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(54) **METHODS AND KITS FOR DETECTION OF
MULTIPLE PATHOGENS**

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(57) **ABSTRACT**

The present invention relates to methods of simultaneously detecting multiple infectious organisms, including methods of simultaneously determining the presence or absence in a sample of one or more members of a group of pathogens including at least one viral pathogen and at least one non-viral pathogen. The present invention also discloses and claims kits for carrying out the methods of the invention.

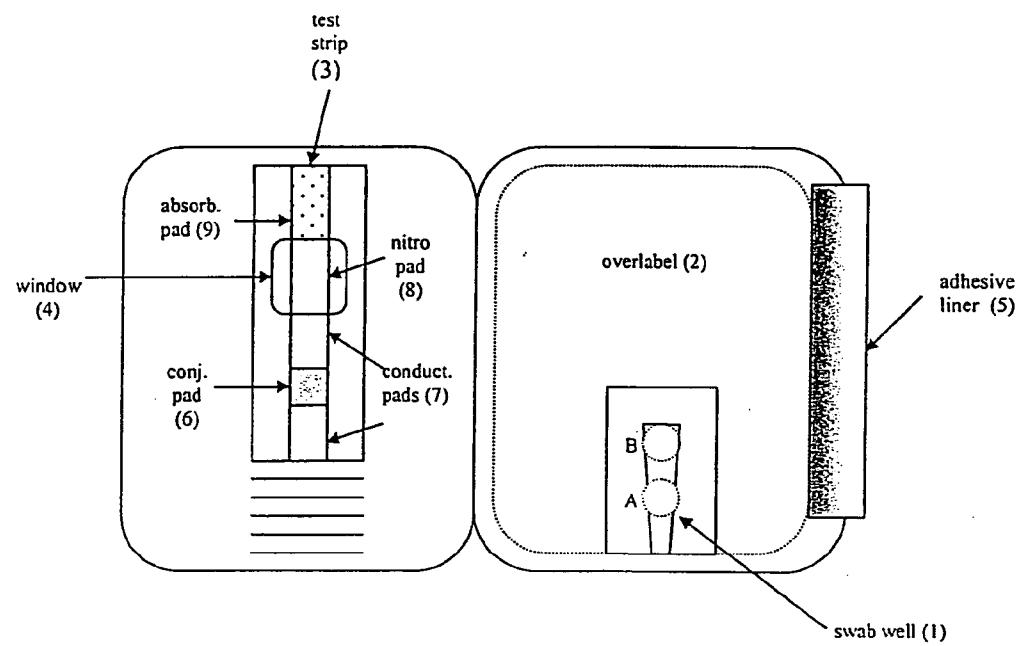


Figure 1

METHODS AND KITS FOR DETECTION OF MULTIPLE PATHOGENS**BACKGROUND OF THE INVENTION****FIELD OF THE INVENTION**

[0001] The present invention relates generally to the field of medical diagnostics, and particularly to methods for simultaneously detecting multiple infectious organisms, including methods for simultaneously detecting viral and bacterial pathogens.

[0002] Infection of an individual by more than one pathogenic organism is a common phenomenon. Mixed viral infections (that is, concurrent infection by more than one type of virus) are not unusual (Waner (1994) *Clin. Microbiol. Rev.*, 7:143-151). Mixed viral and bacterial infections have also been well documented, see, for example, Hietala et al (1989) *Pediatr. Infect. Dis. J.*, 8:683-686, and Korppi (1999) *Pediatr. Pulmonol. Suppl.*, 18: 110, which are incorporated by reference in their entirety herein.

[0003] Viral infections of the respiratory tract are among the most common human diseases, and include, but are not limited to, infection by respiratory syncytial virus (RSV) types A and B, influenza A, B, and C viruses, human parainfluenza viruses (HPIV) 1, 2, and 3, human metapneumovirus (hMPV), measles virus, adenoviruses (AV), rhinoviruses, coronaviruses, and enteroviruses. See, for example, Mackie (2003) *Paediatr. Respir. Rev.*, 4:84-90; and Kahn (2003) *Curr. Opin. Infect. Dis.*, 16:255-258, which are incorporated by reference in their entirety herein. Immuno-compromised patients are vulnerable to respiratory infections by additional viruses, such as herpesviruses (Mackie (2003) *Paediatr. Respir. Rev.*, 4:84-90). Studies of immunocompetent patients have shown that a high percentage (>30%) of patients with RSV or rhinovirus infections showed serological evidence of concomitant infection by another virus. An analysis of mixed viral infections reported that respiratory syncytial virus, influenza viruses, adenoviruses, and parainfluenza viruses were the most common viruses in the mixed respiratory infections, with the combination of RSV and influenza being the most frequent co-infection (Waner (1994) *Clin. Microbiol. Rev.*, 7:143-151). Coinfection by human metapneumovirus and respiratory syncytial virus has also been reported to be common (Greensill et al (2003) *Emerg. Infect. Dis.*, 9:372). Mixed viral-bacterial and viral-fungal respiratory infections are also well documented. See, for example, Korppi et al. (1989) *Pediatr. Infect. Dis. J.*, 8:687-692; Korppi et al. (1990) *Scand. J. Infect. Dis.*, 22:307-312; Korppi et al. (1991) *Acta Paediatr. Scand.*, 80:413-417; Korppi et al. (1993) *Eur. J. Pediatr.*, 152:24; Hament et al. (1999) *FEMS Immunol. Med Microbiol.*, 26:189-195; and Tristram et al. (1988) *Arch. Pediatr. Adolesc. Med.*, 142:834-836, which are incorporated by reference in their entirety herein.

[0004] Acute otitis media, though generally thought of as a bacterial infection of the middle ear, can be associated with or influenced by viral pathogens (Heikkinen and Chonmaitree (2003) *Clin. Microbiol. Rev.*, 16:230-241; Canafax et al. (1998) *Pediatr. Infect. Dis. J.*, 17:149-156; and Ruuskanen et al. (1989) *Pediatr. Infect. Dis. J.*, 8:94 which are incorporated by reference in their entirety herein). Similarly, an antecedent infection by certain respiratory viruses, espe-

cially influenza A, appears to increase incidence and severity of *Streptococcus pneumoniae*-caused otitis media (see, for example, Tong et al. (2000) *Ann. Otol. Rhinol. Laryngol.*, 109:1021-1027; Tong et al. (2001) *Infect. Immun.*, 69:602-606; and Tong et al. (2002) *Infect. Immun.*, 70:4292-4301, which are incorporated by reference in their entirety herein).

[0005] Meningitis and other central nervous system infections may be viral or bacterial in etiology. Multiple infective agents have been demonstrated (see, for example, Eglin et al. (1984) *Lancet*, 2(8409):984; Squadrini et al. (1977) *Lancet*, 1(8026):1371; and Krasinski et al. (1987) *Am. J. Epidemiol.*, 125:499-508, which are incorporated by reference in their entirety herein), with mixed bacterial infections in meningitis having been relatively more commonly reported in the medical literature than mixed viral-bacterial infections (Sferra and Pacini (1988) *Pediatr. Infect. Dis. J.*, 7:552-556, which is incorporated by reference in its entirety herein). Eukaryotic pathogens, such as *Giardia lamblia*, *Entamoeba histolytica*, *Naegleria fowleri*, *Cyclospora caytanensis*, *Toxoplasma gondii*, *Cryptosporidium parvum*, *Acanthamoeba* species, microsporidia, and other protozoan pathogens, can also cause central nervous system infections.

[0006] Infections of the digestive tract are caused by many pathogens, including viruses, bacteria, and protozoans (Leclerc et al. (2002) *Crit. Rev. Microbiol.*, 28:371-409, which is incorporated by reference in its entirety herein). Mixed infections, including mixed viral-bacterial infections, have been associated with gastroenteritis (see, for example, Cramblett and Siewers (1965) *Pediatrics*, 35:885-898; Zavate et al. (1988) *Virologie*, 39:131-136; Bettelheim et al. (2001) *Comp. Immunol. Microbiol. Infect. Dis.*, 24:135-142, which are incorporated by reference in their entirety herein).

