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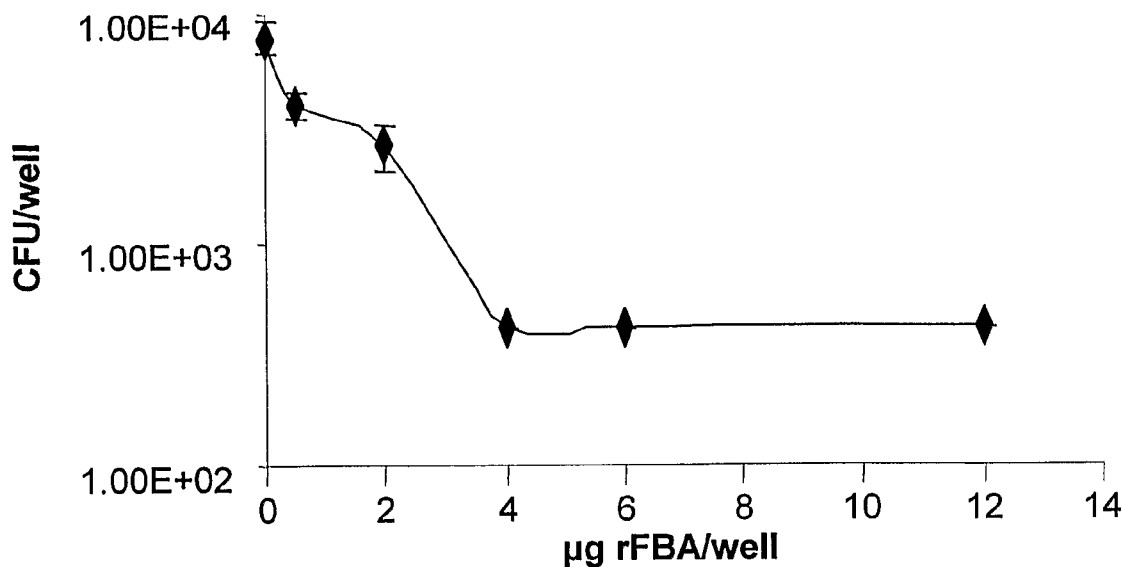
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(54) Title: METHODS AND COMPOSITIONS FOR PREVENTING AND TREATING *STREPTOCOCCUS PNEUMONIAE* INFECTION



(57) Abstract: A method of preventing and/or treating *S. pneumoniae* infection in mammalian subjects, wherein said method comprises administering to said subjects a composition comprising one or more agents that are capable of inhibiting the binding of the *S. pneumoniae* cell wall protein FBA to the respiratory tract cells of said subject.

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METHODS AND COMPOSITIONS FOR PREVENTING AND TREATING
STREPTOCOCCUS PNEUMONIAE INFECTION

Field of the Invention

The present invention relates to compositions and methods for use in preventing and treating infection of mammalian subjects by *Streptococcus pneumoniae*. More specifically, the present invention provides various compositions that may be used to prevent the adhesion of *S. pneumoniae* to mammalian host cells.

Background of the Invention

S. pneumoniae is a gram-positive bacterial organism that colonizes the upper respiratory tract and causes life-threatening diseases such as pneumonia, bacteremia and meningitis throughout the world. Currently, more than one million children die each year from pneumococcal-associated diseases.

S. pneumoniae colonizes the nasopharynx asymptotically, leading to a clinically-silent carriage state. Carriage is most prevalent in children and about 70-80% of children attending day care centers carry *S. pneumoniae* at any given time. In adults, the carriage rate may vary according to environmental conditions. The pathogenic mechanisms that convert this benign state of carriage into clinical disease are unknown. It is generally assumed, however, that in common with other bacterial species, development of the carriage state is dependent on recognition of host cells by *S. pneumoniae* and attachment thereto. It is also suspected that either the appearance of a new strain, unfamiliar to the immune system, or activation of the immune system, with

another viral or bacterial pathogen, enables the invasion and spread of the bacteria into sterile tissues thereby converting the benign carriage state into a life-threatening or potentially life-threatening clinical disease.

Following attachment, the bacteria may invade the cells and tissues, thereby leading to the aforementioned disease states associated with *S. pneumoniae*. Bacterial attachment involves interaction between one or more bacterial surface proteins, known as adhesins, and one or more host cell receptors. By means of blocking the recognition and attachment stage it may be possible to prevent the carriage stage and hence avert the development of the abovementioned disease states. Identification of bacterial adhesins involved in attachment to host mucosal layer are of prime interest in understanding the molecular basis of bacterial interaction with the host.

Currently, there is growing resistance of *S. pneumoniae* to antibiotics. In addition, the currently available vaccines offer only limited protection against infection with this organism. Consequently, an urgent need exists for clinically-safe and efficacious methods of preventing the adhesion of *S. pneumoniae* to host cells.

Summary of the Invention

The inventors have now found that fructose bisphosphate aldolase (FBA) functions as a bacterial adhesin, and advantageously, lacks human orthologs. In addition, the inventors have also found that it is possible to prevent the infection of a mammalian subject with *S. pneumoniae*, and/or treat a pre-existing infection due to this bacterium, by means of administering an agent that is capable of inhibiting

the binding of the *S. pneumoniae* cell wall protein FBA to the respiratory tract cells of said subject.

It is to be understood that the aforementioned agent may be any agent that is capable of interfering with the binding of FBA to its receptor(s) in the mammalian host cells. Consequently, in one aspect of the present invention, said agent may be a compound or composition that binds to the FBA binding site (or receptor) on the host cells, for example FBA itself, a modified form of FBA (including mutant forms, chemically modified forms, fusion proteins comprising FBA or portions thereof), domains and subdomains of FBA and fragments of FBA. Alternatively, the aforementioned agent may be a compound or composition that prevents the bacterial cell-mammalian cell adhesion by means of binding to the FBA that is present on the *S. pneumoniae* cell wall. Such agents include (but not limited to) antibodies directed to one or more epitopes present on the FBA molecule, peptides having sequence identity or homology to one or more regions of the mammalian protein to which FBA binds (i.e. the FBA receptor) and soluble forms of the FBA receptor itself (including truncated forms, chemically modified forms, mutations and so on). It is to be emphasized that the terms 'FBA receptor' and 'FBA binding partner' are used herein to refer to any protein or other molecule or molecular assembly present in or on the host cells to which FBA is capable of binding.

The present invention is thus primarily directed to a method for preventing and/or treating *S. pneumoniae* infection in mammalian subjects by means of administering to said subjects a composition comprising one or more agents that are capable of inhibiting, reversing or preventing the binding of the *S.*

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pneumoniae cell wall protein FBA to the respiratory tract cells of said subject.

Preferably, the mammalian subject is a human subject.

While the method of the present invention are suitable for application to any suitable human subject, in a preferred embodiment, the method is used in a subject who is prone to, or is suffering from, a medical condition selected from the group consisting of: asthma, bronchospasm, bronchitis, bronchiolitis, and pneumonia.

In one aspect of the invention, the agent used to prevent and/or treat the infection is a composition comprising one or more peptides, wherein said peptide(s) have sequence homology to a portion of the human Flamingo homolog 1 cadherin molecule. The administration of this composition may also be used to treat an existing *S. pneumoniae* infection: The peptide for use in the present invention comprises at least five, and preferably at least ten amino acids, wherein said peptide has sequence identity of at least 60%, and preferably at least 90%, with a sub-domain of the human Flamingo homolog 1 protein (Swiss-Prot primary accession no. Q9NYQ7). The degree of sequence identity of two sequences is defined herein below.

Without wishing to be bound by theory, it is believed that the aforementioned peptides are able to exert their desired effect by means of binding to the FBA molecules present on the surface of the bacteria, thereby inhibiting the adhesion of said bacteria to the mucosal and other human cells lining the respiratory tract mucosal cells of the subject being treated.

In one preferred embodiment, the present invention is directed to a method of preventing and/or treating *S. pneumoniae* infection in mammalian subjects by means of administering to said subjects a composition comprising one or more peptides having sequence identity of at least 60% to the amino acid sequence spanning residues 2891-2903 of the member of the cadherin family known as human Flamingo homolog 1 (hFmil) (Swiss-Prot primary accession no. Q9NYQ7; NCBI reference number gi:22095552). (This protein is also variously referred to as cadherin EGF LAG seven pass G-type receptor 3 precursor and CELR3_human.)

In another preferred embodiment, the aforementioned peptides have a sequence identity of at least 70% to the amino acid sequence spanning residues 2891-2903 of the member of the cadherin family known as human Flamingo homolog 1 (hFmil) (Swiss-Prot primary accession no. Q9NYQ7).

In yet another preferred embodiment, the aforementioned peptides have a sequence identity of at least 90% to the amino acid sequence spanning residues 2891-2903 of the member of the cadherin family known as human Flamingo homolog 1 (hFmil) (Swiss-Prot primary accession no. Q9NYQ7).

It is to be emphasized that the peptides used in the present invention (as defined hereinabove and hereinbelow) may comprise, in addition to amino acid sequences homologous to the hFmil sequence, flanking sequences of various lengths. Such flanking sequences may be sequences homologous to the native upstream and downstream sequences that flank residues 2891-2903 of the aforementioned hFmil sequences. Alternatively, the flanking sequences may be either

modifications of said native flanking sequences or unrelated thereto.

In one preferred embodiment of this aspect of the invention, the aforementioned composition comprises one or more peptides selected from the group consisting of:

a) Asp-Ser-Asp-Ser-Asp-Ser-Asp-Leu-Ser-Leu-Glu-Glu-Glu (SEQ ID NO 1);

b) Ala-Asp-Ser-Asp-Ser-Asp-Ser-Asp-Leu-Ser-Leu-Glu-Glu-Glu-Arg (SEQ ID NO 2).

In one particularly preferred embodiment of this aspect of the invention, the aforementioned composition comprises a single peptide having the sequence:

Ala-Asp-Ser-Asp-Ser-Asp-Ser-Asp-Leu-Ser-Leu-Glu-Glu-Glu-Arg
(SEQ ID NO 2)

The peptide-containing compositions used in the above-defined method may be administered either systemically (e.g. by intramuscular or intravenous injection, or by oral administration) or locally (e.g. by inhalation). In a particularly preferred embodiment, the compositions are administered by nasal inhalation. The compositions, in this case, are preferably formulated either in the form of nasal drops or as a nasal spray. The compositions may also be formulated as creams, lotions, ointments or the like for topical administration the skin or mucosal membranes.

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The present invention is also directed to the aforementioned peptide-containing pharmaceutical compositions themselves. In addition to the aforementioned active peptides, the compositions of the present invention may further comprise other pharmaceutically active agents including decongestants and antibiotic agents. Said compositions may also comprise bronchodilators, anti-inflammatory steroids, leukotriene antagonists, histamine receptor antagonists and other active pharmaceutical agents used to treat and/or prevent asthma and bronchospasm.

In another aspect, the present invention is directed to the use of one or more peptides as broadly defined above, and more specifically, peptides having sequence identity of at least 60% to the amino acid sequence spanning residues 2891-2903 of the member of the cadherin family known as human Flamingo homolog 1 (hFm1) (Swiss-Prot primary accession no. Q9NYQ7), in the preparation of a medicament.

In one preferred embodiment, the aforementioned peptides have a sequence identity of at least 70% to the amino acid sequence spanning residues 2891-2903 of the member of the cadherin family known as human Flamingo homolog 1 (hFm1) (Swiss-Prot primary accession no. Q9NYQ7).

In another preferred embodiment, the aforementioned peptides have a sequence identity of at least 90% to the amino acid sequence spanning residues 2891-2903 of the member of the cadherin family known as human Flamingo homolog 1 (hFm1) (Swiss-Prot primary accession no. Q9NYQ7).

In addition, the above-defined peptides used in the preparation of a medicament may also comprise flanking sequences as defined hereinabove.

In a preferred embodiment the present invention is directed to the use of one or more peptides selected from the group consisting of:

a) Asp-Ser-Asp-Ser-Asp-Ser-Asp-Leu-Ser-Leu-Glu-Glu-Glu (SEQ ID NO 1);

b) Ala-Asp-Ser-Asp-Ser-Asp-Ser-Asp-Leu-Ser-Leu-Glu-Glu-Glu-Arg (SEQ ID NO 2);

in the preparation of a medicament.

In a particularly preferred embodiment, the peptide used to prepare the medicament is

Ala-Asp-Ser-Asp-Ser-Asp-Ser-Asp-Leu-Ser-Leu-Glu-Glu-Glu-Arg (SEQ ID NO 2).

In one preferred embodiment, the aforementioned medicament is used to prevent infection of human subjects with *S. pneumoniae*. In another preferred embodiment, the medicament is used to treat a pre-existing *S. pneumoniae* infection.

The present invention also encompasses a method for vaccinating a human subject against infection with *S. pneumoniae*, wherein said method comprises inoculating the subject with a composition comprising one or more of the aforementioned peptides and one or more immunological adjuvants. Any suitable adjuvant having regulatory approval

for use in human subjects may be used. One example of such a suitable adjuvant is alum.

The present invention also includes within its scope a vaccine composition comprising one or more of the aforementioned peptides together with one or more immunological adjuvants.

The invention is also directed to the use of one or more of the aforementioned peptides in the preparation of a vaccine composition. In one preferred embodiment, said vaccine composition is suitable for use in the prevention of disease and carrier states caused by *S. pneumoniae*.

In another aspect, the present invention is directed to an isolated peptide having sequence identity of at least 60% to the amino acid sequence spanning residues 2891-2903 of the member of the cadherin family known as human Flamingo homolog 1 (hFm1) (Swiss-Prot primary accession no. Q9NYQ7).

In one preferred embodiment, the isolated peptide has sequence identity of at least 70% to the amino acid sequence spanning residues 2891-2903 of the member of the cadherin family known as human Flamingo homolog 1 (hFm1) (Swiss-Prot primary accession no. Q9NYQ7).

In another preferred embodiment, the isolated peptide has sequence identity of at least 90% to the amino acid sequence spanning residues 2891-2903 of the member of the cadherin family known as human Flamingo homolog 1 (hFm1) (Swiss-Prot primary accession no. Q9NYQ7).

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In one preferred embodiment of this aspect of the invention, the isolated peptide has the following sequence:

Asp-Ser-Asp-Ser-Asp-Ser-Asp-Leu-Ser-Leu-Glu-Glu-Glu (SEQ ID NO 1).

In another preferred embodiment, the isolated peptide has the following sequence:

Ala-Asp-Ser-Asp-Ser-Asp-Ser-Asp-Leu-Ser-Leu-Glu-Glu-Glu-Arg (SEQ ID NO 2).

The present invention also encompasses a method for preventing and/or treating *S. pneumoniae* infection in mammalian subjects by means of administering to said subjects a composition comprising one or more agents selected from the group consisting of FBA, modified FBA, truncated FBA, FBA domain, subdomain or fragment, and a fusion protein comprising FBA or a modified or truncated form or domain, subdomain or fragment thereof.

In one preferred embodiment, the aforementioned composition comprises FBA. In one particularly preferred embodiment, the composition comprises recombinant FBA (rFBA).

In another preferred embodiment, the composition comprises a domain, subdomain, fragment, truncated form or chemically modified form of FBA.

The compositions used in the aforementioned method may be administered either systemically (e.g. by intramuscular or intravenous injection, or by oral administration in the form of capsules, tablets etc.) or topically (e.g. nasal spray,

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nasal drops, topical formulations for delivery via the skin or mucous membranes etc.). The compositions of the present invention may further comprise other pharmaceutically active agents including decongestants and antibiotic agents, bronchodilators, anti-inflammatory steroids, leukotriene antagonists, histamine receptor antagonists and other active pharmaceutical agents used to treat and/or prevent asthma and bronchospasm.

The present invention is also directed to the use of an agent selected from FBA, modified FBA, truncated FBA, FBA domains, subdomains or fragments, and fusion proteins comprising FBA or modified or truncated forms or domains, subdomains or fragments thereof in the preparation of a medicament. In one preferred embodiment, the invention provides the use of recombinant FBA in the preparation of a medicament.

In one preferred embodiment, the aforementioned medicament is used to prevent infection of human subjects with *S. pneumoniae*. In another preferred embodiment, the medicament is used to treat pre-existing *S. pneumoniae* infection.

The present invention further encompasses a method for preventing and/or treating *S. pneumoniae* infection in mammalian subjects by means of administering to said subjects a composition comprising antibodies directed against one or more epitopes present on the FBA molecule.

The compositions used in the aforementioned method may be administered either systemically (e.g. by intramuscular or intravenous injection, or by oral administration in the form of capsules, tablets etc.) or topically (e.g. nasal spray, nasal drops, topical formulations for delivery via the skin

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or mucous membranes etc.). The compositions of the present invention may further comprise other pharmaceutically active agents including decongestants and antibiotic agents, bronchodilators, anti-inflammatory steroids, leukotriene antagonists, histamine receptor antagonists and other active pharmaceutical agents used to treat and/or prevent asthma and bronchospasm.

The present invention is also directed to the use of anti-FBA antibodies in the preparation of a medicament.

In one preferred embodiment, the aforementioned medicament is used to prevent infection of human subjects with *S. pneumoniae*. In another preferred embodiment, the medicament is used to treat pre-existing *S. pneumoniae* infection.

The present invention also encompasses a method for treating sepsis caused by *S. pneumoniae* in mammalian subjects, wherein said method comprises administering to said subjects a composition comprising one or more peptides as hereinabove described.

The compositions mentioned above may include one or more pharmaceutically acceptable carriers and excipients.

All the above and other characteristics and advantages of the present invention will be further understood from the following illustrative and non-limitative examples of preferred embodiments thereof.

Brief Description of the Drawings

Figure 1 depicts the identification of the recombinant FBA-HAT fusion protein after electrophoretic separation. **Figure 1A** shows a coomassie blue-stained 1D PAGE gel separation of a

bacterial lysate, with the 33.5 kDa FBA-HAT band indicated by an arrow. Figures 1B and 1C, each show a single 33.5 kDa band on western blots after probing with anti-HAT and anti-rFBA antibodies, respectively.

Figure 2 graphically depicts the dose-dependent inhibition of bacterial adhesion to A459 cells by 11 selected phages with the highest ability to bind rFBA. The inhibition due to each of said 11 phages is separately shown in Figures 2A to 2K, while Figure 2L depicts the effect of a phage lacking an insert (also exemplifying the negative phages).

