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(54) Title: SMALL PROTECTIVE EPITOPES OF THE *YERSINIA PESTIS* V ANTIGEN AND DNA VACCINES ENCODING THESE

(57) Abstract: This invention provides an isolated and purified DNA fragment which encodes for the fragment defined essentially by amino acids 135 to 262 of the sequence of the V antigen of *yersinia pestis*. The DNA fragments may be used to express small protective epitopic fragments of the V antigen, for use as a vaccine or they may be used directly as DNA vaccines. Therapeutic antibodies may be raised against the small epitopic fragments. Pharmaceutical compositions comprisin the vaccines of the invention are described and claimed.

Small Protective Epitopes of the *Yersinia pestis* V Antigen and DNA Vaccines Encoding These

This invention relates generally to vaccines for the treatment of infection by *yersinia pestis*. In particular, the invention relates to small epitopic fragments of the *yersinia pestis* V antigen, their use as vaccines against plague and to DNA vaccines which encode for the small epitopic fragments. Methods of treatment using these recombinant protein and DNA vaccines also form part of the invention.

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 and pneumonic forms, the latter being highly transmissible between humans via the aerosol route and often proving fatal, even where antibiotics are administered. Naturally occurring cases in endemic areas and its potential use as a bio-weapon fuel the need for the development of efficacious vaccines.

The current plague vaccine (USP) is a formaldehyde-killed whole cell formulation preserved in phenol. However, the production of killed vaccines from highly pathogenic bacteria requires high-containment facilities, making the vaccine very expensive and hazardous to make. In addition, side effects are common and it has been reported that side effects increase with the number of booster doses of vaccine. Other plague vaccines include the live attenuated EV76 strain, which is neither available nor licensed for use in humans. EV76 is Pgm- (deleted in the pigmentation genes) rendering it non-pathogenic. However, vaccination gave rise to serious side

effects and there was a danger of reversion to virulence. Furthermore, the efficacy of the vaccine was questionable.

More recent advances in plague vaccine research have focused on the delivery of protein subunits in the hope that the inherent problems of whole cell vaccines can be overcome. Two major proteins have been investigated. F1 capsular antigen is thought to be the major immunostimulatory component of killed whole cell vaccines and has been found to be protective as a recombinant protein. However, acapsular, or F1 negative, strains of Y. pestis exist to which protection would not be afforded by an F1 vaccine. A further antigenic protein, LcrV (Low calcium response V or V antigen), has also been shown to give protection. The combination of the F1 and V antigens as separate proteins in a single vaccine or combined via an amino acid linker in a fusion protein are now in early clinical trials in the UK and USA.

However, a smaller epitope-based vaccine is desirable because it may remove potential problems associated with immunodominant, non-protective epitopes contained within larger antigens, which can act as a 'decoy' to the immune system leading to the generation of non-protective responses. The LcrV protein is thought to contain a number of protective epitopes that map to a central region of the protein. The ability to define small protective epitopes within the V antigen could provide a more efficient vaccine for plague. Furthermore, a small epitopic fragment could form the basis of effective multivalent vaccines, since fewer epitopes are presented for the immune response to recognise, resulting in a more efficient and targeted response. Ideally, small (less than 200 amino acids) epitopic fragments of different protective antigens could be formulated into a multivalent protein or DNA-based vaccine.

The full sequence of V antigen is well known in the art and is disclosed, for example, in US Patent no 5,985,285 and international patent application PCT/GB96/00571, published as WO96/28551.

The inventors have identified small epitopic fragments of V antigen, which are protective against challenge with fully virulent *yersinia pestis*. These small epitopic fragments are advantageous in that they can be expressed in traditional expression systems as recombinant proteins and used directly as vaccines. Alternatively the small epitopes may be delivered in "naked" DNA form, as DNA vaccines. These small epitopic fragments have the potential to form part of a multivalent proteinacious or DNA vaccine.

Accordingly, in a first aspect, the invention provides an isolated and purified DNA fragment which encodes for the fragment defined essentially by amino acids 135 to 262 of the sequence of the V antigen of *yersinia pestis*. It will be understood in the art that more than one DNA sequence encodes for the above protective epitopic fragment and that such DNA fragments may optionally include short additional sequences which encode for a purification or detection tag, such as a His or GST tag. It will be understood in the art that the sequences of the invention may comprise such a tag to aid expression or purification and equally that the skilled may choose to remove the tag, for example, if administering the DNA fragment directly as a vaccine. The presence of absence of such a tag is not intended to limit the scope of the invention.

A preferred sequence is that defined in SEQ. ID no.1.(which comprises a GST tag).

In a preferred embodiment the DNA fragment is codon optimised for expression in a mammal. Examples of such codon optimised sequences are SEQ ID no.2 and SEQ ID no.3.

Of course, any of the DNA fragments described herein may be used for the in-vitro expression of recombinant protein. Accordingly, recombinant proteins consisting essentially of amino acids 135 to 262 of the sequence of the V antigen of *yersinia pestis* form a second aspect of the invention.

Both recombinant proteins consisting essentially of amino acids 135 to 262 of the sequence of the V antigen of *yersinia pestis*, and the DNA fragments which encode for the recombinant proteins provide protection when administered to mammals prior to infection with *yersinia pestis*. Accordingly, the recombinant proteins and the DNA sequences encoding them are useful in the manufacture of vaccines for the treatment and/or prophylaxis of plague.

It will be apparent to the skilled person that the recombinant proteins described herein may be used to raise antibodies. Such antibodies are useful in the treatment of plague infection and may be used separately to, in combination with or in addition to the vaccines described herein. Accordingly antibodies raised against the recombinant proteins form another aspect of the invention.

In a further aspect the invention provides a method of preventing *yersinia pestis* infection in a mammal, including man, comprising administering to the mammal a prophlactically effective amount of the isolated and purified DNA fragments or polypeptides described above. Similarly, the invention provides a method of treating *yersinia pestis* infection in a mammal comprising administering to the mammal a

therapeutically effective amount of the antibodies raised against the small eopitopic fragments of the V antigen.

The invention will now be described by way of example with reference to the accompanying drawings in which;

Figure 1 shows the protein vaccination schedule: a) vaccination; b) tail blood sample; c) second vaccination; d) tail blood sample; e) challenge;

Figure 2 shows the DNA vaccination schedule: a) First DNA vaccination; b) tail blood sample; c) Second DNA vaccination; d) tail blood sample; e) Third DNA vaccination, or protein boost; f) tail blood sample; g) challenge;

Figure 3 shows Western blot of V fragments probed with mAb 7.3. Proteins were loaded at 9 µg per lane;

Figure 4 shows Western blot of V fragments probed with mAb 29.3. Proteins were loaded at 9 µg per lane;

Figure 5 shows survival of mice immunised with two doses of GST-tagged V antigen fragments i.m. in alhydrogel adjuvant. Mice were challenged i.p. with 5 x 102 cfu *Yersinia pestis* GB on day 0. * $p<0.005$ compared to naïve control group;

Figure 6 shows Total IgG levels against a)V antigen and b)GST in vaccinated mice. Values are shown in ng/ml serum. Dose 1 values derived from pooled serum samples, dose 2 values are geometric mean from individual serum samples. Values below the detection limits of 39 ng/ml for dose 1 and 312 ng/ml for dose 2 were included as half the detection limit for analysis purposes;

Figure 7 shows geometric mean levels of IgG1 and IgG2a in groups of eight mice vaccinated with two doses of GST-tagged V-antigen fragments in alhydrogel adjuvant. Antibody levels below the detection limit have been included at half the detection limit of 78 ng/ml for analysis purposes.

Figure 8 shows survival of mice immunised with three doses of GST-tagged V antigen fragment DNA vaccine or two doses of DNA vaccine followed by a homologous protein booster i.m. in alhydrogel adjuvant. Mice were challenged i.p. with 13 cfu *Yersinia pestis* GB on day 0. * $p<0.005$ compared to naïve control group.

Figure 9 shows Total IgG levels against a)V antigen and b)GST in DNA vaccinated mice. Values are shown in ng/ml serum. Dose 1 & 2 values derived from pooled serum samples, dose 3 values are geometric mean from individual serum samples. Values below the detection limits of 39 ng/ml for doses 1 & 2 and 312 ng/ml for dose 3 were included as half the detection limit for analysis purposes.