[0007] Viral infections can precede, and overlap with, bacterial infections. In cases of respiratory diseases, it is believed that viral infection predisposes the patient to bacterially caused disease (see, for example, Hament et al. (1999) *FEMS Immunol. Med Microbiol.*, 26:189-195; Korppi (2002) *APMIS*, 110:515). There is also evidence that a preceding infection by adenovirus and possibly influenza B virus may likewise predispose a subject to bacterial meningitis (Krasinski et al. (1987) *Am. J. Epidemiol.*, 125:499-508). Similarly, viral infection of the respiratory tract often precedes and exacerbates cases of otitis media, and prevention of viral infection by immunization has now been recognized as an important way of preventing acute otitis media (Heikkinen and Chonmaitree (2003) *Clin. Microbiol. Rev.*, 16:230-241). Conversely, coinfection (or superinfection) by a bacterial pathogen may make a viral infection more severe or protracted (see, for example, Jarstrand & Tunenvall (1975) *Scand. J. Infect. Dis.*, 7:243-247; Krell et al. (2003) *Infection*, 5:353-358; Thomas et al. (2003) *Pediatr. Infect. Dis. J.*, 22:201-202, which are incorporated by reference in their entirety herein). The determination of all pathogens, whether viral, bacterial, or eukaryotic (including unfungal and protozoan pathogens), that may be involved in the etiology of a disease condition is therefore essential in allowing prompt treatment with the appropriate antiviral, antibacterial, or other antibiological agents. Rapid and accurate diagnosis is especially important in diseases that have a high fatality rate or serious sequelae, such as occurs in meningitis. Accurate differentiation between viral infections from non-viral infections (especially bacterial infections) is important for avoiding the inappropriate use of antibiotics

and the associated adverse effects of antibiotics and risk of developing antibiotic resistance. Diagnosis of viral infections uncomplicated by non-viral infections gives the clinician the option of prescribing antiviral drugs.

BRIEF SUMMARY OF THE INVENTION

[0008] The present invention relates to methods of simultaneously detecting multiple infectious organisms. In particular, the present invention relates to methods of simultaneously determining the presence or absence in a sample of one or more members of a group of pathogens including at least one viral pathogen and at least one non-viral pathogen. The present invention also discloses and claims kits for carrying out the methods of the invention.

[0009] One aspect of the present invention includes a method of simultaneously determining the presence or absence in a sample of one or more members of a group of pathogens including at least one viral pathogen and at least one non-viral pathogen, the method including, for each member of the group of pathogens, performing at least one assay for at least one epitope derived from the member, wherein the at least one assay includes: (a) providing at least one binding agent capable of specifically binding to the at least one epitope derived from the member; (b) contacting the at least one binding agent directly to the sample; (c) allowing the at least one binding agent provided to specifically bind to and form a complex with the epitope; and (d) detecting the complex, wherein the detection is positive if the concentration of the member in the sample is greater than or equal to a reference concentration, and the detection is negative if concentration of the member is less than the reference concentration.

[0010] Another aspect of the present invention includes assays and kits for carrying out the method of the invention. Such assays and kits may be designed for detection of one or more members of a group of pathogens (including at least one viral pathogen and at least one non-viral pathogen), for example, a group of pathogens whose members can individually or severally cause similar symptoms of disease.

BRIEF DESCRIPTION OF THE DRAWINGS

[0011] FIG. 1 depicts a non-limiting example of a device for performing assays of the present invention, as described in the Examples. This device includes a hinged cardboard housing equipped with a window to allow the viewing of multiple test line results. The device has a recess on one side, containing a preformed plastic well (1) for receiving a swab sample; an overlabel (2); a preassembled test strip (3), prepared as described in Example 1; a viewing window (4); and an optional, lightly adhesive liner (5). The preassembled test strip (3) includes a conjugate pad (6) positioned between conductive pads (7) that direct liquid flow. The conjugate pad contains binding agents that have been temporarily immobilized onto or into the conjugate pad. The test strip also includes a membrane (8) containing the multiple test line reading zone, and an absorbent pad (9). The multiple test line reading zone includes a test line for each of the pathogens detectable by the device.

DETAILED DESCRIPTION OF THE INVENTION

[0012] Introduction

[0013] The present invention recognizes the need for, and provides, methods that answer the need for a rapid and accurate determination of the presence or absence of one or more pathogens, for example, determination of whether a subject is infected with a viral pathogen, a non-viral pathogen, or both. The methods can be of use in determining the type of treatment appropriate to an infection, such as whether or not a subject should be treated with antivirals, antibacterials, or other antibiologics. The methods are preferably inexpensive and technically simple, and most preferably do not require expensive or complex instrumentation or equipment. Preferably, such methods do not entail the time and expense of culturing, and most preferably are rapid enough to allow use as a point-of-care diagnostic, such as when used in a clinician's office to provide result within the time of a patient's visit.

[0014] As a non-limiting introduction to the breadth of the present invention, the present invention includes several general and useful aspects, including:

[0015] 1) A method of simultaneously determining the presence or absence in a sample of one or more members of a group of pathogens including at least one viral pathogen and at least one non-viral pathogen, the method including, for each member of the group of pathogens, performing at least one assay for at least one epitope derived from the member, wherein the at least one assay includes: (a) providing at least one binding agent capable of specifically binding to the at least one epitope derived from the member; (b) contacting the at least one binding agent directly to the sample; (c) allowing the at least one binding agent provided to specifically bind to and form a complex with the epitope; and (d) detecting the complex, wherein the detection is positive if the concentration of the member in the sample is greater than or equal to a reference concentration, and the detection is negative if concentration of the member is less than the reference concentration.

[0016] 2) Assays and kits for carrying out the method of the invention.

[0017] Further objectives and advantages of the present invention will become apparent as the description proceeds and when taken in conjunction with the accompanying drawings. To gain a full appreciation of the scope of the present invention, it will be further recognized that various aspects of the present invention can be combined to make desirable embodiments of the invention.

[0018] Throughout this application various publications are referenced. The disclosures of these publications are hereby incorporated by reference, in their entirety, in this application. Citations of these documents are not intended as an admission that any of them are pertinent prior art. All statements as to the date or representation as to the contents of these documents is based on the information available to the applicant and does not constitute any admission as to the correctness of the dates or contents of these documents.

[0019] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this

invention belongs. Generally, the nomenclature used herein and the manufacture or laboratory procedures described below are well known and commonly employed in the art. The technical terms used herein have their ordinary meaning in the art that they are used, as exemplified by a variety of technical dictionaries. Where a term is provided in the singular, the inventors also contemplate the plural of that term. The nomenclature used herein and the procedures described below are those well known and commonly employed in the art. Where there are discrepancies in terms and definitions used in references that are incorporated by reference, the terms used in this application shall have the definitions given herein. Other technical terms used herein have their ordinary meaning in the art that they are used, as exemplified by a variety of technical dictionaries (for example, Chambers Dictionary of Science and Technology, Peter M. B. Walker (editor), Chambers Harrap Publishers, Ltd., Edinburgh, UK, 1999, 1325 pp.). The inventors do not intend to be limited to a mechanism or mode of action. Reference thereto is provided for illustrative purposes only.

[0020] I. Method for Simultaneously Detecting Viral and Non-Viral Pathogens

[0021] The present invention includes a method of simultaneously determining the presence or absence in a sample of one or more members of a group of pathogens including at least one viral pathogen and at least one non-viral pathogen, the method including, for each member of the group of pathogens, performing at least one assay for at least one epitope derived from the member, wherein the at least one assay includes: (a) providing at least one binding agent capable of specifically binding to the at least one epitope derived from the member; (b) contacting the at least one binding agent directly to the sample; (c) allowing the at least one binding agent provided to specifically bind to and form a complex with the epitope; and (d) detecting the complex, wherein the detection is positive if the concentration of the member in the sample is greater than or equal to a reference concentration, and the detection is negative if concentration of the member is less than the reference concentration.