Figure 3 graphically depicts the dose-dependent inhibition of the adhesion of encapsulated and non-encapsulated *S. pneumoniae* strains to A459 cells due to the addition of the 15 amino acid flamingo-related peptide. Figures 3A to D present, respectively, the adhesion inhibition data for *S. pneumoniae* encapsulated serotype 3 strains WU2, unencapsulated serotype 3 strain 3.8DW, encapsulated serotype 2 strain D39 and unencapsulated serotype 2 strain R6.

Figure 4 graphically illustrates the inhibitory effect of the flamingo-related peptide on *S. pneumoniae* colonization of the lungs and nasopharynx in mice following intranasal inoculation of *S. pneumoniae* cells that had been pre-incubated with the peptide. Figures 4A and 4B present the results for colonization of a peptide-treated bacterial inoculum of 5×10^5 cells in the nasopharynx and lungs, respectively. Figures 4C and 4D similarly present the results for colonization of a peptide-treated bacterial inoculum of 10^6 cells in the nasopharynx and lungs, respectively. Moreover, this treatment prevented the development of pneumonia. Figures 4E and 4F are similar

experiments as 4C and 4D but in the control experiments *S. pneumoniae* were treated with PsipD, a bacterial protein not involved in adhesion, which did not affect bacterial adhesion while FCRP inhibited significantly nasopharyngeal and lung colonization.

Figure 5 graphically illustrates the inhibitory effect of recombinant FBA on the adhesion of *S. pneumoniae* strains WU2 (Figure 5A) 3.8DW (Figure 5B) to cultured A549 lung carcinoma cells. Figure 5C demonstrates that unrelated protein PsipD is unable to inhibit bacterial adhesion.

Figure 6 graphically illustrates the concentration-dependent inhibitory effect of anti rFBA antibodies (present in immunized rabbit sera) on the adhesion of *S. pneumoniae* strains WU2 (6A) and 3.8 (6B) to cultured A549 cells. Figure 6C demonstrates that anti-PsipD antibodies are unable to inhibit bacterial adhesion.

Figure 7 shows the results of experiments performed in order to confirm the localization of FCR on the surface of A549 cells and the direct binding of rFBA to FCRP.

Figure 8 graphically illustrates the results of an in-vivo study where mice were inoculated intranasally with PBS treated bacteria and bacteria treated with 1/10 and 1/100 dilution of rabbit derived anti rFBA antibodies.

Detailed Description of Preferred Embodiments

The inventors have unexpectedly found that certain peptides related to a sub-domain of the human flamingo cadherin

molecule are capable of inhibiting the adhesion of *S. pneumoniae* to mammalian cells. It is to be noted that this subdomain (including the 13 amino acid sequence defined hereinabove as SEQ ID NO 1) is also present in other proteins, including the Anchor protein (SwissProt Accession No. Q5Y190), said Anchor protein having 78% identity to the Flamingo cadherin sequence. The present invention constitutes the various practical applications arising from this discovery, including, but not limited to the following:

1) The Flamingo Cadherin (FC) derived synthetic peptide may be used in *S. pneumoniae* preventive medicine:

- a. In many cases *S. pneumoniae* infection follows prior viral infection or allergic episodes. Inclusion of the FC peptide in nasal drops, nasal sprays, inhalations and creams directed to reduce congestion prevents the ensuing development of clinical *S. pneumoniae* diseases.
- b. One particular use of the FC peptide is in patients who are especially prone to *S. pneumoniae* infections (such as patients with asthma or with an immunocompromised immune system). Thus, inclusion of the FC peptide in the nasal drops, nasal sprays, inhalations, and creams directed to reduce congestion is used to prevent the developing of *S. pneumoniae* clinical disease.

2) Treatment with nasal drops, nasal sprays inhalation and creams to reduce bacterial spread and increase the therapeutic effectiveness of antibiotic treatments.

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3) Systemic (for example oral or injected) application to prevent bacterial spread and the development of life threatening diseases that are caused by *S. pneumoniae*.

4) Usage of the peptide for immunization in the presence of adjuvant or conjugated to a carrier protein with adjuvant properties, with the aim of developing antibodies that might neutralize and prevent *S. pneumoniae* colonization and disease development.

The preferred peptides for use in the present invention are defined herein on the basis of their sequence homology to the amino acid sequence spanning residues 2891-2903 of the member of the cadherin family known as human Flamingo homolog 1 (hFm1) (Swiss-Prot primary accession no. Q9NYQ7. The degree of sequence identity of two sequences may be defined, for the purpose of the present invention as follows:

$$\text{Sequence identity (\%)} = 100 \times (N_{\text{total}} - N_{\text{diff}}) / N_{\text{total}},$$

Wherein N_{total} is the total number of amino acid residues in one of the two peptide sequences being compared, and wherein N_{diff} is the number of amino acid differences found between the two sequences when aligned.

The peptides for use in the present invention may be prepared by any suitable means. Generally, it is most convenient to chemically synthesize the peptide(s) having the desired sequence(s) by one of the several standard peptide synthesis techniques that are well known in the art.

The first peptide synthesis methods to find commercial use were liquid-phase techniques. While these techniques have

largely been replaced by solid-phase methods, they still find use when large-scale industrial production of peptides is required.

In solid-phase peptide synthesis techniques (e.g. Merrifield, R.B., J. Am. Chem. Soc. 85. 2149-2154, 1963), peptide chains are constructed on linking groups that are immobilized onto inert beads. Following their synthesis, the desired peptide is removed from the beads by use of a non-specific cleavage agent such as trifluoroacetic acid. In order to ensure complete coupling during each round of amino acid addition, a large excess of each amino acid is commonly used. In order to avoid problems of amino acid homopolymerization as a result of the aforementioned reagent excess, it is usual to incorporate protective groups (commonly either Fmoc or Boc) into the growing peptide chain. Prior to the subsequent amino acid addition, however, the protective group needs to be removed in a deprotection reaction step. Thus, in general outline, the solid-phase synthesis of a peptide involves repetition of the following steps:

- Protective group is removed from trailing amino acids in a deprotection reaction
- Deprotection reagents washed away to provide clean coupling environment
- Protected amino acids dissolved in a solvent such as dimethylformamide (DMF) are combined with coupling reagents are pumped through the synthesis column in order to cause protection of the trailing amino acid residue
- Coupling reagents washed away to provide clean deprotection environment

As an alternative to chemical synthetic methods, peptides may also be prepared by means of expression from recombinant DNA constructs in either prokaryotic or eukaryotic hosts (e.g. *E. coli* and yeasts, human cell lines etc.).

As a first stage, the location of the genes of interest may be determined by reference to a database such as the SwissProt database. The selected sequence may, where appropriate, be isolated directly by the use of appropriate restriction endonucleases, or more effectively by means of PCR amplification. Suitable techniques are described in, for example, US patent Nos. 4,683,195, 4,683,202, 4,800,159, 4,965,188, as well as in Innis *et al.* eds., *PCR Protocols: A guide to method and applications*.

Following amplification and/or restriction endonuclease digestion, the desired gene or gene fragment is ligated either initially into a cloning vector, or directly into an expression vector that is appropriate for the chosen host cell type. In the case of the *S. pneumoniae* proteins, *Escherichia coli* is the most useful expression host. However, many other cell types may be also be usefully employed including other bacteria, yeast cells, insect cells and mammalian cell systems.

High-level expression of the desired protein or peptide within the host cell may be achieved in several different ways (depending on the chosen expression vector) including expression as a fusion protein (e.g. with factor Xa or thrombin), expression as a His-tagged protein, dual vector systems, expression systems leading to incorporation of the recombinant protein inside inclusion bodies etc. The recombinant protein will then need to be isolated from the

cell membrane, interior, inclusion body or (in the case of secreted proteins) the culture medium, by one of the many methods known in the art.

All of the above recombinant DNA and protein purification techniques are well known to all skilled artisans in the field, the details of said techniques being described in many standard works including "Molecular cloning: a laboratory manual" by Sambrook, J., Fritsch, E.F. & Maniatis, T., Cold Spring Harbor, NY, 2nd ed., 1989, which is incorporated herein by reference in its entirety.

The peptide-containing compositions used in the above-defined method may be administered to the intended subject either systemically (e.g. by intramuscular or intravenous injection or by oral administration in the form of tablets, capsules, syrups etc.) or locally (e.g. by inhalation). In a particularly preferred embodiment, the compositions are administered by nasal inhalation. The compositions, in this case, are preferably formulated either in the form of nasal drops or as a nasal spray. The compositions may also be formulated as creams, lotions, ointments or the like for topical administration the skin or mucosal membranes.

As mentioned hereinabove, in a particularly preferred, the peptide-containing compositions are formulated as drops or a spray for administration by the nasal inhalation route. In one preferred embodiment, the nasal drops formulation comprises one or more peptides as defined hereinabove dissolved in a sterile 0.9% (w/v) sodium chloride solution. The solution may optionally contain preservatives such as 0.01% benzalkonium chloride, and/or other excipients. The total concentration of the peptide(s) in the solution will

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generally be in the range of 50 - 1000 µg/ml, more preferably 100 - 500 µg/ml. In one preferred dosage regimen, one drop of the formulation is administered twice daily for three days. The formulation may also be administered with antibiotics or other pharmaceutical agents (as described hereinabove), either concurrently or consecutively. In the case of concurrent use, the additional pharmaceutical agent(s) may be formulated separately or may be included in the peptide-containing composition.

In a particularly preferred embodiment, the nasal drops formulation comprises a single peptide having the sequence:

Ala-Asp-Ser-Asp-Ser-Asp-Ser-Asp-Leu-Ser-Leu-Glu-Glu-Glu-Arg
(SEQ ID NO 2).

Further details regarding the preparation and administration of different formulations containing the peptide compositions of the present invention may be found in standard reference works such as Remington's Pharmaceutical Sciences, Mack Publishing Co, Easton, Pa, USA (1980).

In another aspect of the present invention, the peptide-containing compositions defined hereinabove and claimed herein below may be used as vaccines, i.e. they are administered to a subject in such a form and manner that the immune system of said subject responds by producing specific antibodies to the peptide(s).

Generally, the peptides of the present invention are relatively short and therefore need to be administered

together with an immunological adjuvant, in order to act as efficient immunogens.

Vaccines are generally, but not exclusively, administered by means of injection, generally by way of the intramuscular, intradermal or subcutaneous routes. Some vaccines may also be administered by the intravenous, intraperitoneal, nasal or oral routes.

The peptide containing preparations of the invention can be administered as either single or multiple doses of an effective amount of said protein. The term "effective amount" is used herein to indicate that the vaccine is administered in an amount sufficient to induce or boost a specific immune response, such that measurable amounts (or an increase in the measurable amounts) of one or more antibodies directed against the peptides used are detected in the serum or plasma of the vaccinated subject. The precise weight of peptide that constitutes an "effective amount" will depend upon many factors including the age, weight and physical condition of the subject to be vaccinated. The precise quantity also depends upon the capacity of the subject's immune system to produce antibodies, and the degree of protection desired. Effective dosages can be readily established by one of ordinary skill in the art through routine trials establishing dose response curves. Typical dose regime may be in the range between 0.2 mg/kg body to 50 mg/kg body.

The vaccines of the present invention will generally comprise an effective amount of one or more peptides as defined hereinabove as the active component, suspended in an appropriate vehicle. In the case of intranasal formulations, for example, said formulations may include vehicles that

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neither cause irritation to the nasal mucosa nor significantly disturb ciliary function. Diluents such as water, aqueous saline may also be added. The nasal formulations may also contain preservatives including, but not limited to, chlorobutanol and benzalkonium chloride. A surfactant may be present to enhance absorption of the subject proteins by the nasal mucosa.

However, in general, the vaccines of the present invention would normally be administered parenterally, by the intramuscular, intravenous, intradermal or subcutaneous routes, either by injection or by a rapid infusion method. Compositions for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Besides the abovementioned inert diluents and solvents, the vaccine compositions of the invention can also include adjuvants, wetting agents, emulsifying and suspending agents, or sweetening, flavoring, or perfuming agents.

The aforementioned adjuvants are substances that can be used to non-specifically augment a specific immune response. Normally, the adjuvant and the composition are mixed prior to presentation to the immune system, or presented separately, but into the same site of the subject being vaccinated. Adjuvants that may be usefully employed in the preparation of vaccines include: oil adjuvants (for example, Freund's complete and incomplete adjuvants, which may be used in animal experiments only and which are forbidden for human use), mineral salts, alum, silica, kaolin, and carbon,

polynucleotides and certain natural substances of microbial origin.

Further examples of materials and methods useful in the preparation of vaccine compositions are well known to those skilled in the art. In addition, further details may be gleaned from Remington's Pharmaceutical Sciences, Mack Publishing Co, Easton, Pa, USA (1980).

The following examples are provided for illustrative purposes and in order to more particularly explain and describe the present invention. The present invention, however, is not limited to the particular embodiments disclosed in the examples.

Examples

In the working Examples provided hereinbelow, the materials and methods used are as described in the following general 'Materials and Methods' section, unless otherwise stated.

Material and Methods

Reagents: Unless otherwise stated all chemicals and biochemicals of highest purity available were purchased from Sigma-Aldrich (St. Louis, MS, USA).

Bacterial strains, growth conditions and growth medium: Four *S. pneumoniae* strains and two *E.coli* strains were used in this study. Two pairs of genetically unrelated encapsulated *S. pneumoniae* strains and their unencapsulated derivatives were used in this study as previously described (Mizrachi Nebenzahl Y. et al., FEMS Microbioloy Let. 2004, 233:147-152). *E. coli* were grown in Luria-Bertani (LB) broth.

Pneumococci were grown to mid logarithmic growth phase as determined by optical density (OD) in Todd-Hewitt broth (DIFCO Laboratories, Detroit, MI, USA) supplemented by yeast extract (DIFCO Laboratories, Detroit, MI, USA).

Bacterial growth and protein purification: Fructose-bisphosphate aldolase (FBA; SP0605 *Streptococcus pneumoniae* TIGR4) was cloned into the pHAT expression vector (BD Biosciences Clontech, Palo Alto, CA, USA) as previously described (Backhed F, et al., J Biol Chem. 2002; 17:18198-205). Transformed *E. coli* host expression strain BL21(DE3)pLysS were grown overnight and expression of the recombinant his-tagged protein was induced by the addition of 1 mM IPTG to BL21(DE3)pLysS^{+FBA} cells for 5 hours. The cells were harvested by centrifugation, and lysed in lysis buffer (8 M urea, 0.1 M NaH₂PO₄, 0.01 M Tris-Cl pH 8.0). The histidine-tagged recombinant proteins were purified using a Ni-NTA column (Qiagen GMBH, Hilden, Germany). The lysate containing rFBA was incubated with the Ni-NTA column for 1 hour at room temperature. Then the column was washed with wash buffer (8 M urea, 0.1 M NaH₂PO₄, 0.01 M Tris-Cl pH 6.3), and the recombinant proteins were recovered from the column using elution buffer (8 M urea, 0.1 M NaH₂PO₄, 0.01 M Tris-Cl, pH 5.9). Isolation of the proteins was confirmed by Western blot analysis using anti-HAT antibodies (BD Biosciences Clontech, Palo Alto, CA, USA). The recombinant protein preparations were stored in elution buffer at -20°C.

Protein gel electrophoresis and staining: Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the Hoefer mini VE vertical electrophoresis system (Amersham Biosciences, San Francisco, CA, USA). Where required, the proteins were then separated in the second

dimension using 10% polyacrylamide gels. All gels were stained using Coomassie brilliant blue.

Western blot analysis: Protein mixtures were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Bio-Rad Laboratories, Inc, Carlsbad, CA, USA) as described elsewhere (Ling E et al., Clin. Exp. Immunol. 2004;138:290-298). The immunological detection of immobilized proteins was performed as described before (*ibid.*).

Identification of proteins: Proteins spots were excised from the PAGE gel and washed with 150 μ L of wash solution (50% methanol and 5% acetic acid) for 3 hours and dehydrated with 200 μ L of acetonitrile (5 min.). Reduction and alkylation was performed with 30 μ L of 10 mM DTT and 30 μ L of 100 mM iodoacetamide. After rehydration with 200 μ L of 100 mM ammonium bicarbonate (10 min.) gel pieces were dehydrated, completely dried in vacuum centrifuge and subjected to enzymatic cleavage (porcine trypsin; Promega) for 16 h at 37°C in 50 mM ammonium bicarbonate buffer. Extraction of peptides was performed by adding 30 μ L of 50 mM ammonium bicarbonate and 5% formic acid. Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF-MS) analysis was performed using the REFLEX III (Bruker-Daltonik, Bremen, Germany) mass spectrometer. Peptide peak lists were searched against the database of *S. pneumoniae* TIGR4 (www.matrixscience.com).

Recombinant protein production: Recombinant FBA (NP_345117) was expressed as previously described [Ling E, Feldman G, Portnoi M, et al. Glycolytic enzymes associated with the cell surface of *Streptococcus pneumoniae* are antigenic in humans

and elicit protective immune responses in the mouse. Clin Exp Immunol 2004;138:290-8] Pneumococcal immunogenic protein D (PsipD NP_346607) was expressed similarly using the following forward primer: GGATCCTTGAAAAAGAAGGAACTATC and reverse primer: GAATCCAATTCTTCCTTGTAGTCGT. This protein is a *S. pneumoniae* cell wall derived protein not involved in bacterial adhesion to human cells and is being used as a control throughout. The protein is designated PsipD throughout the specification.

Recombinant Fructose Bisphosphate Aldolase (rFBA) Protein purification: Recombinant Fructose Bisphosphate Aldolase (FBA; SP0605 *Streptococcus pneumoniae* TIGR4) was purified from IPTG treated BL21(DE3)pLysS^{+FBA} using the Ni-NTA columns as previously described (Backhed F, et al., J Biol Chem. 2002; 17:18198-205. The purified protein was separated by 1D PAGE (Figure 1A) and the single band of 33.5kDa, representing the FBA-HAT fusion protein identity, was analyzed and identity verified by MALDI-TOF analysis (Mascot score 146, Z score 2.43, sequence coverage 55%) demonstrating a 99% compatibility. The identity of the protein was further verified in western blots using Rabbit anti HAT antibodies (Figure 1B) and anti rFBA antibodies produced in immunized mice (Figure 1C). The identity and purity of the recombinant protein was verified for each preparation.

