μl of 10 μg/ml solutions of V antigen or GST-tagged V antigen fragments (triplicate wells). ELISAs were carried out as previously described [19] using serially diluted mAb 7.3 as the primary antibody. Results were expressed as the greatest reciprocal dilution at which the absorbance remained >0.1 above background.

2.5. Formulation of inoculations

Protein inoculation doses were formulated as 100 μl doses containing 267 pmol purified protein (equivalent to 10 μg of recombinant V antigen) and 0.25% (w/v) alhydrogel (Brenntag, Frederikssund, Denmark) in PBS. DNA vaccine plasmids were transformed into *E. coli* Top 10F' and prepared using an endotoxin-free mega prep kit (Qiagen, Crawley, UK). Purified DNA was then loaded onto 1.0 μm gold carrier particles and dried onto Gold Coat plastic tubing (Biorad, Hemel Hempstead, UK) as per the manufacturer's instructions. The amount of DNA per dose was calculated by redissolving sample doses into Tris–EDTA buffer and quantifying spectrophotometrically.

2.6. Animal challenge studies

Groups of 8–12-week old female BALB/c mice ($n = 8$) were inoculated via the intramuscular (i.m.) route with one of the six proteins or with adjuvant only. A naïve control group was also included. Two i.m. doses were given, 21 days apart, with blood samples taken from a tail vein on days 20 and 34 for immunological analysis. Fourteen days after the final vaccination, 6 randomly selected mice per group were challenged via the intraperitoneal (i.p.) route with approximately 5.1×10^2 cfu (51 MLD [27]) of *Y. pestis* GB in 100 μl PBS (actual dosage calculated *post priori* from the remaining inoculum). Survivors were culled 14 days post-challenge. In a second experiment, groups of BALB/c mice ($n = 8$) were inoculated with either three intradermal (i.d.) doses of DNA vaccine (Helios gene gun (Biorad) discharged at 300 psi onto freshly shaven abdominal skin) or two doses of DNA vaccine (via gene gun) and a booster dose of corresponding protein (via the i.m. route) at 2-week intervals. Control groups received administrations of empty DNA vector followed by adjuvant only. The actual doses of DNA administered were: empty vector DNA-5.26 μg; Vfull-1.91 μg; V1–2.28 μg; V5–1.27 μg. A naïve control group was also included. Blood samples were taken from tail veins for immunological analysis 13 days after each inoculation. On day 49, 6 randomly selected mice per group were challenged via the i.p. route with 13 cfu (1.3 MLD) of *Y. pestis* GB in 100 μl PBS (dosage calculated *post priori* from the remaining inoculum). Survivors were culled 14 days post-challenge. All experiments were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986. Animals which showed signs of disease were culled at humane endpoints.

2.7. Assay of antibody levels in mouse serum samples

Serum samples were subjected to ELISA to quantify the levels of anti-V antigen IgG, anti-GST IgG, and the ratio of anti-V antigen IgG1:IgG2a. Pooled or individual serum samples were assayed in triplicate as previously described using plates coated with recombinant V antigen [19]. Standard curves were calculated using mouse serum total IgG (Sigma). Results were calculated from the standard curves. The geometric mean was calculated where individual samples were assayed.

2.8. Statistical analysis

Statistical analysis of survival data was carried out using the Kaplan–Meyer log rank test. Antibody data was analysed using ANOVA of $\log_{10}$ titres. Values below the detection limits of 39 ng/ml for primary dose data and 312 ng/ml for final dose data were included as half the detection limit for analysis purposes.

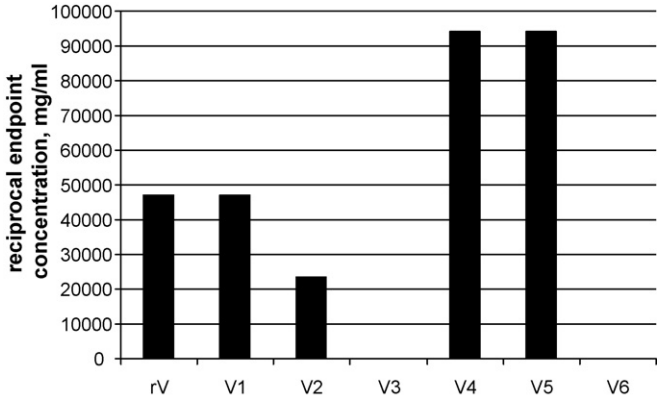

Fig. 2. Reactogenicity of a protective anti-V antigen monoclonal antibody to V-antigen fragments. Elisa plates were coated with 10 μg/ml proteins and mAb was serially diluted in triplicate. The endpoint concentration was reached at the greatest dilution which gave an $A_{414\,nm}$ of at least 0.1 above background.

