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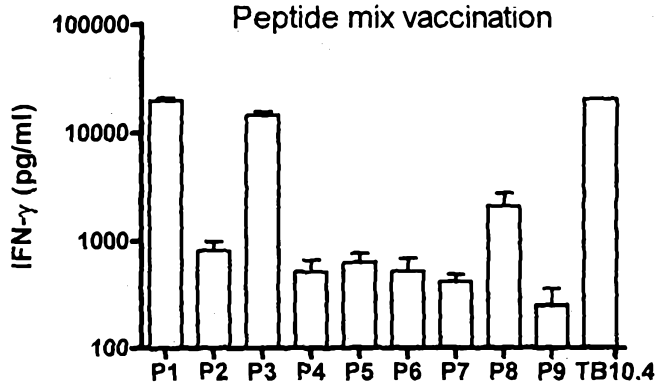

(57) Abstract: The present invention teaches a convenient way of inducing a broad recognition of dominant and subdominant responses to epitopes of any given antigen of importance for prophylaxis or treatment of a chronic disease by immunizing with pools of overlapping fragments (synthetic peptides e.g. 10-30 mers with 2-20 aa overlap) of the desired antigen in appropriate adjuvants. The T cell repertoire is primed to include not only the immunodominant epitope recognized when the intact molecule is used for immunization and induced by the chronic infection itself, but induce a much broader and balanced response to a number of the subdominant epitopes as well. The resulting T-cell response to subdominant epitopes is important for protection against chronic diseases that on their own induces a response focused only towards immunodominant epitopes. The major advantage of the present invention is that it requires no prior knowledge of the precise localisation and identity of the subdominant epitopes and their recognition in a human population, but expands the T-cell repertoire and thereby the total number of epitopes recognized by specific T cells primed by vaccination from a few immunodominant epitopes to multiple of epitopes of vaccine relevance. For chronic disease controlled by humoral immunity the T helper cell response primed by the peptide mix may conveniently be boosted by the full size protein for maximum induction of an antibody response as well.

WO 2008/000261 A3

Expanding the T cell repertoire to include subdominant epitopes by vaccination with antigens delivered as protein fragments or peptide cocktails

Field of invention

The present invention discloses a vaccine against chronic diseases such as a bacterial, viral or parasitic infection or cancer comprising a peptide mixture of overlapping peptides spanning the whole amino acid sequence of a protein that is expressed during the chronic phase of the disease such as a chronic infection caused by a bacteria, a persistent virus or parasite or from proteins expressed in malignant tumours, a method of making such vaccines and prophylaxis and treatment of chronic disease.

General Background

Compared to the limited number of diseases where vaccines are currently available a very large number have so far escaped attempts to develop efficient vaccines. A common characteristica for many of these infectious diseases as well as cancer is that they develop slowly and manifest themselves as chronic diseases where the disease is maintained for years in the face of an existing host immune response. This often eventually results in immunopatothology that is some cases such as Clamydia thracomatis is the real cause of the human disease such as inflammatory scarring of the oviduct resulting in infertility. For some diseases such as M. tuberculosis infection (TB) a vaccine exist (BCG) but although the vaccine may prevent the acute manifestations of the disease the bacteria is not cleared and a chronic or latent disease therefore established. TB runs essentially through 3 phases. During the acute phase, the bacteria proliferate in the organs, until the immune response increases to the point at which it can control the infection, whereupon the bacterial load peaks and starts declining. After this, a chronic or latent phase is established where the bacterial load is kept stable at a low level. In this phase *M. tuberculosis* goes from active multiplication to a state of slow or non-replicating persistence which can last for many years. However in some TB cases, the infection can suddenly reactivate and overt disease will be the outcome. The factors that lead to this reactivation is largely unknown. In other cases such as Clamydia the infection may

remain asymptomatic but the ongoing inflammatory process cause later clinical manifestations such as infertility.

The immune response to many of these difficult diseases include both a humoral and a cell-mediated immune (CMI) response component. The CMI response is directed to a hierachy of T cell antigens and epitopes from the pathogen. The epitopes are amino acid (aa) stretches of 7-9 aa (MHC I) and 12-15 aa (MHC II) (1). In chronic viral and bacterial disease such as HIV, TB as well as in cancer, the hierachy of epitope responses changes over time to a few immunodominant epitopes which gradually constitute a large part of the total T cell response, whereas a large number of other epitopes that all have the potential to bind the MHC class I or II antigen presentation molecules are subdominant or even cryptic resulting in T cell responses at levels close to or below the detection level (2-6). If induced by vaccination (without the competition from dominant epitopes) responses to such subdominant epitopes have been reported to be protective eg. in TB (7), indicating that the epitopes are indeed expressed during the natural infection and can be recognized by effector cells on the invading pathogen. That such responses may have advantages compared to responses to immundominant epitopes have been suggested by studies in HIV where escape mutants lacking immunodominant epitopes, and therefore not seen by the immune system, is a major concern for current vaccine development (8).

The utilization of subdominant T cell epitopes in the design of vaccines have so far been hampered by two major roadblocks; i) The need for a large panel of different epitopes to cover a diverse human population due to the variation of individual epitopes recognized by individuals with different HLA composition; ii) The need to identify subdominant epitopes to which only low level T cell responses close to or below the detection level of the immunological assays (eg ELISPOT) are found.

Olsen et al (7) described that a vaccine based on one subdominant epitope of ESAT6 can protect against TB. However, a mix of overlapping peptides spanning the entire region of ESAT6 was not used in this study.

In WO01016163 a vaccine against virus comprising a peptide mix consisting of peptides that activate T cells regardless of their HLA genotype is described. This application teaches the use of peptide mixes from Hepatitis B to enable a broad coverage when applied for the vaccination of a genetically diverse human population thereby

circumventing the non-reponders found when immunizing with single peptides. This invention does not teach the peptide driven expansion of T cells directed against subdominant T cell epitopes relevant for the preventive and therapeutic vaccination against chronic diseases as taught in the present invention.

In WO03011331 a prime-boost vaccine is disclosed. To prevent an increased response to dominant epitopes and decreased response to subdominant epitopes priming is achieved by a DNA or viral vector encoding a string of epitopes. Following the priming stage, the epitopes are used individually, in separate constructs or carried on separate vehicles, to boost the response as opposed to being administered as a single poly-epitope DNA or viral construct. In contrast, the present invention use stretches of amino acid sequences, spanning a whole protein, in a peptide mixture with an overlap of 6-20 amino acids for priming and optionally boosting with the whole protein as an adjuvanted subunit vaccine or expressed in viral delivery systems for maximal induction of humoral responses as well.

It is generally observed that in chronic diseases the hierarchy of epitope responses change over time to constitute responses towards only a few dominant epitopes. However, as responses against subdominant epitopes have proven protective (7) and since a response against these epitopes are not promoted by the chronic disease itself, a chronic disease represents an obvious target for an invention that induce an immune response towards a broad range of epitopes, including both dominant and most importantly, subdominant epitopes.

Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in the field relevant to the present disclosure as it existed before the priority date of each claim of this application.

Summary of the invention

The present invention discloses vaccines inducing a broad recognition of dominant and subdominant responses to any given antigen. The vaccine comprises overlapping fragments of the desired antigen in appropriate adjuvants. The T cell repertoire is thereby expanded to include not only the immunodominant epitope recognized when the intact molecule is used for immunzation or induced by the chronic infection itself, but also to induce a much broader and balanced response to a number of the subdominant epitopes as well. The major advantage of the present invention is that it requires no prior knowledge of the precise localization or identity of the subdominant epitopes and their

recognition in a human population but expands the T-cell repertoire and thereby the total number of target specific T cells primed by vaccination from a few immunodominant epitopes to multiple epitopes. As teached by the present invention targeting chronic diseases with a broad range of responses to subdominant epitopes harness the immunity to these diseases dramatically.

In a first aspect, the present invention provides a vaccine against a chronic disease comprising a peptide mixture consisting of adjacent overlapping peptides spanning the whole amino acid sequence of a protein that is expressed during the chronic phase of the disease, also comprising an adjuvant where the adjuvant is a cationic liposome.

In a second aspect, the present invention provides a method of preparing a vaccine according to the first aspect, where the peptide mixture is prepared by proteolytic cleavage of the protein with two or more proteolytic cleavage agents.

In a third aspect, the present invention provides a method for prophylaxis or treatment of a chronic disease in an animal, including a human being, comprising administering to the animal the vaccine according to the first aspect.

Detailed disclosure of the invention

Disclosed herein is a vaccine against a chronic disease such as a bacterial, viral or parasitic infection or cancer comprising a peptide mixture consisting of adjacent overlapping peptides spanning the whole amino acid sequence of a protein that is expressed during the chronic phase of the disease.

Also disclosed herein is use of a mixture of overlapping peptides derived from an antigenic protein and/or the nucleic acid encoding these peptides for a vaccine against a chronic disease such as a bacterial, viral or parasitic infection or cancer.