Immunization of mice with rFBA: Seven weeks old BALB/c female mice (Harlan Laboratories, Israel) were housed in sterile conditions under 12 hour light/dark cycles and fed Purina Chow and tap water *ad libitum*. Animal experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the Ben-Gurion University of the Negev, Beer Sheva, Israel. Mice were immunized

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intraperitoneally with 25µg of rFBA and 75 µl of Inject Alum adjuvant (Pierce Biotechnology Inc, Rockford, IL, USA) on days 0 (primary immunization) and 21 (booster). Control mice were sham immunized with adjuvant. Blood samples were collected from mice one week prior to immunization and one week after booster immunization. The sera were pooled for immunological assays.

Phage display library: The filamentous bacteriophages fd, which carry combinatorial random phage library, was kindly provided by Prof. J. Gershony (Tel Aviv, Israel) (Enshell-Seijffers D *et al.*, Nucleic Acids Res. 2001 29(10):E50-0).

50µg rFBA in Tris buffered Saline (TBS) were added into 6 well plates (Corning, Inc. Corning, NY, USA) and incubated overnight at 4°C. Following blocking with 0.25% gelatin, TBS containing the phage library was added to the well and incubated overnight at 4°C. The unadhered phages were removed and the adhered phages were eluted with 0.1N HCL, 0.1%BSA in TBS pH 2.2 and neutralizing (6M Tris-HCL pH 9.1). The rFBA adhered phages, were grown in *E. coli* strain DH5α in Terrific broth and transferred to LB agar containing 100µg/ml tetracycline for selection of phage carrying bacteria. Following incubation the plates were centrifuged to isolate the phages. The phages were precipitated with 50µl poly ethylene glycol buffer (40mM PEG, 3M NaCl), centrifuged for 40 minutes at 6000g and resuspended in TBS.

To identify the nucleic acid sequence in positive (i.e. FBA-binding) phages, the DH5α *E. coli* carrying the phages were grown overnight at 37°C in 5 ml LB broth. Following centrifugation the supernatant was removed and the DNA was recovered from the pellet using Q1Aprep spin M13 (Qiagene

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GmPh, Hilden, Germany). Sequencing was done by Daniel Biotec (Israel) using the primer: 5' TTTCACGTTGAAAATCTCC 3' (HyLabs, Rehovot, Israel).

To verify the ability of the phages to bind rFBA, the phages were transferred to nitrocellulose membrane using a Dot Blot apparatus (Biometra, Gottingen, Germany), and treated sequentially with rFBA in 30ml, mouse anti rFBA antibodies (1:5,000) and a secondary antibody (Peroxidase conjugated affinity pure goat anti mouse IgG; Jackson laboratories, West Groove, PA, USA) in a western blot like assay. The results were obtained by exposing the membrane to films (AGFA, Geveart, Belgium).

Inhibition of S. pneumoniae adhesion to A549 cells by addition of phages: A549 cells (type II epithelial lung carcinoma cells; ATCC, Rockville, MD, USA) were grown in DMEM medium supplemented with 10% foetal calf serum with penicillin and streptomycin (100µg/ml each) at 37°C in a humidified incubator. A549 cells (2.5×10^4 /well) were cultured on fibronectin coated plates, to increase A549 cell adhesion to the wells and prevent their detachment during washings, in the absence of antibiotic for 24 hours at which time the cells reached confluence (5×10^4 cells/well on average). The plates were blocked with 1% gelatine for 1 hour and 10^6 CFU bacteria were added per well. The adhesion of *S. pneumoniae* to uncoated plates was negligible (< 80CFU/ml). The number of bacteria that adhered to fibronectin coated plates (8000 ± 100 SD CFU/ml on average) was subtracted afterwards to determine the net number of bacteria that adhered to the A549 cells only. rFBA at the denoted concentrations (ranging from 5-30 µg/ml) was added to the

cultured cells and incubated for an hour. Following extensive washings *S. pneumoniae* (10^6 CFU) were added for 1 hour incubation. To identify phages able to interfere in bacterial adhesion the bacteria were preincubated with the phages for 1 hour centrifuged and added to the cultured cells. Viability of the bacteria was tested prior to and after the incubation with the phages. Following 1 hour incubation, the wells were extensively washed (x5), for the removal of non adherent bacteria. The A549 cells were liberated by 0.25% trypsin-EDTA for 5 minutes at 37°C and plated, in serial dilutions, onto Blood agar plates. The Blood agar plates were incubated for 18 hours at 37°C. The experiments were performed in triplicates and repeated three times on different occasions.

Inhibition of S. pneumoniae nasal and lung colonization with flamingo cadherin-related peptide: Seven weeks old BALB/c female mice (Harlan Laboratories, Israel) were housed in sterile conditions under 12 hour light/dark cycles and fed Purina Chow and tap water *ad libitum*. Animal experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the Ben-Gurion University of the Negev, Beer Sheva, Israel. For respiratory challenge with virulent *S. pneumoniae* mice were anaesthetized with Isoflurane (R_xElite, Meridian, ID, USA) and inoculated intranasally (i.n.) with the denoted colony forming units (CFU) bacteria (in 25 µl PBS) prior and after treatment with the flamingo cadherin derived peptide at the denoted concentrations. At 3, 6, 24 and 48 hours following inoculation the nasopharynx and the lung were excised, homogenized and plated onto blood agar plates. The experiments were performed with groups of 4 mice at each treatment and each time point and the experiments were

repeated on three different occasions.

Inhibition of S. pneumoniae adhesion to A549 cells following addition of rFBA: A549 cells (type II epithelial lung carcinoma cells; ATCC, Rockville, MD, USA) were grown in DMEM medium supplemented with 10% foetal calf serum with penicillin and streptomycin (100µg/ml each) at 37°C in a humidified incubator. A549 cells (2.5×10^4 /well) were cultured on fibronectin coated plates, to increase A549 cell adhesion to the wells and prevent their detachment during washings, in the absence of antibiotic for 24 hours at which time the cells reached confluence (5×10^4 cells/well on average). The plates were blocked with 1% gelatine for 1 hour and 10^6 CFU bacteria were added per well. The adhesion of *S. pneumoniae* to uncoated plates was negligible (< 80CFU/ml). The number of bacteria that adhered to fibronectin coated plates (8000 ± 100 SD CFU/ml on average) was subtracted afterwards to determine the net number of bacteria that adhered to the A549 cells only. rFBA at the denoted concentrations (ranging from 5-30µg/ml) were added to the cultured cells and incubated for an hour. Following extensive washings *S. pneumoniae* (10^6 CFU) were added for 1 hour incubation. To identify phages able to interfere in bacterial adhesion the bacteria were preincubated with the phages for 1 hour centrifuged and added to the cultured cells. Viability of the bacteria was tested prior and after the incubation with the phages. Following 1 hour incubation, the wells were extensively washed (x5), for the removal of non adherent bacteria. The A549 cells were liberated by 0.25% trypsin-EDTA for 5 minutes at 37°C and plated, in serial dilutions, onto Blood agar plates. The Blood agar plates were incubated for 18 hours at 37°C. The

experiments were performed in triplicates and repeated three times on different occasions.

Inhibition of encapsulated S. pneumoniae to A549 lung carcinoma cells with anti rFBA antibodies: Bacteria were treated at the denoted concentrations with anti-rFBA serum for 30 minutes centrifuged and added to the cultured A549 cells for 60 minutes incubation. Excess bacteria were removed and the cells detached with trypsin and plated onto Blood agar plates. The experiments were performed in triplicates and repeated on three different occasions.

Immunization of mice with rFBA and PsipD: Seven week old BALB/cOlaHsd (BALB/c) female mice (Harlan Laboratories, Israel) were immunized intraperitoneally with 25µg of rFBA [Ling et al., *supra*] or 25µg of PsipD or 25µg of Flamingo Cadherin receptor derived synthetic peptide (FCRP) fused to keyhole limpet hemocyanin (KLH; Sigma-Aldrich, Ltd. Rehovot, Istrael) with Inject Alum adjuvant (Pierce Biotechnology Inc, Rockford, IL, USA) at days 0 and 21. Blood samples were collected prior to and one week after booster immunization. All animal experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the Ben Gurion University of the Negev, Beer Sheva, Israel.

Immunization of rabbits: Three month old albino rabbits were immunized with 200µg rFBA or 200µg FCRP-KLH fusion protein in complete Freund's adjuvant subcutaneously and intramuscularly. Booster immunizations were performed with incomplete Freund's adjuvant.

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Flow cytometry and antibodies: A549 cells were incubated with mouse anti-FCRP-KLH serum, mouse anti-PsipD serum or control mouse serum, washed and stained with FITC-conjugated-F(ab')₂-Goat-anti-mouse-IgG+IgM (Jackson ImmunoResearch, West Grove, PA). Flow cytometry was performed using a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA). Data files were acquired and analyzed using BD CELLQuest™ 3.3 software.

Western blots: rFBA and FCRP were separated by 1D-SDS PAGE, transferred to nitrocellulose and probed with FCRP or rFBA, respectively. Rabbit anti-FCRP-KLH serum or rabbit anti rFBA was added respectively. In both cases, anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA) was used for final identification. In the control experiment preimmune serum was used as first antibody.

Bioinformatic analysis: The nucleic acid sequences of the inserted peptides in the positive phages were aligned against the human genome in the NCBI database using the Blast software. Prediction of membrane-spanning regions and their orientation was done using TMHMM server v 2.0 (<http://www.cbs.dtu.dk/services/TMHMM-2.0>).

Statistical analysis: The Spearman regression analysis was used to verify the ability of phages and peptides to inhibit bacterial adhesion to A549 lung epithelial cells in a concentration dependent manner. Further verification was done using the two-way ANOVA for repeated measurements.

Example 1

Identification of biologically active rFBA binding phages

In order to identify rFBA binding phages a random phage display library (FMC12C) was screened by the panning method, as described hereinabove. The rFBA binding phages were grown and retested for their ability to bind rFBA using a western blot like assay. Using this screening method some phages, including the phage without insert, were found not to bind rFBA, others were partially positive and 30 phages, which displayed the highest ability to bind rFBA were selected for further studies.

The 30 phages that bound rFBA were amplified in DH α *E. coli* and tested for their ability to interfere in *S. pneumoniae* adhesion to A549 lung derived cells. Unencapsulated *S. pneumoniae* serotype 3 strain 3.8DW bacteria were incubated with the selected phages using the denoted dilutions prior to their addition to the A549 cells. Eleven out of the 30 phages demonstrated a significant inhibition of unencapsulated *S. pneumoniae* serotype 3 strain 3.8DW adhesion to the cultured A549 cells starting at 1:10000 (10^8 phages/ml) dilution ($p < 0.001$) and reached 86%-96% inhibition at 1:10 dilution (10^{11} phages/ml) of the phages. These 11 phages inhibited bacterial adhesion in a dose dependent manner ($r \leq -0.778$, $p < 0.001$; Figure 2A-K). The negative phages and the phage lacking a peptide insert inhibit only 20% of *S. pneumoniae* adhesion to the A549 cells (exemplified by phage without insert; Figure 2L). Although this inhibition was significant, the extent of inhibition by these phages was significantly lower ($p < 0.001$) than the inhibitory activity of the 11 positive phages.

Example 2Identification of FBA target protein

To identify FBA target protein, the peptide-inserts in the envelope protein of the 11 phages found to be capable of interfering with *S. pneumoniae* adhesion to the cultured epithelial cells were amplified and sequenced. The nucleotide sequences were translated into the corresponding amino acid sequences as shown in the following table:

Table I

Inhibitory sequence	Phage number
Gly Asp Val Glu Asp Phe Gly Gly Asp	1
Glu Glu stop codon	2
Glu Glu Tyr Ala Asp	3
Asp Glu Glu Gly Cys Asp Cys Tyr	4
Ser Tyr Glu Glu Ser	5
Asp Glu Gly	6
Asp Ser Ser Asp Cys Asp Leu Ala Trp Glu Glu Glu	7
Gly Gly Gly Ala Trp Glu Ser Asp Trp Glu Ser Asp	8
Ser Glu Ser Glu	9
Asp Ser Tyr Gly Ser	10
Gly Gly Ala Glu	11

All the sequences were combined and aligned against the human genome in the NCBI data base using the BLAST software. Two membrane proteins were identified, each containing peptides homologous to the phage derived peptide sequences: the Flamingo cadherin protein and the opioid receptor protein.

gi|22095552|sp|Q9NYQ7|CLR3 HUMAN

Cadherin EGF LAG seven-pass G-type receptor 3 precursor
 (Flamingo homolog 1) (hFm1) (Multiple epidermal growth
 factor-like domains 2) (Epidermal growth factor-like 1)

Length = 3312

Score = 23.5 bits (48), Expect = 249

Identities = 9/13 (69%), Positives = 10/13 (76%), Gaps = 1/13
 (7%)

Query: 1 DS-SDCDLAWEEE 12

DS SD DL+ EEE

Sbjct: 2891 DSDSDSDLSEEEE 2903

Using the TMHMM software the homologous areas in the Flamingo cadherin and the opioid receptor were analyzed. The area of homology to the FBA binding peptides were found to reside in the extracellular domain of Flamingo cadherin, with a 69% homology. However, the area of homology to the opioid growth factor receptor appears at the N-terminal intracellular domain with only 45% homology to the FBA binding peptides.

The homologous area in the Flamingo-Cadherin (FC) protein to the FBA binding peptides was found between amino acids 2891-2903.

Alignment analysis using the RELIC software revealed that 9 out of the 11 peptide sequences from the positive phages matched different regions of FCR (Table II). The insert peptide in phage number 7 aligned to FCR in three different areas with the highest homology.

Table II: Homology of peptide inserts to Flamingo Cadherin Receptor.

Phage number	insert sequence	Homology to Cadherin
1	GDVEDFGGD	GDVEDFGGD 28LSQEELGGG ³⁶
3	EEYAD	EEYAD 2298EEYAA ²³⁰²
5	SYEES	SYEES 2812LFEES ²⁸¹⁶
6	DEG	DEG 1095DEG ¹⁰⁹⁷
7	DSSDCDLAWEEE	DSSDCDLAWEEE 1466TGEDCELDTEAG ¹⁴⁷⁸
		DSSDCDLAWEEE 1629SVDDCDVAVALQ ¹⁶⁴¹
		DS-SDCDLAWEEE 2891DSDSDSLSLEEE ²⁹⁰³
8	GGGAWESDWESD	GGGAWESDWESD 57GGGALALCPSS ⁶⁸
9	SESE	SESE 2911SESE ²⁹¹⁴
10	DSYGS	DSYGS 2666EGYGN ²⁶⁷⁰
11	GGAE	GGAE 1617GGAQ ¹⁶²⁰

Example 3**Synthesis of 15 amino acid FC-derived peptide**

A 15 amino acid peptide from Flamingo-cadherin, based on the 13 residue homologous area described in Example 2, with two additional flanking amino acids (one N-terminal and one C-terminal, both present in the native FC sequence), was synthesized using the Sigma Genosys peptide synthesis service (Sigma Chemical Co., St. Louis, Missouri).

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According to the technical protocols provided by Sigma, the peptide synthesis technique used is based on the use of Fmoc chemistry under continuous flow conditions using PEG-Polystyrene resins. Peptides are synthesized in a C terminus-to-N terminus direction, following which they are removed from said resin and de-protected using a TFA/DTT/H₂O/Triisopropyl silane (88/5/5/2) cleavage cocktail. Peptides are then precipitated from the cleavage cocktail using cold diethyl ether. The precipitate is then washed three times with the cold ether and then dissolved in a buffer solution containing H₂O/ACN/HOAc (75/20/5). Finally, the washed peptides are lyophilized from the aforementioned buffer solution. The purity of the synthesized peptides is assessed using reversed phase chromatography on a Supelco Bio Wide Pore column, and by MALDI-TOF mass spectrometry, and if required, further purification of the peptide is performed by reversed phase chromatography.

The sequence of the 15 amino acid residue peptide synthesized using the above method is:

H-Ala-Asp-Ser-Asp-Ser-Asp-Ser-Asp-Leu-Ser-Leu-Glu-Glu-Glu-Arg-OH

Localization of FCR on the surface of A549 cells was verified by flow cytometry using mouse anti-FCRP-KLH serum followed by goat anti mouse IgM+IgG FITC. The majority (92%) of A549 cells are stained positively by anti-FCRP-KLH serum, whereas the cells are not stained by the pre-immune serum, by another control serum (anti-PsipD serum; Figure 7A) or by anti-KLH antibodies (Figure 7B).

To determine more directly whether FCRP binds to rFBA, Western blot like assays were performed where rFBA is separated using 1D SDS-PAGE, transferred to a nitrocellulose membrane and probed with FCRP. Binding of FCRP was detected by incubating the membrane sequentially with rabbit anti-FCRP-KLH serum and anti-rabbit IgG. Using this protocol, a band of 33.5kDa was shown to bind FCRP, corresponding to the molecular weight of the rFBA-HAT fusion protein (Figure 7C). In the control (Figure 7D) the first antibody was a pre-immune serum. In the converse experiment, when FCRP is separated on SDS-PAGE, a band of approximately 1.5kDa corresponding to the molecular weight of FCRP is bound by rFBA (Figure 7 E). In the control (Figure 7F) the first antibody was a pre-immune serum.

Example 4

The biological activity of the Flamingo cadherin (FC) derived peptide in vitro

The ability of FC derived synthetic peptide to interfere in *S. pneumoniae* adhesion to A549 cells was analyzed. FC peptide ability to interfere in *S. pneumoniae* adhesion to A549 cells of encapsulated *S. pneumoniae* serotype 3 strain WU2 and serotype 2 strain D39 and their unencapsulated derivatives 3.8DW and R6, respectively was tested. FC inhibited significantly in a dose dependent manner ($r=-0.844$, $p<0.001$) the adhesion of the *S. pneumoniae* strain WU2 to A549 cells. Maximal adhesion was achieved at 15 μ g/well FC peptide (80%; Figure 3A). Inhibition of the non-encapsulated mutant strain 3.8DW was dose dependent ($r=-0.551$, $p<0.05$; Figure 3B) and reached maximal adhesion inhibition of 41% with 20 μ g/well of FC derived peptide. Furthermore, the peptide inhibited both the D39 and the R6 strains adhesion to A549

significantly ($p < 0.001$) at $5\mu\text{g}/\text{well}$. In both strains, the inhibition of bacterial adhesion was concentration dependent ($r = -0.768$, $p < 0.001$; $r = -0.833$, $p = 0.001$, respectively; Figure 3C and 3D). FC peptide inhibited 90% of *S. pneumoniae* strain D39 adhesion to A549 cells significantly at $20\mu\text{g}/\text{well}$ ($p < 0.001$). FC peptide inhibited 75% of the unencapsulated strain R6 at $15\mu\text{g}/\text{well}$.

