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(54) Title: GROES AND GROEL HOMOLOGS OF FRANCISELLA TULARENSIS

(57) Abstract: A polypeptide comprising at least one component of an Hsp60 obtainable from *Francisella tularensis*, and in particular the GroEL protein, or a fragment thereof, or a variant of any of these, which polypeptide, when administered to an animal, produces an immune response which is protective against *F. tularensis* infection, for use in prophylactic or therapeutic vaccination against *F. tularensis*. The polypeptide may be administered directly to the host, or nucleic acid encoding the polypeptide may be administered in a form in which they might be expressed in vivo. Vaccines based upon these polypeptides suitably include an additional component and in particular an adjuvant which promotes a cellular immune response.

GROES AND GROEL HOMOLOGS OF FRANCISELLA TULARENSIS

This invention relates to a polypeptide useful in the prophylactic or therapeutic treatment of diseases caused by *Francisella tularensis,* and to vaccines and therapies using this.

There are two identified blovars of *F. tularensis* classified according to the severity of disease they cause. Type A strains are fully virulent (e.g. Schu4), whereas Type B strains are less virulent in man, but cause tularemia in mice (e.g. HN63). The avirulent type B stain, live vaccine strain (LVS), causes disease in mice and is considered to be comparable to tularemia in man. It is therefore generally used as a model of the disease.

It has been shown that a humoral or antibody response protects mice from challenge with LVS, but that this is not effective against challenge with more virulent strains (Tarnvik A. 1989. Revs. Infectious Diseases 11:440-451). It is also well documented that a cell mediated immune (CMI) response is required for protection against infection in man (Tarnvik A. 1989. Revs. Infectious Diseases 11:440-451). Therefore for a sub-unit vaccine to be effectual, it must contain an appropriate antigen and raise a CMI response.

However, research towards the development of a sub-unit tularemia vaccine has so far failed to identify a protective antigen.

It is known that *F. tularensis,* in common with many other bacteria produces heat shock-induced proteins including members of the highly conserved hsp60 family. These include proteins which are homologous to the GroEL and GroES chaperone proteins of *Escherichia coli*. The sequence of *F. tularensis* GroEL protein (SEQ ID NO 1) and *F. tularensis*

GroES protein (SEQ ID NO 2) is shown in Figure 1 hereinafter. These two proteins are understood to form an Hsp60 complex.

The present invention relates to polypeptides which are useful as prophylactic and therapeutic vaccines against *F.tularensis*.

Accordingly, the present invention provides a polypeptide comprising at least one component of an Hsp60 obtainable from *Francisella tularensis*, or a fragment thereof, or a variant of any of these, which polypeptide, when administered to a animal (such as a mammal), produces an immune response which is protective against *F. tularensis* infection, for use in prophylactic or therapeutic vaccination against *F. tularensis*.

In particular, the polypeptide of the invention comprises the GroEL protein of SEQ ID NO 1 or a fragment thereof, or a variant of either of these. Suitably the polypeptide cross-reacts with anti-groEL antibody (available from Sigma).

However, in alternative embodiments, the polypeptide may comprise the GroES protein of SEQ ID NO 2 or a complex of GroEL and GroES proteins.

Hsp60 is constitutively expressed in *F. tularensis* and conserved between strains. It has been shown to be upregulated in response to stress (Ericsson M, et al., (1994). Infection & Immunity 62(1):178-83) making it a useful vaccine against a range of *F. tularensis* strains.

The applicants have found that it is possible to elute and purify the GroEL protein of SEQ ID NO 1 as a single protein from a *F. tularensis* bacterial lysate. Immunisation with this protein alone provided some protection against LVS

infection in mice, thus indicating that a protein sub-unit can offer protection against systemic tularemia infection.

Fragments of the polypeptides include deletion mutants and polypeptides where small regions of the polypeptide are joined together. The fragments should contain at least one antigenic region however so that they continue to produce a protective immune response.