[0022] The method of the present invention may be applied to any sample that is suspected of having one or more members of a group of pathogens including at least one viral pathogen and at least one non-viral pathogen. Such samples may include pathology or diagnostic samples, experimental or research samples, or environmental samples. A pathology or diagnostic sample may be from a subject, such as a human subject or a veterinary subject, especially a human or veterinary subject suspected of having a disease caused by an infectious organism. Human subjects may be infants, children, and adult humans of any age. Veterinary subjects may be immature or mature animals of economic, domestic, or research interest (including, but not limited to, dogs, cats, cattle, sheep, goats, swine, rabbits, rats, mice, fish, birds, and non-human primates). Diseases of interest may be any disease that is believed to be caused by at least one pathogen, particularly where the at least one pathogen may be viral or non-viral or a mixture of pathogens. Such diseases include, but are not limited to, respiratory tract infections (such as, but are not limited to, pneumonia, influenza and influenza-like illnesses, sinusitis, bronchitis, tonsillitis, pharyngitis, laryngitis, croup, bronchitis, chronic obstructive pulmonary disease), acute otitis media, conjunctivitis, meningitis and other central nervous

system infections, and digestive tract infections (such as gastroenteritis or diarrhoea). Where a disease includes infection by multiple pathogens, such infections may be co-infections or superinfections, and may occur concurrently or in succession (with or without overlap of infections).

[0023] Where the sample is from a human subject, the sample may include whole cells, tissues, organs, biopsies, biological materials and fluids (for example, blood, serum, plasma, urine, cerebrospinal fluid, synovial fluid, sputum, saliva, semen, tears, and feces), swabs, washes, lavages, discharges, or aspirates (for example, nasal, oral, nasopharyngeal, oropharyngeal, esophageal, gastric, rectal, or vaginal, swabs, washes, ravages, discharges, or aspirates), and extracts or derivatives thereof. For use in the method of the present invention, samples may need minimal preparation (for example, collection into a suitable container), or more extensive preparation (such as, but not limited to, removal, inactivation, or blocking of undesirable material, such as contaminants, undesired cells or cellular material, or endogenous enzymes; treatment with buffers, detergents, surfactants, enzymes, denaturants, reductants, oxidizers, or other reagents; subjection to heat, cold, pressure, vacuum, or other physical treatments; filtration, centrifugation, size selection, or affinity purification; cell fixation, permeabilization, or lysis; and concentration or dilution). Samples may be treated with one or more preparation techniques or one or more preparation reagents. In one embodiment, a sample may use one preparation technique or reagent to enable or facilitate assaying for one or more viral pathogens, and another preparation technique or reagent to enable or facilitate assaying for one or more non-viral pathogens. In a more preferred embodiment, an identical preparation technique (for example, collection of the sample and treatment with the same reagent or reagents) is suitable for treating all members of the group of pathogens of interest. Most preferably, such an identical preparation technique is simple, rapid, and inexpensive.

[0024] The method of the present invention can simultaneously determine the presence or absence in a sample of one or more members of a group of pathogens including at least one viral pathogen and at least one non-viral pathogen. By "simultaneously determine" is meant to determine substantially at the same time or substantially within the same narrow time frame. A narrow time frame may be less than about one second, or less than about 1 minute, or less than about 5 minutes, or less than about 15 minutes. Most preferably, the presence or absence in a sample of all of the members of the group of pathogens can be determined within a relatively short period of time, such as during the time of a patient's consultation with a physician or other health care provider, thus permitting the timely prescription of appropriate therapy.

[0025] The group of pathogens is generally selected from at least one viral pathogen and at least one non-viral pathogen that each are capable of causing similar or identical symptoms of disease, for example, symptoms of upper respiratory infection, symptoms of meningeal inflammation, or symptoms of gastrointestinal distress. The method can be especially useful in distinguishing viral infections from bacterial or eukaryotic infections, and can additionally be valuable in identifying the particular pathogenic species, type, group, or strain.

[0026] Viral pathogens include, but are not limited to, respiratory syncytial virus (RSV) types A and B, influenza A, B, and C viruses, human parainfluenza viruses (hPIV) 1, 2, and 3, human metapneumovirus (hMPV), cytomegaloviruses, adenoviruses (AV), rhinoviruses, coronaviruses, enteroviruses, herpesviruses, enteric adenoviruses, rotavirus groups A, B, and C, astroviruses, sapoviruses, toroviruses, caliciviruses (including noroviruses, Norwalk-like viruses, and Norwalk viruses), herpes viruses, human immunodeficiency viruses, varicella-zoster viruses, polioviruses, arboviruses, mumps viruses, measles viruses, pox viruses, vaccinia viruses, Epstein-Barr viruses, rubella viruses, hantaviruses, echoviruses, coxsackieviruses, and polymyxoviruses. Non-viral pathogens include pathogenic bacteria (including mycoplasmas) and eukaryotic pathogens (including fungi and protozoans). Pathogenic, or potentially pathogenic bacteria of interest include, but are not limited to, (1) bacteria that can be associated with respiratory infections, such as *Acinetobacter* species, viridans streptococci, beta-hemolytic streptococci (including group A beta-hemolytic streptococci such as *Streptococcus pyogenes*), non-hemolytic streptococci, *Streptococcus pneumoniae*, staphylococci (including coagulase-negative staphylococci and *Staphylococcus aureus*), micrococci, *Corynebacterium* species (including *Corynebacterium diphtheriae*), *Neisseria* species (including *Neisseria meningitidis* and *Neisseria gonorrhoeae*), *Cryptococcus neoformans*, *Mycoplasma* species (including *Mycoplasma pneumoniae*), *Haemophilus influenzae* (both serotypable and non-typable), *Haemophilus parainfluenzae*, *Chlamydia pneumonia*, *Moraxella (Branhamella) catarrhalis*, enterobacteria, *Lactobacillus* species, *Veillonella* species, *Mycobacterium* species, *Pseudomonas* species, *Klebsiella* species (including *Klebsiella ozaenae* or *Klebsiella pneumoniae*), *Bordatella pertussis*, *Eikenella corrodens*, *Bacteroides* species, *Pectostreptococcus* species, *Actinomyces* species, *Nocardia* species, spirochaetes; (2) bacteria that can be associated with digestive tract infections, such as *Bacillus* species (including *Bacillus cereus*), *Staphylococcus aureus*, *Clostridium perfringens*, *Clostridium difficile*, *Campylobacter jejuni*, *Listeria monocytogenes*, pathogenic *Escherichia coli*, *E. coli* O157:H7, *Salmonella* species, *Shigella* species, *Vibrio* species, *Yersinia* species, *Aeromonas* species, *Pleisiomonas* species, and *Helicobacter pylori*; and (3) bacteria that can be associated with meningitis and other central nervous system infections, such as group B streptococci (including *Streptococcus pyogenes*), *Streptococcus agalactiae*, *Streptococcus mitis*, non-group B streptococci, *Streptococcus pneumoniae*, *Staphylococcus* species (including *Staphylococcus epidermidis* and *Staphylococcus aureus*), *Listeria monocytogenes*, *Klebsiella pneumoniae*, *Serratia marcescens*, *Enterococcus faecium*, *Enterococcus faecalis*, *Proteus mirabilis*, *Mycobacterium tuberculosis*, *Escherichia coli*, *Legionella pneumophila*, *Corynebacterium* species, *Haemophilus* species (including *Haemophilus influenzae*), *Neisseria* species (including *Neisseria meningitidis* and *Neisseria gonorrhoeae*), *Moraxella lacunata*, pseudomonads (including *Pseudomonas aeruginosa*), *Borrelia burgdorferi*, and *Chlamydia* species. Eukaryotic pathogens of interest include, but are not limited to, *Histoplasma capsulatum*, *Coccidioides immitis*, *Blastomyces dermatitidis*, *Paracoccidioides brasiliensis*, a *Candida* species, an *Aspergillus* species, a *Mucor* species, *Cryptococcus neoformans*, *Pneumocystis carinii*, and other fungal pathogens, and *Giardia*

lamblia, *Entamoeba histolytica*, *Naegleria fowleri*, *Cyclospora cayetanensis*, *Toxoplasma gondii*, *Cryptosporidium parvum* and other cryptosporidia, *Acanthamoeba* species, microsporidia, and other protozoan pathogens (see, for example, Marshall et al. (1997) *Clin. Microbiol. Rev.*, 10:67-85, which is incorporated by reference in its entirety herein).