Figure 10 shows In vitro expression of DNA vaccines. M: marker, E: empty vector, V: full-length antigen DNA vaccine, V1: V1 DNA vaccine, V5: V5 DNA vaccine, rV: recombinant V antigen, GST: GST tag protein. a: GST-V fusion protein, b: GST-V5 fusion protein, c: recombinant V antigen (no GST tag).

Figure 11 shows Geometric mean levels of IgG1 and IgG2a in groups of eight mice vaccinated with DNA vaccines with and without protein boost. Antibody levels greater than the limit of this assay (>640000 ng/ml) have been included as 640000 ng/ml for the purposes of this analysis. Levels below the detection limit of 78 ng/ml have been included as half the detection limit.

Example

Materials and Methods

In silico analysis

The crystallographically defined structure of Y. pestis LcrV was used in conjunction with molecular modelling and visualisation techniques to predict the effects of structural modification. The file 1R6F was extracted from the Protein Databank (www.rcsb.org). All modelling and visualisation was carried out using SYBYL 7.1 (Tripos Ltd, Milton Keynes, UK). The structure was visualised 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. Probable structures of previously produced experimental constructs aa 135-275, 168-325, 135-245 and 135-275 Δ218-234 were generated computationally for comparative purposes. All models were based on the conjecture that the folding of remaining secondary structures would be maintained in a similar fashion to that of the original whole LcrV. The rationale for construct choices was based on retaining secondary structure in a similar fashion to that of the whole LcrV molecule, minimal areas of exposed lipophilicity and the maintenance of co-ordination of clear intra-molecular hydrogen bonding.

2. Bacterial strains and plasmids

PCR primers were designed to incorporate restriction sites to amplify the identified LcrV fragments by PCR (see Table 1). The regions of interest were amplified using standard PCR conditions. Internal deletions were created using overlap extension PCR. Amplicons were cloned in frame with the GST tag in pGEX6P-1 (GE Healthcare, Amersham, UK). Restriction digestion of the vector and amplified DNA were performed and the molecules ligated using T4 DNA ligase (Roche, Burgess Hill, UK). Plasmids were routinely maintained in E. coli Top10F' (Invitrogen, Paisley, UK). Glycerol stocks were stored at -70°C. For expression of recombinant proteins plasmids were transformed into chemically competent E. coli BL21 (DE3) pLysS (Invitrogen). DNA vaccine plasmids were constructed incorporating DNA sequences codon-optimised for expression in the mouse. Codon-optimised DNA was synthesised

de novo by Geneart AG (Regensburg, Germany). Synthesised DNA was cloned in to the vector pSTU2 (Bennett et al., 1999).

3. Production of recombinant proteins.

Cultures were grown to mid-log phase and then isopropyl-beta-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1mM to induce expression of the recombinant proteins. Cells were harvested via centrifugation after 3 hours. The harvested cells were stored at -20°C until required. Cell pellets 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 removed by centrifugation followed by filtration through a 0.45 µm syringe filter. FPLC was used to purify proteins via affinity chromatography. Proteins were loaded onto a GSTrapFF column (GE Healthcare) and eluted with a buffer containing 50mM Tris-HCl and 10mM reduced glutathione. Proteins were dialysed against PBS overnight and then assayed for protein content using the bicinchoninic acid (BCA) method. Aliquotted protein solutions were stored at -70°C.

Table 1: PCR primers used in this study

Primer	Sequence	Purpose
pGEX135F	5' CGG ATC CAT TTT GAA AGT GAT TGT TG 3'	*Bam*HI site
pGEX168F	5' CGG ATC CTC AGT TAT TCA AGC CGA AA 3'	*Bam*HI site
pGEX175F	5' CGG ATC CAA TAA GCA TCT GTC TAG TA 3'	*Bam*HI site
pGEX262R	5' CGT CGA CTC AAT CTT TAT TAT AAG AG 3'	*Sal*I site
pGEX268R	5' CGT CGA CTC AGT GAG ATA ATT CAT TA 3'	*Sal*I site
pGEX275R	5' CGT CGA CTC AAT CCG AGC AGG TGG TG 3'	*Sal*I site
IntF	5' AAA ATT CTC GAG AAA GTC TCG ATA AAG GAC 3'	Overlap extension

IntR	5' GTC CTT TAT CGA GAC TTT CTC GAG AAT TTT 3'	Overlap extension

4. Immunoassay of V antigen fragments.

Protein samples were subjected to polyacrylamide gel electrophoresis (PAGE) and transferred by Western blot to nitrocellulose membranes. The membranes were blocked overnight in 2% bovine serum albumin (BSA) in PBS. The monoclonal anti-V antibodies mAb7.3 and mAb29.3 (Hill et al., 1997) were used as primary antibodies and anti-mouse-HRP (AbD Serotech, Kidlington, UK) was used as the secondary antibody. Avidin-HRP was included to detect biotinylated markers. Diamino benzidine tetrahydrochloride (DAB, Sigma, Gillingham, UK) was used to develop the blots.

5. Production of protein vaccines

The purified GST-V fragments were passed through an endotoxin removal column (Endotrap, Lonza, Slough, UK) seven times in series to remove endotoxin, as per the manufacturer's instructions. The proteins were then re-quantified and run on PAGE gels to confirm protein recovery. Endotoxin levels in the proteins were assayed using the QCL-1000 Chromogenic LAL Kit (Lonza). Vaccine doses were formulated as 100 µl doses containing 267 pmol protein (10 µg rV equivalent) and 0.25% alhydrogel (Brenntag, Frederikssund, Denmark) w/v in PBS. Vaccines were made up as required and allowed to adsorb to the alhydrogel adjuvant at 4°C overnight.

6. Production of DNA vaccines.

Plasmids were transformed into E. coli 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) using a tubing prep station (BioRad) and using polyvinylpyrollidone (PVP) as an adhesive. The tubing was cut into lengths for use with the Helios gene gun system (BioRad). The amount of DNA per dose was calculated by redissolving sample doses into TE buffer and quantifying spectrophotometrically.

7. Animal challenge studies

All experiments were carried out in accordance with the Animals (Scientific Procedures) Act 1986. Animals which showed signs of disease were culled at humane endpoints. a) Protein vaccine study Groups of BALB/c mice (n=8) were vaccinated intramuscularly (i.m.) with one of the six proteins or adjuvant only. A naïve control group was also included. Two doses were given, as per the schedule shown in Figure 1, with blood samples taken from a tail vein for immunological analysis.
On day 42 mice were challenged intraperitoneally (i.p.) with 5.1 x 102 cfu (5.1 x 102 MLD) *Yersinia pestis* GB in 100 µl PBS (actual dosage calculated post priori from the remaining inoculum). Survivors were culled 14 days post challenge. b) DNA vaccine study Eight groups of BALB/c mice (n=8) were vaccinated with either three doses of DNA vaccine (Helios gene gun (Biorad) discharged at 300 psi onto freshly shaven abdominal skin) or two doses of DNA vaccine (gene gun) and a booster dose of protein (i.m.). Control groups received empty DNA vector/alhydrogel. A naïve control group was also included. The amount of DNA per dose is shown in Table 2. Animals were vaccinated, and blood samples taken for immunological analysis, as per the schedule described in Figure 2. On day 49 mice were challenged intraperitoneally (i.p.) with 13 cfu (13 MLD) *Yersinia pestis* GB in 100 µl PBS (actual dosage calculated post priori from the remaining inoculum). Survivors were culled 14 days post challenge.

Table 2: Amount of plasmid DNA per vaccine cartridge

Plasmid	DNA/cartridge (µg)
pSTU (empty)	5.26
pSTU GSTVfull	1.91
pSTU GSTV1	2.28
pSTU GSTV5	1.27

8. Assay of antibody levels in mouse serum samples

Blood samples were allowed to clot overnight at 4°C and were then centrifuged in a bench microcentrifuge for 5 minutes at 13000 rpm. Serum was aspirated and stored at -20°C. ELISA was used to quantify the levels of anti-V IgG, anti-GST IgG, and the ratio of IgG1:IgG2a. Plates were coated with recombinant V antigen or GST as appropriate or goat anti-mouse Fab (Sigma) for the construction of standard curves. After washing off excess coating antigen with PBS containing 1% v/v Tween20 (PBS-T), plates were blocked by the addition of 2% skim milk powder in PBS (blotto) and stored at 4°C until needed. Pooled or individual serum samples were assayed in triplicate, diluted in blotto. Standard curves were calculated using mouse serum total IgG (Sigma). Serum/antibodies were allowed to bind for 1 h at 37°C, prior to washing off with PBS-T. HRP-conjugated goat anti-mouse IgG/IgG1/IgG2a (AbD Serotech) diluted 1:2000 in blotto was added. The excess HRP-conjugated antibodies were washed off with PBS-T and the substrate 2,2'-azino bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS, Sigma) was added. Following incubation at room temperature for 15 minutes the A410nm was read using a plate reader. Results were calculated from the standard curves and expressed as ng/ml.