The peptides are 10 to 30 amino acids long, preferably 12-20 amino acids long where the overlap with the adjacent peptide is 6-20 amino acids, preferably 10-12 amino acids.

The antigenic protein which the peptide mixture spans is chosen among proteins that are expressed during the chronic phase of a disease and induces a cell mediated immune response in the case of chronic disease.

Preferably the protein is selected from a bacteria such as a virulent mycobacteria, e.g. by *Mycobacterium tuberculosis*, *Mycobacterium africanum* or *Mycobacterium bovis, Mycobacterium leprae* or *Chlamydia trachomatis* or a virus such as hepatitis B or C or a parasite such as *Leishmania* or the malaria causing parasite *Plasmodium falciparum* or from molecules expressed in malignant tumours .

The peptides are not restricted to but preferably from a protein selected from *M. tuberculosis* such as ESAT6, Ag85A, Ag85B or TB10.4 or from Chlamydia trachomatis

such as CT184, CT521, CT443, CT520, CT521, CT375, CT583, CT603, CT610 or CT681 or from a hepatitis B or C or from *Plasmodium falciparum* such as Msp1, Msp2, Msp3, Ama1, GLURP, LSA1, LSA3 or CSP.

Disclosed herein is a method for preparing a peptide mixture according to the invention by proteolytic cleavage of the protein with two or more proteolytic cleavage agents such as proteolytic enzymes such a trypsin, V-8 protease, AspN or chymotrypsin or chemical agents such as CNBr or BNPS-skatole.

The peptide mixture according to the invention can be used for preparing a vaccine against a chronic disease such as a bacterial, viral or parasitic infection or cancer. The vaccine can optionally comprise a delivery system such as an adjuvant. The adjuvant is preferably a based on cationic liposomes such as dimethyldioctadecylammonium bromide /Trehalose dibehenate (DDA/TDB) . The peptide mixture used for vaccination can be mixed with preformed liposomes or each peptide can be mixed with the preformed liposomes, the individual peptides formulated in the liposomes are then mixed before immunization.

Each peptide in the peptide mixture can preferably be individually mixed with the liposome prior to making the peptide mixture for optimal interaction with individual antigen presenting cells from the immune system thereby ensuring maximum responses to all possible epitopes in the molecule.

Also disclosed herein is a method and vaccine for prophylaxis or therapeutic treatment of a chronic disease in an animal, including a human being, comprising administering to the animal the vaccine of the invention. Optionally the prophylaxis or treatment is boosted by administering a second vaccine comprising the full size protein spanned by the peptide mixture in an adjuvant or expressed in a viral delivery systems or as a pure DNA vaccine for optimal boosting a CMI as well as a humoral response.

The invention further discloses a vaccine in which the amino acid sequence is lipidated or conjugated directly to TLR agonist such as CPG so as to allow a self-adjuvanting effect of the polypeptide

The preferred embodiment of the invention is a vaccine comprising a peptide mixture of the invention preferably with an adjuvant as described above.

Definitions

Chronic disease

A chronic disease is a long-lasting or recurring disease. The term *chronic* describes the course of the disease, or its rate of onset and development. A chronic course is distinguished from a recurrent course; recurrent diseases relapse repeatedly, with periods of remission in between. Chronic infections can be caused by bacteria e.g. Mycobacteria sp. or Chlamydia s.p among others, by virus e.g. Hepatitis or HIV, by a parasite e.g. a malaria causing parasite or Leishmania or by diseases such as cancer, diabetes etc.

Peptides

The word "peptide" in the present invention should have its usual meaning. That is an amino acid chain of any length being a part or fragment of a protein, wherein the amino acid residues are linked by covalent peptide bonds.

The peptide may be chemically modified by being glycosylated, by being lipidated (e.g. by chemical lipidation with palmitoyloxy succinimide as described by (9), labeling with PAM3Cys (18) or with dodecanoyl chloride as described by (10)), by comprising prosthetic groups, or by containing additional amino acids such as e.g. a his-tag or a signal peptide or by direct conjugation to TLR agonist (e.g.as described by (11)).

Each peptide may thus be characterised by specific amino acids and be encoded by specific nucleic acid sequences. It will be understood that such sequences include analogues and variants produced by recombinant or synthetic methods wherein such polypeptide sequences have been modified by substitution, insertion, addition or deletion of one or more amino acid residues in the recombinant polypeptide and still be immunogenic in any of the biological assays described herein. Substitutions are preferably "conservative". These are defined according to the following table. Amino acids in the same block in the second column and preferably in the same line in the third column may be substituted for each other. The amino acids in the third column are indicated in one-letter code.

ALIPHATIC	Non-polar	GAP
		ILV
	Polar-uncharged	CSTM
		NQ
	Polar-charged	DE
		KR
AROMATIC		HFWY

A peptide mixture is liquid mixture of fragments of a protein.

A preferred peptide mixture within the present invention is based on a protein from *M. tuberculosis* such as ESAT6, Ag85A, Ag85B or TB10.4 or from Chlamydia trachomatis such as CT184, CT521, CT443, CT520, CT521 or CT375 or from a hepatitis virus or from *Plasmodium falciparuman* such as momp, omp, msp1, msp3, ama1 or glurp. It may also be a peptide mixture or proteolytic digest based on a fusion molecule e.g. as previously described as a relevant vaccine constructs against TB in PCT/DK2006/000356. In general all peptide mixtures of proteins inducing a CMI response which can be used in vaccines against chronic disease can be used to induce an increased prophylactic or therapeutic response as a vaccine

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations thereof such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers.

Although the minimum length of a T-cell epitope has been shown to be at least 6 amino acids, it is normal that such epitopes are constituted of longer stretches of amino acids. Hence, it is preferred that the polypeptide fragment of the invention has a length of at least 7 amino acid residues, such as at least 8, at least 9, at least 10, at least 12, at least 14, at least 16, at least 18, at least 20, at least 22, at least 24, and at least 30 amino acid residues. Hence, in important embodiments of the inventive method, it is preferred that the polypeptide fragment has a length of at most 50 amino acid residues, such as at most 40, 35, 30, 25, and 20 amino acid residues. It is expected that the peptides having a length of between 10 and 20 amino acid residues will prove to be most efficient as MHC

class II epitopes and therefore especially preferred lengths of the polypeptide fragment used in the inventive method are 18, such as 15, 14, 13, 12 and even 11 amino acid residues. It is expected that the peptides having a length of between 7 and 12 amino acid residues will prove to be most efficient as MHC class I epitopes and therefore especially preferred lengths of the polypeptide fragment used in the inventive method are 11, such as 10, 9, 8 and even 7 amino acid residues.

Epitopes

By T cell epitopes is understood a sequence of amino acids that is recognized by specific T cells through their T cell receptor after presentation by an antigen presenting cell in the context of either MHC classI or II.

A dominant epitope is a sequence of amino acids that, when part of a protein, induce a high T cell response and often the majority of the response to an antigen is directed to a few T dominant T cell epitopes.

A subdominant epitope is a sequence of amino acids that when part of a protein does not induce a strong T cell response, even though the epitopes are immunogenic and able to induce a significant T cell response when isolated from the protein.

By mixture of overlapping polypeptides or protein fragments is understood a mixture of 10 to 30 mers, with a 6-20 amino acid overlap, spanning an entire protein.

Variants

A common feature of the polypeptides of the invention is their capability to induce an immunological response as illustrated in the examples. It is understood that a variant of a polypeptide of the invention produced by substitution, insertion, addition or deletion may also be immunogenic as determined by any of the assays described herein.

Immune individual

An immune individual is defined as a person or an animal, which has cleared or controlled an infection.

Immune response

The immune response may be monitored by one of the following methods:

- An in vitro cellular response is determined by induction of the release of a relevant cytokine such as IFN-γ or the induction of proliferation in lymphocytes withdrawn from an animal or human being currently or previously infected with virulent mycobacteria or immunized with the relevant peptide mixture.The induction being performed by the addition of the peptide mixture or the immunogenic portion of the mixture to a suspension comprising from $2x10^5$ cells to $4x10^5$ cells per well. The cells being isolated from either the blood, the spleen, the lymph nodes, the liver or the lung and the addition of the polypeptide or the immunogenic portion resulting in a concentration of not more than 20 μg per ml suspension and the stimulation being performed from two to five days. For monitoring cell proliferation the cells are pulsed with radioactive labeled Thymidine and after 16-22 hours of incubation detecting the proliferation is measured by liquid scintillation counting. A positive response is defined as being a response more than background plus two standard deviations. The release of IFN-γ can be determined by the ELISA method, which is well known to a person skilled in the art. A positive response being a response more than background plus two standard deviations. Other cytokines than IFN-γ could be relevant when monitoring the immunological response to the polypeptide, such as IL-12, TNF-α, IL-4, IL-5, IL-10, IL-6, TGF-β. Another and more sensitive method for detecting the immune response is the ELISpot method, in which the frequency of IFN-γ producing cells is determined. In an ELIspot plate (MAHA, Millipore) precoated with anti-murine IFN-γ antibodies (PharMingen) graded numbers of cells isolated from either blood, spleen, or lung (typically between 1 to 4×10^5 cells /well) are incubated for 24-32 hrs in the presence of the peptide mixture or the immunogenic portion resulting in a concentration of not more than 20 μg per ml. The plates are subsequently incubated with biotinylated anti-IFN-γ antibodies followed by a streptavidin-alkaline phosphatase incubation. The IFN-γ producing cells are identified by adding BCIP/NBT (Sigma), the relevant substrate giving rise to spots. These spots can be enumerated using a dissection microscope. It is also a possibility to determine the presence of mRNA coding for the relevant cytokine by the use of the PCR technique. Usually one or more cytokines will be measured utilizing for example PCR, ELISPOT or ELISA. It will be appreciated by a person skilled in the art that a significant increase or decrease

in the amount of any of these cytokines induced by a specific peptide mixture can be used in evaluation of the immunological activity of the polypeptide.