Suitable fragments of the polypeptide have one or more amino acids deleted from the sequence and may be as small as 6 amino acids in length, provided they contain at least one antigenic determinant of the Hsp60 protein and preferably the GroEL protein of *F. tularensis*. Suitably the fragments will comprise at least 15, more suitably at least 30 and preferably at least 60 amino acids

The expression "variant" refers to sequences of amino acids which differ from the base sequence from which they are derived in that one or more amino acids within the sequence are substituted for other amino acids. Amino acid substitutions may be regarded as "conservative" where an amino acid is replaced with a different amino acid with broadly similar properties. Non-conservative substitutions are where amino acids are replaced with amino acids of a different type.

By 'conservative substitution' is meant the substitution of an amino acid by another one of the same class; the classes being as follows:

CLASS	EXAMPLES OF AMINO ACID
Nonpolar:	Ala, Val, Leu, Ile, Pro, Met, Phe, Trp
Uncharged polar:	Gly, Ser, Thr, Cys, Tyr, Asn, Gln
Acidic:	Asp, Glu
Basic:	Lys, Arg, His

As is well known to those skilled in the art, altering the primary structure of a peptide by a conservative substitution may not significantly alter the activity of that peptide because the side-chain of the amino acid which is inserted into the sequence may be able to form similar bonds and contacts as the side chain of the amino acid which has been substituted out. This is so even when the substitution is in a region which is critical in determining the peptides conformation.

Non-conservative substitutions are possible provided that these do not interrupt with the immunogenicity of the polypeptide.

Broadly speaking, fewer non-conservative substitutions will be possible without altering the biological activity of the polypeptide. Suitably variants will be at least 85% homologous, preferably at least 90% homologous to the base sequence. Variants include allelic variants as well as proteins which are encoded by nucleic acid sequences which hybridise to DNA sequences which encode the polypeptide of the invention under stringent conditions as explained hereinafter. Preferably, such hybridisation occurs at high stringency conditions, for example those illustrated in "Molecular Cloning", A Laboratory Manual" by Sambrook, Fritsch and Maniatis, Cold Spring Habor Laboratory Press, Cold Spring Harbor, N.Y.

Examples of high stringency conditions include hybridisation in 0.1 x SSC at about 65°C. SSC is the name of a buffer of 0.15M NaCl, 0.015M trisodium citrate.

The term "homologous" as used herein means that two sequences, when aligned, have similar (identical or conservatively replaced) amino acids in like positions or regions, and where identical or conservatively replaced amino acids are those which do not alter the activity or function of the protein as compared to the starting protein. For example, two amino acid sequences which are at least 85% homologous to each other have at least 85% similar (identical or conservatively replaced amino residues) in a like position when aligned. Suitably there will be no more than 5 suitably no more than 3 gaps in the alignment, and preferably, each gap would affect no more than about 25, and preferably no more than 15 amino acid residues.

Homology may be determined using methods well known in the art (see, for example, Deveraux *et al.* 1984, *Nucleic Acids Research* 12, 387-395, Wilbur, W.J. and Lipman, D.J. "Rapid Similarity Searches of Nucleic Acid and Protein Data Banks." Proceedings of the National Academy of Sciences USA 80, 726-730 (1983) and Myers E.and Miller W. "Optimal Alignments in Linear Space". Comput. Appl. Biosci. 4:11-17(1988)). One programme which may be used in determining homology is the MegAlign Lipman-Pearson one pair method (using default parameters) which can be obtained from DNAstar Inc, 1228, Selfpark Street, Madison, Wisconsin, 53715, USA as part of the Lasergene system.

The term "polypeptide" as used herein includes long chain peptides including proteins.

Preferably, the polypeptide of the invention comprises a protein of SEQ ID NO 1.

Nucleic acids which encode a polypeptide as described above will be known or isolable from *F. tularensis* libraries, or readily deducible from a knowledge of the genetic code. The nucleic acid may be DNA or RNA, and where it is aDNA molecule, it may comprise a cDNA or a genomic DNA. These nucleic acids may themselves be useful in therapy as elements of vaccine vectors, which when administered to an animal, result in expression of the desired polypeptide *in vivo*.

Thus vaccines may comprise for example a DNA or cDNA vaccine which encodes a polypeptide as described above.