9. In vitro expression of DNA vaccines

COS7 cells were grown in DMEM (Gibco [Invitrogen], Paisley, UK) supplemented with 10% foetal calf serum (Sigma) and 100 U/ml penicillin, 0.1 mg/ml streptomycin and 2mM glutamine (Sigma). Cells were seeded in 6 well plates at a density of 4 x 105 per

well 24 h before transfection. Plasmid DNA (1.5 µg per well) was used to transfect cells using Polyfect Reagent (Qiagen) according to the manufacturer's instructions. Cells were harvested 24 h after transfection and resuspended into 200 µl PBS. Crude cell lysate was then subjected to SDS-PAGE. Western blot using mAb 7.3 was used to detect expression from DNA vaccines.

10. Statistical analysis

Statistical analysis of survival curves was carried out using the Kaplan-Meyer log rank test. Antibody data was analysed using ANOVA.

Results and Discussion

In silico modelling of V antigen fragments

The analysis, in combination with data from previous studies resulted in the identification of five fragments of LcrV, smaller than aa 135-275, which may retain the ability to bind the protective monoclonal antibodies, and these are given in Table 3.

Table 3: Suggested LcrV 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

Immunoassay of V antigen fragments

Purified, GST-tagged protein samples were subjected to analysis by Western blot using the protective monoclonal antibodies mAb7.3 and mAb 29.3 (Figures 3 & 4). Both antibodies bound most of the fragments. Fragments V3 and V6 were not recognised by either of the protective antibodies, suggesting that the epitopes had been lost. This 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 and in the β-sheets removed by the deletion of a.a. 218-234. Deleting the region between a.a. 135 and 168, whilst still allowing binding, may reduce the affinity of the protein for the antibody as the bands in this lane are less dense than for the other fragments. Amino acids in this region may make a more minor contribution to conformational stability or the residues making up the binding region of the antibody. V4 and V5 both bound antibody equally as well as the control fragment, V1. This would suggest that removal of the entire loop remnant does not expose a problematic lipophillic patch.

Protection afforded by V antigen fragments

Different V fragments were used to immunise mice which were later challenged with plague. Endotoxin levels per dose varied but were between approximately 15 and 150 EU/dose (data not shown). Blood samples were taken 13 days after each immunisation such that antibody responses could be quantified and compared with survival. Survival of immunised animals is shown in Figure 5.

Animals receiving the V2 fragment which retained partial binding to the protective monoclonal antibodies succumbed to disease. This suggests that despite some ability of this fragment to bind protective antibody, it is not sufficient to generate a protective response. This observation is backed by the analysis of serum antibody levels (Figure 6a & Table 4). All groups, regardless of whether they produced anti-V antibodies or not, produced a response to the GST tag indicating that the proteins had been properly presented to the immune system (Figure 6b & Table 4). The response was almost completely TH2 rather than TH1 mediated, as demonstrated by high levels of IgG1 compared to minimal levels of IgG2a (Figure 7). This demonstrates that a predominantly humoral response is being mounted rather than a cell mediated/inflammatory response.

Table 4: Statistical analysis of antibody levels following the second dose in mice receiving protein vaccines. Analysis carried out using ANOVA. Shaded cells show non-significant differences (P>0.01)

Comparison	P (Anti-V)	P (Anti-GST)
V1 / naïve	1.6×10^{-16}	1.2×10^{-4}
V2 / naïve	8.4×10^{-8}	2.7×10^{-6}
V3 / naïve	4.9×10^{-2}	9.4×10^{-4}
V4 / naïve	6.4×10^{-17}	2.4×10^{-3}
V5 / naïve	4.4×10^{-16}	9.6×10^{-5}
V6 / naïve	7.5×10^{-10}	6.33×10^{-10}
Naïve	-	-

Protection afforded by V antigen fragment DNA vaccines

Selected V fragments which bound protective monoclonal antibodies were made into DNA vaccines. A full-length V antigen DNA vaccine has previously been shown to stimulate anti-V antibodies in mice but in that study no challenge was carried out to

determine whether the antibody response was protective. In this study, GST tagged full length V antigen, V1 and V5 sequences were codon- optimised for expression in mice and cloned into the mammalian expression vector pSTU2. Mice were given either three doses of DNA vaccine via gene gun or two DNA vaccine doses followed by a homologous protein boost i.m. formulated as for the protein vaccine study. Blood samples were taken after each immunisation to quantify antibody responses. Mice were challenged i.p. with Y. pestis GB. Survival of immunised animals is shown in Figure 8.

Mice vaccinated with the V1 DNA vaccine succumbed to disease and there was only one survivor in the group which received V1 DNA vaccine and a protein boost. This was not expected as the same fragment given in the protein vaccine regimen was seen to be protective. The analysis of antibody levels of mice in this group showed that there was approximately 10-fold less antibody produced to V antigen and only two individuals made a detectable level of antibody to the GST tag (Figure 9). The survivor in the group which also received a protein boost generated a high level of anti-V antibody, probably in response to the protein boost rather than the DNA vaccinations. Statistical analysis by ANOVA showed the level of antibody generated to both V and GST not to be raised compared to the relevant control groups (Table 5). To investigate why the vaccine did not elicit any response, in vitro DNA vaccine expression was carried out. COS7 cells were transfected with DNA vaccines and allowed to express protein for one day before the cells were harvested and lysed and the proteins analysed by SDS-PAGE/Western blot. Both the vaccines that gave protection gave strong bands in a position corresponding to the size of the cloned gene products when the blot was developed using mAb 7.3 (Figure 10, a & b). However, no band of the correct size was observed for the V1 DNA vaccine. The plasmid was investigated further. Using the enzymes used to clone in the GSTV1

insert, a restriction digest of the plasmid was performed. No DNA insert was digested out of the plasmid. Consultation with the company which synthesised the gene revealed that a plasmid containing the correct DNA sequence but cloned in the wrong orientation and on the wrong enzyme sites had been sent accompanied by quality assurance documentation that belonged with a plasmid with the gene cloned in the right direction.

Table 5: Statistical analysis of antibody levels following the third dose in mice receiving DNA vaccines. Analysis carried out using ANOVA. Shaded cells show insignificant differences (P>0.01)

Comparison	P (Anti-V)	P (Anti-GST)
Vfull DNA / vector DNA	1.1×10^{-6}	9.3×10^{-5}
V1 DNA / vector DNA	2.7×10^{-2}	6.1×10^{-2}
V5 DNA / vector DNA	5.6×10^{-6}	5.9×10^{-6}
Vfull DNA + protein / vector DNA + alhydrogel	3.3×10^{-10}	8.2×10^{-3}
V1 DNA + protein / vector DNA + alhydrogel	3.3×10^{-10}	6.9×10^{-3}
V5 DNA+ protein / vector DNA + alhydrogel	4.2×10^{-10}	7.4×10^{-9}
Naïve / vector DNA	3.8×10^{-8}	6.2×10^{-2}
Naïve / vector DNA + alhydrogel	2.3×10^{-2}	

Mice vaccinated with full-length V-antigen DNA vaccine and the V5 fragment DNA vaccine (with or without protein boost) exhibited significantly greater survival than the control groups. Two groups were completely protected (those receiving three doses of GST-tagged full length V antigen expressing vaccine and those receiving two doses of GST-tagged V5 fragment expressing vaccine followed by a protein boost.) The corresponding groups (Vfull with a protein boost and V5 DNA alone) gave 4/6 protection. In the vector DNA only group, two mice survived the 14 day post-challenge period. These individuals were starting to exhibit signs of disease by this point and it was expected that a humane endpoint would soon have been reached if the

experiment were allowed to proceed for any further time. In all groups the IgG1:IgG2a ratio was indicative of a TH2 response rather than a cell-mediated TH1 response (Figure 11).

Summary

Various fragments of the V antigen were found to be protective when given as GST-tagged proteins. V1 (aa 135-275), V4 (aa 135-268) and V5 (aa 135-262) all gave significantly better survival than the naïve control group. DNA vaccines were constructed expressing selected fragments and administered in a solely DNA vaccine or a DNA prime-protein boost regimen. Some vaccines were fully protective. A DNA vaccine of the V5 (aa 135-262) fragment was found to offer complete protection after three doses and a significant improvement in survival after two doses and a protein booster dose. This compares equally well to a vaccine encoding the full-length V antigen which gave complete protection after the prime-boost regimen and partial protection after the three DNA doses.