- An *in vitro* cellular response may also be determined by the use of T cell lines derived from an immune individual or an infected person where the T cell lines have been driven with either live bacteria, extracts from the bacterial cell or culture filtrate for 10 to 20 days with the addition of IL-2. The induction being performed by addition of not more than 20 μg peptide mixture per ml suspension to the T cell lines containing from $1x10^5$ cells to $3x10^5$ cells per well and incubation being performed from two to six days. The induction of IFN-γ or release of another relevant cytokine is detected by ELISA. The stimulation of T cells can also be monitored by detecting cell proliferation using radioactively labeled Thymidine as described above. For both assays a positive response being a response more than background plus two standard deviations.

- An *in vivo* cellular response may be determined as a positive DTH response after intradermal injection or local application patch of at most 100 μg of the polypeptide or the immunogenic portion to an individual who is clinically or subclinically infected with a virulent bacterium, a positive response having a diameter of at least 5 mm 72-96 hours after the injection or application.

- An *in vitro* humoral response is determined by a specific antibody response in an immune or infected individual. The presence of antibodies may be determined by an ELISA technique or a Western blot where the peptide mixture or the immunogenic portion is absorbed to either a nitrocellulose membrane or a polystyrene surface. The serum is preferably diluted in PBS from 1:10 to 1:100 and added to the absorbed peptide mixture and the incubation being performed from 1 to 12 hours. By the use of labeled secondary antibodies the presence of specific antibodies can be determined by measuring the OD e.g. by ELISA where a positive response is a response of more than background plus two standard deviations or alternatively a visual response in a Western blot.

- Another relevant parameter is measurement of the protection in animal models induced after vaccination with the peptide mixture in an adjuvant or after DNA vaccination. Suitable animal models include primates, guinea pigs or mice, which are challenged with an infection. Readout for induced protection could be decrease of the bacterial load in target organs compared to non-vaccinated animals, prolonged survival times compared to non-vaccinated animals and diminished weight loss compared to non-vaccinated animals.

Preparation methods

In general, antigens and DNA sequences encoding such antigens, may be prepared using any one of a variety of procedures.

The peptide mixture can be produced synthetically when the peptide fragment have fewer than about 100 amino acids, and generally fewer than 50 amino acids and may be generated using techniques well known to those ordinarily skilled in the art, such as commercially available solid-phase techniques where amino acids are sequentially added to a growing amino acid chain.

In the construction and preparation of plasmid DNA encoding the peptide mixture as defined by the invention for DNA vaccination a host strain such as *E. coli* can be used. Plasmid DNA can then be prepared from cultures of the host strain carrying the plasmid of interest, and purified using e.g. the Qiagen Giga -Plasmid column kit (Qiagen, Santa Clarita, CA, USA) including an endotoxin removal step. It is preferred that plasmid DNA used for DNA vaccination is endotoxin free.

Protease digest of antigens

A set of overlapping peptides can be made by proteolytic cleavage of the intact protein which can be expressed as a recombinant tagged protein in e.g. E. coli followed by purification by column chromatography such as metal chelate chromatography. Two or more proteolytic cleavage agents can be selected that will generate different fragments and thereby overlapping peptide cocktail. Proteolytic enzymes such as trypsin, V-8 protease, AspN or chymotrypsin can be used or chemical agents like CNBr or BNPS-skatole. The number of cleavage sites and the length of the fragments generated are determined by the amino acid sequence of the protein and the specific cleavage agent, e.g. Asp-N hydrolyzes proteins at the N-terminal side of aspartic acid and cysteic acid residues. The V-8 protease cleaves at the carboxyl side of glutamic acid in ammonium

bicarbonate buffer at pH 7.8. For proteolytic enzymes coupling of the enzyme to beads before cleavage is possible (16), and this coupling will allow removal of the enzyme after completion of the cleavage by centrifugation of the beads. Alternatively, the protease can be removed from the digestion mixture by chromatographic methods such as gel filtration or reversed-phase HPLC. After digestion of the protein, mass spectrometry analysis of the digest is performed to confirm that cleavage of the protein has taken place as predicted. Finally, the two digestion mixtures can be combined to form a mixture of overlapping peptides.

Protein Vaccine

A vaccination with a recombinant protein will induce a T cell response towards a limited number of dominant peptide epitopes within this protein. In contrast, vaccinating with a mix of overlapping peptides, spanning the entire amino acid sequence of the protein, will generate a T cell response against an increased number of epitopes being both dominant and sub-dominant peptide epitopes.

The invention pertains to a vaccine composition comprising a peptide mixture according to the invention. In order to ensure optimum performance of such a vaccine composition it is preferred that it comprises an immunologically and pharmaceutically acceptable carrier, vehicle or adjuvant.

An effective vaccine, wherein a peptide mixture of the invention is recognized by the animal, will in an animal model be able to decrease bacterial load in target organs, prolong survival times and/or diminish weight loss after challenge with an infectious organism, compared to non-vaccinated animals either when given as a preventive or therapeutic vaccine.

Suitable vehicles are selected from the group consisting of a diluent and a suspending agent. The adjuvant is preferably selected from the group consisting of DDA, Quil A, poly I:C, aluminium hydroxide, Freund's incomplete adjuvant, IFN-γ, IL-2, IL-12, monophosphoryl lipid A (MPL), Treholose Dimycolate (TDM), TDB and muramyl dipeptide (MDP).

An adjuvant is defined as a substance that non-specifically enhances the immune response to an antigen. Depending on the nature of the adjuvant it can promote a cell-mediated immune response, a humoral immune response or a mixture of the two. Since the enhancement of the immune response is non-specific, it is well understood in the field that the same adjuvant can be used with different antigens to promote responses against different targets e.g. with an antigen from *M. tuberculosis* to promote immunity against *M. tuberculosis* or with an antigen derived from a tumor, to promote immunity against tumors of that specific kind.

"Liposomes" are defined as closed vesicles structures made up of one or more lipid bilayers surrounding an aqueous core. Each lipid bilayer is composed of two lipid monolayers, each of which has a hydrophobic "tail" region and a hydrophilic "head" region. In the bilayer, the hydrophobic "tails" of the lipid monolayers orient toward the inside of the bilayer, while the hydrophilic "heads" orient toward the outside of the bilayer. Liposomes can have a variety of physicochemical properties such as size, lipid composition, surface charge, fluidity and number of bilayer membranes. According to the number of lipid bilayers liposomes can be categorized as unilamellar vesicles (UV) comprising a single lipid bilayer or multilamellar vesicles (MLV) comprising two or more concentric bilayers each separated from the next by a layer of water. Water soluble compounds are entrapped within the aqueous phases/core of the liposomes opposed to lipophilic compounds which are trapped in the core of the lipid bilayer membranes.

The peptide mixture used for vaccination can be mixed with preformed liposomes as previously described (WO2006002642 which is hereby incorporated as reference) or each peptide can be mixed with the preformed liposomes in the same manner, the individual peptides formulated in the liposomes are then mixed before immunization.

The standard preparation of liposomes is by dissolving the lipids in an organic solvent which is then evaporated to dryness leaving a thin lipid film on the inside of the test tube. The dry lipid film is then hydrated in an appropriate amount of aqueous phase and the mixture is heated to above the phase transition temperature of the lipids and allowed to "swell". The resulting liposomes which consist of multilamellar vesicles (MLV's) are dispersed by shaking the test tube.

Different principles for interaction of a peptide or peptide mixtures to liposomes exist. One method is surface association (by electrostatic or hydrophobic interactions) of the peptides with the liposomes by incubation of the peptides with preformed liposomes (19). It is also possible to to make a covalent coupling of peptides to the surface of the liposomes by chemical crosslinking (e.g. as described in reference 20). In addition, the peptides can be encapsulated in the liposomes by different methods. One method is to add the peptides directly into the lipid film followed by rehydration. Another method describes adding the peptides to the buffer used for rehydration of the liposomes from the lipid film. In addition, the peptides can be encapsulated by the dehydration-rehydration method (21) in which a peptide is encapsulated by freeze drying followed by rehydration of the lyophilized liposomes. Alternatively the antigen is encapsulated using the freeze and thaw technique described by Pick (22) and by Bally et al. in U.S. Pat. No. 4,975,282. In this technique vesicles are mixed with the protein antigen and repeatedly snap frozen in liquid nitrogen and warmed to temperatures above the main phase transition temperature of the relevant lipids. The vesicles may be further processed to remove any non-entrapped antigen e.g. by washing and centrifuging.