Example 5

The biological activity of the Flamingo cadherin derived peptide in vivo

To find whether FC peptide is capable of inhibiting *S. pneumoniae* colonization, mice were inoculated intranasally prior and after treatment with the FC-derived peptide at the denoted concentration. *S. pneumoniae* serotype 3, strain WU2 bacteria were treated with FC peptide, excess peptide was removed and the bacteria were inoculated intranasally. Inoculation of the mice with 5×10^5 *S. pneumoniae* treated with $20\mu\text{g}/\text{ml}$ FC peptide inhibited colonization in the nasopharynx significantly at 3, 6 24 and 48 hours following inoculation ($p < 0.01$; Figure 4A). Treatment of the 5×10^5 *S. pneumoniae* with $40\mu\text{g}/\text{ml}$ FC peptide prevented *S. pneumoniae* colonization of the nasopharynx at all time points tested ($p < 0.01$; Figure 4A). Treatment of 5×10^5 *S. pneumoniae* with 20 or $40\mu\text{g}/\text{ml}$ FC peptide inhibited significantly colonization of the lung at 3 and 6 hours post inoculation ($p < 0.001$; Figure 4B). Using this size of inoculum, a natural clearance of bacteria from the lungs occurred 24 hours and 48 hours following inoculation.

A similar experiment was also performed with a higher initial inoculum. Inoculation of the mice with 10^6 *S. pneumoniae*

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serotype 3 strain WU2 treated with 20µg/ml FC inhibited colonization in the nasopharynx significantly at 3, 6 24 and 48 hours following inoculation ($p < 0.05$; Figure 4C). Treatment of this inoculum size with 40µg/ml FC peptide demonstrated a significantly higher ability to inhibit *S. pneumoniae* colonization of the nasopharynx ($p < 0.01$; Figure 4C). Treatment of 10^6 *S. pneumoniae* with 40µg/ml FC peptide significantly inhibited colonization of the lung at 3, 6 and 24 hours post inoculation ($p < 0.05$; Figure 4D). However, 48 hours post inoculation, while a significant increase in bacterial numbers could be found in mice inoculated with untreated bacteria, signifying the development of pneumonia, no bacteria could be detected in the lungs of mice inoculated with 20 or 40µg/ml FC peptide-treated bacteria ($p < 0.001$; Figure 4D).

Treatment of bacteria with an unrelated (PsipD) full length protein did not significantly inhibit bacterial adhesion in a dose dependent manner (data not shown).

FCRP inhibited bacterial colonization in the nasopharynx (Figure 4E) and the lungs (Figure 4F) significantly ($p < 0.05$) in comparison to bacteria treated with the unrelated protein rPsipD.

Example 6

Inhibition of *S. pneumoniae* adhesion to A549 lung carcinoma cells by rFBA

rFBA, at the indicated concentrations, was added to cultured A549 lung carcinoma cells. Following incubation the excess

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protein was removed and *S. pneumoniae* were added to the cells. Excess bacteria were removed and the cells were released, plated onto blood agar plates and the adhered bacteria were enumerated. rFBA inhibited *S. pneumoniae* encapsulated serotype 3 strain WU2 and its unencapsulated mutant 3.8DW adhesion significantly in a concentration dependent manner ($r=-0.712$; $p<0.001$ and $r=-0.646$, $p<0.001$, respectively). rFBA inhibited 63% of *S. pneumoniae* strain WU2 adhesion significantly at $0.5\mu\text{g}/\text{well}$ ($p<0.001$) and 92% of *S. pneumoniae* strain 3.8DW at $12\mu\text{g}/\text{well}$ ($p<0.001$; Figure 5A and 5B respectively). Inhibition of encapsulated serotype 3 strain WU2 adhesion to A549 cells was obtained only at $40\mu\text{g}/\text{ml}$ of PsipD (an unrelated bacterial protein) with a low correlation ($r=-0.419$; $p<0.001$; Figure 5C). These results differed significantly from the inhibition of the bacteria adhesion with rFBA ($p<0.001$).

Example 7

Inhibition of encapsulated *S. pneumoniae* adhesion to A549 lung carcinoma cells with anti rFBA antibodies

S. pneumoniae serotype 3, strains WU2 and 3.8DW, were treated with serum obtained from immunized rabbits, and with preimmune sera as controls at the indicated concentrations. The excess sera were removed following incubation by centrifugation. No effect on bacterial viability was observed with either the immune or the preimmune sera. The assay for inhibition of *S. pneumoniae* adhesion to cultured cells was performed as described in the materials and methods section hereinabove. Anti rFBA antibodies inhibited *S. pneumoniae* strains WU2 and 3.8 adhesion to A549 cells significantly in a concentration dependent manner ($r=-0.884$,

$p < 0.001$ and $r = -0.841$, $p < 0.001$, respectively; Figures 6A and 6B). No effect on the A549 cells viability was observed with either the preimmune or immune sera at the dilutions used in these experiments (data not shown). Anti-PsipD antibodies did not affect bacterial adhesion to 549 cells significantly ($r = 0.159$; $p < 0.603$; Figure 6C). Notably, none of the sera influenced bacterial viability (no serum $6.8 \times 10^4 \pm 2900$, pre-immune serum $6.9 \times 10^4 \pm 3300$, and immune serum $7.1 \times 10^4 \pm 3500$ CFU).

Example 8

S. pneumoniae reduced virulence upon treatment with antibodies to rFBA in vivo

7 weeks old BALB/c mice were inoculated intranasally with PBS treated bacteria and bacteria treated with 1/10 and 1/100 dilution of rabbit derived anti rFBA antibodies. While animals inoculated with the PBS succumbed within 5 days, a significant increase ($p < 0.05$) in survival were obtained in mice inoculated with 1/10 or 1/100 dilution of the anti rFBA antibodies (40% and 20% respectively). The results are shown in Figure 8, where the abscissa indicates the time (in days) following administration of the antibodies and the ordinate indicates percentages of survival of the mice.

While specific embodiments of the invention have been described for the purpose of illustration, it will be understood that the invention may be carried out in practice by skilled persons with many modifications, variations and adaptations, without departing from its spirit or exceeding the scope of the claims.

Claims

1. A method of preventing and/or treating *S. pneumoniae* infection in mammalian subjects, wherein said method comprises administering to said subjects a composition comprising one or more agents that are capable of inhibiting the binding of the *S. pneumoniae* cell wall protein FBA to the respiratory tract cells of said subject.
2. A method according to claim 1, wherein the composition used comprises at least one peptide which contains at least five amino acids, said peptide having sequence identity of at least 60% with a sub-domain of the human Flamingo homolog 1 protein (Swiss-Prot primary accession no. Q9NYQ7).
3. A method according to claim 2, wherein the composition used comprises at least one peptide which contains at least ten amino acids, said peptide having sequence identity of at least 60% with a sub-domain of the human Flamingo homolog 1 protein (Swiss-Prot primary accession no. Q9NYQ7).
4. A method according to claims 2 or 3, wherein the composition used comprises one or more peptides having sequence identity of at least 90% with a sub-domain of the human Flamingo homolog 1 protein (Swiss-Prot primary accession no. Q9NYQ7).
5. A method according to claim 1, wherein said method comprises administering to the subject a composition comprising one or more peptides having sequence identity of at least 60% to the amino acid sequence spanning residues 2891-2903 of the human Flamingo homolog 1 protein (Swiss-Prot primary accession no. Q9NYQ7).

6. The method according to claim 5, wherein the composition used comprises one or more peptides having sequence identity of at least 90% to the amino acid sequence spanning residues 2891-2903 of the human Flamingo homolog 1 protein (Swiss-Prot primary accession no. Q9NYQ7).

7. The method according to claim 5, wherein the one or more peptides are selected from the group consisting of:

a) Asp-Ser-Asp-Ser-Asp-Ser-Asp-Leu-Ser-Leu-Glu-Glu-Glu (SEQ ID NO 1);

b) Ala-Asp-Ser-Asp-Ser-Asp-Ser-Asp-Leu-Ser-Leu-Glu-Glu-Glu-Arg (SEQ ID NO 2).

8. The method according to claim 7, wherein the composition administered comprises a single peptide having the sequence: Ala-Asp-Ser-Asp-Ser-Asp-Ser-Asp-Leu-Ser-Leu-Glu-Glu-Glu-Arg (SEQ ID NO 2).

9. The method according to claim 1, wherein said method comprises administering to said subjects a composition comprising one or more agents selected from the group consisting of FBA, modified FBA, truncated FBA, FBA domains, subdomains or fragments, and a fusion protein comprising FBA or a modified or truncated form or domain, subdomain or fragment thereof.

10. The method according to claim 9, wherein the composition comprises FBA.

11. The method according to claim 1, wherein said method comprises administering to said subjects a composition comprising antibodies directed against one or more epitopes present on the FBA molecule.
12. The method according to any one of claims 1 to 11, wherein the composition is administered systemically.
13. The method according to any one of claims 1 to 12, wherein the composition is administered by local administration.
14. The method according to claim 13, wherein the composition is administered by nasal inhalation.
15. The method according to claim 13, wherein the composition is topically administered to the skin and/or mucosal membranes.
16. The method according to any one of the preceding claims, wherein the mammalian subject is a human subject.
17. The method according to claim 16, wherein the human subject is immunocompromised.
18. The method according to claim 16, wherein the human subject is prone to, or is suffering from, a medical condition selected from the group consisting of: asthma, bronchospasm, bronchitis, bronchiolitis, and pneumonia.
19. The method according to any one of the preceding claims, wherein the composition is co-administered with one or more

additional pharmaceutical agents selected from the group consisting of: decongestants, antibiotics, bronchodilators, anti-inflammatory steroids, leukotriene antagonists and histamine receptor antagonists.

20. A pharmaceutical composition comprising one or more peptides having sequence identity of at least 60% to the amino acid sequence spanning residues 2891-2903 of the human Flamingo homolog 1 protein (Swiss-Prot primary accession no. Q9NYQ7).

21. The pharmaceutical composition according to claim 20, wherein the one or more peptides have sequence identity of at least 90% to the amino acid sequence spanning residues 2891-2903 of the human Flamingo homolog 1 protein (Swiss-Prot primary accession no. Q9NYQ7).

22. The pharmaceutical composition according to claim 20, wherein the one or more peptides are selected from the group consisting of:

a) Asp-Ser-Asp-Ser-Asp-Ser-Asp-Leu-Ser-Leu-Glu-Glu-Glu (SEQ ID NO 1);

b) Ala-Asp-Ser-Asp-Ser-Asp-Ser-Asp-Leu-Ser-Leu-Glu-Glu-Glu-Arg (SEQ ID NO 2).

23. The pharmaceutical composition according to claim 22, wherein said composition comprises a single peptide having the sequence:

Ala-Asp-Ser-Asp-Ser-Asp-Ser-Asp-Leu-Ser-Leu-Glu-Glu-Glu-Arg (SEQ ID NO 2).

24. The pharmaceutical composition according to claim 20, wherein said composition further comprises one or more additional pharmaceutically active agents selected from the group consisting of: decongestants, antibiotics, bronchodilators, anti-inflammatory steroids, leukotriene antagonists and histamine receptor antagonists.

25. Use of at least one peptide which contains at least five amino acids, said peptide having sequence identity of at least 60% with a sub-domain of the human Flamingo homolog 1 protein (Swiss-Prot primary accession no. Q9NYQ7), in the preparation of a medicament for preventing and/or treating *S. pneumoniae* infection in mammalian subjects.

26. The use according to claim 25, wherein the peptide contains at least ten amino acids.

27. The use according to claims 25 or 26, wherein the peptide has sequence identity of at least 90% with a sub-domain of the human Flamingo homolog 1 protein (Swiss-Prot primary accession no. Q9NYQ7).

28. Use of one or more peptides having sequence identity of at least 60% to the amino acid sequence spanning residues 2891-2903 of the human Flamingo homolog 1 protein (Swiss-Prot primary accession no. Q9NYQ7), in the preparation of a medicament.

29. The use according to claim 28, wherein the one or more peptides have a sequence identity of at least 90% to the amino acid sequence spanning residues 2891-2903 of the human

Flamingo homolog 1 protein (Swiss-Prot primary accession no. Q9NYQ7).

30. The use according to claim 28, wherein the one or more peptides are selected from the group consisting of:

a) Asp-Ser-Asp-Ser-Asp-Ser-Asp-Leu-Ser-Leu-Glu-Glu-Glu (SEQ ID NO 1);

b) Ala-Asp-Ser-Asp-Ser-Asp-Ser-Asp-Leu-Ser-Leu-Glu-Glu-Glu-Arg (SEQ ID NO 2).

31. The use according to claim 30, wherein the peptide is Ala-Asp-Ser-Asp-Ser-Asp-Ser-Asp-Leu-Ser-Leu-Glu-Glu-Glu-Arg (SEQ ID NO 2).

32. A method for vaccinating a human subject against infection with *S. pneumoniae*, wherein said method comprises inoculating the subject with a composition comprising one or more of the peptides defined in any of claims 2 to 8, together with one or more immunological adjuvants.

33. Use of one or more of the peptides defined in any one of claims 2 to 8 in the preparation of a vaccine composition.

34. The use according to claim 33, wherein the vaccine composition is suitable for use in the prevention of disease and carrier states caused by *S. pneumoniae*.

35. An isolated peptide having sequence identity of at least 60% to the amino acid sequence spanning residues 2891-2903 of the human Flamingo homolog 1 protein (Swiss-Prot primary accession no. Q9NYQ7).

36. The isolated peptide according to claim 35, wherein said peptide has sequence identity of at least 90% to the amino acid sequence spanning residues 2891-2903 of the human Flamingo homolog 1 protein (Swiss-Prot primary accession no. Q9NYQ7).

37. The isolated peptide according to claim 35, wherein said peptide has the following sequence:

Asp-Ser-Asp-Ser-Asp-Ser-Asp-Leu-Ser-Leu-Glu-Glu-Glu (SEQ ID NO 1).

38. The isolated peptide according to claim 35, wherein said peptide has the following sequence:

Ala-Asp-Ser-Asp-Ser-Asp-Ser-Asp-Leu-Ser-Leu-Glu-Glu-Glu-Arg (SEQ ID NO 2).

39. A pharmaceutical composition comprising one or more agents selected from the group consisting of FBA, modified FBA, truncated FBA, FBA domain, subdomain or fragment, and a fusion protein comprising FBA or a modified or truncated form or domain, subdomain or fragment thereof.

40. The pharmaceutical composition according to claim 39, wherein the agent is recombinant FBA.

41. The pharmaceutical composition according to claim 39 or claim 40, wherein said composition further comprises one or more additional pharmaceutically active agents selected from the group consisting of: decongestants, antibiotics,

bronchodilators, anti-inflammatory, steroids, leukotriene antagonists and histamine receptor antagonists.

42. Use of an agent selected from the group consisting of FBA, modified FBA, truncated FBA, FBA domains, subdomains or fragments, and fusion proteins comprising FBA or modified or truncated forms or domains, subdomains or fragments thereof in the preparation of a medicament.

43. The use according to claim 42, wherein the agent is recombinant FBA.

44. A pharmaceutical composition comprising antibodies directed against one or more epitopes present on the FBA molecule.

45. Use of anti-FBA antibodies in the preparation of a medicament.

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Fig. 1A

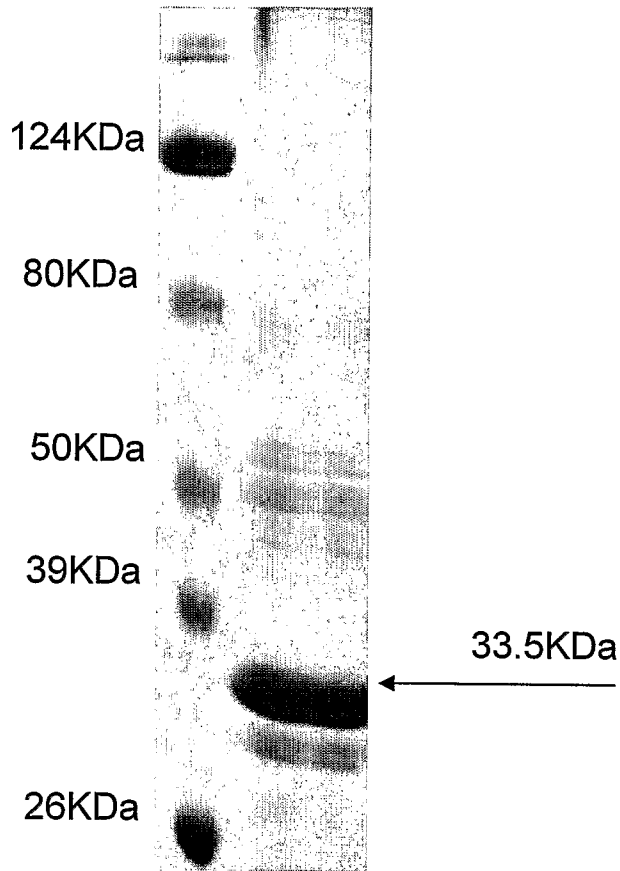


Fig. 1B

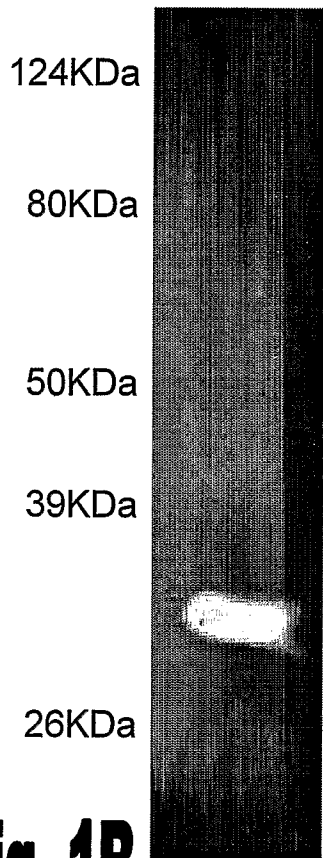
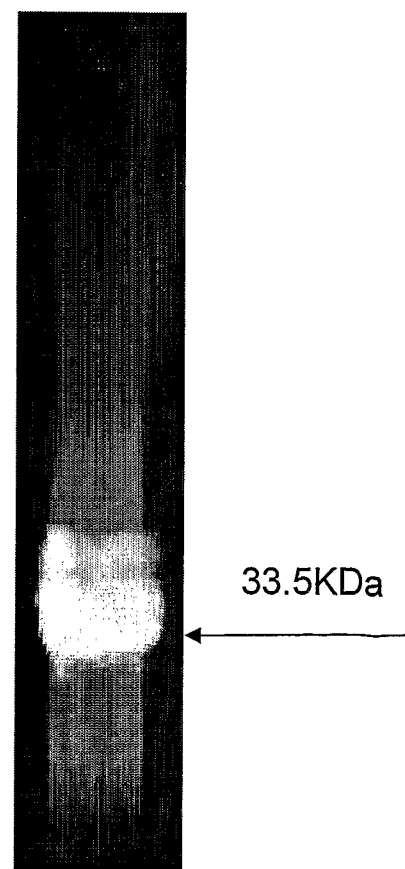