SEQ. ID no. 1

ATGTCCCCTATACTAGGTTATTGGAAAATTAAGGGCCTTGTGCAACCCACTCGACTTC
TTTTGGAATATCTTGAAGAAAAATATGAAGAGCATTTGTATGAGCGCGATGAAGGTGA
TAAATGGCGAAACAAAAAGTTTGAATTGGGTTTGGAGTTTCCCAATCTTCCTTATTAT
ATTGATGGTGATGTTAAATTAACACAGTCTATGGCCATCATACGTTATATAGCTGACA
AGCACAACATGTTGGGTGGTTGTCCAAAAGAGCGTGCAGAGATTTCAATGCTTGAAGG
AGCGGTTTTGGATATTAGATACGGTGTTTCGAGAATTGCATATAGTAAAGACTTTGAA
ACTCTCAAAGTTGATTTTCTTAGCAAGCTACCTGAAATGCTGAAAATGTTCGAAGATC
GTTTATGTCATAAAACATATTTAAATGGTGATCATGTAACCCATCCTGACTTCATGTT
GTATGACGCTCTTGATGTTGTTTTATACATGGACCCAATGTGCCTGGATGCGTTCCCA
AAATTAGTTTGTTTTAAAAAACGTATTGAAGCTATCCCACAAATTGATAAGTACTTGA
AATCCAGCAAGTATATAGCATGGCCTTTGCAGGGCTGGCAAGCCACGTTTGGTGGTGG
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TTGAAAGTGATTGTTGATTCAATGAATCATCATGGTGATGCCCGTAGCAAGTTGCGTG
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SEQ. ID no. 2

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ACCCTGAAGGTGGACTTCCTGAGCAAGCTGCCCGAGATGCTGAAGATGTTCGAGGACC
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AAGCTGGTGTGTTTCAAGAAGCGGATCGAGGCCATCCCTCAGATCGACAAGTACCTGA
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ACAAGATCCTGGAGAAGATGCCCCAGACCACCATCCAGGTGGACGGCAGCGAGAAGAA
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SEQ. ID no.3

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AGTACGAGGAGCACCTGTACGAGCGGGATGAGGGCGACAAGTGGCGGAACAAGAAGTT
CGAGCTGGGCCTGGAGTTCCCTAACCTGCCCTACTACATCGACGGCGACGTGAAGCTG
ACACAGAGCATGGCCATCATCCGGTACATCGCCGACAAGCACAACATGCTGGGCGGCT
GCCCTAAGGAGCGGGCCGAGATCTCTATGCTGGAGGGCGCCGTGCTGGATATCAGATA
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GCTGTACATGGACCCCATGTGCCTGGATGCCTTCCCCAAGCTGGTGTGTTTCAAGAAG
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CCTGGAAGTGCTGTTTCAGGGCCCTCTGGGCTCTATCCTGAAGGTGATCGTGGACAGC
ATGAACCACCACGGCGACGCCAGATCTAAGCTGAGAGAGGAGCTGGCTGAGCTGACAG
CCGAGCTGAAGATCTACAGCGTGATCCAGGCCGAGATCAACAAGCACCTGAGCAGCTC
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TCTGGGCTCCGAGAACAAGAGAACAGGCGCCCTGGGCAACCTGAAGAACAGCTACAGC
TACAACAAGGACTGAACTAG**TTCGCGATAGATAGATAGGCGGCCGCG**

Claims

1) An isolated and purified DNA fragment which encodes for a protective epitopic fragment defined essentially by amino acids 135 to 262 of the sequence of the V antigen of *yersinia pestis*.

2) An isolated and purified DNA fragment according to claim 1 which is of SEQ ID no.1.

3) An isolated and purified DNA fragment according to claims 1 or 2 wherein the sequence of the DNA fragment has been codon optimised for expression in a mammal

4) An isolated and purified DNA fragment according to claim 3 wherein the DNA fragment has SEQ ID no.2 or SEQ ID no.3.

5) A recombinant protein consisting essentially of amino acids 135 to 262 of the sequence of the V antigen of *yersinia pestis.*

6) A recombinant protein consisting essentially of amino acids 135 to 262 of the sequence of the V antigen of *yersinia pestis* for use in the manufacture of a medicament for the treatment of plague.

7) An antibody raised against the recombinant protein of claim 5

8) A method of preventing *yersinia pestis* infection in a mammal, including man, comprising administering to the mammal a prophlactically effective amount of the isolated and purified DNA fragment of any of claims 1 to 4 or the polypeptide of claim 5.

9) A method of treating *yersinia pestis* infection in a mammal comprising administering to the mammal a therapeutically effective amount of the antibody of claim 5.

10) An isolated and purified DNA fragment, a recombinant protein, an antibody or a method of treating or preventing infection by *yersinia pestis* substantially as described hereinbefore described with reference to the example and accompanying drawings.