Such a vaccine may be constructed using cells. Preferably, the cell is a eukaryotic cell, such as J774 or a recombinant bacterial cell, such as recombinant *Salmonella* although other systems are available.

A nucleic acid as described above may be incorporated into a vector, which is used to transform a cell, preferably a eukaryotic cel. The vector is one which is adapted to express the protein or peptide as described above *in situ*. The vector may contain the usual expression control functions such as promoters, enhancers and signal sequences, as well as a selection marker in order to allow detection of successful transformants. The selection of these will depend upon the precise nature of the vector chosen and will be known to or readily determinable by a person skilled in the art.

The vaccine may alternatively be in the form of a so-called "naked DNA" vaccine or in DNA vaccine where the vector consists of a DNA plasmid which is adapted to express the protein or peptide in situ.

In yet another alternative, the nucleic acid in included in a viral vector as are well known in the art. Where the vector is a viral vector, it is suitably attenuated to minimise any

harmful effects associated with the virus on the host. Preferably, the viral vector is derived from vaccinia virus, as it has many properties which make it a suitable vector for vaccination, including its ability to efficiently stimulate humoral as well as cell-mediated immune responses.

The polypeptides of the invention produce an immunogenic response in a animal to which it is administered which is protective against infection by *Francisella tularensis* making them useful as a basis for a prophylactic or therapeutic vaccine. Vaccines are suitably in the form of pharmaceutically acceptable compositions.

Thus the present invention further provides a pharmaceutical composition comprising either (a) a polypeptide as defined above, or (b) a nucleic acid as described above which is incorporated into a vector, such that it is expressed in a host to which said vector is administered; in combination with a pharmaceutically acceptable carrier or diluent.

Preferably the composition will comprise a polypeptide as defined above.

The compositions may be in a form suitable for intra-muscular, intra-venous, mucosal or parenteral application. Mucosal applications include intra-nasal or oral applications.

Suitable carriers are well known in the art and include solid and liquid diluents, for example, water, saline or aqueous ethanol. The liquid carrier is suitably sterile and pyrogen free.

The compositions may be in the form of liquids suitable for infusion or injection, or syrups, suspensions or solutions, as well as solid forms such as capsules, tablets, or reconstitutable powders.

The compositions of the invention may further additional other active components. For example, the other component may comprise an adjuvant which enhances the host's immune response, and/or the polypeptide may be combined with an antigen giving protective immunity against a different pathogen to form a multivalent vaccine in order to increase the benefit-to-risk ratio of vaccination.

In a particularly preferred embodiment, the other active component comprises an adjuvant which enhances the host's immune response and in particular promotes a cellular immune response, such as a CD8+, a CD4+ and/or a Th1 response.

Adjuvants which may achieve these effects include cytokines such as interleukins and interferons. In particular, the other component comprises a cytokine such as an interleukin, which acts as a Th-1 adjuvant. A particularly preferred interleukin for inclusion in the vaccines of the invention is IL-12, which has been shown to drive the expansion of a protective Th-1 cell response during early murine tularemia (Golovliov I, et al.. (1995). Infection and Immunity 63(2):534-8).

For example, the applicants have found that protection is greatly enhanced by the inclusion of the Th-1 adjuvant IL-12 to include protection against the more virulent type B strains. As illustrated hereinafter, a fully virulent type B strain (HN63) was used as the challenge strain to demonstrate protection beyond that provided by a humoral response. It is believed that this improved survival is due to stimulation of cell mediated immunity.

Polypeptides as described above may be prepared by various means including chemical synthesis, isolation from natural sources followed by any chemical modification if required, or using recombinant DNA technology. Preferably, the polypeptide is prepared by a method which comprises incorporating a

nucleotide sequence which encodes said polypeptide into a recombinant expression vector, transforming a host cell with said vector, and culturing said cell and recovering the peptide from the culture. The host cell may be eukaryotic or prokaryotic, but is conveniently a prokaryotic cell such as *E. coli*.

The dosage of the pharmaceutical compositions of the invention used as vaccines will depend upon the nature of the animal (such as mammals and in particular humans) being immunised as well as the precise nature and form of the vaccine. This will be determined by the clinician responsible. However in general, when using a virus vector such as a vaccinia virus vectors, dosages of the vector may be in the range of from 10^4-10^{12}pfu (pfu = particle forming units).