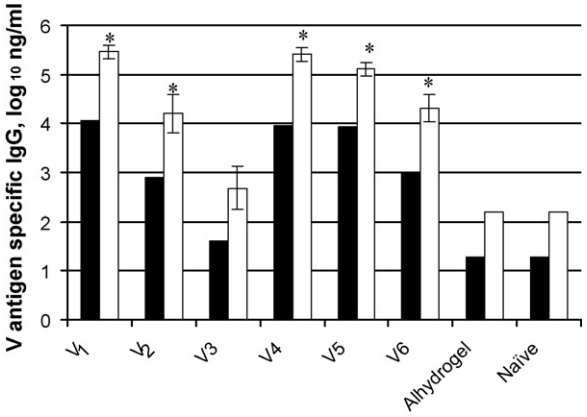

Fig. 3. Total IgG levels induced against V antigen in immunized mice. Groups of eight Balb/c mice were immunized intramuscularly with two doses of 267 pmol of GST-V antigen fragments V1–V6 on days 0 and 21. Serum samples were removed on day 20 and pooled (black bars) and day 34 and assayed individually (white bars). $^{*}p < 0.05$ compared to the naïve group (ANOVA). Error bars represent the 95% confidence interval.

3. Results

3.1. Immunoassay of V antigen fragments

To determine whether the V antigen fragments retained the epitope required for protective antibody binding, purified recombinant GST-tagged V antigen protein samples were subjected to analysis by ELISA using the protective monoclonal antibody mAb 7.3 (Fig. 2). Triplicate assays showed no variation. The antibody bound most of the fragments. However, fragments V3 and V6 were not recognized by the protective antibody, suggesting that the epitopes had been lost. Fragments V4 and V5 both reacted with the antibody when used at a greater dilution than fragment V1 or the full-length V antigen, demonstrating that in these proteins the epitopes remained intact. In comparison, fragment V2 showed a lower level of reactogenicity with the antibody.

3.2. Protection afforded by V antigen fragments

In order to assess the efficacy of the V antigen fragments in evoking protective immune responses, different purified recombinant GST-tagged V antigen fragments were used to immunize mice which were subsequently challenged with *Y. pestis*. Groups of mice were inoculated via the i.m. route with the proteins adjuvanted with Alhydrogel on days 0 and 21. Blood samples were taken on days 20 and 34 such that antibody responses could be quantified and compared with survival data. The survival of immunized animals following challenge via the i.p. route with 51 MLD *Y. pestis* GB on day 42 is shown in Table 2. Animals which were immunized with V antigen fragments that retained binding to protective monoclonal antibodies (V1, V4, V5) all showed a greater number of survivors when compared to the naïve control group. This data corresponds with previous experiments in which GST-V or GST-V1 gave 6/6 or 5/6 survivors respectively against a subcutaneous challenge of 1.1×10^5 MLD [19]. In comparison, animals receiving the V2 fragment, which retained partial binding to the protective monoclonal antibodies, succumbed to the infection. This suggests that, despite some ability of this V2 fragment to bind protective antibody, it is not sufficient to generate a protective response. The survival data is supported by the analysis of serum antibody levels in the immunized mice (Fig. 3), since the highest levels of anti-V antigen IgG were found in animals given fragments V1, V4 or V5. Since fragments V2 and V3 were unable to provide protective efficacy, this data suggests that residues in the a.a. 135–168 region are critical for retaining the protective epitope within a.a. 135–275. In all groups IgG1 was found at greater levels than IgG2a (data not shown).

Table 2
Survival of mice immunized via the i.m. route with two doses of GST-tagged V antigen fragments in alhydrogel adjuvant. Mice were challenged via the i.p. route with approximately 5×10^2 cfu of *Yersinia pestis* GB and monitored for 14 days after challenge.