Fig. 1C



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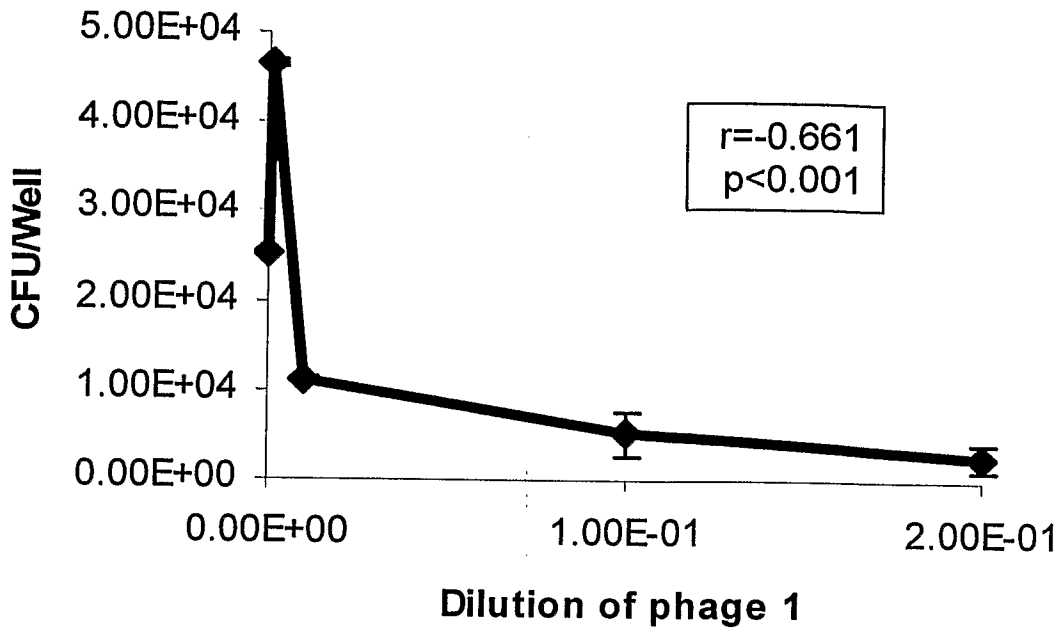


Fig. 2A

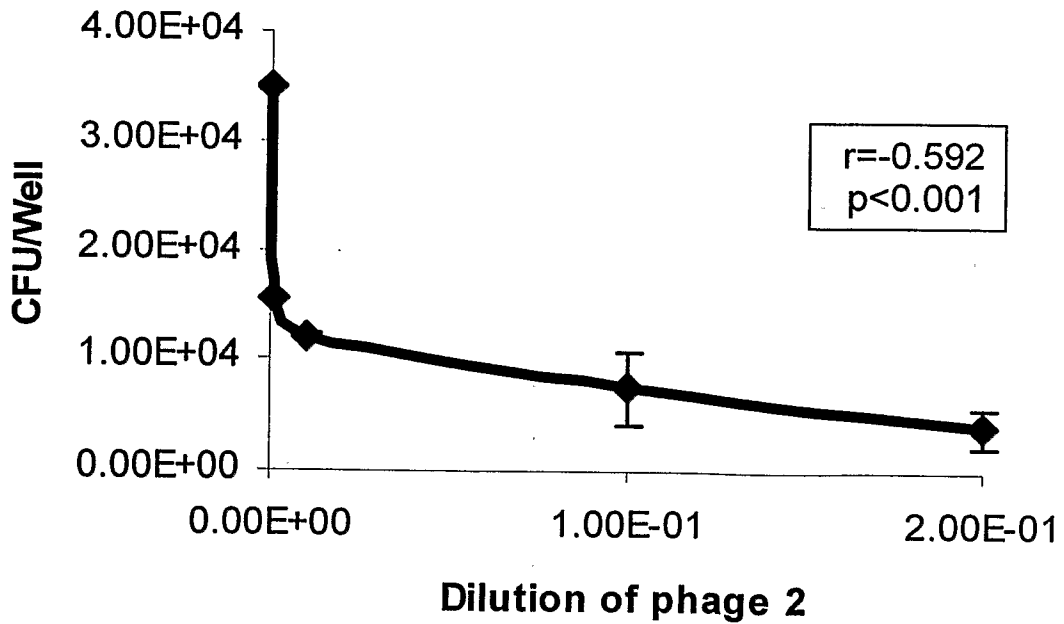


Fig. 2B

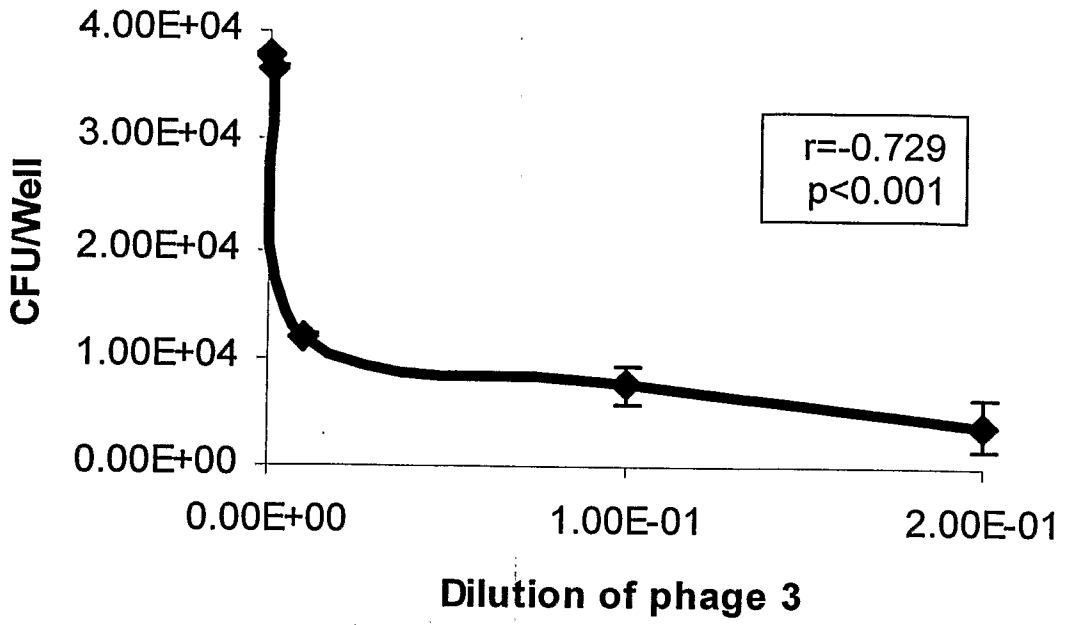


Fig. 2C

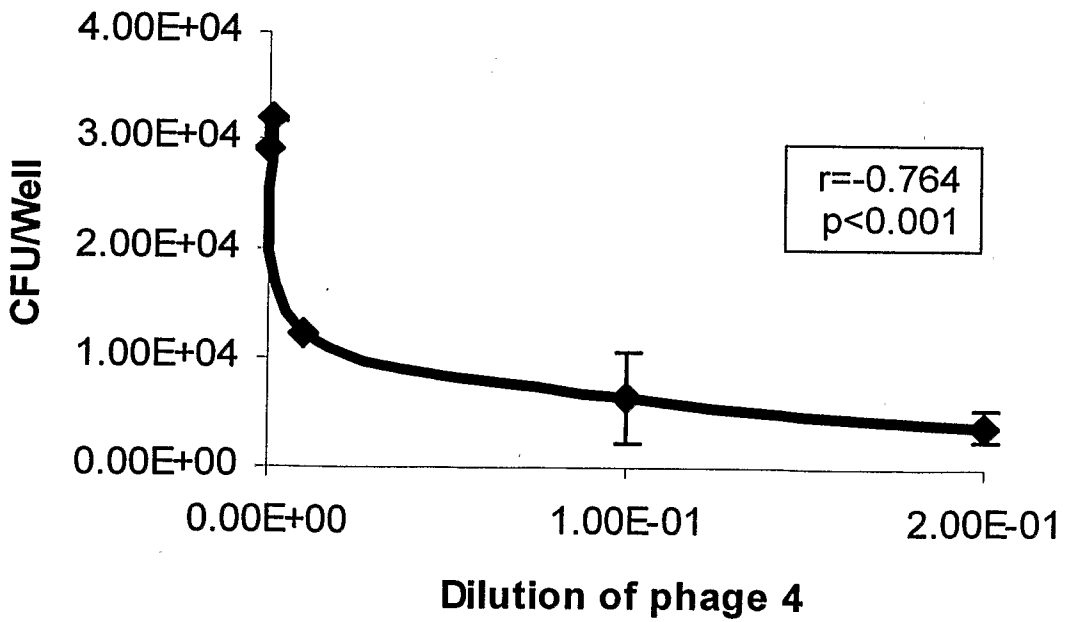


Fig. 2D

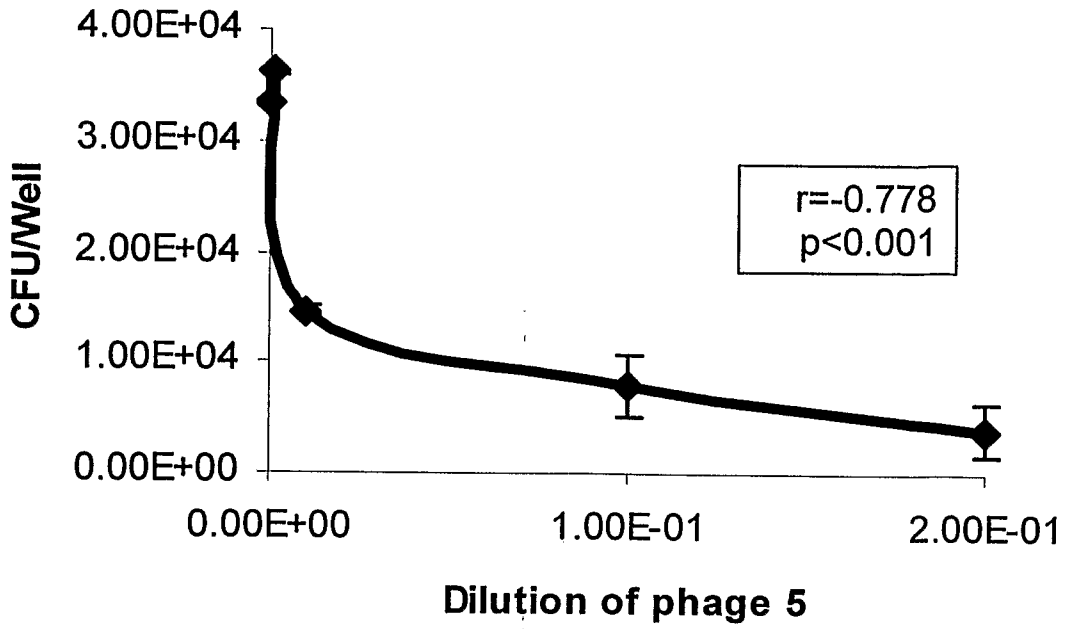


Fig. 2E

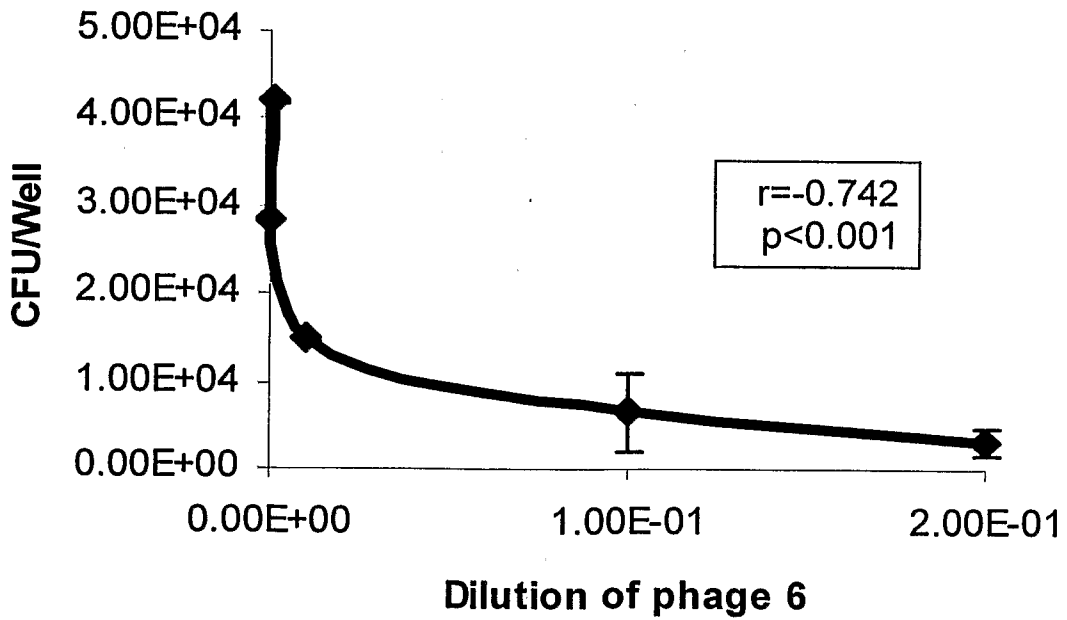


Fig. 2F

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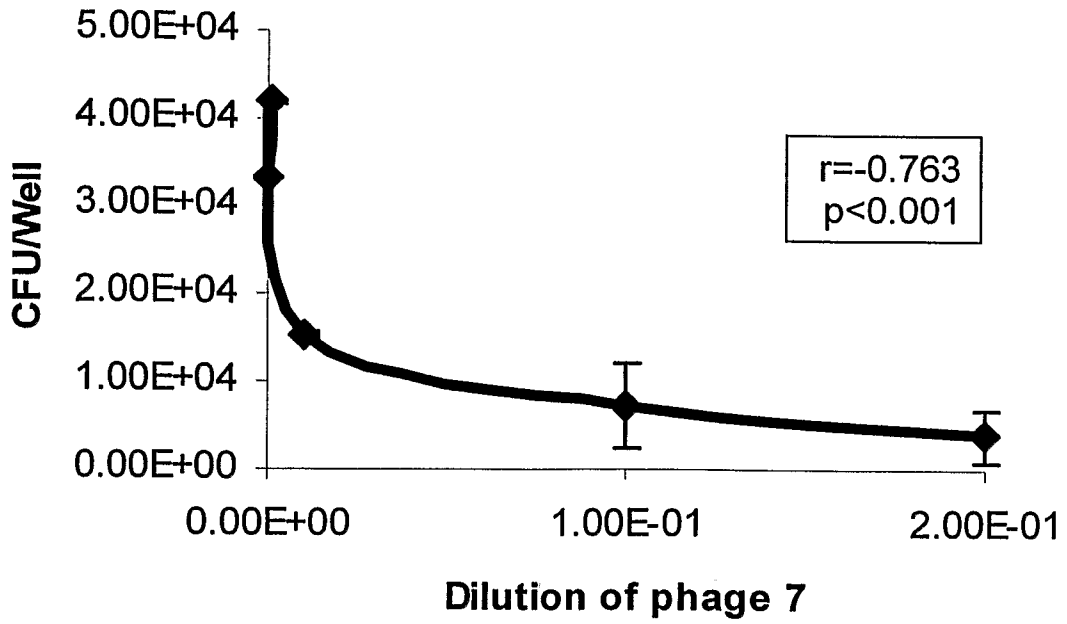