[0027] The method includes, for each member of the group of pathogens of interest, performing at least one assay for at least one epitope derived from the member of the group of pathogens. In some embodiments of the method, an assay may be performed for more than one epitope derived from the member, for example, in order to verify with increased certainty the presence or absence of the member, or in order to distinguish between different strains or types of a given member. More than one assay may be performed for a given epitope of interest. Each at least one assay may be performed (1) in parallel with but separately from another assay, (2) sequentially prior to or following another assay, or (3) coincidentally with another assay. Thus, as described below under the heading "II. Assays and kits for simultaneously detecting viral and non-viral pathogens", the method of the present invention can be performed using kits consisting of a single test device that assays all epitopes of interest, or of multiple test devices, wherein each such single or multiple test devices can make use of single or multiple paths of fluid flow.

[0028] The term "epitope" is used here to generally encompass a molecular or multi-molecular structure or site that is specifically recognized and can be bound by a binding agent, such binding generally involving non-covalent interactions. Examples of such generally non-covalent binding of a binding agent to an epitope include, but are not limited to, the binding of an antibody or antibody-like molecule to an antigen, the binding of a T-cell receptor to an antigen, the binding of a receptor to a ligand, the binding of an aptamer to a peptide or other target, the binding of an enzyme to a co-factor or to an enzymatic substrate, and the binding of avidin to biotin. An epitope may represent a much larger antigen or analytical target; for example, an epitope consisting of a small peptide may represent a large protein or proteinaceous complex, or an epitope consisting of a polysaccharide may represent an entire cell. Portions of the molecular structure not directly in contact with the binding agent may affect the binding of an epitope to a binding agent. Binding of the binding agent to the epitope may additionally include covalent bonding, for example, where a disulfide or other covalent bond is formed between the binding agent and the epitope. The at least one epitope derived from the member can be any suitable epitope, including, but not limited to, peptides, polypeptides, proteins, glycoproteins, carbohydrates, lipids, glycolipids, lipoproteins, nucleic acids, antigens, enzymes, receptors, cell wall components, whole cells or intact virions of the member, fragments of the member, substances (such as primary metabolites, secondary metabolites, toxins, enzymes, and exopolymers) produced or secreted by the member, and combinations thereof. Epitopes may include an entire molecule or part of a molecule, or may include all or parts of more than one molecule.

[0029] The at least one epitope can optionally be modified, for example, by physical or chemical modification. Modification of the at least one epitope can include any suitable

modification, including, but not limited to, treatment with chemical reagents or enzymes, oxidation or reduction, labelling with a detectable or bindable or reactable label, and covalent or non-covalent attachment of the epitope to a separate moiety, molecule, molecular structure, surface, or combination thereof. Non-limiting examples of modification of the at least one epitope include addition of a chemically reactive functional group to the epitope, removal of portions of the epitope (for example, by deglycosylation or by reductive or enzymatic cleavage of covalent bonds), and introduction of a bindable moiety (for example, labelling the epitope with a bindable biotin or other ligand). In some embodiments, the at least one epitope may occur naturally free in solution or suspension, or be made thus, for example, by treatment with appropriate chemical reagents, enzymes, or physical treatments. In other embodiments, the at least one epitope may occur naturally attached to a molecular structure, a molecular complex, a membrane, a cellular component, a whole cell or virion, a porous or solid substrate, or a combination thereof, or be made thus by covalent or non-covalent means or a combination thereof.

[0030] Each at least one assay for at least one epitope derived from the member of the group of pathogens includes the step of providing at least one binding agent capable of specifically binding to the at least one epitope derived from the member. Binding agents can be virtually any molecule or combination of molecules capable of recognizing and binding the at least one epitope. Such binding agents can include, without limitation, peptides, polypeptides, antibodies, antibody fragments, fusion proteins, chimeric or hybrid molecules, nucleic acids, nucleic acid mimics (for example, peptide nucleic acids), carbohydrates, cell surface antigens, receptors, ligands, or combinations thereof.

[0031] In one preferred embodiment, the at least one binding agent includes an antibody (monoclonal or polyclonal, natural, modified, or recombinant) or an antibody fragment (such as an Fab fragment or single-chain antibody variable region fragment). Antibodies may be natural, modified, or recombinant. Antibody fragments include, but are not limited to, $F(ab')_2$ fragments, Fab' fragments, Fab fragments, Fv fragments, and complementarity determining regions (CDRs). Recombinant antibodies include, but are not limited to, single-chain antibody variable region fragments (scFv), miniantibodies (Müller et al. (1998) *FEBS Lett.*, 432:45-49), antibody fusion proteins, and the like (see, for example, "Antibody Engineering", R. Kontermann and S. Dübel, editors, Springer-Verlag, Berlin Heidelberg, 790 pp.). Antibodies can be monovalent or polyvalent, such as divalent (Plückthun and Pack (1997) *Immunotechnology*, 3:83-105; Pack et al. (1995) *J. Mol. Biol.*, 246:28-34). Antibodies can be monospecific or polyspecific, such as bispecific (Müller et al. (1998) *FEBS Lett.*, 432:45-49). Methods of preparing, modifying, and using such antibodies or antibody fragments are known in the art (see, for example, "Antibodies: A Laboratory Manual", E. Harlow and D. Lane, editors, Cold Spring Harbor Laboratory, 1988, 726 pp; "Monoclonal Antibodies: A Practical Approach", P. Shepherd and C. Dean, editors, Oxford University Press, 2000, 479 pp.; and "Chicken Egg Yolk Antibodies, Production and Application: IgY-Technology (Springer Lab Manual)", by R. Schade et al., editors, Springer-Verlag, 2001, 255 pp.). All references cited in this paragraph are incorporated by reference in their entirety herein.

[0032] The at least one binding agent can include an antigen, such as an antigen capable of specifically binding to an antibody that recognizes an epitope derived from the member of the group of pathogens. In other embodiments, the binding agent can include a nucleic acid or nucleic acid mimic aptamer that binds a target such as a peptide or small molecule, or a receptor that binds a ligand, or a ligand that binds a receptor. The binding agent may be capable of binding to a mimotope, such as a peptide, that mimics an epitope derived from the member of the group of pathogens (see, for example, Kieber-Emmons (1998) *Immunol. Res.*, 17:95-108; Shin et al. (2001) *Infect. Immun.*, 69:3335-3342; Beenhouwer et al. (2002) *J. Immunol.*, 169:6992-6999; Hou and Gu (2003) *J. Immunol.*, 170:4373-4379; and Tang et al. (2003) *Clin. Diagn. Lab. Immunol.*, 10:1078-1084, which are incorporated by reference in their entirety herein).

[0033] Where the at least one epitope is optionally modified, for example, by physical or chemical modification, the at least one binding agent can be capable of specifically binding to the modified at least one epitope. In one embodiment, the binding agent may specifically bind to a modification of the at least one epitope, such as to a bindable moiety on the at least one epitope. In another embodiment, the binding agent may bind to the epitope by a non-covalent interaction, and then become affixed to the epitope by a covalent interaction.

[0034] The at least one binding agent can optionally include a functional group (such as a chemically reactive moiety or cross-linking moiety) or a detectable label. Detectable labels include, but are not limited to, fluorophores, luminophores, dyes, pigments, members of resonance energy transfer pairs, detectable nuclei (including radioactive isotopes and non-radioactive isotopes), spin labels, lanthanides, magnetic labels, detectable nucleic acids, metals, particles (such as, but not limited to, beads, fibers, or particles made of gold or other metals, magnetic or paramagnetic substances, glass, silicates, ceramics, latex, polymers, or composites), enzymes, antigenically recognizable structures (for example, digoxin or digoxigenin), and bindable moieties (for example, receptors, ligands, polyhistidine tags, biotin, or avidin). Methods to introduce such functional groups or detectable labels are known in the art (see, for example, R. P. Haugland, "Handbook of Fluorescent Probes and Research Products", 9th edition, J. Gregory (editor), Molecular Probes, Inc., Eugene, Oreg., USA, 2002, 966 pp.; Seitz and Kohler (2001), *Chemistry*, 7:3911-3925; Pierce Technical Handbook, Pierce Biotechnology, Inc., 1994, Rockford, Ill.; and Pierce 2003-2004 Applications Handbook and Catalog, Pierce Biotechnology, Inc., 2003, Rockford, Ill., which are incorporated by reference in their entirety herein).