Group	Survivors
V1	4/6*
V2	0/6
V3	0/6
V4	5/6*
V5	3/6*
V6	0/6
Naïve	0/6

* $p < 0.005$ by ANOVA compared to naïve control group.

3.3. Protection afforded by DNA vaccines

Since reducing antigen size enables the amount of material inserted into DNA vaccines to be minimized, with the ultimate aim of improving multivalent DNA vaccines, we produced a DNA vaccine plasmid containing the reduced antigen fragment V5 that could also be evaluated for protection against *Y. pestis*. In this work, the GST-tagged V5 fragment and full-length V antigen sequences were codon-optimized for expression in mice and cloned into the mammalian expression vector pSTU2. Mice were given either three doses of DNA vaccine at 2-week intervals via the gene gun or two DNA vaccine doses followed by a homologous protein boost via the i.m. route. Blood samples were taken after immunization to quantify antibody responses and assays demonstrated that all immunized mice showed high anti-V antigen IgG responses with similar magnitude responses in those animals given DNA vaccines expressing the V5 fragment or the full-length V antigen (Table 3). The IgG1:IgG2a ratio was strongly biased towards IgG1 (data not shown). The survival of immunized animals challenged on day 49 via the i.p. route with *Y. pestis* GB is shown in Table 3. In the group of mice given vector DNA only, two mice survived the 14-day post-challenge period, although these individuals were starting to exhibit signs of disease. In comparison, mice vaccinated with either the DNA vaccine encoding full-length V antigen or the DNA vaccine encoding the V5 fragment (either with or without the

Table 3
Survival of mice immunized with three doses of GST-tagged V antigen fragment DNA vaccine or two doses of DNA vaccine followed by a homologous protein booster in Alhydrogel adjuvant. Mice were challenged via the i.p. route with 13 cfu of *Yersinia pestis* GB.

Group	Survivors	Pre-challenge anti-V IgG, $\log_{10}$ ng/ml serum (95% CI)
Vfull DNA	6/6*	4.09 (1.47)
Vfull DNA + protein boost	4/6*	3.71 (1.46)
V5DNA	4/6*	4.00 (1.37)
V5 DNA + protein boost	6/6*	4.02 (1.61)
Vector DNA	2/6	1.83 (2.18)
Vector DNA + adjuvant boost	0/6	1.75 (2.01)
Naïve	0/6	1.59 (0)

* $p < 0.005$ by ANOVA compared to naïve control group. For antibody data all groups showed greater anti-V IgG responses when compared to the relevant control group (ANOVA, $p < 0.05$) (titres less than the detection limit are included as half the detection limit for analysis purposes).

protein boost) exhibited significantly greater survival against challenge than the control groups ($p < 0.005$). Mice receiving the DNA vaccine encoding the V5 fragment were 60% protected (4/6 survivors), or 100% protected (6/6 survivors) if also given a protein boost, demonstrating the utility of the V5 fragment in DNA vaccination.

4. Discussion

Through designing and expressing fragments of the V antigen we have been able to make judgements regarding the residues of the protein important for protective antibody binding. In this work, we modelled *in silico* five V antigen fragments within a.a. 135–275 of V antigen that may retain the ability to bind protective monoclonal antibodies. We then produced experimentally these protein fragments as fusions to GST and evaluated the ability of the fragments to bind protective monoclonal antibodies and to provide protection against experimental *Y. pestis* infection in mice.

Our modelling suggests that coordination of the latter part of helix α7 with the region between β-sheets β3 and β6 (a.a. 165–262) is important for structural integrity. It is known that removal of β-sheet β3 (as in fragment 186–326 [7]) or β6 (as in fragment 135–245) abolishes binding of the protective monoclonal antibody mAb 7.3, though only the latter removes the critical residue N255, and both deletions impact upon the hydrophobic bond coordination stabilising the local structure [19]. Thus the anti-parallel pairing of β3 and β6 appears to be critical for anchoring the structural integrity of the epitope region. Visualisation of the hydrophobic residue packing around residues Val169 and Ile170 reveals these residues to be closely coordinated with residues 190–200, 215–220 and 236–239 in the epitope region fold. The proposed pentameric coordination of V antigen at the injectisome tip [13] results in the central globular domain being at the tip with the two long helices (α7 and α12) being innermost. The resulting structure would be likely to expose the proposed antibody binding site and bound antibody would act to block interactions of the injectisome.