Fig. 2G

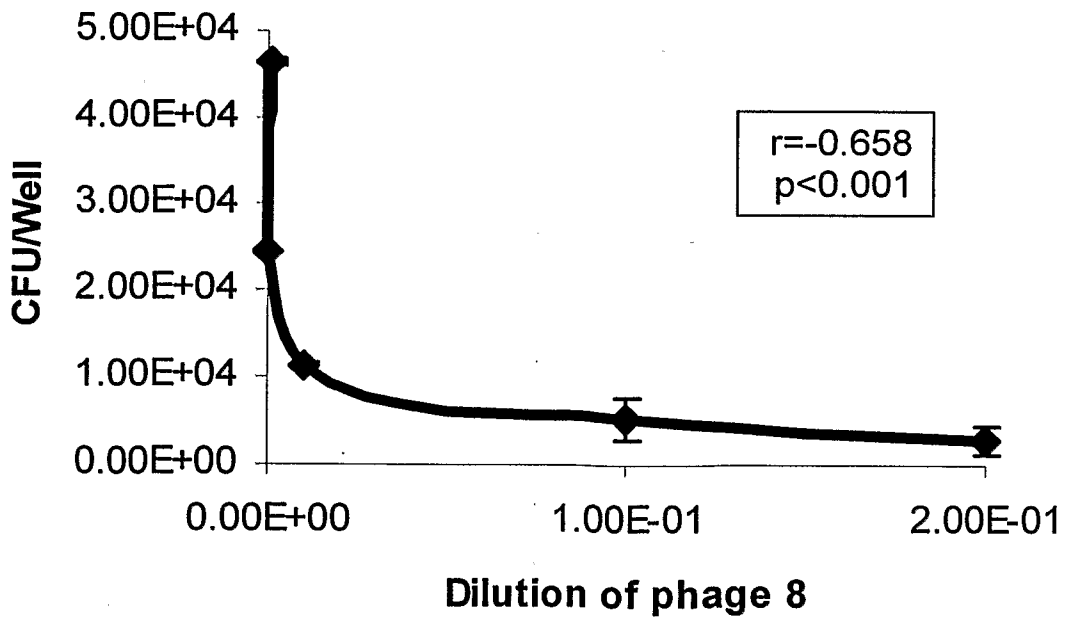


Fig. 2H

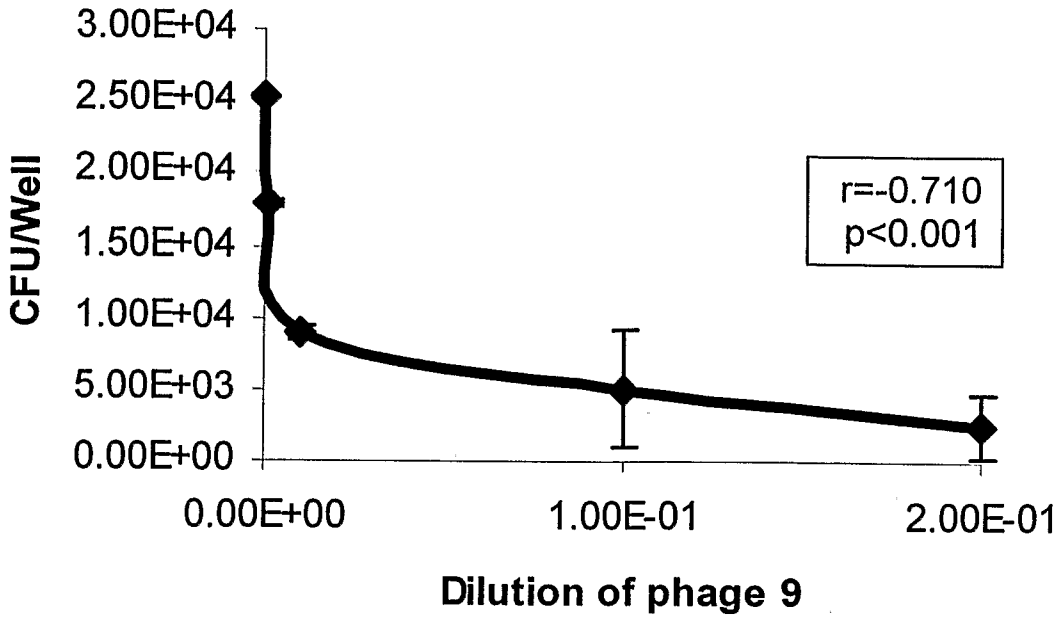


Fig. 2I

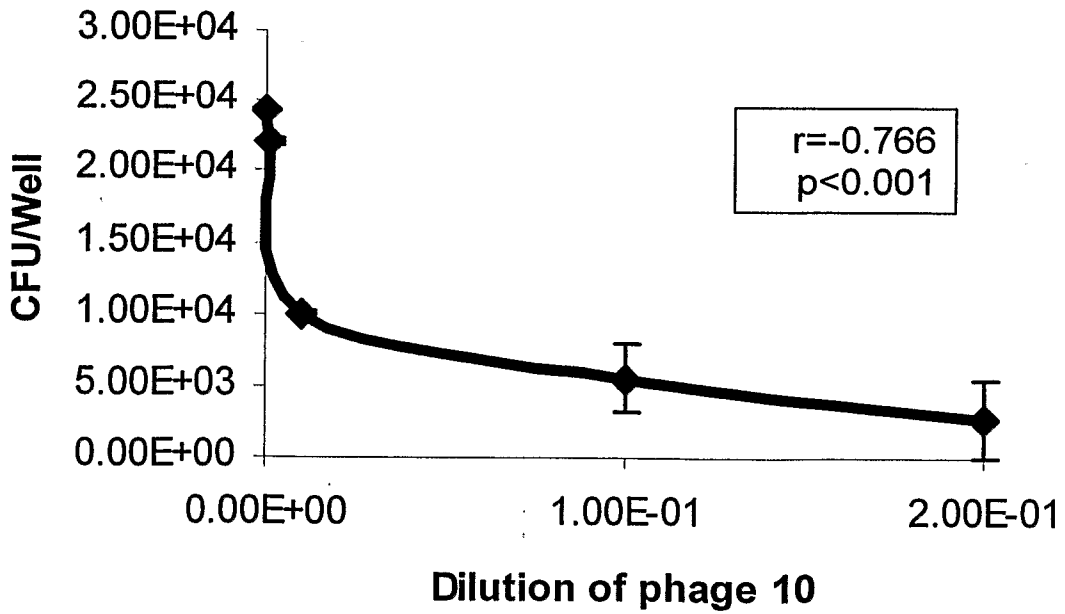


Fig. 2J

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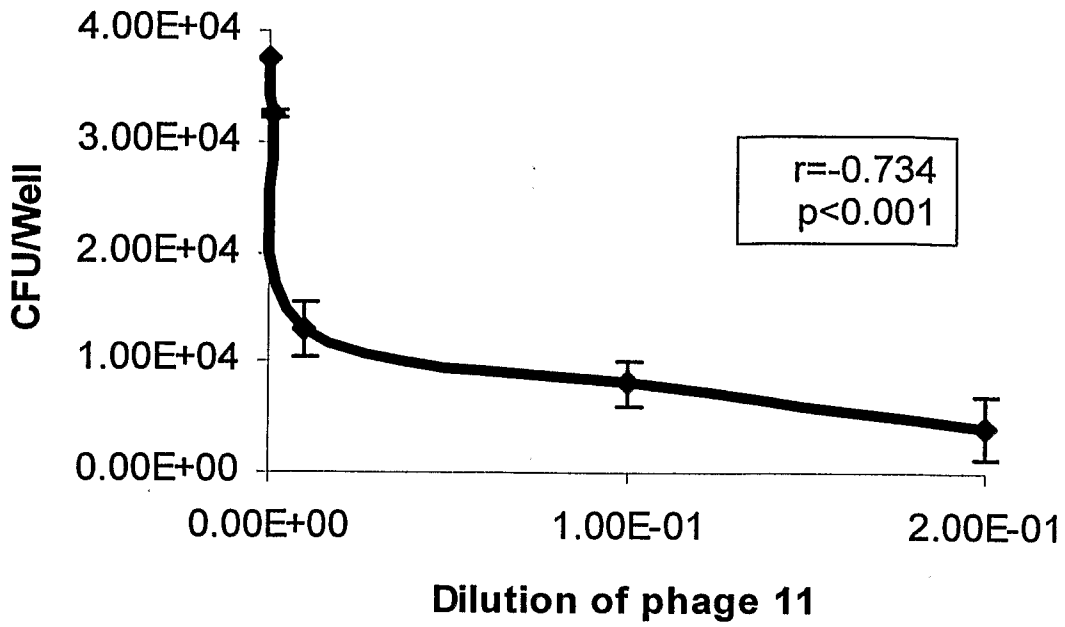


Fig. 2K

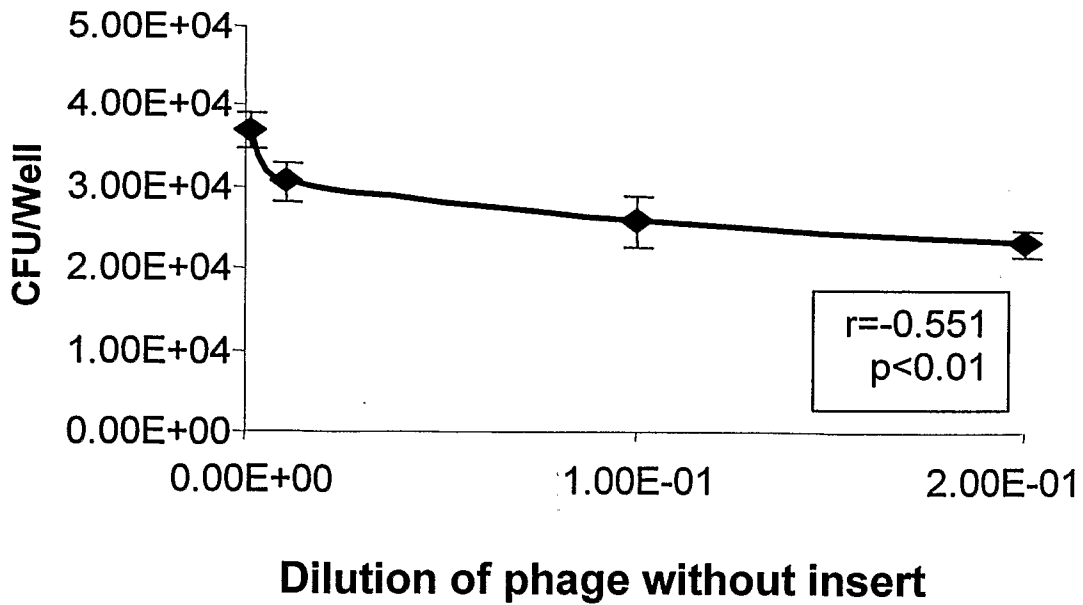


Fig. 2L

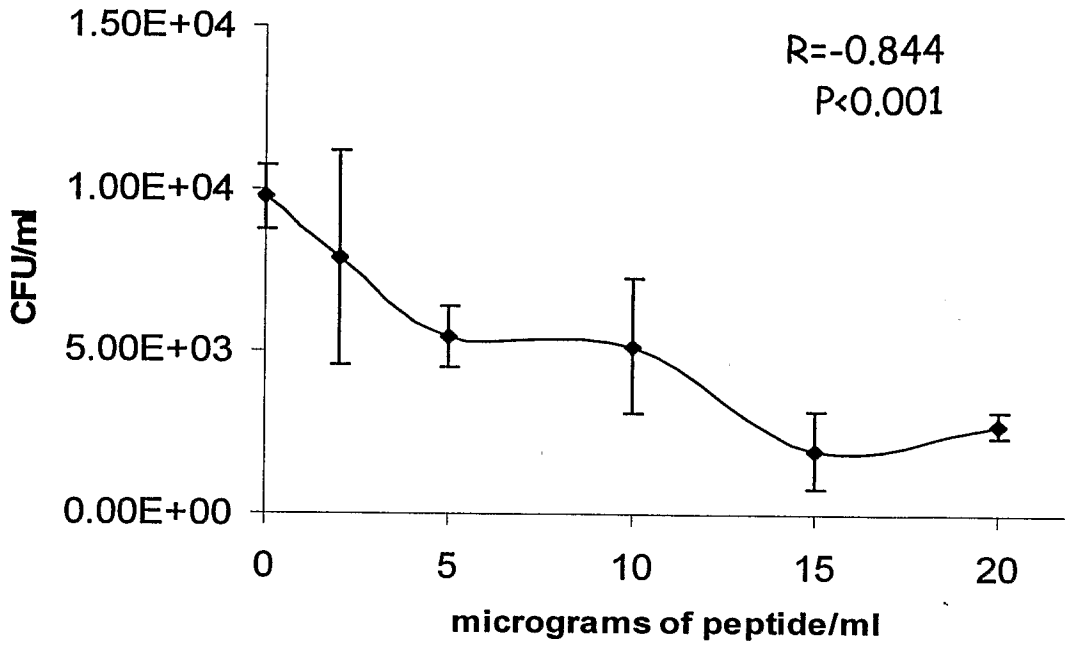


Fig. 3A

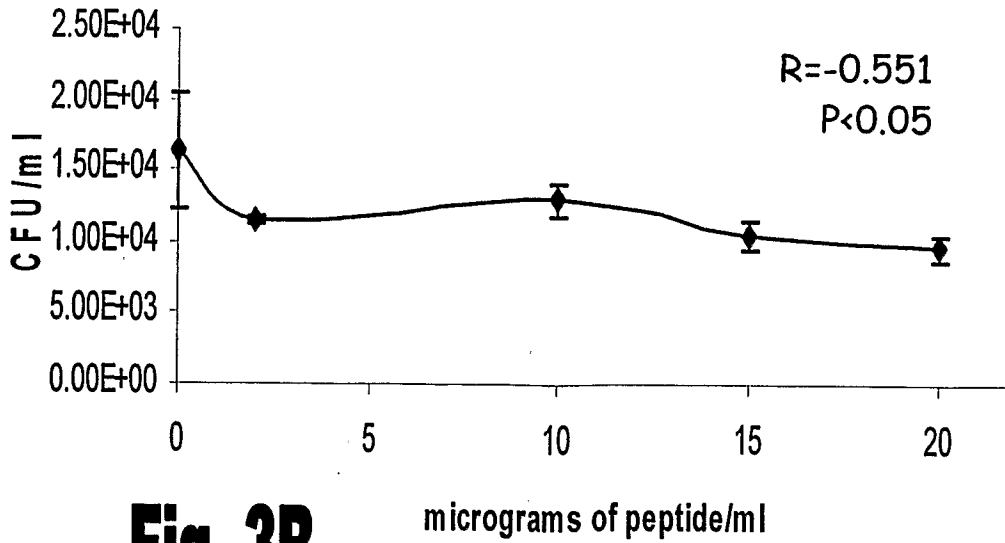


Fig. 3B

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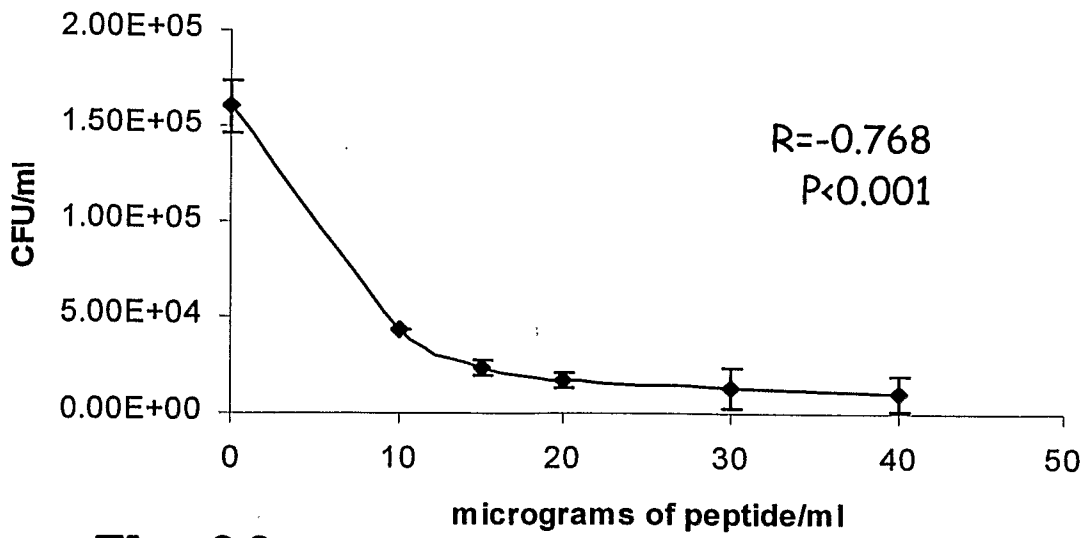


Fig. 3C

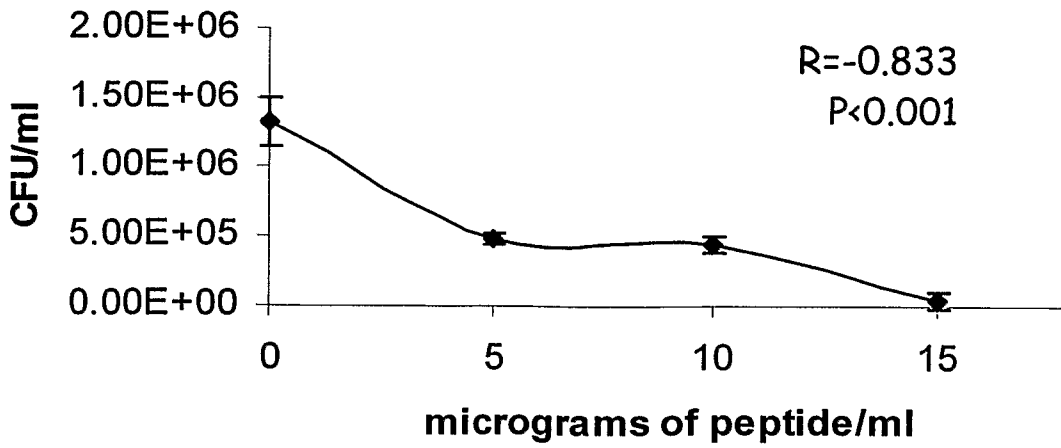


Fig. 3D

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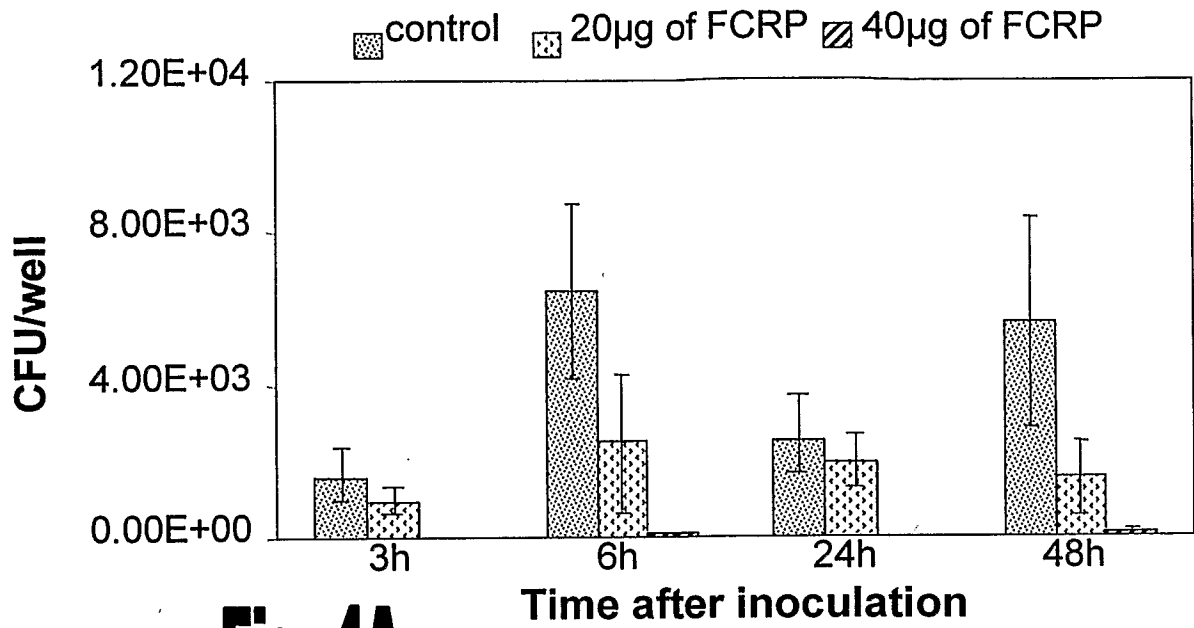