In a further aspect, the invention provides the use of a polypeptide as described above in the prevention or treatment of infection by *F. tularensis*.

For example, the polypeptide may be used in the preparation of a medicament for the prevention or treatment of infection by *F. tularensis*.

In addition the invention provides the use of a nucleic acid as described above in the prevention or treatment of infection by *F. tularensis*. The use of a nucleic acid as described above in the preparation of a medicament for the prevention or treatment of infection by *F. tularensis* forms another aspect of the invention.

In yet a further aspect, the invention provides a method for preventing or treating infection by *F. tularensis*, said method comprising administering to an animal, a polypeptide as described above. The polypeptide may be administered directly in the form of a pharmaceutical composition.

Alternatively a nucleic acid encoding said polypeptide is administered to an animal in a form in which it is expressed *in situ*. Suitable forms or vectors are described above.

Embodiments of the invention will now be described, with reference to the following non-limiting examples and with reference to the accompanying drawings in which:

Figure 1 shows the amino acid sequences of the *F.tularensis* GroEL and *F.tularensis* GroES proteins;

Figure 2 shows an ECL western blot of A) eluted Hsp60 B) LVS lysate C) Schu 4 lysate D) molecular weight marker after probing with anti-groEL antibody;

Figure 3 is a graph showing the survival of mice immunised as described in Example 2 below following intraperitoneal challenge with 10^4 MLD's of live vaccine strain;

Figure 4 is a graph showing the survival of mice immunised as described in Example 2 below following intraperitoneal challenge with 100 MLD's of the more virulent HN63 strain; and

Figure 5 shows a silver-stained SDS PAGE showing: lane A and E molecular weight markers, lane B eluted GroEL, lane C LVS and lane D HN63.

Example 1

Francisella tularensis LVS was grown on blood cysteine glucose agar for 72 hours, harvested into PBS, washed and heat killed at 65°C for 1 hour. The bacteria were lysed by boiling in Laemmli denaturing sample buffer and the proteins were separated using SDS-PAGE. GroEL was identified by western blotting using a monoclonal antibody to GroEL (Sigma) and by molecular weight determination.

Once located GroEL was excised from large format unstained gels and electroeluted into buffer using the Hoefer gel eluter (Amersham Pharmacia) set at 70 volts for 2 hours and following manufacturers instructions. The eluted product was pooled and purified by diluting (more than x20) in volatile buffer (ammonium bicarbonate 3.95g/l, SDS 1g/l) and centrifuged over a dialysis membrane (MWC 10,000 Vivascience). The protein was then diluted (x20) in sterile water and centrifuged further, until significant concentration was achieved. Any excess SDS was removed by cold precipitation followed by microcentrifugation.

Sections of gel above 60kDa, known not to contain protein bands were excised, eluted, purified and concentrated in a similar manner; and used to provide control vaccinations. The samples were protein assayed by the BCA protein assay using BSA as a standard.

Silver staining to the gel (Figure 5) showed that the eluted purified protein is a single protein (lane B) of approx. 60kDa, and that this protein occurs not only in LVS (from which it was eluted), but also within all the strains.

The identity of the eluted protein was confirmed by SDS-PAGE and western blotting using the anti-GroEL antibody and visualised by ECL detection (Pharmacia) as shown in Figure 2. This confirms the identity of this protein as a GroEL homologue of Hsp60.

Example 2

Mice immunisation and challenge regime

Groups of 5 Balb/c mice were immunised on days 0 and 14 as detailed in Table 1. Groups were challenged on day 28 with either 10^4 MLD's of LVS intraperitoneally or 100 MLD's of HN63 subcutaneously. Mice were monitored for 14 days and survival to a humane end point was recorded.

Table 1: immunisation regime

	25μg GroEL per 100μl saline	0.05μg IL-12
Group 1	+	+
Group 2	+	-
Group 3	-	-

Figures 3 and 4 show the survival of mice immunised with 50μg (in total) of this protein and challenged with either LVS or the more virulent HN63. In both challenges all of the control mice died (groups 3), and there was partial protection from immunisation with Hsp6 alone (groups 2).