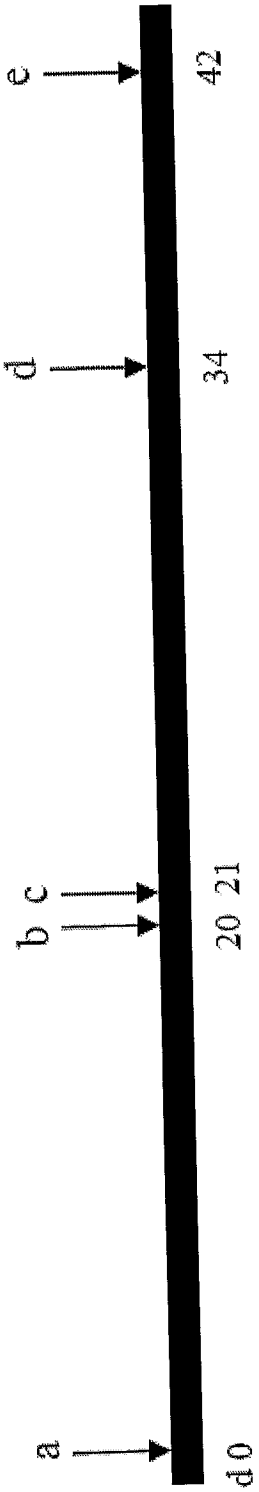


Fig 1

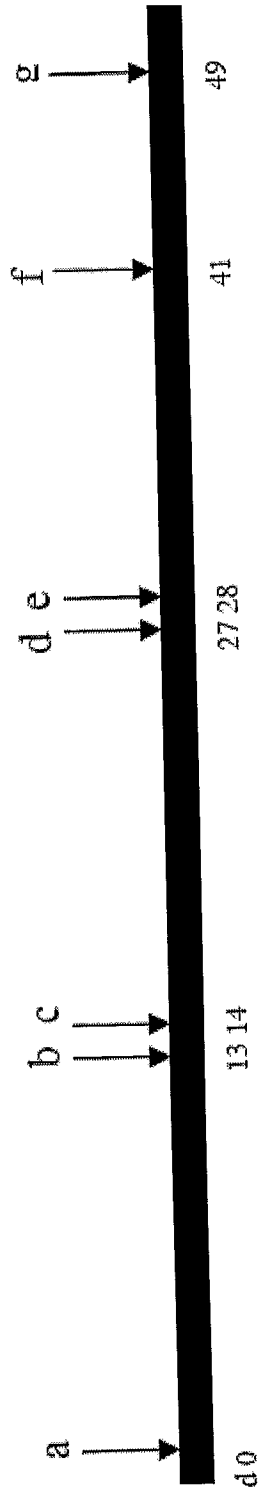


Fig 2

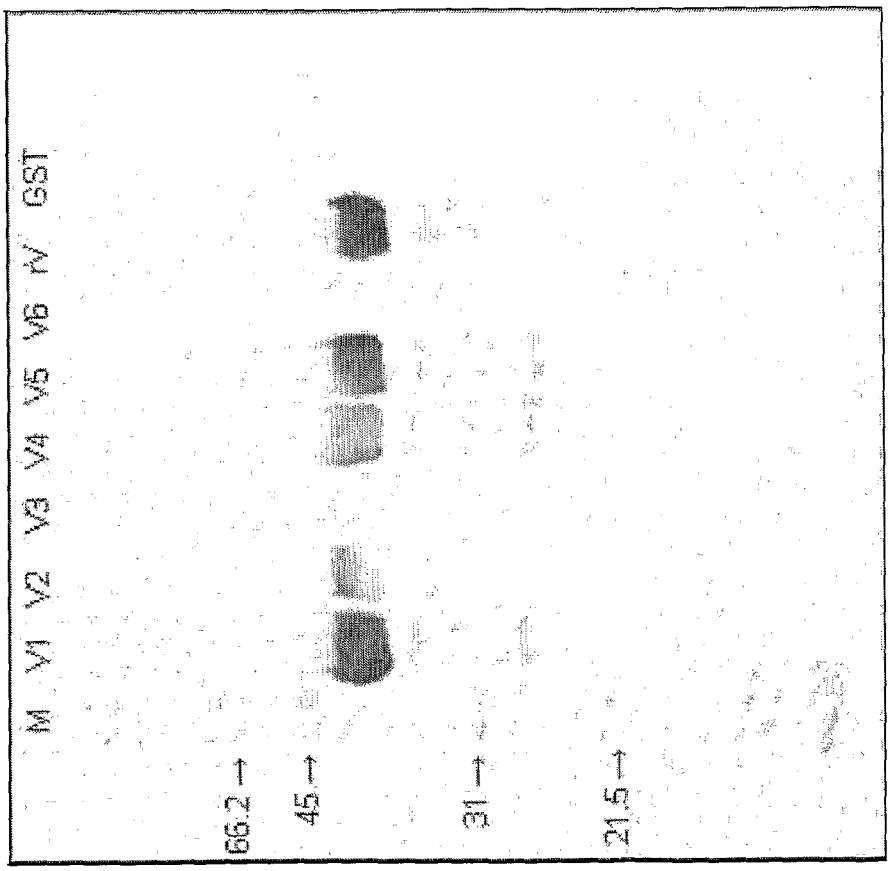


Fig. 3

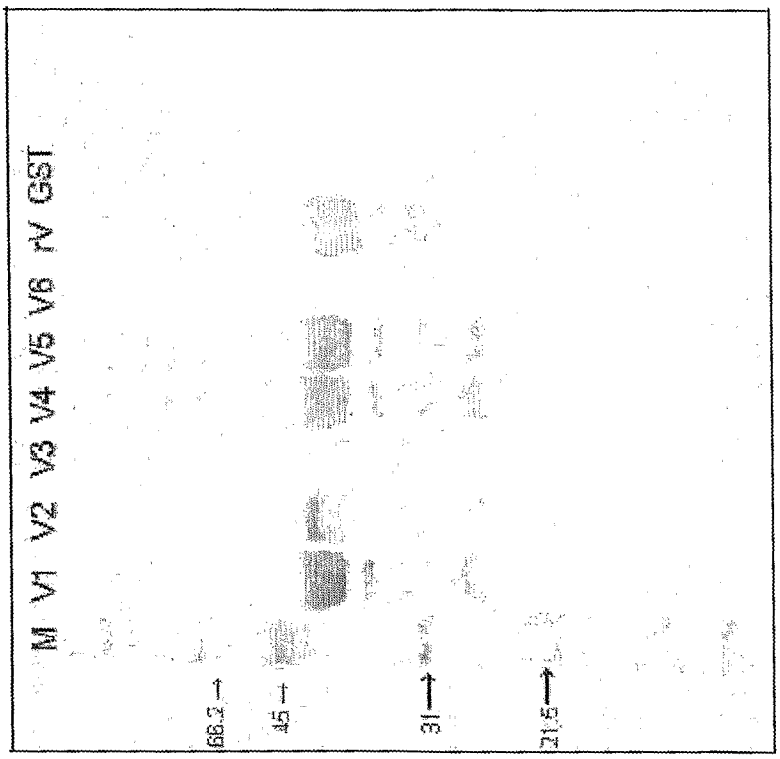


Fig 4

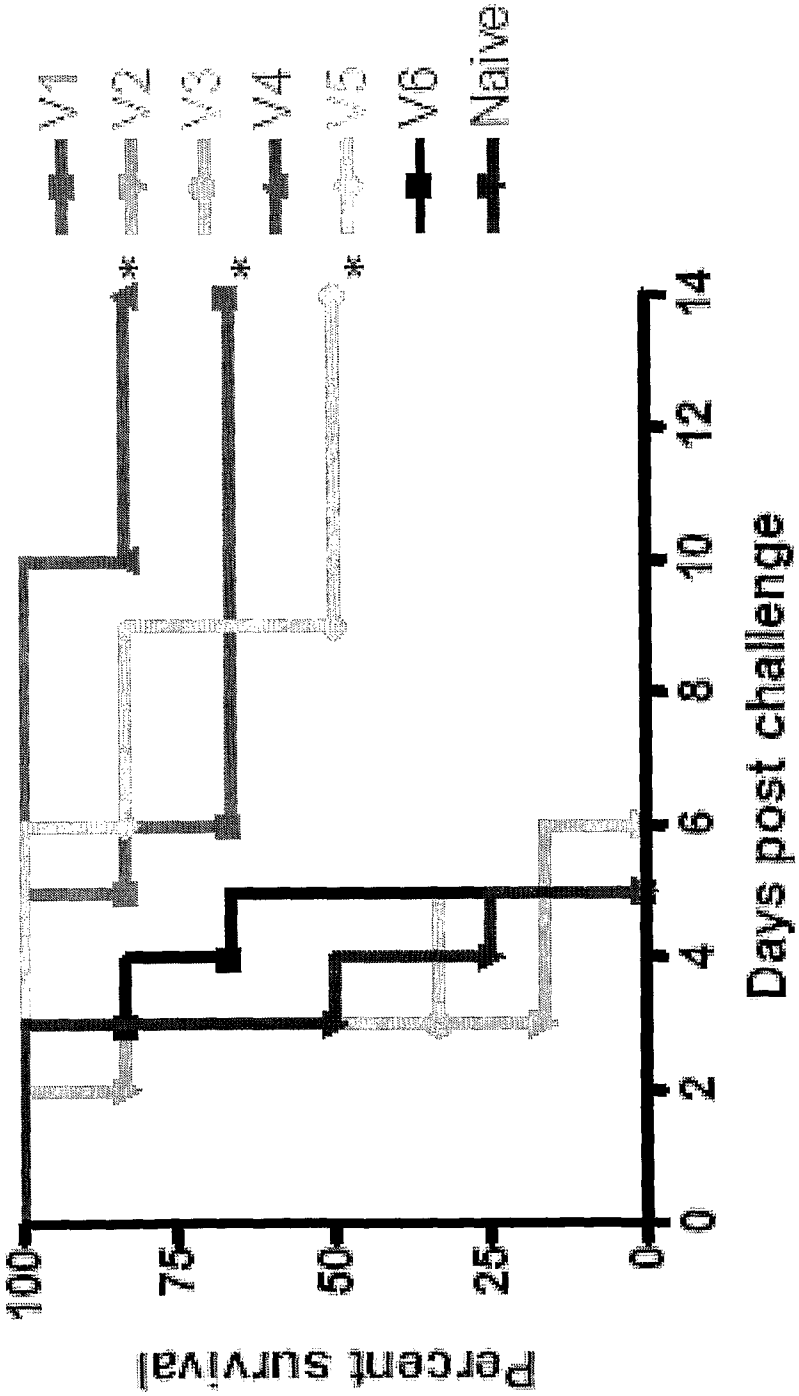

W1
W2
W3
W4
W5
W6
Naïve
*
*
*
Percent survival
100
75
50
25
0
0
2
4
6
8
10
12
14
Days post challenge