Finally, the peptide mixture can then be delivered by the liposomes in two ways. The peptides can either be mixed before the interaction with the liposomes or the peptides can be mixed after interaction of the individual peptides with the liposomes as described above.

The peptides can also be encapsulated in the liposomes by adding the peptides to the buffer used for rehydration of the liposomes from a lipid film or on freeze dried form.

The polypeptide may also be chemically modified by being glycosylated, by being lipidated (e.g. by chemical lipidation with palmitoyloxy succinimide as described by Mowat et al. 1991, labeling with PAM3Cys (18) or with dodecanoyl chloride as described by Lustig et al. 1976), by comprising prosthetic groups or by direct conjugation to TLR agonist (eg.as described by Seder 2006).

Preparation of vaccines which contain peptide sequences as active ingredients is generally well understood in the art, as exemplified by U.S. Patents 4,608,251; 4,601,903; 4,599,231 and 4,599,230, all incorporated herein by reference.

Other methods of achieving adjuvant effect for the vaccine include use of agents such as aluminum hydroxide or phosphate (alum), synthetic polymers of sugars (Carbopol), aggregation of the protein in the vaccine by heat treatment, aggregation by reactivating with pepsin treated (Fab) antibodies to albumin, mixture with bacterial cells such as C. parvum or endotoxins or lipopolysaccharide components of gram-negative bacteria, emulsion in physiologically acceptable oil vehicles such as mannide mono-oleate (Aracel A) or emulsion with 20 percent solution of a perfluorocarbon (Fluosol-DA) used as a block substitute may also be employed. Other possibilities involve the use of immune modulating substances such as cytokines or synthetic IFN-γ inducers such as poly I:C in combination with the above-mentioned adjuvants.

Another interesting possibility for achieving adjuvant effect is to employ the technique described in (17) (which is hereby incorporated by reference herein). In brief, a relevant antigen such as an antigen of the present invention can be conjugated to an antibody (or antigen binding antibody fragment) against the Fcγ receptors on monocytes/macrophages.

The vaccines are administered in a manner compatible with the dosage formulation, and in such amount as will be therapeutically effective and immunogenic. The quantity to be administered depends on the subject to be treated, including, e.g., the capacity of the individual's immune system to mount an immune response, and the degree of protection desired. Suitable dosage ranges are of the order of several hundred micrograms active ingredient per vaccination with a preferred range from about 0.1 μg to 1000 μg, such as in the range from about 1 μg to 300 μg, and especially in the range from about 10 μg to 50 μg. Suitable regimens for initial administration and booster shots are also variable but are typified by an initial administration followed by subsequent inoculations or other administrations.

The manner of application may be varied widely. Any of the conventional methods for administration of a vaccine are applicable. These are believed to include oral application on a solid physiologically acceptable base or in a physiologically acceptable dispersion, parenterally, by injection or the like. The dosage of the vaccine will depend on the route of administration and will vary according to the age of the person to be vaccinated and, to a lesser degree, the size of the person to be vaccinated.

The vaccines are conventionally administered parenterally, by injection, for example, either subcutaneously or intramuscularly. Additional formulations which are suitable for other modes of administration include suppositories and, in some cases, oral formulations. For suppositories, traditional binders and carriers may include, for example, polyalkalene glycols or triglycerides; such suppositories may be formed from mixtures containing the active ingredient in the range of 0.5% to 10%, preferably 1-2%. Oral formulations include such normally employed excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, and the like. These compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders and advantageously contain 10-95% of active ingredient, preferably 25-70%.

In many instances, it will be necessary to have multiple administrations of the vaccine. Especially, vaccines can be administered to prevent an infection. When administered to prevent an infection, the vaccine is given prophylactically, before definitive clinical signs or symptoms of an infection are present. Since the current vaccines e.g. BCG appears to induce an effective, but short-lived immune response, prophylactic vaccines may also be designed to be used as booster vaccines. Such vaccines are given to individuals who have previously received a vaccination, with the intention of prolonging the period of protection.

In instances where the individual has already become infected or is suspected to have become infected, the previous vaccination may have provided sufficient immunity to prevent primary disease, but as discussed previously, boosting this immune response will not help against the latent infection. In such a situation, the vaccine has a particular advantage as a therapeutic vaccine designed for efficacy against the latent stage of infection.

Importantly in chronic diseases such as TB, cancer, hepatitis and HIV, the long term equilibrium between host and pathogen often results in immune responses focused towards a few immuno-dominant epitopes. Inducing a broad balanced response towards a range of epitopes within a given protein can not be achieved by immunizing with the recombinant protein, which would only lead to a response towards a limited number of

dominant epitopes. However in contrast, the present invention teaches that vaccinating with a mix of overlapping peptides does induce a T cell immune response towards a range of epitopes, including subdominant epitopes, within a given protein. The present invention and the induction of responses to subdominant epitopes therefore has a particular advantage in these diseases because it can induce an immune response against protective epitopes that are not induced by the chronic disease itself, or by vaccinating with the given protein in a recombinant full lengh form. By conventional preventive vaccination or post-exposure in a therapeutic manner the application of the peptide mixture vaccine technology is superior and with much higher activity than conventional vaccines based on full size molecules against these chronic diseases.

Furthermore, for chronic diseases where humoral immunity is important it is possible to induce an optimal broad T cell response and a maximal B cell response towards the same protein. In this situation primary immunization is done with a mix of overlapping peptides (in an adjuvant) spanning the entire sequence of a given protein and the boosting is achieved with a second vaccine comprising the same protein in recombinant form in an adjuvant. In this way the broad T cell response against both dominant and subdominant epitopes will enable maximal T helper cell activity and thereby a very strong antibody response. The resulting response is a broad T cell and maximal antibody response towards the same antigen, with particular use against chronic diseases.

Figure Legends

Figure 1.

A. Overview of the ESAT-6 overlapping peptides. B. Amino acid sequence of Δ15-ESAT-6

Figure 2. Immunogenicity of ESAT6 and Δ15ESAT6 in splenocytes

Groups of F1 (Balb/cxC57BL/6) mice were subcutaneously vaccinated three times at two-week intervals with either saline, ESAT6 or Δ15ESAT6 in DDA/TDB. Three weeks after the final vaccination, spleen cells were analyzed by ELISA for IFN-gamma secretion following stimulation with 1 microgram/ml ESAT6, Δ15ESAT6 or one of the 13 overlapping

peptides covering the ESAT6 sequence (P1-P13 as indicated in the figure, also shown in figure 1).

Figure 3. Protective efficacy of ESAT6 and Δ15ESAT6
Groups of F1 (Balb/cxC57BL/6) mice were subcutaneously vaccinated three times at two-week intervals with either saline, BCG, or DDA/TDB with ESAT6 or Δ15ESAT6. Six weeks after the last vaccination the mice were challenged with virulent M.tb. Six weeks post-challenge, the mice were killed and the bacterial burden (CFU) was measured in the lung.

Figure 4. *TB10.4 overlapping peptides, P1-P9*

Figure 5. *Vaccination with recombinant TB10 followed by in vitro stimulation with individual peptides P1-P9. In vitro* IFN-γ responses of cells from mice vaccinated three times with DDA/TDB-TB10.4 in DDA/TDB. Cells taken two weeks after final vaccination from blood and stimulated with 0.5ug/ml of the indicated peptide.

Figure 6. *Recognition of TB10-4 peptides P1-P9 following vaccination with individual peptides.*
In vitro IFN-γ responses of cells from mice vaccinated three times with individual TB10.4 peptides in DDA/TDB. Cells taken two weeks after final vaccination from blood and stimulated with 0.5ug/ml of the same peptide used for the vaccination and secretion of IFN-γ was determined by ELISA .

Figure 7. *Protective ability of TB10-4 peptides P1-P9.* Bacterial burden in vaccinated mice (expressed as $\log_{10}$ in CFU protection) challenged by the aerosol route with virulent M.tb six weeks after the last vaccination. Six weeks post-challenge, the mice were killed and the bacterial burden (CFU) was measured in the lung. (* $P<0.05$ compared to non-vaccinated mice, ANOVA and Tukey's test).

Figure 8. *Recognition of TB10-4 peptides P1-P9 after vaccinating with TB10-4 peptide mix.* In vitro IFN-γ responses of cells from mice vaccinated three times with DDA/TDB-TB10.4-peptide mix. Cells taken two weeks after final vaccination from blood and stimulated with 0.5ug/ml peptide or TB10.4 protein as indicated.