[0035] The at least one binding agent may be free in solution, or may be temporarily or permanently affixed, directly or indirectly, onto a separate moiety, molecule, molecular structure, or surface. In one non-limiting example of direct immobilization, the at least one binding agent can be temporarily immobilized by drying or otherwise transiently binding onto a surface or into a matrix, wherein addition of a fluid can cause the binding agent to become mobile. Temporary immobilization may include the use of covalent or non-covalent means. In one embodiment, the binding agent can be temporarily immobilized by a covalent bond that can be cleaved, for example, by reduction or

enzymatic reaction or by other physical or chemical treatment. In another embodiment, the binding agent may be temporarily immobilized by non-covalent means including, but not limited to, specific interactions (such as between the binding agent and a molecule that recognizes and binds the binding agent), physical adsorption, hydrophobic or hydrophilic interaction, magnetic forces, ionic interactions, electrostatic interactions, van der Waals forces, and combinations thereof. In another non-limiting example of direct immobilization, the binding agent can be permanently immobilized by covalent or non-covalent attachment to a porous or non-porous surface, such as to a bead, fiber, particle, matrix, membrane, microplate well, tube, chip, or slide. In a non-limiting example of indirect immobilization, the at least one binding agent can be affixed by covalent or non-covalent means (such as by passive adsorption or avidin/biotin binding) to particles (for example, beads or fibers or particles of latex, gold or other metals, or magnetic or paramagnetic materials), and the binding agent-bearing particles temporarily or permanently immobilized onto a surface or within a matrix. In another non-limiting example of indirect immobilization, the at least one binding agent can be affixed using a linking moiety, such as a cross-linking molecule or a multivalent molecule (such as avidin), to a separate moiety, molecule, molecular structure, matrix, or surface.

[0036] In one embodiment, the at least one binding agent binds monovalently to the epitope of interest. In another embodiment, the binding agent binds multivalently, for example bivalently and optionally bispecifically, to the epitope (or mimotope) of interest, and may bind more than a single epitope together in a multi-epitope (and optionally multi-binding agent) complex. In some embodiments, one unit of the binding agent can bind to one unit of the epitope of interest, and in other embodiments, more than one unit of the binding agent can bind to one unit of the epitope of interest and form a multi-binding agent (and optionally multi-epitope) complex.

[0037] The at least one binding agent can be used in more than one form or type, for example, where the binding agent is an antibody or antibody fragment and is used in a sandwich assay that involves an unlabelled binding agent to immobilize the epitope and a detectably labelled binding agent that binds the same epitope. In methods of the invention that use such sandwich assays, any suitable order of the binding steps may be used. Thus, in one non-limiting example, the epitope may be captured and immobilized by a first form of a binding agent, followed by a second form of the binding agent binding to the immobilized epitope to form the detectable complex. In another non-limiting example, a first form of a binding agent binds the epitope to form a detectable complex, followed by immobilization of the complex by capture by a second form of the binding agent.

[0038] The at least one binding agent's ability to specifically bind to an epitope derived from the member of the group of pathogens can be improved by means known in the art, for example, by selection of a peptide sequence based on panning methods (see, for example, Coomber (2001) *Methods Mol. Biol.*, 178:133-145; Zhou et al. (2002) *Proc. Natl. Acad. Sci. USA*, 99:5241-5246; Fehrsen and du Plessis (1999) *Immunotechnology*, 4:175-184; Deng et al. (1994) *J. Biol. Chem.*, 269:9533-9538; Burioni et al. (1998) *Res.*

Virol., 149:327-330; Boel et al. (1998) *Infect. Immun.*, 66:83-88; and Parsons et al. (1996) *Protein Eng.*, 9:1043-1049, which are incorporated by reference in their entirety herein).

[0039] Improvement of the at least one binding agent's ability to bind to an epitope derived from the member of the group of pathogens (or to a mimotope mimicking the epitope) can use display methods as are known in the art, including displaying on a polypeptide (Kamb, et al., U.S. Pat. No. 6,025,485; Christmann et al., 1999, *Protein Eng.*, 12:797; Abedi et al., 1998, *Nucleic Acids Res.*, 26:623; Peelle et al., 2001, *J. Protein Chem.*, 20:507), a phage (He, 1999, *J. Immunol. Methods*, 231:105; Smith, 1985, *Science*, 228:1315), a ribosome (Schaffitzel et al., 1999, *J. Immunol. Methods*, 231:119; Roberts, 1999, *Curr. Opin. Chem. Biol.*, 3:268), an mRNA (Wilson et al., 2001, *Proc. Natl. Acad. Sci.*, 98:3750), or a yeast cell surface (Yeung and Wittrup, 2002, *Biotechnol. Prog.*, 18:212; Shusta et al., 1999, *J. Mol. Biol.*, 292:949), a bacterial cell surface (Leenhouts et al., 1999, *Antonie Van Leeuwenhoek*, 76:367; Christmann et al., 2001, *J. Immunol. Methods*, 257:163), or a bacterial spore surface (Wittrup, 2001, *Curr. Opin. Biotechnol.*, 12:395; Boder and Wittrup, 1998, *Biotechnol. Prog.*, 14:55). All references cited in this paragraph are incorporated by reference in their entirety herein.

[0040] Each at least one assay for at least one epitope derived from the member of the group of pathogens also includes the step of contacting the at least one binding agent directly to the sample. The binding agent is contacted directly to the sample, which may have undergone prior minimal or more extensive preparation, but which has not been subjected to culturing or to nucleic acid amplification for the member of the group of pathogens.

[0041] Each at least one assay for at least one epitope derived from the member of the group of pathogens also includes the step of allowing the at least one binding agent provided to specifically bind to and form a complex with the epitope. The binding of the at least one binding agent to the epitope can be by any suitable means, including, but not limited to, covalent binding, non-covalent binding, antibody-antigen recognition, receptor-ligand binding, aptamer-nucleic acid binding, physical adsorption, electrostatic forces, ionic interactions, hydrogen bonding, hydrophilic-hydrophobic interactions, van der Waals forces, magnetic forces, and combinations thereof. Where more than one binding agent is used (such as, for example, in a sandwich assay employing two antibodies or other binding agents that bind the same epitope, or that bind different epitopes derived from the same pathogen of interest), the multiple binding agents can bind to the epitope by more than one means. Preferably, the binding agent binds to the epitope with sufficient specificity to give minimal or no non-specific or cross-reactive binding between the binding agent and an epitope derived from sources other than the member of interest (such as from cells or tissues of the human subject, or from other infectious or non-infectious species or strains not of interest). The specific binding of the binding agent to the epitope preferably results in a complex of sufficient stability to be detected.

[0042] Where the at least one epitope is modified, the modification of the at least one epitope can occur: (1) at any time or times prior to, during, or after the time when the at

least one binding agent is contacted with the sample, (2) at any time or time prior to, during, or after the time when the at least one binding agent specifically binds to and forms a complex with the epitope, (3) at any time or times prior to, during, or after the time when the complex is detected, or (4) in any combination of (1), (2), and (3). Modification of the at least one epitope can occur in more than one way and at more than one time. In a non-limiting example, the at least one epitope is modified by covalent or non-covalent attachment to a porous or solid substrate (such as a fibrous support, a nitrocellulose membrane, a siliceous surface, a magnetic bead, or the like) prior to the time when the at least one binding agent (such as an antibody or antibody fragment, optionally modified, for example, by labelling with a detectable label or by permanent or temporary, covalent or non-covalent, attachment to a porous or non-porous surface) is contacted with the sample and specifically binds to and forms a complex with the epitope. In another non-limiting example, the at least one epitope is modified (for example, by introduction of a chemically reactive moiety or by a detectable label) after it is bound to and complexed with the at least one binding agent. In another non-limiting example, the at least one epitope is modified by introduction of a bindable moiety, then bound to and complexed by the at least one binding agent, then modified again by covalent or non-covalent attachment (for example, by means of the introduced bindable moiety) to a porous or solid support.

[0043] Each at least one assay for at least one epitope derived from the member of the group of pathogens also includes the step of detecting the complex, wherein the detection is positive if the concentration of the member in the sample is greater than or equal to a reference concentration, and the detection is negative if concentration of the member is less than the reference concentration.