Fig. 4A

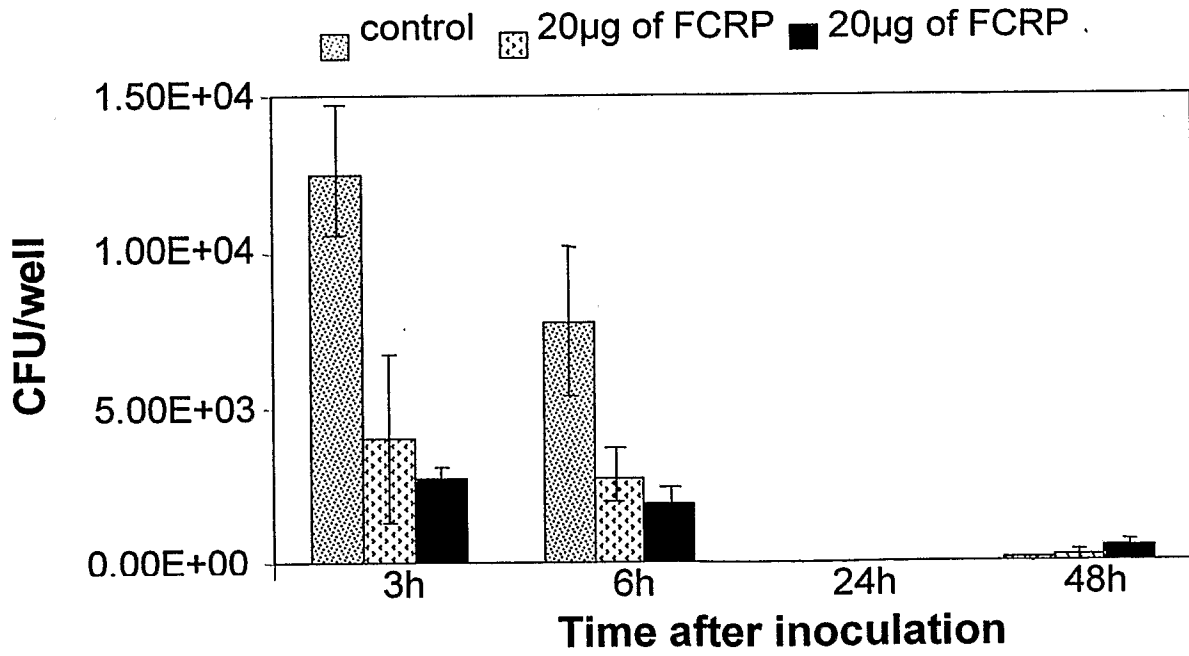


Fig. 4B

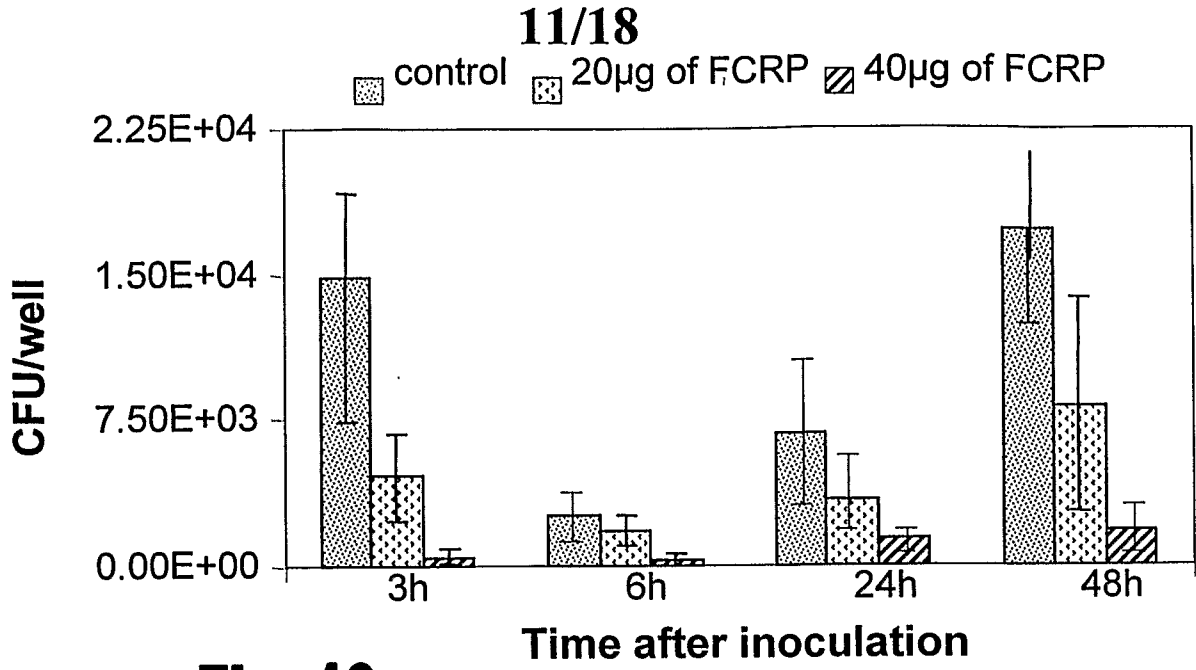


Fig. 4C

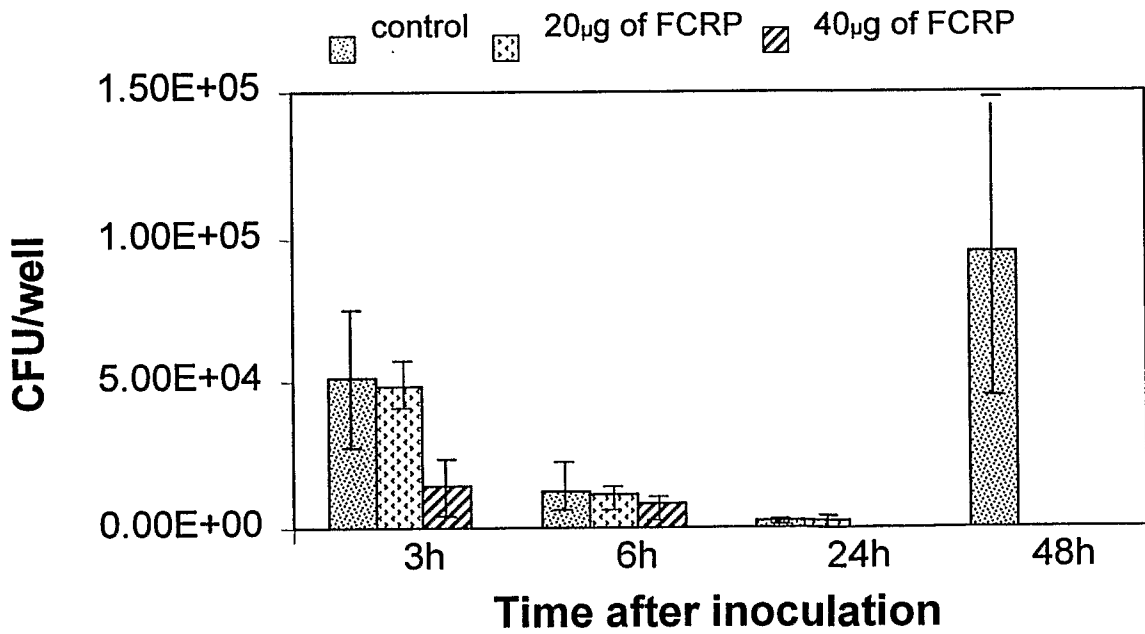


Fig. 4D

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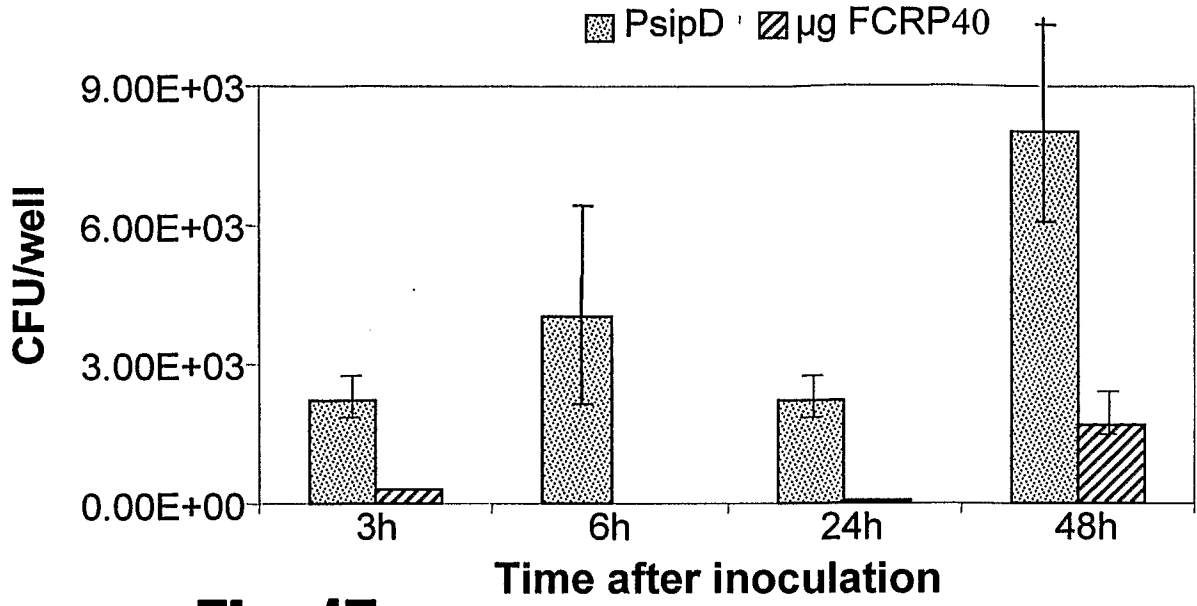


Fig. 4E

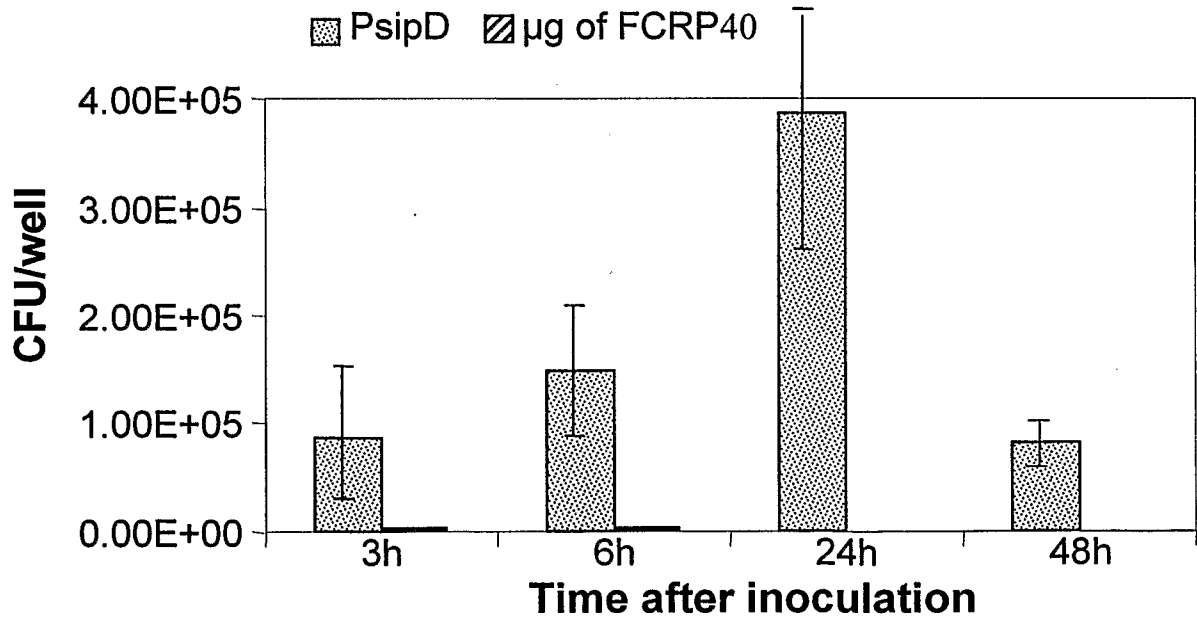


Fig. F

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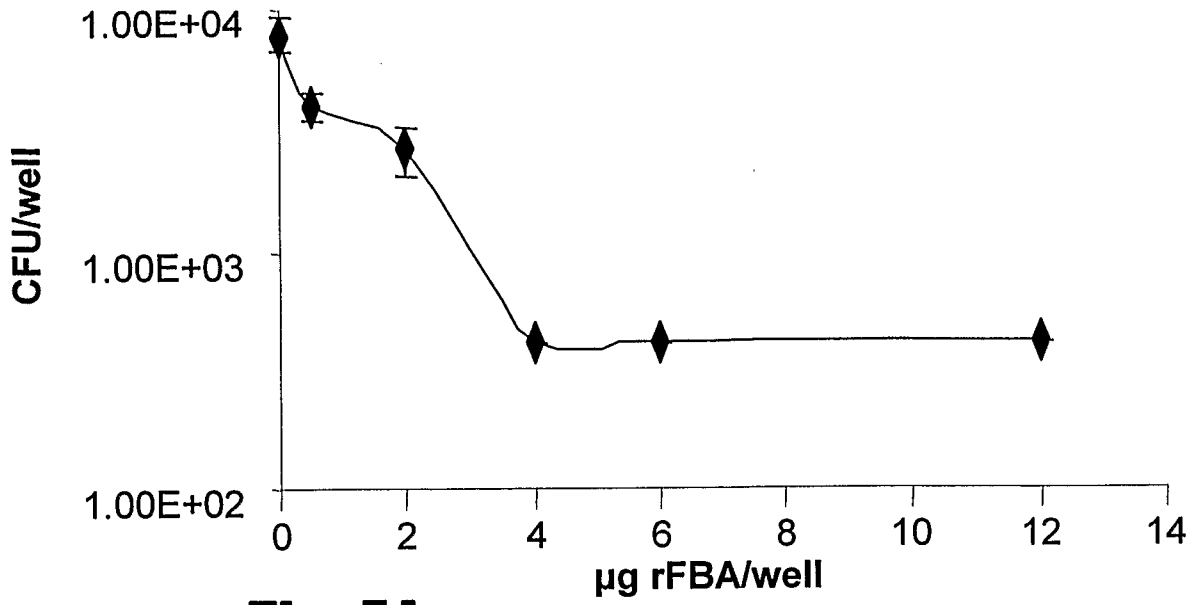


Fig. 5A

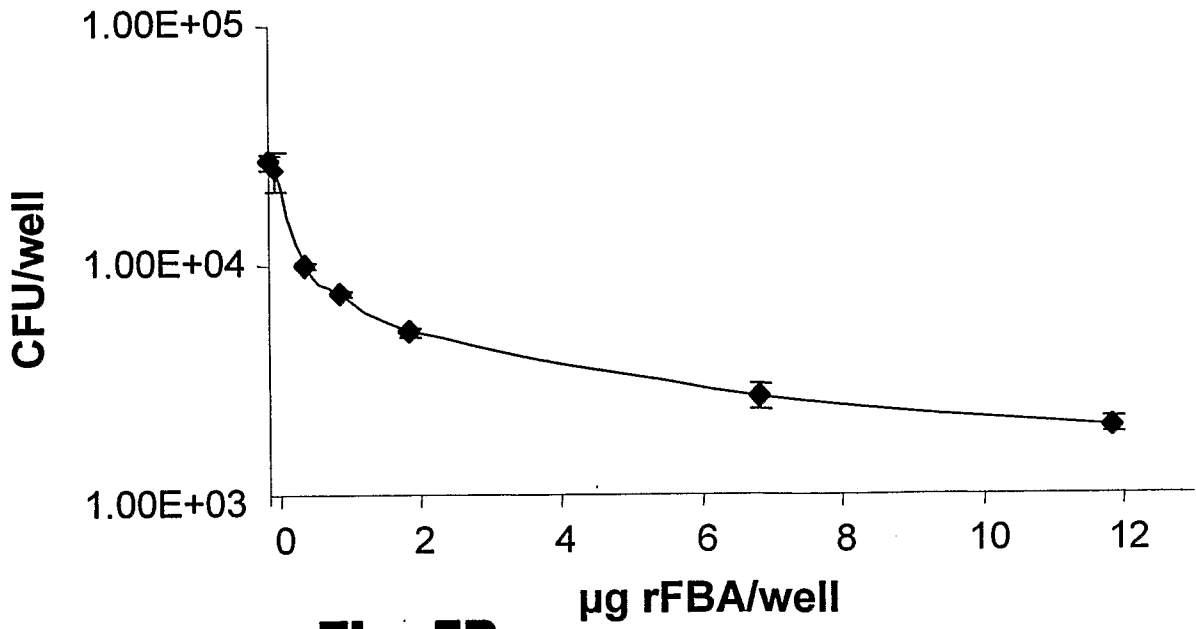


Fig. 5B

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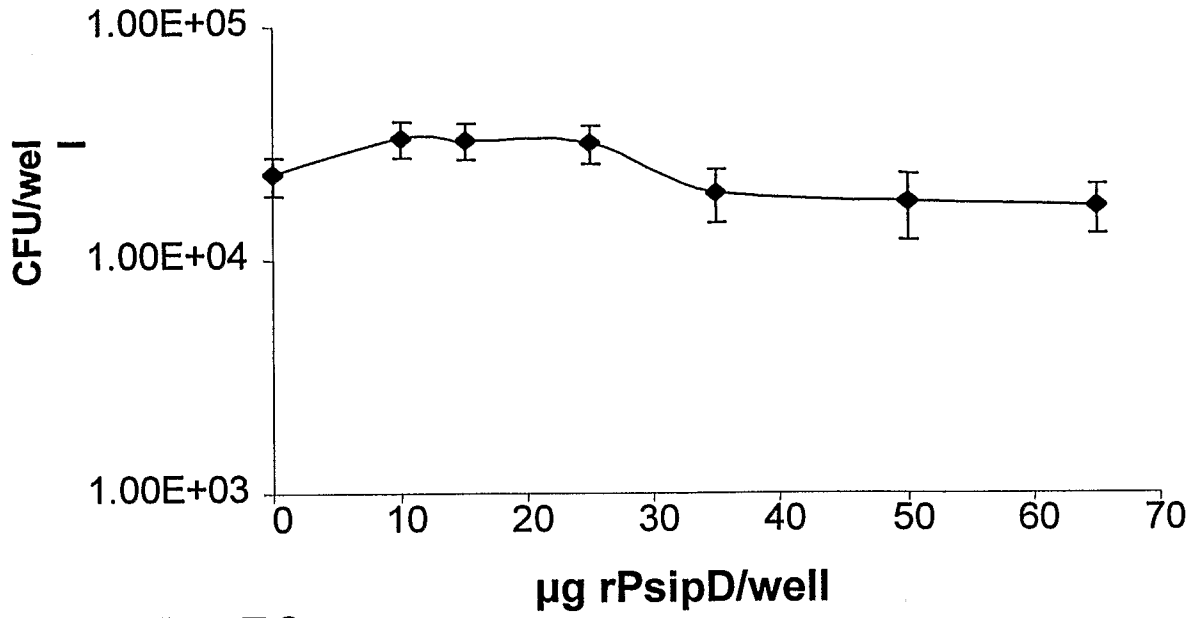