Figure. 9. *Bacterial burden in TB10.4 or TB10.4-peptide vaccinated mice infected with M.tb*

Bacterial burden in vaccinated mice (expressed as $\log_{10}$ in CFU) compared to non-vaccinated controls challenged by the aerosol route with virulent M.tb ten weeks after the first vaccination. Six weeks post-challenge, the mice were killed and the bacterial burden (CFU) was measured in the lung. (* PO.05, ANOVA and Tukey's test).

Figure 10.

Overview of the CT521 overlapping peptides.

Figure 11

Mice were vaccinated three times at 2-week intervals with a mix of all the ESAT-6 peptides (P1-P13), and the immune response as measured by secretion of IFN-gamma, was investigated by culturing blood cells with each of the individual ESAT-6 peptides P1-P13.

Figure 12

Mice were vaccinated three times at 2-week intervals with either ESAT-6 or ESAT-6-peptide mix (P1-P13). 6 weeks after the last vaccination, the mice were subjected to an aerosol challenge with virulent M.tb. 10 weeks after the challenge, the mice were killed and the bacterial numbers were determined in the lungs.

Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in the field relevant to the present disclosure as it existed before the priority date of each claim of this application.

Examples

Example 1:

ESAT-6.

To examine to which degree the *Mycobacterium tuberculosis* expressed antigen ESAT-6 contains dominant and subdominant epitopes mice were vaccinated with the recombinant protein ESAT-6 3 times at 2 weeks interval and cells taken two weeks after final vaccination from blood and stimulated with the indicated ESAT-6 peptides (Fig. 1A) where after secretion of IFN-gamma, as assessed by ELISA, was determined. The results showed an induction of IFN-gamma producing T cells specific for P1 and to a lesser

degree P2. Removing the immunodominant epitope P1 from ESAT6 (giving the construct named "Δ15-ESAT-6 in which the amino acids 1-15 have been deleted" (Fig. 1B)) led to immune recognition of new epitopes, P2 and in particular P3 (Fig. 2). This demonstrated that P1 is a dominant epitope, and that P2 and P3 constitute subdominant (but immunogenic) epitopes.

We next examined whether the subdominant epitopes were able to confer protection against infection with M.tb. Mice were subcutaneously vaccinated three times at two-week intervals with either saline, BCG, or DDA/TDB with ESAT6 or Δ15ESAT6. Six weeks after the last vaccination the mice were challenged with virulent M.tb. Six weeks post-challenge, the mice were killed and the bacterial burden (CFU) was measured in the lung.

Protection experiments showed that Δ15-ESAT-6 was more protective than ESAT-6 (Fig. 3), indicating that the subdominant peptides (epitopes) P2 and P3 were indeed able to induce an immune response that mediated protection against infection with M.tb.

We next examined whether vaccination with a mix of all overlapping ESAT-6 peptides would lead to a broader recognition of P1-P13 compared to mice vaccinated with the recombinant protein ESAT-6. Mice were vaccinated three times at 2-week intervals with a mix of all the peptides, and the immune response was investigated by culturing blood cells with each of the individual ESAT-6 peptides P1-P13 (Fig. 11).

The results showed that, in contrast to vaccinating with recombinant protein ESAT-6, vaccinating with a mix of ESAT-6 peptides (P1-P13) led to a broader recognition of the peptides (Fig. 11).

To examine whether the broader response towards ESAT-6 was reflected in the protection against infection with *M. tuberculosis*, as compared to the protein induced by vaccination with the recombinant protein ESAT-6. mice were vaccinated three times at 2-week intervals with either ESAT-6 or ESAT-6-peptide mix. 6 weeks after the last vaccination, the mice were subjected to an aerosol challenge with virulent M.tb. 10 weeks after the challenge, the mice were killed and the bacterial numbers were determined in the lungs.

The results showed that mice vaccinated with ESAT-6 peptide mix not only exhibited a broader recognition of ESAT-6, but were also significant more protected against infection with M.tb compared to mice vaccinated with the recombinant protein ESAT-6 (Fig. 12).

Thus, vaccinating with a mix of ESAT-6 peptides leads to a broader recognition of ESAT-6 epitopes which in turn induce a significant higher protection against infection with M.tb, compared to when vaccinating with the recombinant protein ESAT-6.

Example 2:

TB10.4.

We next analyzed another protein, expressed by M.tb., TB10.4. Mice were vaccinated 3 times at 2 weeks interval with recombinant TB10.4, and cells were taken two weeks after final vaccination from blood and stimulated with 0.5ug/ml of the indicated TB10.4 peptides (Fig. 4) and secretion of IFN-gamma, as assessed by ELISA, was determined.

The results showed that vaccinating with TB10.4 mainly induced P3 specific T cells (Fig.5). P3 therefore constituted a dominant epitope.

Example 3:

To analyze whether the lack of T cells responding to peptides P1, P2, P4, P5, P6, and P9 (and to some degree P7 and P8) was due to these peptide epitopes being subdominant or not immunogenic we next vaccinated with the individual TB10.4 peptides (P1-P9). Following vaccination purified lymphocytes were stimulated in vitro the same peptides used for vaccination and secretion of IFN-γ was determined by ELISA. The results showed that other, subdominant (when vaccinating with the recombinant protein TB10.4) peptides were also strongly immunogenic. In particular vaccinating with peptide 1 or 3, or to a lesser degree P7, P8, or P9 induced a specific T cell response (Fig. 6).

In addition, the subdominant peptides, in particular P1, P7, P8, P9 all protected against infection with M.tb. (Fig. 7). Vaccinating with the dominant peptide epitope P3 also induced significant protection.

Example 4:

Having determined the existence of subdominant epitopes when vaccinating with the recombinant protein TB10.4 we next examined whether vaccination with a mix of all overlapping TB10.4 peptides would lead to a broader recognition of P1-P9 compared to

mice vaccinated with the recombinant protein TB10.4. Mice were vaccinated three times at 2-week intervals with a mix of all the peptides, and the immune response was investigated by culturing blood cells with each of the individual TB10.4 peptides P1-P9 (Fig. 8).

The results showed that, in contrast to vaccinating with recombinant protein TB10.4, vaccinating with a mix of TB10.4 peptides (P1-P9) led to a much broader recognition of the peptides. In particular, P1, P3, and P8 were all strongly recognized (Fig. 8).

Example 5:

To examine whether the broader response towards TB10.4 was reflected in the protection against infection with *M. tuberculosis*, as compared to the protein induced by vaccination with the recombinant protein TB10.4. mice were vaccinated three times at 2-week intervals with either TB10.4 or TB10.4-peptide mix. 6 weeks after the last vaccination, the mice were subjected to an aerosol challenge with virulent M.tb. 6 weeks after the challenge, the mice were killed and the bacterial numbers were determined in the lungs.

The results showed that mice vaccinated with TB10.4-peptide mix not only exhibited a broader recognition of TB10.4, but were also significant more protected against infection with M.tb compared to mice vaccinated with the recombinant protein TB10.4 (Fig. 9). Thus, vaccinating with a mix of TB10.4 peptides leads to a broader recognition of TB10.4 epitopes which in turn induce a significant higher protection against infection with M.tb, compared to when vaccinating with the recombinant protein TB10.4.

Example 5:

ct521

Mice was vaccinated 3 times at 2 weeks interval with recombinant CT521 or a mix of CT521 overlapping peptides (Fig. 10), and cells taken two weeks after final vaccination from blood was stimulated with 0.5ug/ml of each of the CT521 peptides. Secretion of IFN-gamma, as assessed by ELISA, was determined to examine whether vaccinating with a mix of CT521 peptides leads to a broader recognition of CT521, compared to vaccinating with the recombinant CT521 protein.

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2007264205 20 Mar 2013

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A vaccine against a chronic disease comprising a peptide mixture consisting of adjacent overlapping peptides spanning the whole amino acid sequence of a protein that is expressed during the chronic phase of the disease, also comprising an adjuvant where the adjuvant is a cationic liposome.

2. The vaccine according to claim 1, where the adjuvant is dimethyldi-octadecylammonium bromide/trehalose dibehenate (DDA/TDB).

3. A vaccine according to claim 1 or claim 2, where each peptide is incorporated individually into liposomes prior to making the mixture.

4. A vaccine according to any one of claims 1 to 3, where the peptides are 10 to 30 amino acids long.

5. A vaccine according to any one of claims 1 to 4, where the peptides are 12-20 amino acids long.

6. A vaccine according to any one of claims 1 to 5, where the overlap with the adjacent peptide is 6-20 amino acids.

7. A vaccine according to any one of claims 1 to 6, where the overlap with the adjacent peptide is 10-12 amino acids.

8. A vaccine according to any one of claims 1 to 7, where the protein is selected from:

- a bacteria; or
- a virus; or
- a parasite; or
- molecules expressed in malignant tumours.

9. The vaccine according to claim 8, wherein the bacteria is a virulent mycobacteria or *Chlamydia trachomatis*.

10. The vaccine according to claim 9, wherein the virulent mycobacteria is selected from: *Mycobacterium tuberculosis*, *Mycobacterium africanum*, *Mycobacterium bovis* and *Mycobacterium leprae*.