[0044] Detection of the complex can be direct, such as by detection of a label on the at least one binding agent. Alternatively, detection of the complex can be indirect, by any suitable means, including, but not limited to, the use of a second binding agent, such as a secondary antibody bearing a detectable label, or such as a detectably-labelled avidin moiety, where the complex includes a bindable biotin. Useful detectable labels include, but are not limited to, fluorophores, luminophores, dyes, pigments, members of resonance energy transfer pairs, detectable nuclei, spin labels, lanthanides, magnetic labels, detectable nucleic acids, metals, particles (such as, but not limited to, beads, fibers, or particles made of gold or other metals, magnetic or paramagnetic substances, glass, silicates, ceramics, latex, polymers, or composites), enzymes, products of enzymatic reactions, antigenically recognizable structures, and bindable moieties (such as avidin, biotin, antigens, antibodies and the like, ligands, and receptors).

[0045] In some embodiments, detection of the complex may make use of instrumentation. Instrumentation that may be suitable for use in the method of the invention includes, but is not limited to, spectrophotometric instrumentation (such as instruments capable of ultraviolet, visual, infrared, Raman, luminescent, fluorescent, and phosphorescent light detection), instrumentation for electrochemical detection (such as coulometry, voltammetry, potentiometry, and specific ion detection), gravimetric instrumentation, mass spectrometers, electrophoretic equipment, chromatographic equipment, surface plasmon resonance detectors, magnetic reso-

nance detectors, cameras or microscopes (light, electron, atomic force), charge-coupled devices, thermal analysis instrumentation, and combinations thereof. Some embodiments may also use computerized methods to detect or amplify detection of the complex, for example, using computers to integrate a signal over time, to interpolate between known data points, or to increase signal-to-noise ratios. In other embodiments, detection of the complex can be made without the need for instrumentation, for example, by simple visualization by the naked eye without using magnification, physical amplification of the visual signal, or computerized amplification of a digitized signal. Embodiments not needing expensive or complex equipment are preferred for their simplicity and ease of use in clinical settings, particularly when it is impracticable or uneconomical to use embodiments that require specialized technical training.

[0046] Detection of the complex is positive if the concentration of the member of the group of pathogens in the sample is greater than or equal to a reference concentration. Conversely, detection of the complex is negative if the concentration of the member of the group of pathogens in the sample is less than the reference concentration. The reference concentration selected for a given member of the group of pathogens depends on several factors, including, but not limited to, the nature of the binding agent and of the epitope derived from the member, the type of sample, and, where the sample is from a subject, the type of subject (for example, an adult or a child). A reference concentration can be any suitable concentration, and can be established by routine testing. Detection can be linear (such as spectrophotometric measurement of product formation by an enzymatic reaction) or non-linear (such as visual detection of a gold label). Detection is optionally at least semi-quantitative, for example, judged to be greater than or equal to, or less than, a reference value. Detection can be optionally quantitative, wherein a positive detection signal can be correlated to a range of concentrations of the member of the group of pathogens.

[0047] In one embodiment of the invention, the sample is from a subject suspected of being diseased by at least one of the members of the group of pathogens. Subjects may be human or veterinary. In some cases, a subject may be an asymptomatic "carrier" of a member of the group of pathogens, that is to say, otherwise healthy but colonized, generally at relatively lower concentrations, by the member, where a relatively higher concentration of the member is associated with symptoms of a disease caused by that pathogen. In such a case, a desirable reference concentration is a concentration below which a sample from a subject who either is not colonized by the member in question, or who is colonized by the member but otherwise healthy, gives a negative detection result. This same reference concentration is preferably a concentration at or above which a sample from a subject who is colonized and diseased by the member gives a positive detection result.

[0048] Thus, in one embodiment of the invention, a positive detection result indicates that the subject is at least colonized by the member of the group of pathogens, or is colonized and diseased by that pathogen. In one alternative embodiment of the invention, a negative detection result preferably indicates that the subject is not colonized by the member of the group of pathogens to a level associated with a disease caused by that pathogen.

[0049] In yet another embodiment of the invention, the sample is from a subject suspected of being diseased by at least one of the members of the group of pathogens, wherein a very low concentration of the at least one member is sufficient to indicate that the subject is likely to be diseased by that at least one member. In such an embodiment, the reference concentration for that at least one member may be very low, optionally approaching or equal to zero.

[0050] A desirable reference concentration preferably yields a positive predictive value (that is to say, the probability that the subject with a positive detection result is diseased by the member of the group of pathogens) of at least about 80%, more preferably of at least about 90%, and most preferably of at least about 95%. A desirable reference concentration preferably yields a negative predictive value (that is to say, the probability that the subject with a negative detection result is not diseased by the member of the group of pathogens) of at least about 80%, more preferably of at least about 90%, and most preferably of at least about 95%.

[0051] II. Assays and Kits for Simultaneously Detecting Viral and Non-Viral Pathogens

[0052] The method of the present invention may be carried out by means of a suitable assay. Non-limiting examples of suitable assays for performing the method include dipstick or test strip assays, flow-through assays, chromatographic assays, affinity separation assays, lateral flow assays, latex agglutination assays, radioimmunometric assays, enzyme-linked immunosorbent assays, fluorescence assays, luminescence assays, dot blot assays, and combinations thereof. Assays can be run in any suitable format, including, but not limited to, test strips, dipsticks, membranes, filters, micro-titer plates, tubes, chips, slides, and flow-through chambers. Preferably, the assay is rapid, most preferably sufficiently rapid to produce results within a relatively brief period of time, such as within the time of a subject's consultation with a physician or other health-care provider.

[0053] Kits can be designed for convenience in performing the method, according to the assay used. Kits can include, in addition to a means for performing the assay, means for collecting and appropriately treating a sample (such as appropriate collection containers, a swab, a means to aspirate a sample, a means to biopsy a sample, wash solutions or buffers, chemical or enzymatic reagents, filters, centrifuge tubes, and the like). In some embodiments, the means for collecting and appropriately treating a sample may include more than one means for collecting or more than one means of treating a sample. More preferably, a single means for collecting and a single means for treating a sample are sufficient for performing the method. Kits can include materials (such as gloves and other personal safety equipment, biohazard disposal containers, or decontamination materials) that aid in the safe handling of potentially hazardous samples. Kits can include instructions for the use of the kit, for example, instructions in the form of a brochure, leaflet, pamphlet, booklet, or audiovisual materials.

[0054] Non-limiting examples of kits of the invention follow:

[0055] 1. Kit for influenza and influenza-like illnesses (see, for example, Centers for Disease Control (2001) *Morbidity Mortality Weekly Report*, 50(44):984-986, which is

incorporated by reference in its entirety herein), designed to detect one or more members of a group of pathogens including at least one viral pathogen (such as, but not limited to, an influenza virus, a rhinovirus, a respiratory syncytial virus, an adenovirus, a parainfluenza virus, a coronavirus, and a metapneumovirus) and at least one non-viral pathogen (such as, but not limited to, *Streptococcus pneumoniae*, *Chlamydia pneumoniae*, and *Mycoplasma pneumoniae*). Suitable samples for analysis include, but are not limited to, nasopharyngeal, oropharyngeal, or esophageal swabs or washes or discharges and the like, as well as blood, urine, sputum, or saliva, and extracts or derivatives thereof.

[0056] 2. Kit for pneumonia, designed to detect one or more members of a group of pathogens including at least one viral pathogen (such as, but not limited to, an influenza virus, a rhinovirus, a respiratory syncytial virus, an adenovirus, a parainfluenza virus, a coronavirus, a hantavirus, a cytomegalovirus, and a metapneumovirus) and at least one non-viral pathogen (such as, but not limited to, a group A streptococcus, *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Klebsiella pneumoniae*, a *Staphylococcus* species, *Haemophilus influenzae*, *Chlamydia pneumoniae*, *Mycoplasma pneumoniae*, a *Pseudomonas* species, *Histoplasma capsulatum*, *Coccidioides immitis*, *Blastomyces dermatitidis*, *Paracoccidioides brasiliensis*, *Candida* species, *Aspergillus* species, *Mucor* species, *Cryptococcus neoformans*, and *Pneumocystis carinii*). Suitable samples for analysis include, but are not limited to, nasopharyngeal, oropharyngeal, or esophageal swabs or washes or discharges and the like, as well as blood, urine, sputum, or saliva, and extracts or derivatives thereof.