Fig. 5

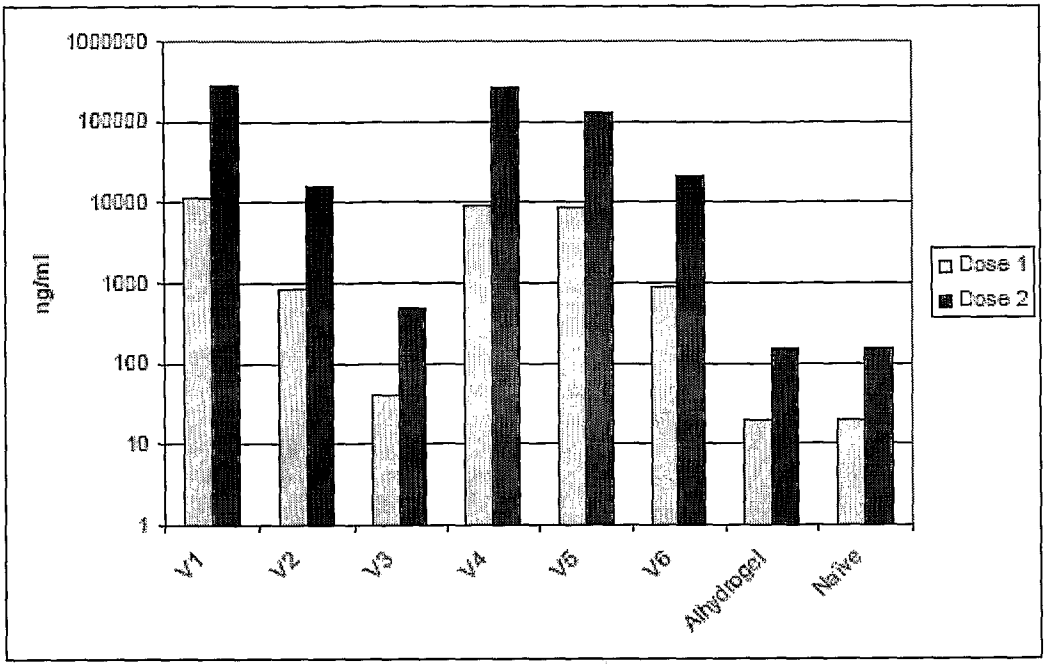

1000000
100000
10000
1000
100
10
1
ng/ml
V1
V2
V3
V4
V5
V6
Alhydrogel
Naive
Dose 1
Dose 2

Fig. 6A

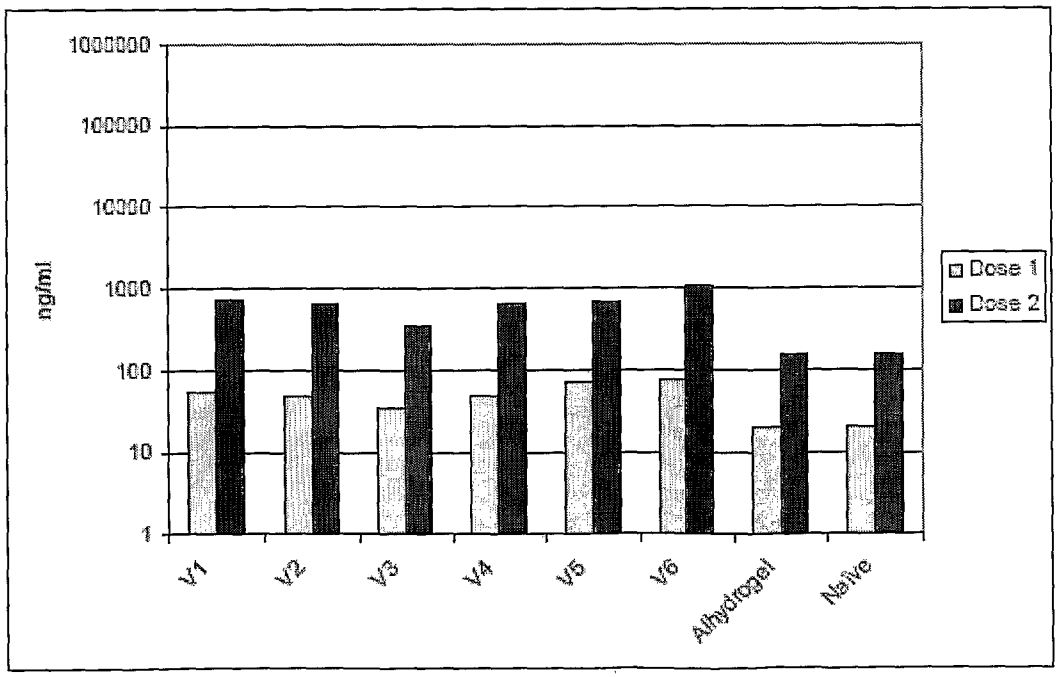

1000000
100000
10000
1000
100
10
1
ng/ml
V1
V2
V3
V4
V5
V6
Alhydrogel
Naive
Dose 1
Dose 2