Fig. 5C

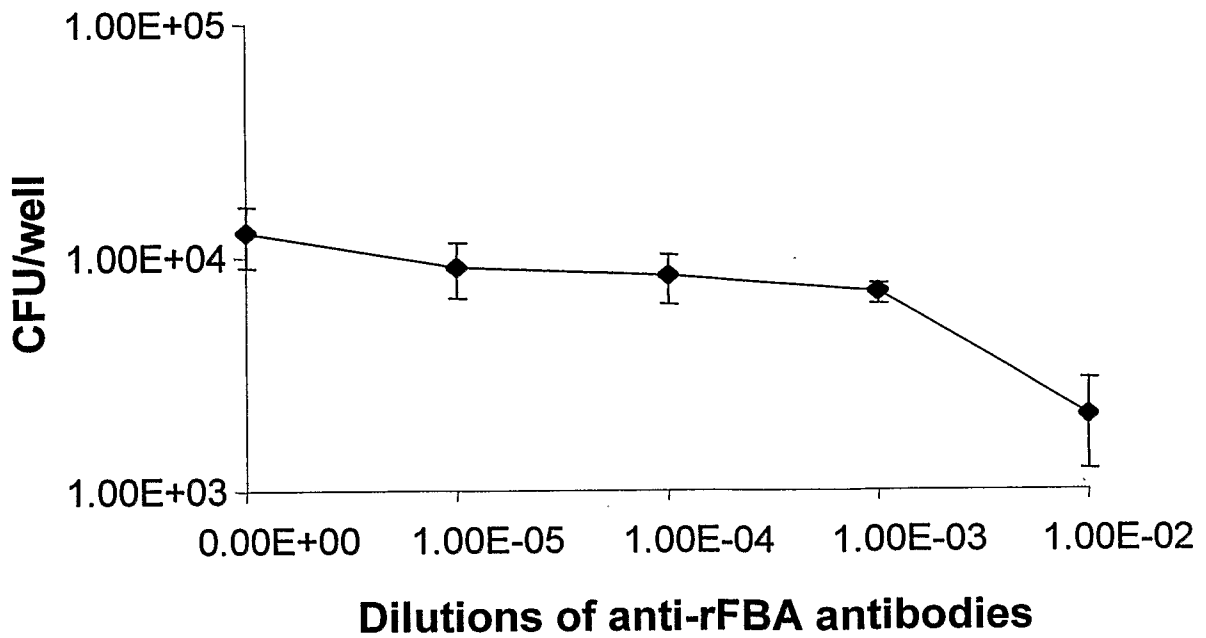


Fig. 6A

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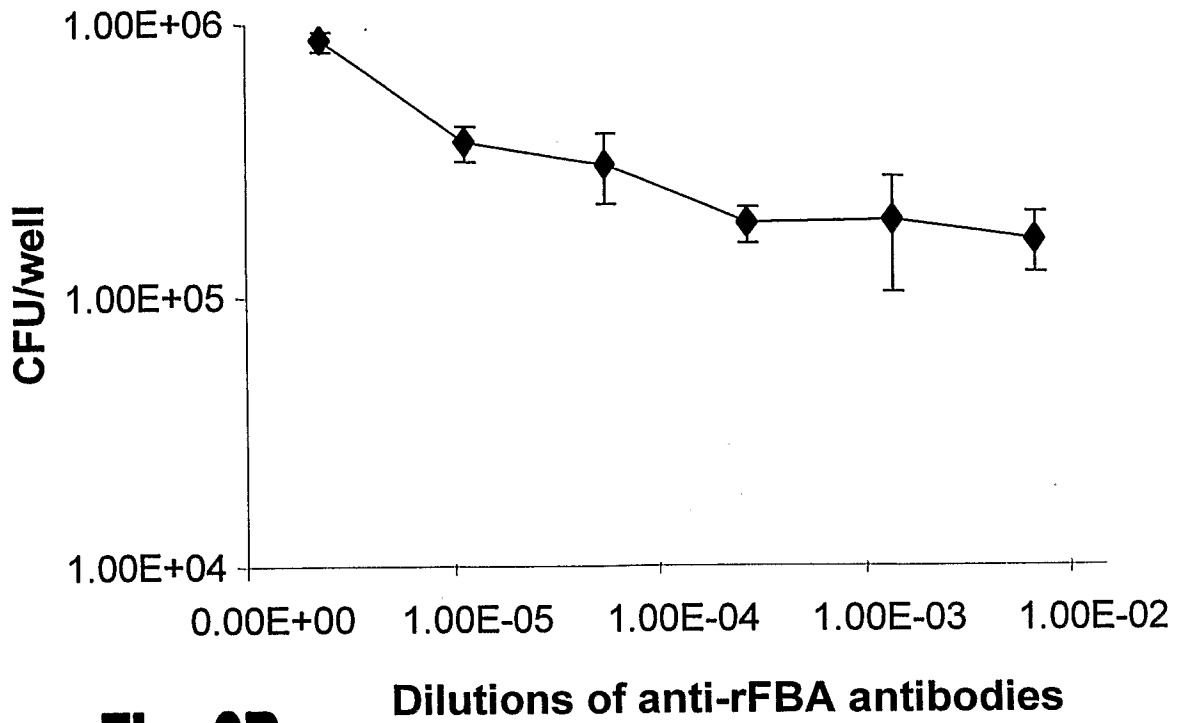


Fig. 6B

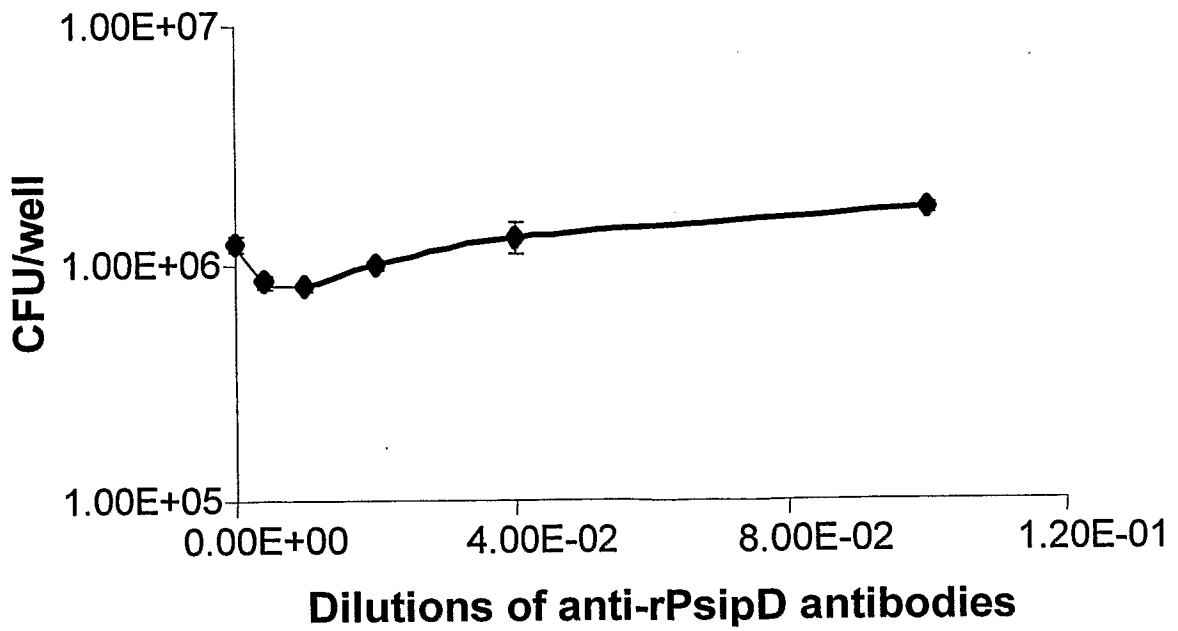


Fig. 6C

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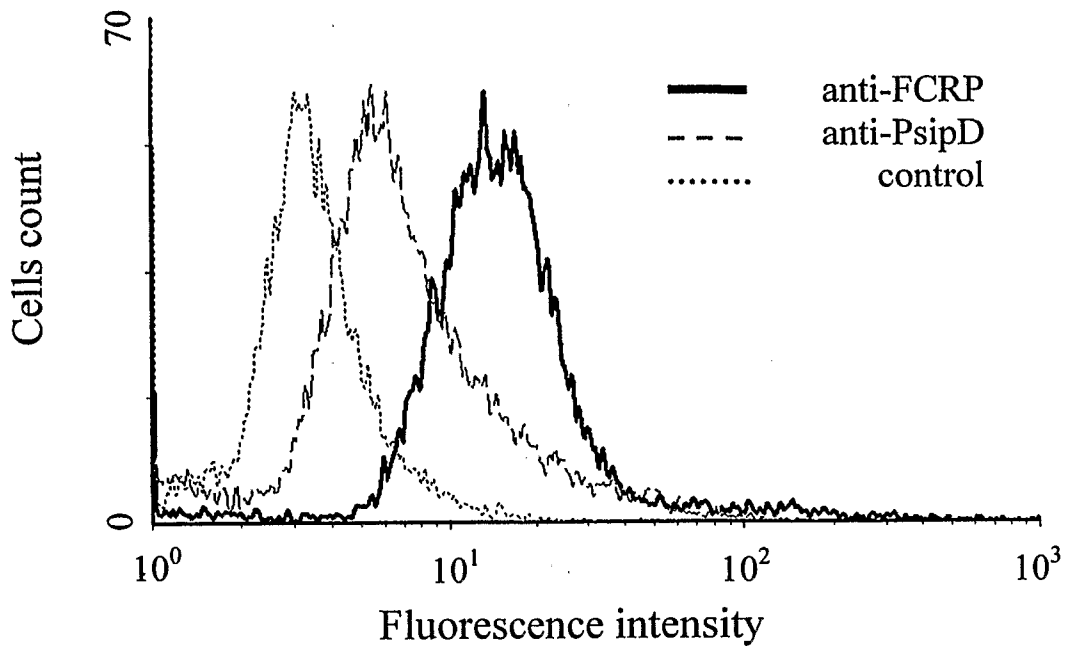


Fig. 7A

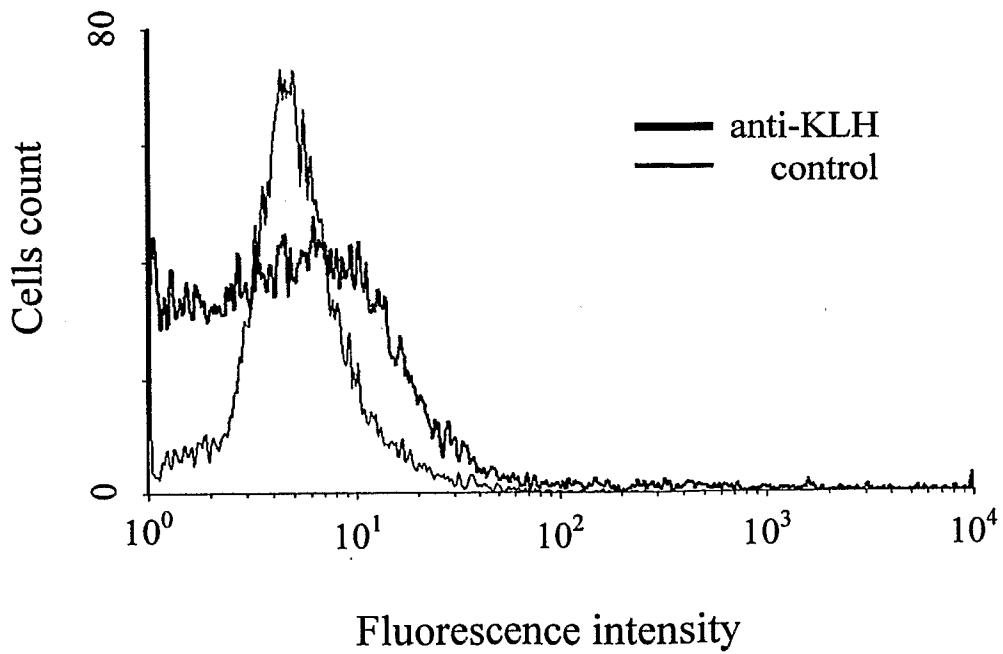
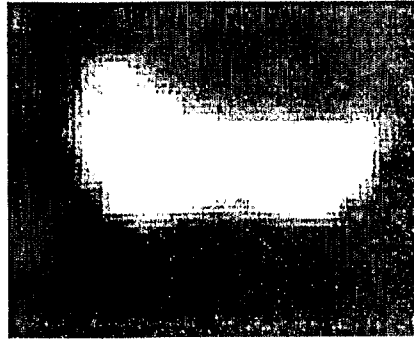


Fig. 7B

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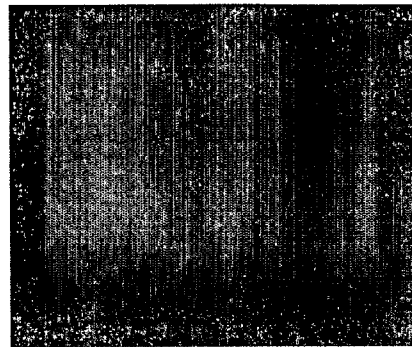
Fig. 7C

33.5 kDa



← FBA+FCRP

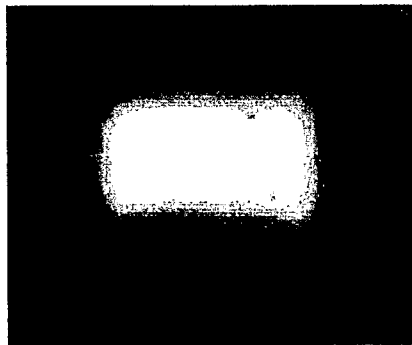
Fig. 7D



FBA (Control)

Fig. 7E

1.5 kDa



← FCRP+FBA

Fig. 7F



FCRP (Control)

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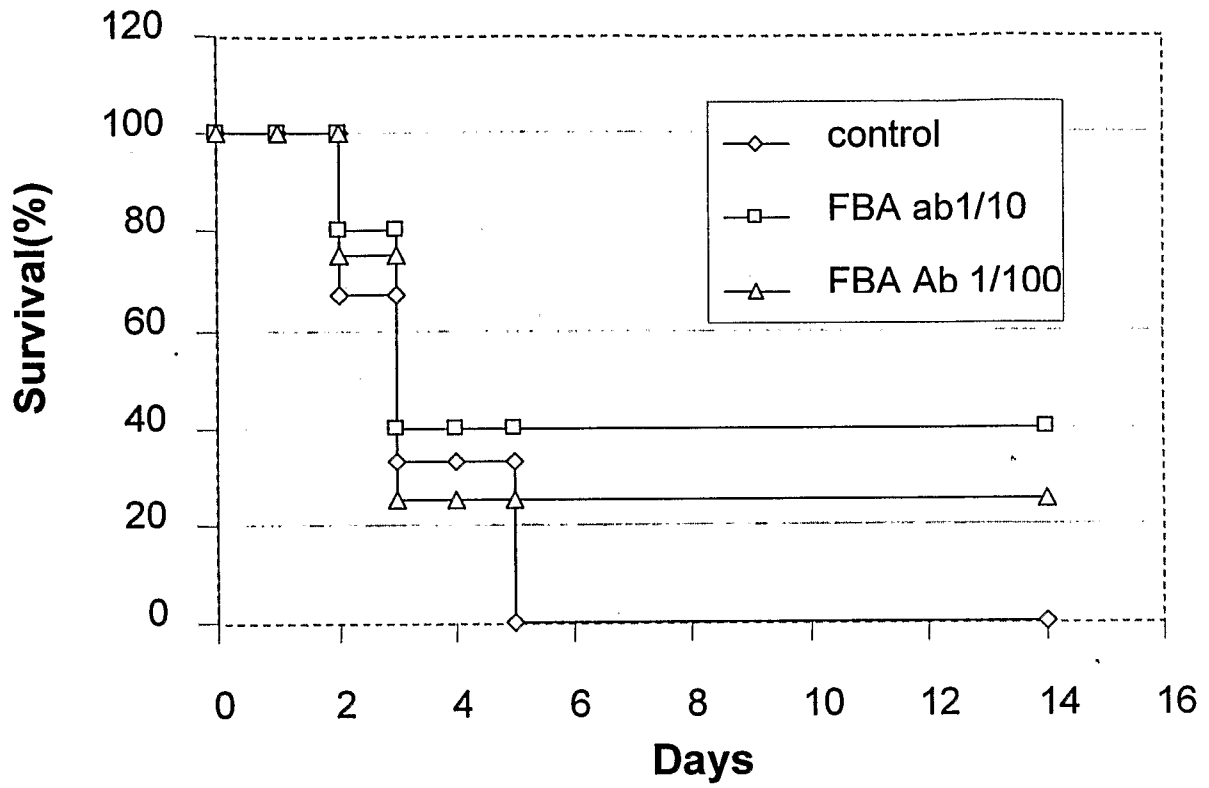


Fig. 8