[0057] 3. Kit for bronchitis, designed to detect one or more members of a group of pathogens including at least one viral pathogen (such as, but not limited to, an influenza virus, a rhinovirus, a respiratory syncytial virus, an adenovirus, a parainfluenza virus, a coronavirus, a hantavirus, a cytomegalovirus, and a metapneumovirus) and at least one non-viral pathogen (such as, but not limited to, *Mycoplasma* species such as *Mycoplasma pneumoniae*, *Chlamydia pneumoniae*, *Bordatella pertussis*, Group A *Streptococcus*, *Streptococcus pyogenes*, *Moraxella catarrhalis*, *Haemophilus influenzae*, *Haemophilus parainfluenzae*, and *Staphylococcus aureus*). Suitable samples for analysis include, but are not limited to, nasopharyngeal, oropharyngeal, or esophageal swabs or washes or discharges and the like, as well as blood, urine, sputum, or saliva, and extracts or derivatives thereof.

[0058] 4. Kit for sinusitis, designed to detect one or more members of a group of pathogens including at least one viral pathogen (such as, but not limited to, an influenza virus, a rhinovirus, a respiratory syncytial virus, an adenovirus, a parainfluenza virus, a coronavirus, a hantavirus, a cytomegalovirus, and a metapneumovirus) and at least one non-viral pathogen (such as, but not limited to, *Streptococcus* species such as *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Staphylococcus* species such as *Staphylococcus aureus*, and *Neisseria* species, or fungal pathogens). Suitable samples for analysis include, but are not limited to, nasopharyngeal, oropharyngeal, or esophageal swabs or washes or discharges and the like, sinus aspirates or biopsies, blood, urine, sputum, or saliva, and extracts or derivatives thereof.

[0059] 5. Kit for acute otitis media (see, for example, Jones and Wilson (2002), "Otitis Media", on-line article at

<http://www.emedicine.com/ped/topic1689.htm>, accessed 21 Jan. 2004), designed to detect one or more members of a group of pathogens including at least one viral pathogen (such as, but not limited to, an influenza virus, a rhinovirus, a respiratory syncytial virus, an adenovirus, a parainfluenza virus, a coronavirus, and a metapneumovirus) and at least one non-viral pathogen (such as, but not limited to, non-typable *Haemophilus influenzae* and *Moraxella catarrhalis*, *Streptococcus pneumoniae*). Suitable samples for analysis include, but are not limited to nasopharyngeal, oropharyngeal, or esophageal swabs or washes or discharges and the like, middle ear fluid, discharges, aspirates, or biopsies, blood, urine, sputum, or saliva, and extracts or derivatives thereof.

[0060] 6. Kit for conjunctivitis (see, for example, Scott and Luu (2001), "Viral Conjunctivitis", on-line article at www.emedicine.com/oph/topic84.htm, accessed 22 Jan. 2004; and Marlin (2003), "Bacterial Conjunctivitis", on-line article at www.emedicine.com/OPH/topic88.htm, accessed 22 Jan. 2004), designed to detect one or more members of a group of pathogens including at least one viral pathogen (such as, but not limited to, an adenovirus, a herpes virus, such as a herpes simplex virus, varicella-zoster virus, an enterovirus, a coxsackievirus, a molluscum contagiosum poxvirus, a vaccinia poxvirus, an influenza virus, an Epstein-Barr virus, a paramyxovirus, and a rubella virus) and at least one non-viral pathogen (such as, but not limited to, a group A streptococcus, *Streptococcus pyogenes*, *Streptococcus mitis*, *Streptococcus pneumoniae*, *Staphylococcus epidermidis*, *Staphylococcus aureus*, a *Corynebacterium* species, *Haemophilus* species, *Haemophilus influenzae*, *Neisseria* species such as *Neisseria meningitidis* and *Neisseria gonorrhoeae*, *Moraxella lacunata*, pseudomonads such as *Pseudomonas aeruginosa*, and *Chlamydia* species). Suitable samples for analysis include, but are not limited to, nasopharyngeal, oropharyngeal, or esophageal swabs, washes, or discharges and the like, and blood, urine, sputum, tears, eye discharges, or saliva, and extracts or derivatives thereof.

[0061] 7. Kit for central nervous system infections, such as meningitis (see, for example, Incisu and Khosla (2003), "Bacterial Meningitis", on-line article at www.emedicine.com/radio/topic441.htm, accessed 22 Jan. 2004; and Vokshoor and Moore (2004), "Viral Meningitis", on-line article at: www.emedicine.com/neuro/topic607.htm, accessed 22 Jan. 2004) designed to detect one or more members of a group of pathogens including at least one viral pathogen (such as, but not limited to, a herpes virus, a varicella-zoster virus, an enterovirus, a poliovirus, an arbovirus, a mumps virus, a measles virus, an echovirus, a coxsackievirus, a human immunodeficiency virus, and adenovirus) and at least one non-viral pathogen (such as, but not limited to, *Haemophilus influenzae* type b, *Streptococcus pneumoniae*, *Neisseria meningitidis*, group B streptococci, *Streptococcus agalactiae*, *Streptococcus pyogenes*, non-group B streptococci, *Staphylococcus* species, *Listeria monocytogenes*, *Klebsiella pneumoniae*, *Serratia marcescens*, *Pseudomonas aeruginosa*, *Enterococcus faecium*, *Enterococcus faecalis*, *Proteus mirabilis*, *Escherichia coli*, *Mycobacterium tuberculosis*, *Legionella pneumophila*, *Borrelia burgdorferi*). Suitable samples for analysis include, but are not limited to nasopharyngeal, oropharyngeal, or esophageal swabs or

washes or discharges and the like, as well as blood, urine, cerebrospinal fluid, tears, sputum, or saliva, and extracts or derivatives thereof.

[0062] 8. Kit for gastroenteritis (see, for example, Goodgame (2003) "Viral Gastroenteritis", on-line article at www.emedicine.com/med/topic856.htm, accessed 22 Jan. 2004; and Frye et al. (2002) "Bacterial Gastroenteritis", on-line article at www.emedicine.com/MED/topic855.htm, accessed 22 Jan. 2004), designed to detect one or more members of a group of pathogens including at least one viral pathogen (such as, but not limited to, a rotavirus, a calicivirus, such as a norovirus, a Norwalk-like virus, a Norwalk virus, and a sapovirus, an astrovirus, and an adenovirus) and at least one non-viral pathogen (such as, but not limited to, *Escherichia coli*, *E. coli* O157:H7, *Bacillus* species, *Staphylococcus aureus*, *Shigella* species, *Salmonella* species, *Campylobacter* species, *Clostridium difficile*, *Clostridium perfringens*, *Vibrio* species, *Listeria monocytogenes*, *Aeromonas* species, *Yersinia* species, *Plesiomonas* species, *Giardia lamblia*, *Entamoeba histolytica*, *Toxoplasma gondii*, and cryptosporidia). Suitable samples for analysis include, but are not limited to nasopharyngeal, oropharyngeal, esophageal, gastric, or rectal swabs or washes or discharges and the like, as well as blood, urine, sputum, saliva, feces, and extracts or derivatives thereof.

EXAMPLES

Example 1

Preparation of a Test Strip

[0063] This example describes the preparation of a test strip for use in a device for determining the presence or absence in a sample of one or more members of a group of pathogens including at least one viral pathogen and at least one non-viral pathogen.

[0064] The test strip includes a conjugate pad, such as, but not limited to, a pad made of glass fiber material or non-woven polyester (for example, Hollingsworth & Vose 7304, Web Converting, Holliston, Mass., USA), positioned between conductive pads, such as, but not limited to, pads made of cellulosic paper (for example, Ahlstrom 1281 Paper, 90% cellulose fiber, 10% rayon with traces of polyacrylamide, Web Converting, Holliston, Mass., USA) that direct liquid flow. The conjugate pad contains binding agents, such as detectably labelled antibodies, that have been temporarily immobilized by drying onto the conjugate pad. The test strip also includes a nitrocellulose membrane (for example, Millipore STHF nitrocellulose, catalogue number SA3J727H5, Millipore, Inc., Bedford, Mass., USA) or other suitable membrane (such as, but not limited to, a membrane made of nylon or polyethylene sulfone) containing the multiple test line reading zone. The multiple test line reading zone includes a test line for each of the pathogens detectable by the device. Each test line contains an antibody specific for the particular pathogen, permanently immobilized by adsorption in a stripe. The test strip also includes an absorbent pad that serves as a reservoir for liquid and promotes capillary flow of the sample through the conjugate pad and thence through the nitrocellulose membrane. In this embodiment, the immunochromatographic analysis of the pathogens of interest occurs in and along a single path of fluid flow, wherein the fluid flows through each of the test lines.