Survival was enhanced against both strains by the inclusion of IL-12 (groups 1), complete protection was achieved against 10,000 MLD's LVS introduced intraperitoneally (figure 3).

CLAIMS

1. A polypeptide comprising at least one component of an Hsp60 obtainable from *Francisella tularensis*, or a fragment thereof, or a variant of any of these, which polypeptide, when administered to a animal, produces an immune response which is protective against *F. tularensis* infection, for use in prophylactic or therapeutic vaccination against *F. tularensis*.

2. A polypeptide according to claim 1 which is of SEQ ID NO 1, or a fragment thereof, or a variant of either of these, which produces an immune response which is protective against *F. tularensis* infection.

3. A nucleic acid which encodes a polypeptide according to claim 1 or claim 2 for use in prophylactic or therapeutic vaccination against *F. tularensis*.

4. A pharmaceutical composition comprising either (a) a polypeptide according to claim 1 or claim 2, or (b) a nucleic acid according to claim 3 which is incorporated into a vector, such that it is expressed in a host to which said vector is administered; in combination with a pharmaceutically acceptable carrier or diluent.

5. A pharmaceutical composition according to claim 4 which comprises a polypeptide according to claim 1 or claim 2.

6. A pharmaceutical composition according to claim 4 or claim 5 which further comprises an additional pharmaceutically active component.

7. A pharmaceutical composition according to claim 6 wherein the said additional component comprises an adjuvant which promotes a cellular immune response.

8. A pharmaceutical composition according to claim 7 wherein the cellular immune response is a CD8+, a CD4+ or a Th-1 response.

9. A pharmaceutical composition according to claim 7 or claim 8 wherein said adjuvant is a cytokine.

10. A pharmaceutical composition according to claim 9 wherein the cytokine is IL-12.

11. A pharmaceutical composition according to any one of claims 6 to 10 wherein said further component comprises an antigen giving protective immunity against a different pathogen.

12. The use of a polypeptide according to claim 1 or claim 2 in the prevention or treatment of infection by *F. tularensis.*

13. The use of a polypeptide according to claim 1 or claim 2 in the preparation of a medicament for the prevention or treatment of infection by *F. tularensis.*

14. The use of a nucleic acid according to claim 3 in the prevention or treatment of infection by *F. tularensis.*

15. The use of a nucleic acid according to claim 3 in the preparation of a medicament for the prevention or treatment of infection by *F. tularensis*

16. A method for preventing or treating infection by F. tularensis, said method comprising administering to an animal, a polypeptide according to claim 1 or claim 2.

17. A method according to claim 16 wherein a nucleic acid encoding said polypeptide is administered to an animal in a form in which it is expressed *in situ.*