11. The vaccine according to claim 8, wherein the virus is hepatitis B or hepatitis C.

12. The vaccine according to claim 8, wherein the parasite is a Leishmania species or a malaria causing parasite *Plasmodium falciparum*.

13. A vaccine according to any one of claims 1 to 7, wherein the protein is expressed by a bacteria, a virus, a parasite or cancer.

14. A vaccine according to any one of claims 8 to 13, where the peptides are from a protein selected from:

- *M. tuberculosis* proteins; or
- *Chlamydia trachomatis* proteins; or
- a hepatitis virus; or
- *Plasmodium falciparuman* proteins.

15. The vaccine according to claim 14, wherein the *M. tuberculosis* protein is selected from: ESAT6, Ag85A, Ag85B and TB10.4.

16. The vaccine according to claim 14, wherein the *Chlamydia trachomatis* protein is selected from: CT184, CT521, CT443, CT520, CT521, CT375, CT583, CT603, CT610 and CT681.

17. The vaccine according to claim 14, wherein the *Plasmodium falciparuman* protein is selected from: Msp1, Msp2, Msp3, Ama1, GLURP, LSA1, LSA3 and CSP.

18. A vaccine according to any one of claims 1 to 17, where one or more of the peptides are chemically modified.

19. A vaccine according to claim 18, where one or more of the peptides are chemically modified by being glycosylated, lipidated, labelled with PAM3Cys or dodecanoyl chloride, or comprising prosthetic groups, or by containing additional amino acids, or by direct conjugation to TLR agonistlipidated.

20. A vaccine according to any one of claims 1 to 19, where the peptides are delivered encapsulated in the liposomes where the peptide mixture is incorporated into liposomes or each peptide is incorporated individually into liposomes prior to making the mixture.

21. A method of preparing a vaccine according to any one of claims 1 to 20, where the peptide mixture is prepared by proteolytic cleavage of the protein with two or more proteolytic cleavage agents.

22. A method of preparing a vaccine according to claim 21, where the proteolytic cleavage agent is chosen from:

- proteolytic enzymes; and
- chemical agents.

23. The method according to claim 22, wherein the proeolytic enzyme is chosen from: trypsin, V-8 protease, AspN or chymotrypsin.

24. The method according to claim 22, wherein the chemical agent is selected from CNBr and BNPS-skatole.

25. A method for prophylaxis or treatment of a chronic disease in an animal, including a human being, comprising administering to the animal the vaccine according to claim 1-20.

26. A method according to claim 25 where the prophylaxis or treatment is boosted by administering a second vaccine comprising the full size protein spanned by the peptide mixture in adjuvant or expressed in a live delivery system.

A

```
ESAT6      MTEQQWNFAGIEAAASAIQGNVTSIHSLLDEGKQSLTKLAAAWGGSGSEAYQGVQQKWDATTELNNALQNLARTISEAGQAMASTEGNVTGMFA
ESAT6-P1   MTEQQWNFAGIEAAA
ESAT6-P2       NFAGIEAAASAIQGN
ESAT6-P3           ASAIQGNVTSIHSLL
ESAT6-P4               NVTSIHSLLDEGKQS
ESAT6-P5                   SLLDEGKQSLTKLAA
ESAT6-P6                       KQSLTKLAAAWGGSG
ESAT6-P7                           AAWGGSGSEAYQGVQ
ESAT6-P8                               GSEAYQGVQQKWDAT
ESAT6-P9                                   QQKWDATTELNNAL
ESAT6-P10                                      TTELNNALQNLART
ESAT6-P11                                          ALQNLARTISEAGQA
ESAT6-P12                                              TISEAGQAMASTEGN
ESAT6-P13                                                  QAMASTEGNVTGMFA
```

B

Δ15-ESAT6 SAIQGNVTSIHSLLDEGKQSLTKLAAAWGGSGSEAYQGVQQKWDATTELNNALQNLARTI-

SEAGQAMASTEGNVTGMFA

Figure 1.

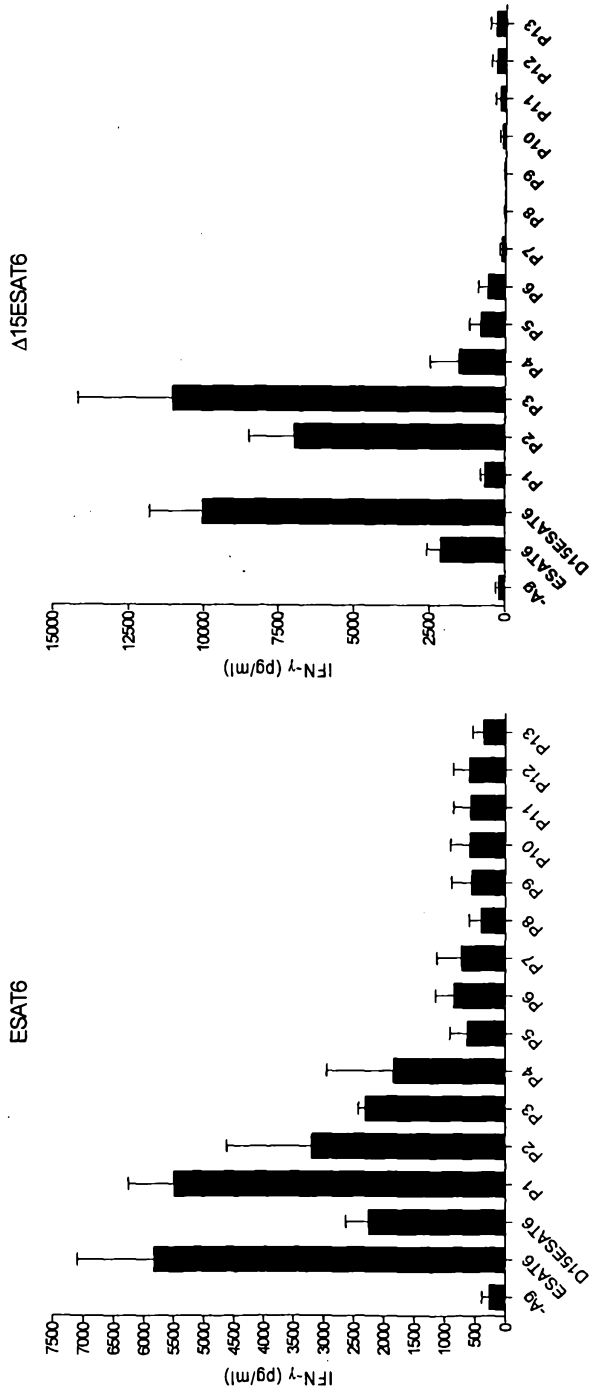
Δ15ESAT6
IFN-γ (pg/ml)
15000
12500
10000
7500
5000
2500
0
Ag
ESAT6
D15ESAT6
P1
P2
P3
P4
P5
P6
P7
P8
P9
P10
P11
P12
P13
ESAT6
IFN-γ (pg/ml)
7500
7000
6500
6000
5500
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
Ag
ESAT6
D15ESAT6
P1
P2
P3
P4
P5
P6
P7
P8
P9
P10
P11
P12
P13

Figure 2

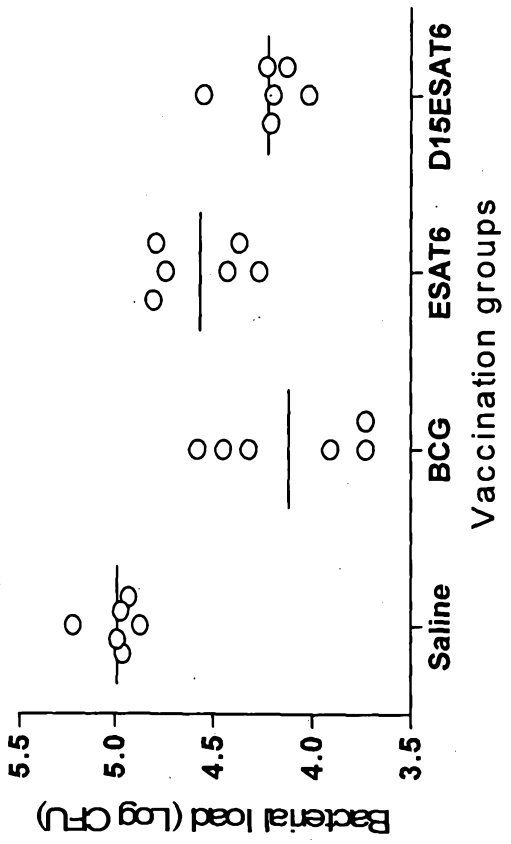
Bacterial load (Log CFU)
5.5
5.0
4.5
4.0
3.5
Saline
BCG
ESAT6
D15ESAT6
Vaccination groups