Fig. 6B

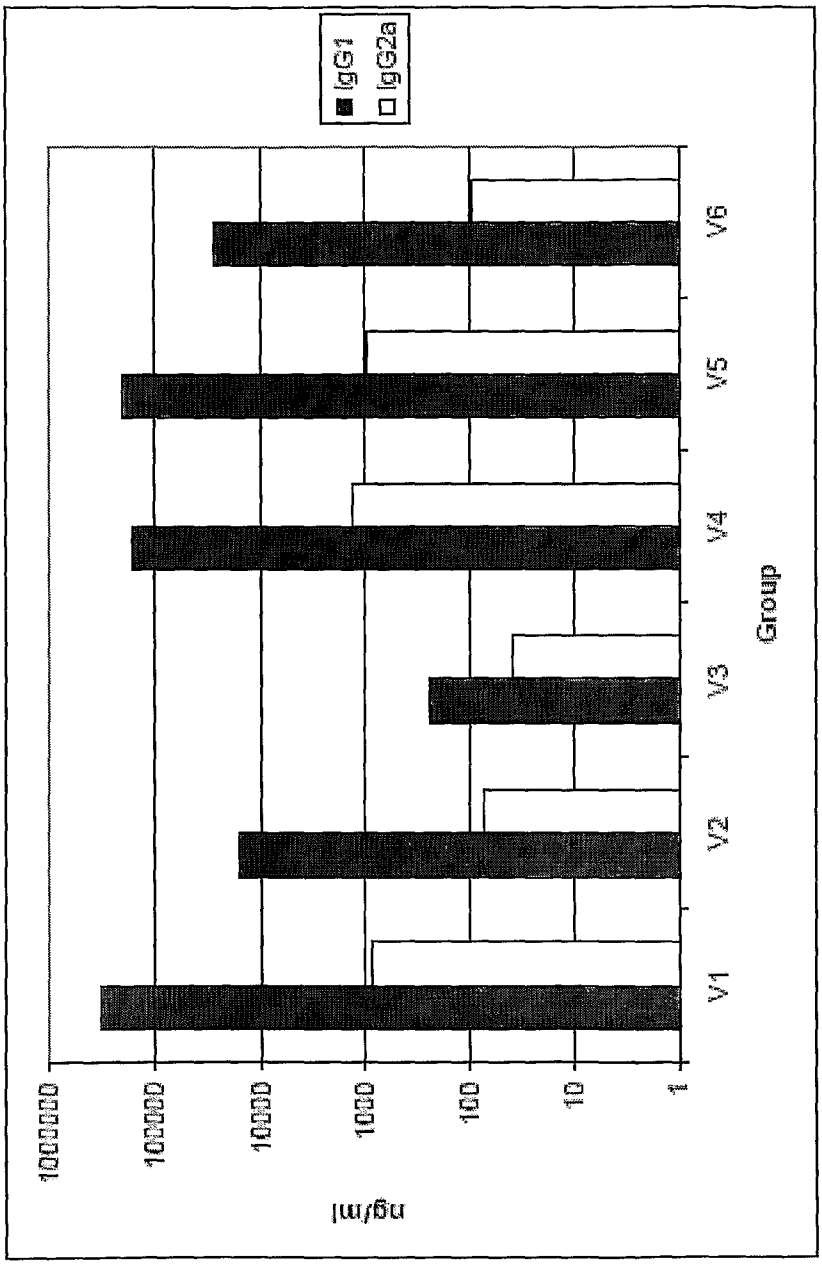

IgG1
IgG2a
ng/ml
1000000
100000
10000
1000
100
10
1
V1
V2
V3
V4
V5
V6
Group

Fig. 7

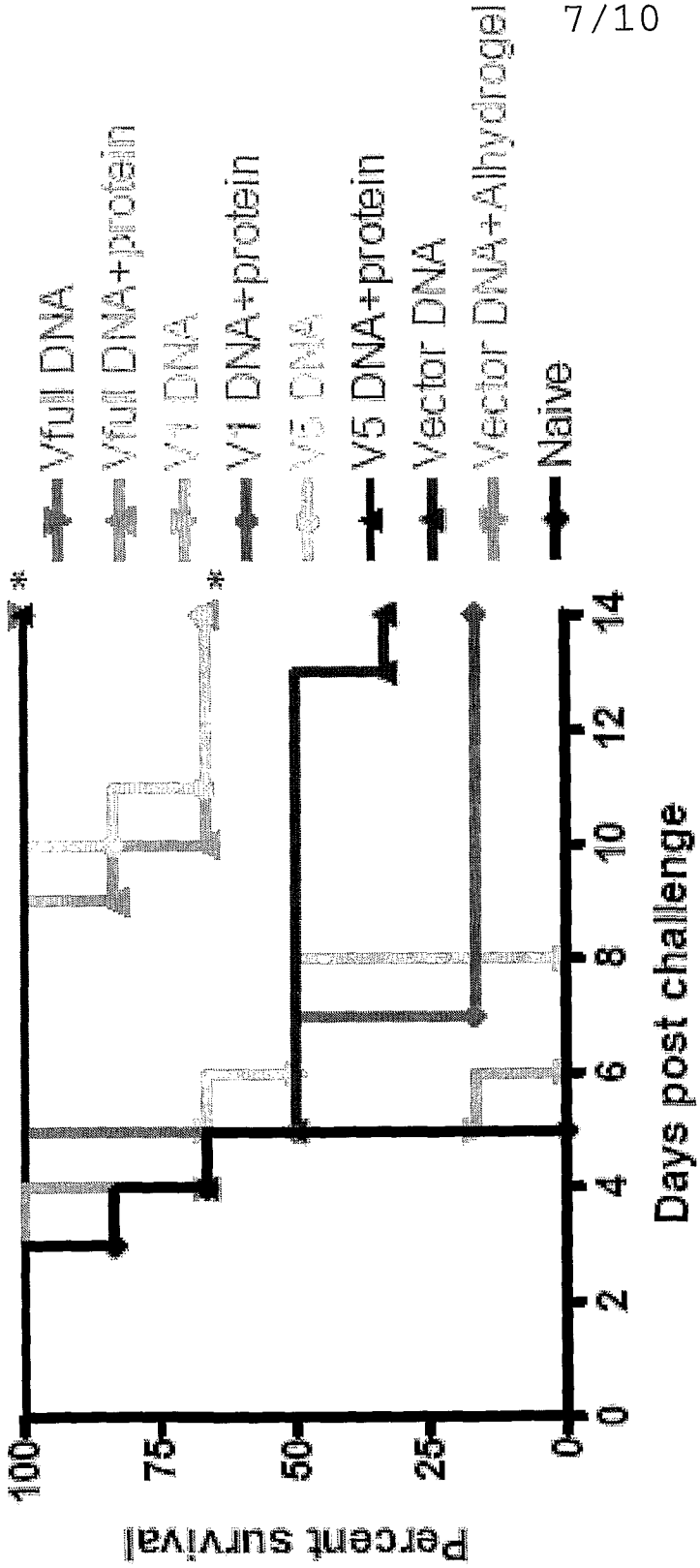

Vfull DNA
Vfull DNA+protein
V1 DNA
V1 DNA+protein
V5 DNA
V5 DNA+protein
Vector DNA
Vector DNA+Alhydrogel
Naive
Percent survival
100
75
50
25
0
0
2
4
6
8
10
12
14
Days post challenge
*
*

Fig. 8

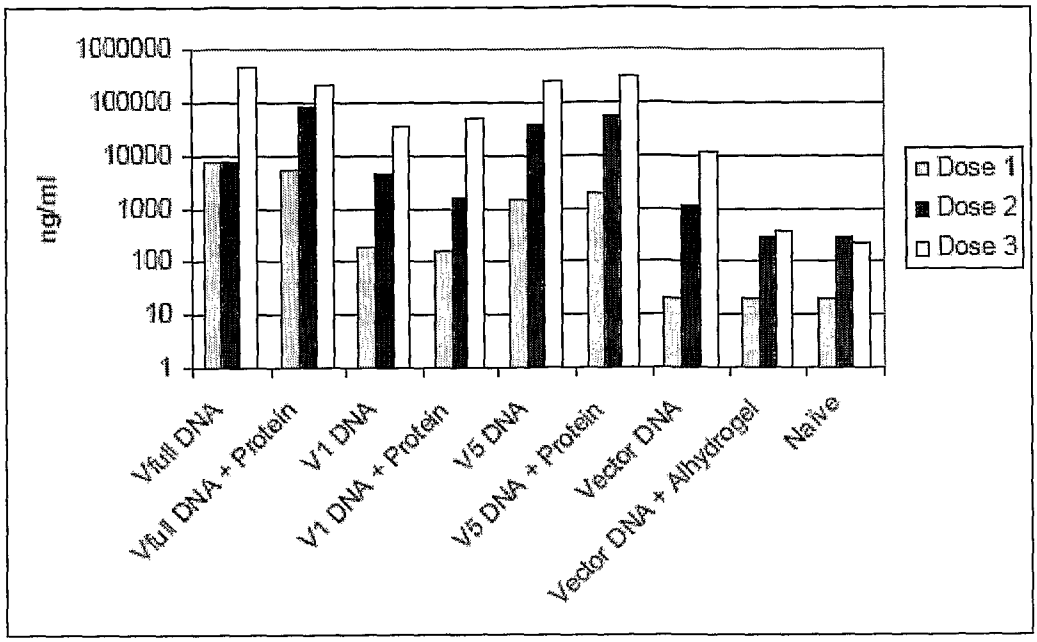

1000000
100000
10000
1000
100
10
1
ng/ml
Dose 1
Dose 2
Dose 3
Vfull DNA
Vfull DNA + Protein
V1 DNA
V1 DNA + Protein
V5 DNA
V5 DNA + Protein
Vector DNA
Vector DNA + Alhydrogel
Naïve

Fig. 9A

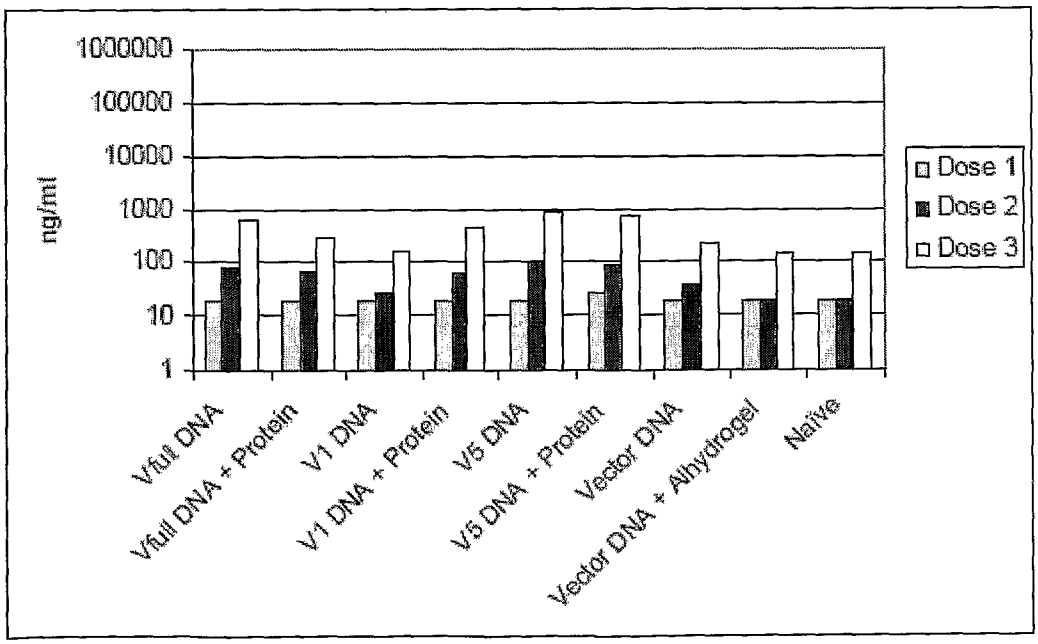

1000000
100000
10000
1000
100
10
1
ng/ml
Dose 1
Dose 2
Dose 3
Vfull DNA
Vfull DNA + Protein
V1 DNA
V1 DNA + Protein
V5 DNA
V5 DNA + Protein
Vector DNA
Vector DNA + Alhydrogel
Naïve