F. tularensis GroEL
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GMM.

SEQ ID NO 1

F. tularensis GroES
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SEQ ID NO 2

Figure 1

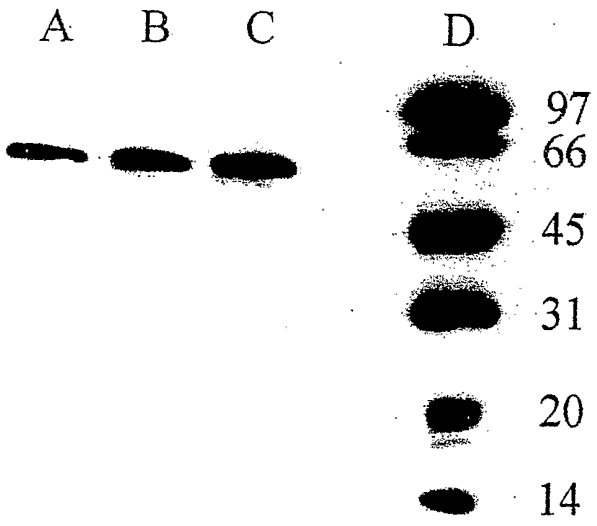
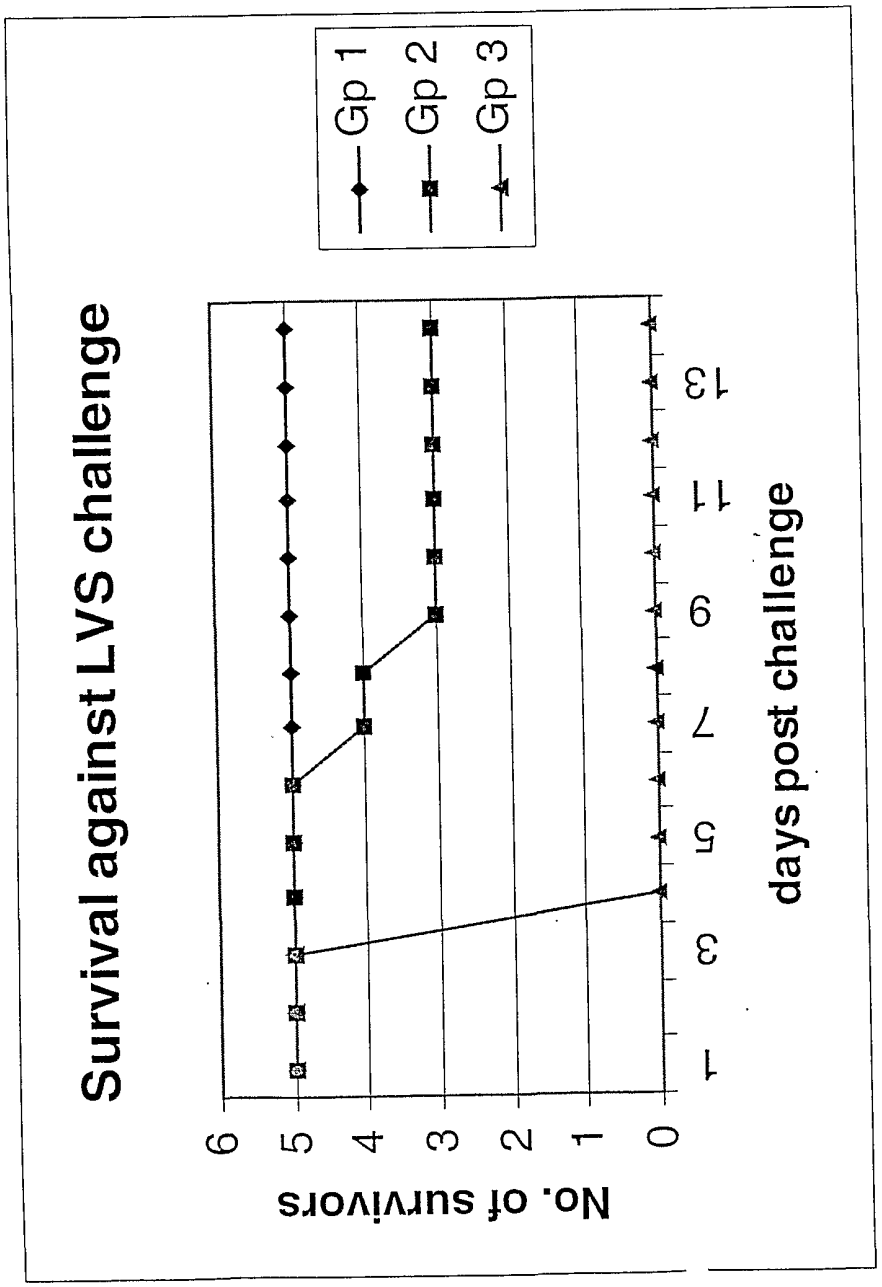


Figure 2


Survival against LVS challenge
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Gp 2
Gp 3
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days post challenge
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11
13