Figure 3

```
TB10.4      MSQIMYNYPAMLGHAGDMAGYAGTLQSLGAEIAVEQAALQSAWQGDTGITYQAWQAQWNQAMEDLVRAYHAMSSTHEANTMAMMARDTAEAAKWGG
TB10.4-P1   MSQIMYNYPAMLGHAGDM
TB10.4-P2               MLGHAGDMAGYAGTLQSL
TB10.4-P3                       YAGTLQSLGAEIAVEQAA
TB10.4-P4                               EIAVEQAALQSAWQGDTG
TB10.4-P5                                       SAWQGDTGITYQAWQAQW
TB10.4-P6                                               YQAWQAQWNQAMEDLVRA
TB10.4-P7                                                       AMEDLVRAYHAMSSTHEA
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TB10.4-P9                                                                       MAMMARDTAEAAKWGG
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Figure 4

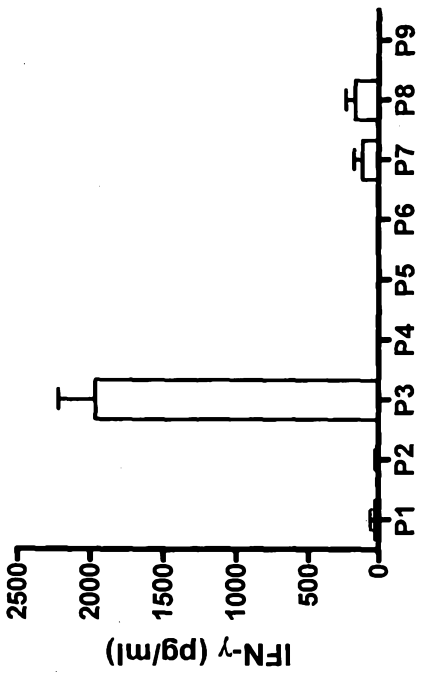


Figure 5

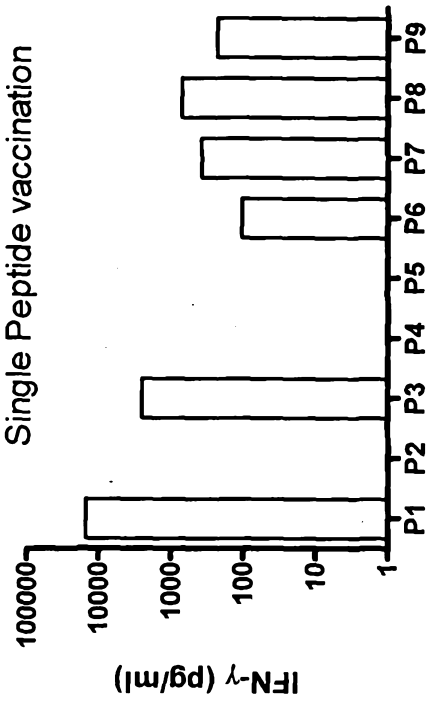

Single Peptide vaccination
IFN-γ (pg/ml)
100000
10000
1000
100
10
1
P1
P2
P3
P4
P5
P6
P7
P8
P9

Figure 6

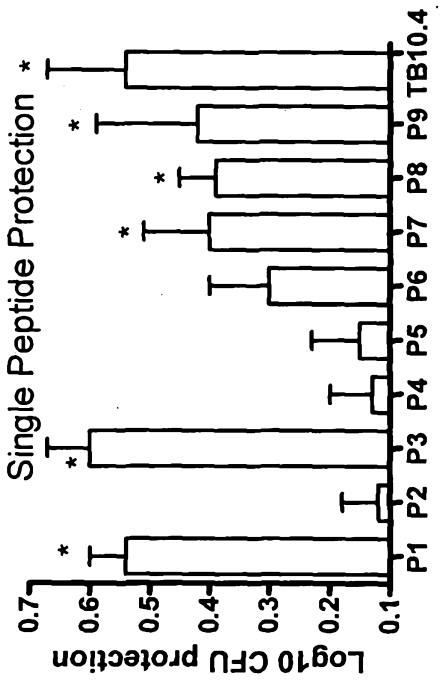

Single Peptide Protection
Log10 CFU protection
0.7
0.6
0.5
0.4
0.3
0.2
0.1
P1 P2 P3 P4 P5 P6 P7 P8 P9 TB10.4
*

Figure 7

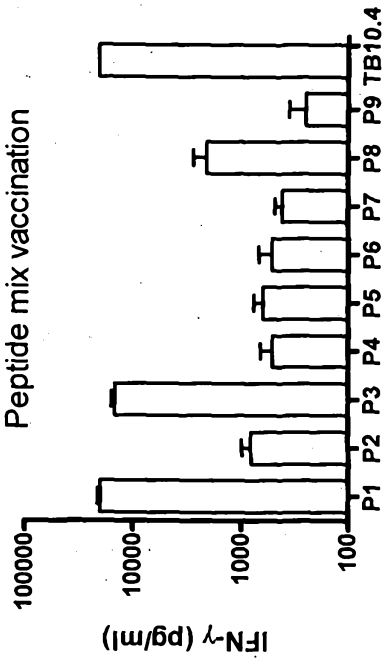

Peptide mix vaccination
IFN-γ (pg/ml)
100000
10000
1000
100
P1
P2
P3
P4
P5
P6
P7
P8
P9
TB10.4

Figure 8

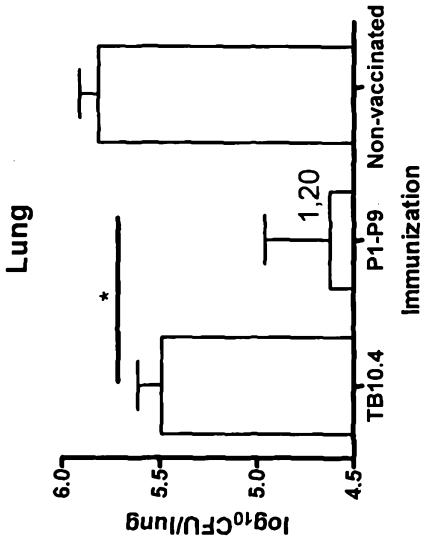

Lung
log10CFU/lung
6.0
5.5
5.0
4.5
*
1,20
TB10.4
P1-P9
Non-vaccinated
Immunization

Figure. 9

```
CT521 :     MLMPKRTKERKQQKGQFAGLSKGATFVDFGEFGMQTLERGMITSRQIEACRVAINRYLKRKGKVWIRVFPDRSVTKKPAETRMGKGKGAPDHWVVVRPGRILFEVANVSKEDAQDALRRAAAKLGIRTRFVKRVERV