Fig. 9B

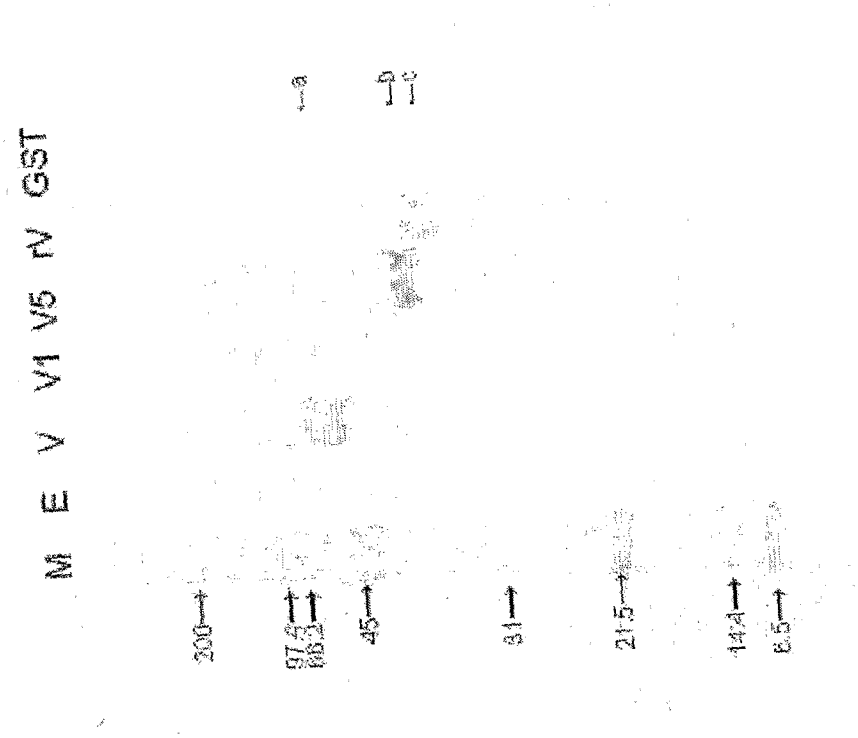


Fig. 10

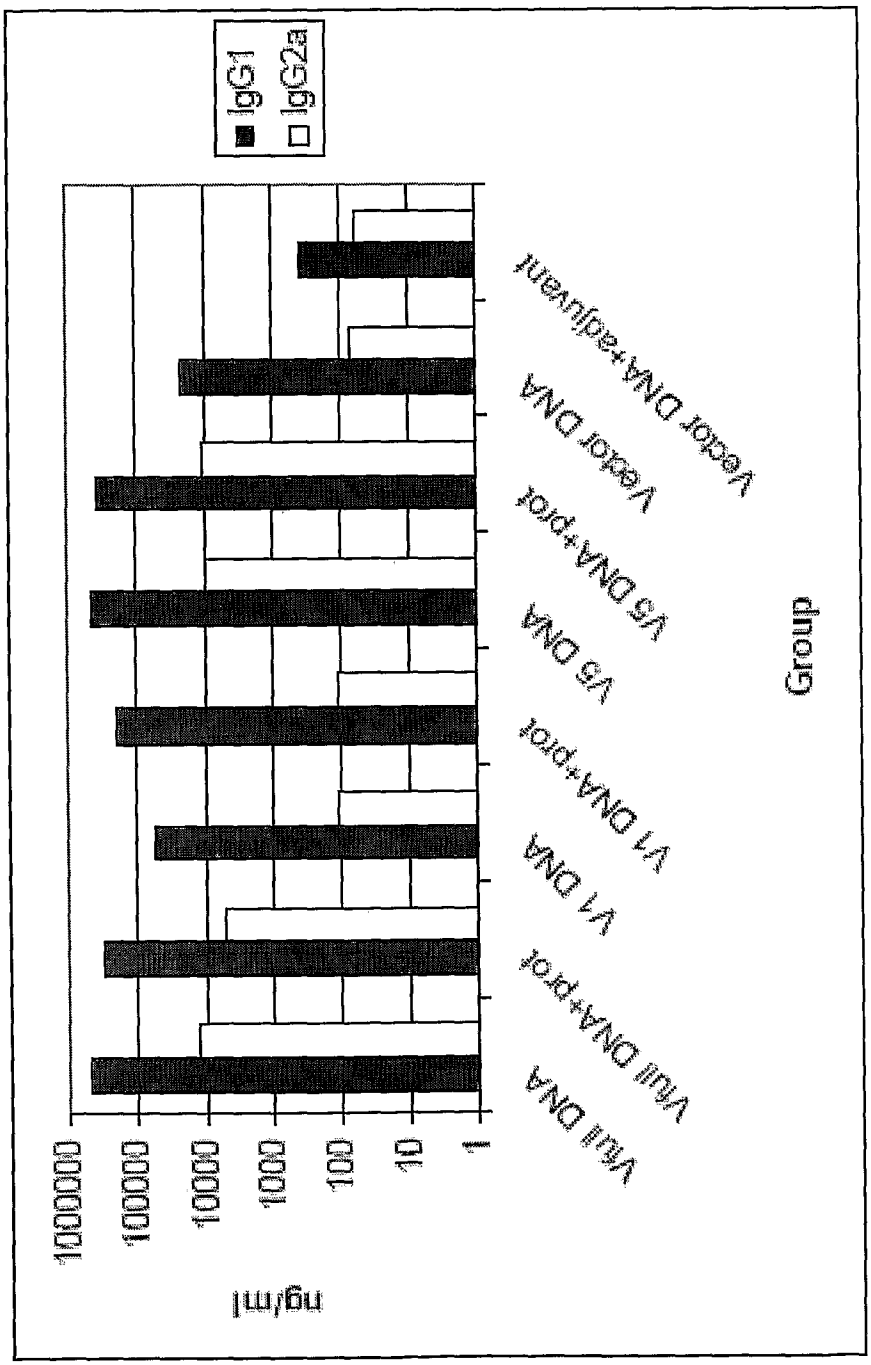

IgG1
IgG2a
ng/ml
1000000
100000
10000
1000
100
10
1
VIaII DNA
VIaII DNA+prot
V1 DNA
V1 DNA+prot
V5 DNA
V5 DNA+prot
Vector DNA
Vector DNA+adjuvant
Group

Fig. 11

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2008/003635

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K14/24 A61K39/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, Sequence Search, WPI Data, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HILL J ET AL: "Regions of Yersinia pestis V antigen that contribute to protection against plague identified by passive and active immunization." INFECTION AND IMMUNITY NOV 1997, vol. 65, no. 11, November 1997 (1997-11), pages 4476-4482, XP002516669 ISSN: 0019-9567 cited in the application the whole document figure 1; table 3	1-10

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
2 March 2009	12/03/2009

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Offermann, Stefanie

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2008/003635

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WEEKS S ET AL: "Anti-V antigen antibody protects macrophages from Yersinia pestis-induced cell death and promotes phagocytosis" MICROBIAL PATHOGENESIS, vol. 32, no. 5, May 2002 (2002-05), pages 227-237, XP002516670 ISSN: 0882-4010 the whole document	1-10
Y	MOTIN V L ET AL: "PASSIVE IMMUNITY TO YERSINIAE MEDIATED BY ANTI-RECOMBINANT V ANTIGEN AND PROTEIN A-V ANTIGEN FUSION PEPTIDE" INFECTION AND IMMUNITY, AMERICAN SOCIETY FOR MICROBIOLOGY. WASHINGTON, vol. 62, no. 10, 1 October 1994 (1994-10-01), pages 4192-4201, XP002006750 ISSN: 0019-9567 the whole document	1-10
Y	US 2006/246084 A1 (BRUBAKER ROBERT R [US] ET AL) 2 November 2006 (2006-11-02) paragraphs [0233], [0257]	1-10
Y	SHIM HO-KI ET AL: "Mechanisms of major histocompatibility complex class II-restricted processing and presentation of the V antigen of Yersinia pestis." IMMUNOLOGY NOV 2006, vol. 119, no. 3, November 2006 (2006-11), pages 385-392, XP002516671 ISSN: 0019-2805 the whole document	1-10
Y	PARENT MICHELLE A ET AL: "Yersinia pestis V protein epitopes recognized by CD4 T cells." INFECTION AND IMMUNITY APR 2005, vol. 73, no. 4, April 2005 (2005-04), pages 2197-2204, XP002516672 ISSN: 0019-9567 the whole document	1-10
A	DEREWENDA ET AL: "The Structure of Yersinia pestis V-Antigen, an Essential Virulence Factor and Mediator of Immunity against Plague" STRUCTURE, CURRENT BIOLOGY LTD., PHILADELPHIA, PA, US, vol. 12, no. 2, 12 February 2004 (2004-02-12), pages 301-306, XP022553545 ISSN: 0969-2126 the whole document	

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/GB2008/003635

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2006246084 A1	02-11-2006	NONE	