Figure 3

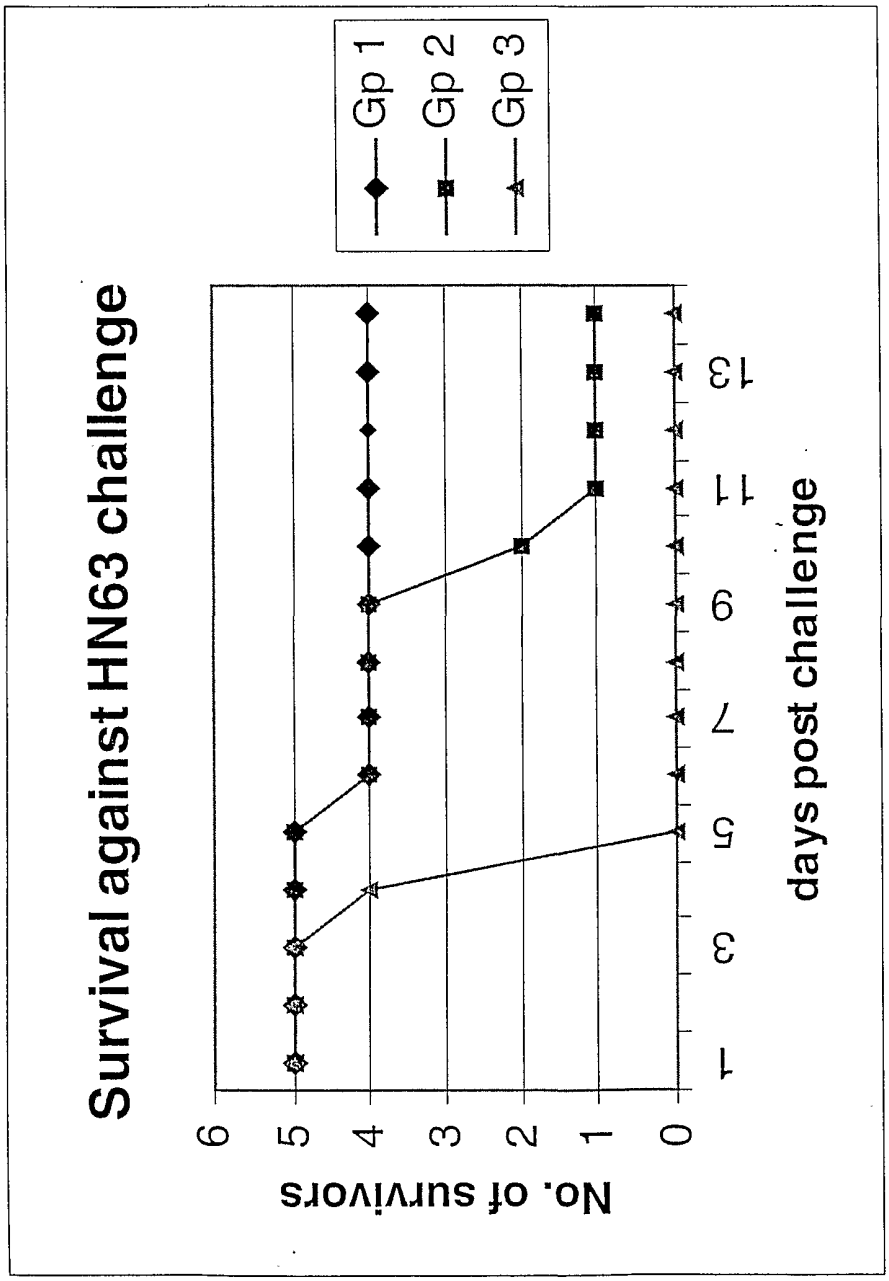
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Figure 4