In our experimental studies, the inability of the V3 fragment to bind the protective monoclonal antibodies suggests that there are important residues involved in antibody binding or in the stabilisation of the epitope structure contained within the a.a. 168–175 region. The β-sheet removed by the deletion of a.a. 218–234 (V6) is also likely to have a role in antibody binding or epitope stabilisation. The epitope is considered likely to be conformational rather than linear since the removal of this internal structure not thought to be involved in direct interaction with antibodies did, in fact, abrogate their binding. Deleting the region of a.a. 135–168 (as in fragment V2), whilst still allowing binding, may reduce the affinity of the protein for the antibody. The contribution to conformational stability of these residues may be due to close packing of helix α7 with helices α8, α10 and α11. Amino acid Ile166 is implicated in the hydrophobic packing region of the epitope fold, so the reduction in affinity of V2 may be as a result of a minor conformational rearrangement. It is possible that maintenance of full levels of binding may be possible in a fragment whose N-terminal is somewhere between a.a. 135–168. In particular, the removal of the N-terminal helix and loop from V1 presents an interesting fragment for investigation. The equal ability of fragments V4 and V5 to bind antibody would suggest that removal of the entire loop remnant does not expose a problematic lipophillic patch.

The survival of mice immunized with the V antigen fragment fusion proteins correlated well with the antibody binding data, with those groups receiving fragments which bound well exhibiting greater levels of survival than those immunized with fragments which did not bind the protective antibodies. The smallest fragment to provide protection was V5 (a.a. 135–262), a fragment that is reduced back to the end of the 6th β-sheet (removing the partial loop structure from a.a. 135–275), giving a minimal protective region identified in this study of 128 a.a. Whilst this fragment induced a lower anti-V antigen antibody titre than the V4 fragment, the level of survival was not significantly decreased when compared to the group receiving the GST-V4 protein. The lack of protection seen in mice immunized with the V2 fragment further supports the observation that a.a. in the 135–168 region are required for full stability of the epitope. This builds on the findings of Motin et al. [17] who suggest that an epitope lies between a.a. 168 and 275.

In this work we sought to identify the smallest fragment of LcrV capable of conferring protection against *Y. pestis* and were able to demonstrate that V5 (a.a. 135–262), when delivered as a DNA vaccine protected 4/6 immunized mice against challenge with *Y. pestis*. Since immunity to *Y. pestis* infection is critically reliant on the induction of protective serum antibodies [4,28], we used the gene gun to deliver the DNA vaccine encoding V5, an approach that has previously been found to be effective in evoking T_H2-type humoral responses [29]. This method of DNA vaccine delivery stimulated very high antibody titres in immunized mice, comparable to those seen in animals receiving the V antigen fragment protein vaccines. In this study two animals in given vector DNA only survived the 14-day challenge period. It should be noted that these animals were starting to exhibit signs of disease which was expected to progress to a humane endpoint should the study have continued (no other surviving animals showed any disease signs). It is possible that the presence of CpG motifs in the plasmid DNA may have stimulated the immune system leading to an extended the time to death. Protective effects against bacterial infection have been observed for up to two weeks following administration of CpG DNA [30] and so it is feasible that low-level effects may still be present three weeks after administration.

In the context of developing a single plasmid-based DNA vaccine capable of conferring protection against *Y. pestis* and a second pathogen, such as *B. anthracis*, these results suggest that the incorporation of a region encoding V5 represents a valuable first step in achieving this aim. Indeed, considerable work will be needed before this promising preliminary data, derived from an inbred laboratory strain of mouse, can be translated into a vaccine capable of protecting an outbreak human population. Indeed, in a parallel study of human volunteers immunized with recombinant V antigen we are in the process of identifying promiscuous CD4 T cell epitopes to be incorporated alongside V5 in a future vaccine construct (unpublished data; [31]).

In summary, this work identified a small protective fragment of the *Y. pestis* V antigen which is protective when administered to mice either as a protein or as a DNA vaccine.

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