CT521-P1:   MLMPKRTKERKQQKGQFAGLSK
CT521-P2:               KGQFAGLSKGATFVDFGEFGMQT
CT521-P3:                       VDFGEFGMQTLERGMITSRQIEA
CT521-P4:                               GMITSRQIEACRVAINRYLKRKG
CT521-P5:                                       AINRYLKRKGKVWIRVFPDRSVT
CT521-P6:                                               IRVFPDRSVTKKPAETRMGKGKG
CT521-P7:                                                       KPAETRMGKGKGAPDHWVVVRP
CT521-P8:                                                               PDHWVVVRPGRILFEVANVSKE
CT521-P9:                                                                       ILFEVANVSKEDAQDALRRAAAK
CT521-P10:                                                                      DALRRAAAKLGIRTRFVKRVERV
```

Figure. 10

Figure 11

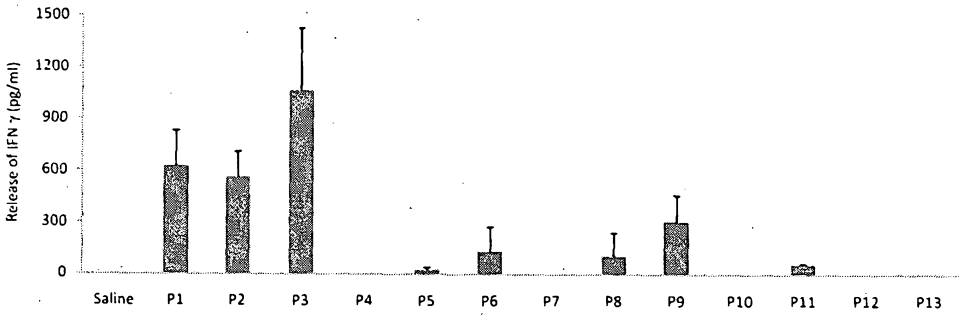
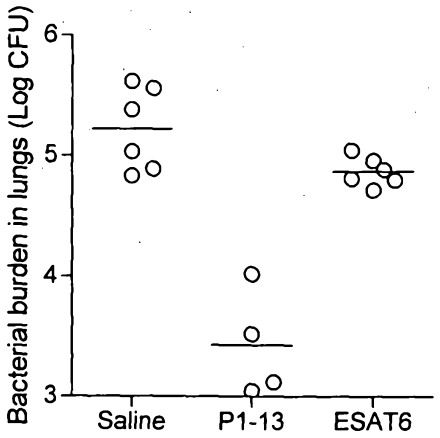


Figure 12



SEQUENCE LISTING

<110> Statens serum institut

<120> Expanding the T cell repertoire to include subdominant epitopes by vaccination with antigens delivered as protein fragments or peptide cocktails

<130> 44272PC01

<150> PCT/DK2007/000312
<151> 2007-06-26

<160> 36

<170> PatentIn version 3.5

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1 5 10 15

Ala Ile Gln Gly Asn Val Thr Ser Ile His Ser Leu Leu Asp Glu Gly
20 25 30

Lys Gln Ser Leu Thr Lys Leu Ala Ala Ala Trp Gly Gly Ser Gly Ser
35 40 45

Glu Ala Tyr Gln Gly Val Gln Gln Lys Trp Asp Ala Thr Thr Glu Leu
50 55 60

Asn Asn Ala Leu Gln Asn Leu Ala Arg Thr Ile Ser Glu Ala Gly Gln
65 70 75 80

Ala Met Ala Ser Thr Glu Gly Asn Val Thr Gly Met Phe Ala
85 90

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1 5 10 15

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Ala Ser Ala Ile Gln Gly Asn Val Thr Ser Ile His Ser Leu Leu
1 5 10 15

<210> 5
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Asn Val Thr Ser Ile His Ser Leu Leu Asp Glu Gly Lys Gln Ser
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<210> 6
<211> 15
<212> PRT
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Ser Leu Leu Asp Glu Gly Lys Gln Ser Leu Thr Lys Leu Ala Ala
1 5 10 15

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<212> PRT
<213> Mycobacterium tuberculosis

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<400> 7

Lys Gln Ser Leu Thr Lys Leu Ala Ala Ala Trp Gly Gly Ser Gly
1 5 10 15

<210> 8

<211> 15
<212> PRT
<213> Mycobacterium tuberculosis

<220>
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Ala Ala Trp Gly Gly Ser Gly Ser Glu Ala Tyr Gln Gly Val Gln
1 5 10 15

<210> 9
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<212> PRT
<213> Mycobacterium tuberculosis

<220>
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<400> 9

Gly Ser Glu Ala Tyr Gln Gly Val Gln Gln Lys Trp Asp Ala Thr
1 5 10 15

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<212> PRT
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<220>
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Gln Gln Lys Trp Asp Ala Thr Thr Glu Leu Asn Asn Ala Leu
1 5 10

<210> 11
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<212> PRT
<213> Mycobacterium tuberculosis

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<400> 11

Thr Thr Glu Leu Asn Asn Ala Leu Gln Asn Leu Ala Arg Thr
1 5 10

<210> 12
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<212> PRT
<213> Mycobacterium tuberculosis

<220>
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Ala Leu Gln Asn Leu Ala Arg Thr Ile Ser Glu Ala Gly Gln Ala
1 5 10 15

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<212> PRT
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<400> 13

Thr Ile Ser Glu Ala Gly Gln Ala Met Ala Ser Thr Glu Gly Asn
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1 5 10 15

Gly Lys Gln Ser Leu Thr Lys Leu Ala Ala Ala Trp Gly Gly Ser Gly
20 25 30

Ser Glu Ala Tyr Gln Gly Val Gln Gln Lys Trp Asp Ala Thr Thr Glu
35 40 45

Leu Asn Asn Ala Leu Gln Asn Leu Ala Arg Thr Ile Ser Glu Ala Gly
50 55 60

Gln Ala Met Ala Ser Thr Glu Gly Asn Val Thr Gly Met Phe Ala
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<210> 16
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1 5 10 15

Asp Met Ala Gly Tyr Ala Gly Thr Leu Gln Ser Leu Gly Ala Glu Ile
20 25 30

Ala Val Glu Gln Ala Ala Leu Gln Ser Ala Trp Gln Gly Asp Thr Gly
35 40 45

Ile Thr Tyr Gln Ala Trp Gln Ala Gln Trp Asn Gln Ala Met Glu Asp
50 55 60

Leu Val Arg Ala Tyr His Ala Met Ser Ser Thr His Glu Ala Asn Thr
65 70 75 80

Met Ala Met Met Ala Arg Asp Thr Ala Glu Ala Ala Lys Trp Gly Gly
85 90 95

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Met Ser Gln Ile Met Tyr Asn Tyr Pro Ala Met Leu Gly His Ala Gly
1 5 10 15

Asp Met

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Met Leu Gly His Ala Gly Asp Met Ala Gly Tyr Ala Gly Thr Leu Gln
1 5 10 15

Ser Leu

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<220>
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<400> 19

Tyr Ala Gly Thr Leu Gln Ser Leu Gly Ala Glu Ile Ala Val Glu Gln
1 5 10 15

Ala Ala

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Glu Ile Ala Val Glu Gln Ala Ala Leu Gln Ser Ala Trp Gln Gly Asp
1 5 10 15

Thr Gly

<210> 21
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<220>
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Ser Ala Trp Gln Gly Asp Thr Gly Ile Thr Tyr Gln Ala Trp Gln Ala
1 5 10 15

Gln Trp

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Tyr Gln Ala Trp Gln Ala Gln Trp Asn Gln Ala Met Glu Asp Leu Val
1 5 10 15

Arg Ala

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<220>
<223> TB10.4-P7

<400> 23

Ala Met Glu Asp Leu Val Arg Ala Tyr His Ala Met Ser Ser Thr His
1 5 10 15

Glu Ala

<210> 24
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<220>
<223> TB10.4-P8

<400> 24

Ala Met Ser Ser Thr His Glu Ala Asn Thr Met Ala Met Met Ala Arg
1 5 10 15

Asp Thr

<210> 25
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Met Ala Met Met Ala Arg Asp Thr Ala Glu Ala Ala Lys Trp Gly Gly
1 5 10 15

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1 5 10 15

Phe Ala Gly Leu Ser Lys Gly Ala Thr Phe Val Asp Phe Gly Glu Phe
20 25 30

Gly Met Gln Thr Leu Glu Arg Gly Trp Ile Thr Ser Arg Gln Ile Glu
35 40 45

Ala Cys Arg Val Ala Ile Asn Arg Tyr Leu Lys Arg Lys Gly Lys Val
50 55 60

Trp Ile Arg Val Phe Pro Asp Lys Ser Val Thr Lys Lys Pro Ala Glu
65 70 75 80

Thr Arg Met Gly Lys Gly Lys Gly Ala Pro Asp His Trp Val Val Val
85 90 95

Val Arg Pro Gly Arg Ile Leu Phe Glu Val Ala Asn Val Ser Lys Glu
100 105 110

Asp Ala Gln Asp Ala Leu Arg Arg Ala Ala Ala Lys Leu Gly Ile Arg
115 120 125

Thr Arg Phe Val Lys Arg Val Glu Arg Val
130 135

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Met Leu Met Pro Lys Arg Thr Lys Phe Arg Lys Gln Gln Lys Gly Gln
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Phe Ala Gly Leu Ser Lys
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1 5 10 15

Gly Glu Phe Gly Met Gln Thr
20

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<220>
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Val Asp Phe Gly Glu Phe Gly Met Gln Thr Leu Glu Arg Gly Trp Ile
1 5 10 15

Thr Ser Arg Gln Ile Glu Ala
20

<210> 30
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<213> Chlamydia trachomatis

<220>
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Gly Trp Ile Thr Ser Arg Gln Ile Glu Ala Cys Arg Val Ala Ile Asn
1 5 10 15

Arg Tyr Leu Lys Arg Lys Gly
20

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<213> Chlamydia trachomatis

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Ala Ile Asn Arg Tyr Leu Lys Arg Lys Gly Lys Val Trp Ile Arg Val
1 5 10 15

Phe Pro Asp Lys Ser Val Thr
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<210> 32
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<213> Chlamydia trachomatis

<220>
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Ile Arg Val Phe Pro Asp Lys Ser Val Thr Lys Lys Pro Ala Glu Thr
1 5 10 15

Arg Met Gly Lys Gly Lys Gly
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<210> 33
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<213> Chlamydia trachomatis

<220>
<223> CT521-P7

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Lys Pro Ala Glu Thr Arg Met Gly Lys Gly Lys Gly Ala Pro Asp His
1 5 10 15

Trp Val Val Val Val Arg Pro
20

<210> 34
<211> 23
<212> PRT
<213> Chlamydia trachomatis

<220>
<223> CT521-P8

<400> 34

Pro Asp His Trp Val Val Val Val Arg Pro Gly Arg Ile Leu Phe Glu
1 5 10 15

Val Ala Asn Val Ser Lys Glu
20

<210> 35
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Ile Leu Phe Glu Val Ala Asn Val Ser Lys Glu Asp Ala Gln Asp Ala
1 5 10 15

Leu Arg Arg Ala Ala Ala Lys
20

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Asp Ala Leu Arg Arg Ala Ala Ala Lys Leu Gly Ile Arg Thr Arg Phe
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Val Lys Arg Val Glu Arg Val
20