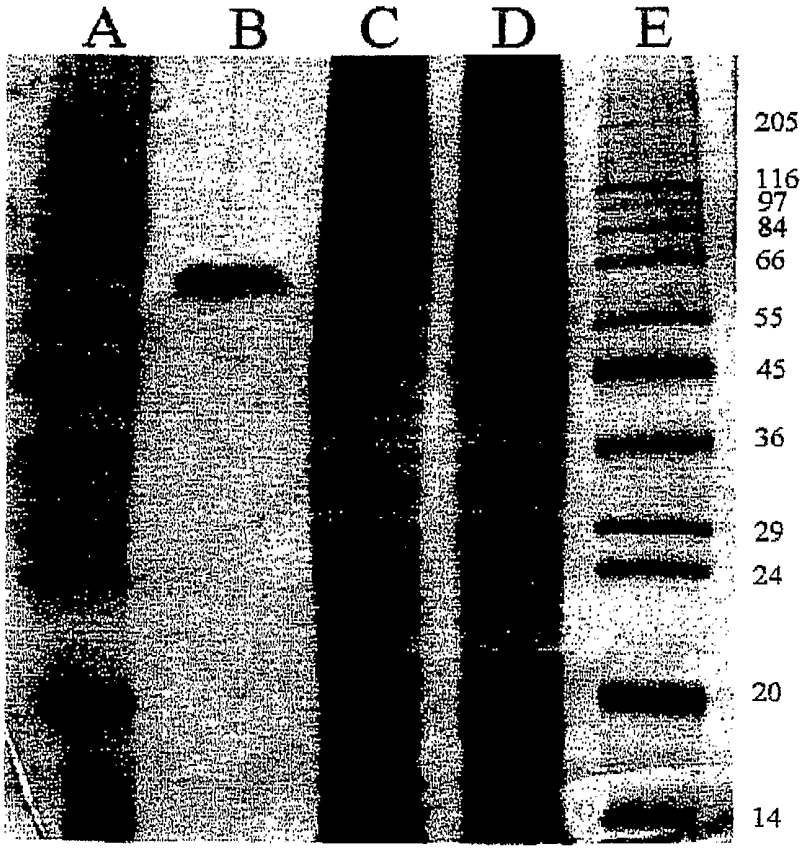

Figure 5

SEQUENCE LISTING

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Met Asn Ile Arg Pro Leu Gln Asp Arg Val Leu Val Arg Arg Ala Glu
1 5 10 15

Glu Glu Lys Lys Ser Ala Gly Gly Ile Ile Leu Thr Gly Asn Ala Gln
20 25 30

Glu Lys Pro Ser Gln Gly Glu Val Val Ala Val Gly Asn Gly Lys Lys
35 40 45

Leu Asp Asn Gly Thr Thr Leu Pro Met Asp Val Lys Val Gly Asp Lys
50 55 60

Val Leu Phe Gly Lys Tyr Ser Gly Ser Glu Val Lys Val Gly Asp Glu
65 70 75 80

Thr Leu Leu Met Met Arg Glu Glu Asp Ile Met Gly Ile Ile Ala
85 90 95

INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/GB 01/03835

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/31 C12N15/63 C07K14/195 A61K39/02 A61K31/711

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)
IPC 7 C12N C07K

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, WPI Data, PAJ, BIOSIS, CHEM ABS Data, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ERICSSON MATS ET AL: "Characterization of the nucleotide sequence of the groE operon encoding heat shock proteins chaperone-60 and -10 of Francisella tularensis and determination of the T-cell response to the proteins in individuals vaccinated with F. tularensis." INFECTION AND IMMUNITY, vol. 65, no. 5, 1997, pages 1824-1829, XP002184369 ISSN: 0019-9567 page 1827, left-hand column, paragraph 2 --- -/--	1-17

[X] Further documents are listed in the continuation of box C. [ ] Patent family members are listed in 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

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 November 2001	13/12/2001

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, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Mata Vicente, T.

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	ERICSSON M ET AL: "Increased synthesis of DnaK, GroEL, and GroES homologs by Francisella tularensis LVS in response to heat and hydrogen peroxide." INFECTION AND IMMUNITY, vol. 62, no. 1, 1994, pages 178-183, XP002184370 ISSN: 0019-9567 page 182, right-hand column, paragraph 1 ---	1-17
P,X	ERICSSON MATS ET AL: "Long-lasting recall response of CD4+ and CD8+ alphabeta T cells, but not gammadelta T cells, to heat shock proteins of Francisella tularensis." SCANDINAVIAN JOURNAL OF INFECTIOUS DISEASES, vol. 33, no. 2, 2001, pages 145-152, XP001041558 ISSN: 0036-5548 abstract ---	1-17
A	WEIGL E ET AL: "Heat shock proteins in immune reactions." FOLIA MICROBIOLOGICA, vol. 44, no. 5, 1999, pages 561-566, XP001041540 ISSN: 0015-5632 page 562, paragraph 2 -----	1-17