Alternative embodiments of this device may be designed, such as a device wherein the above described immunochromatographic analysis of individual pathogens is carried out using discrete paths of fluid flow (each containing one or more test lines or the like), such as, but not limited to, in multiple discrete test strips, or in a test strip wherein fluid flow is compartmentalized by any suitable method of separating multiple paths of fluid flow (for example, by treatment with a hydrophobic reagent that prevents aqueous fluids from flowing across the area treated).

[0065] Antibodies are selected as appropriate to the antigens or pathogens of interest. Affinity purification is carried out as necessary, by methods well known in the art (see, for example, P. Tijssen, "Practice and theory of enzyme immunoassays", pp. 173-189, in "Laboratory techniques in biochemistry and molecular biology", R. Burden and P. VanKnippenberg (editors), Elsevier, Amsterdam, The Netherlands, 1985; "Antibodies: A Laboratory Manual", E. Harlow and D. Lane, editors, Cold Spring Harbor Laboratory, 1988, 726 pp; "Monoclonal Antibodies: A Practical Approach", P. Shepherd and C. Dean, editors, Oxford University Press, 2000, 479 pp.; and "Chicken Egg Yolk Antibodies, Production and Application: IgY-Technology (Springer Lab Manual)", by R. Schade et al., editors, Springer-Verlag, 2001, 255 pp., which are incorporated by reference in their entirety herein).

[0066] Gold particles are conjugated to the selected antibodies. Any method for conjugation may be used, a non-limiting example of which is that described by DeMay in "The Preparation and Use of Gold Probes" (in "Immunocytochemistry: Modern Methods and Applications", 2nd edition, J. M. Polak and S. Van Noorden (editors), Wright, Bristol, England, 1986, 703 pp). In other embodiments, detectable labels other than gold may be used (such as, but not limited to, visible dyes, pigments, magnetic particles or other particulates, enzymes, radioactive isotopes, spin labels, or nucleic acids). The labelled antibodies are mixed with a drying agent (aqueous 5 millimolar sodium tetraborate, pH 8.0, containing 1.0% bovine serum albumin, 0.1% Triton X-100, 2.0% Tween 20, 6.0% sucrose, and 0.02% sodium azide) and temporarily immobilized onto a suitable inert absorbent material (referred to as the "conjugate pad"), such as a non-woven polyester pad able to hold the dried labelled antibodies and to release them when wetted. The conjugate pad is heated sufficiently (for example, to 60 degrees Celsius for 20 minutes) to remove substantially all the liquid, leaving the labelled antibodies temporarily immobilized on the conjugate pad.

[0067] The nitrocellulose membrane is prepared by immobilizing the appropriate capture antibodies in individual stripes (referred to as "capture lines" or "test lines") within the area of the multiple test line reading zone. The capture antibodies, dissolved in a saline solution (0.01 molar phosphate buffered saline, pH 7.4, 1% sucrose, and 0.02% intrawhite dye, 0.05% sodium azide), are applied in stripes. The nitrocellulose is heated sufficiently (for example, to 50 degrees Celsius for 2 minutes) to remove substantially all the liquid, leaving the capture antibodies immobilized on the nitrocellulose membrane, which is then preferably stored desiccated at about 15 to about 30 degrees Celsius to promote permanent absorption of the antibodies to the nitrocellulose.

[0068] The absorbent pad that serves as a liquid reservoir and promotes capillary flow can be made of any suitable material. A non-limiting example of suitable material is cellulosic material sold commercially as Ahlstrom 939 (Ahlstrom, Mount Holly Spring, Pa., USA).

[0069] The conductive pads, conjugate pad, nitrocellulose membrane, and absorbent pad are assembled together into a test strip, in a manner that can permit fluid flow, for example, by assembly on an adhesive strip. Such an assembled test strip can be further incorporated into assay devices, such as those described in Example 2.

Example 2

Immunochemical Assay

[0070] This example describes a non-limiting embodiment of a method for determining the presence or absence in a sample of one or more members of a group of pathogens including at least one viral pathogen and at least one non-viral pathogen.

[0071] In this example, an immunochemical assay makes use of the method of the invention to detect the presence or absence of a viral pathogen (influenza A) and two non-viral pathogens (the pathogenic bacteria *Streptococcus pneumoniae* and *Legionella pneumoniae*). Such an assay would be desirable, for example, for determining whether a subject suspected of having pneumonia is diseased by any of these pathogens, and to establishing suitable therapy (such as the use or non-use of antibiotics). Similar assays can be designed to detect the presence or absence of additional, or different, pathogens. In one non-limiting example, an assay for meningitis may be designed to detect the presence or absence in a cerebrospinal fluid or nasopharyngeal sample of enteroviruses and the non-viral pathogens *Streptococcus pneumoniae*, *Neisseria meningitidis*, and *Haemophilus influenzae*. In another non-limiting example, an assay for gastroenteritis may be designed to detect the presence or absence in a stool sample of enteroviruses and caliciviruses, and the non-viral pathogens *Campylobacter* species, *Salmonella* species, *Shigella* species, *Escherichia coli*, and *Giardia lamblia*.

[0072] The assay is performed using a lateral flow, immunochemical assay device, as described in U.S. Pat. No. 5,877,028 to Chandler et al., "Immunochemical assay device", issued 2 Mar. 1999, and in U.S. patent applications Ser. No. 09/156,486 and Ser. No. 09/518,165, which are incorporated by reference in their entirety herein. This device comprises a hinged cardboard housing equipped with a window to allow the viewing of multiple test line results (FIG. 1).

[0073] The device has a recess on one side, containing a preformed plastic well (1) for receiving a swab sample. An overlabel (2) equipped with two openings is placed over this side of the device with the openings positioned over the well (1) to allow insertion of the swab through the bottom opening (A), so that when the swab is pushed forward, its tip is exposed through the top opening (B). The arrangement of the overlabel, its openings, and the well, combine to hold the swab in the desired position during the assay and to promote the expulsion of sample liquid from the swab.

[0074] On the side opposite to that with the well (1), a preassembled test strip, prepared as described in Example 1,

(3) is adhered in a position that allows viewing of the multiple test line reading zone through the window (4) when the device is closed. Optionally, the device includes a means to keep the device closed, such as a lightly adhesive liner (5). The preassembled test strip (3) includes a conjugate pad (6) positioned between conductive pads (7) that direct liquid flow. See FIG. 1. The conjugate pad contains binding agents that have been temporarily immobilized by drying onto the conjugate pad. In this non-limiting example, the binding agents include mouse anti-influenza A monoclonal antibodies and affinity purified rabbit anti-*Legionella pneumoniae*, and rabbit anti-*Streptococcus pneumoniae* polyclonal antibodies that have been conjugated to a detectable label (gold particles). The test strip also includes a nitrocellulose membrane (8) containing the multiple test line reading zone, and an absorbent pad (9). The multiple test line reading zone includes a test line for each of the pathogens detectable by the device. Each test line contains an antibody specific for the particular pathogen (affinity purified mouse anti-influenza A, rabbit anti-*Legionella pneumoniae*, and rabbit anti-*Streptococcus pneumoniae* antibodies) immobilized by adsorption in a stripe.

[0075] The assay is carried out with the assembled hinged device. A fibrous Dacron swab is inserted through the opening A into the well (1), resulting in the swab tip being exposed through the opening B. Liquid sample (100 microliters) is pipetted through the opening A to wet the swab. Three drops of "Reagent A" (0.05 molar citrate, 0.25 molar phosphate, pH 7.0, containing 1.4% Tween 20, 0.5% sodium dodecyl sulphate, and 0.05% sodium azide) are added to the swab sample through opening A. The hinged device is closed, and kept closed optionally by the adhesive strip. When the device is closed, the sample liquid contacts the bottom conductive pad of the test strip (3), initiating assay flow. The sample liquid then contacts the conjugate pad and solubilizes the gold-labelled antibodies, allowing their movement onto the nitrocellulose membrane, with excess fluid flowing into the absorbent pad. Antigen present in the sample is bound by the gold-labelled antibodies to form a complex, which is bound by the immobilized antibodies in the test lines. A visually detected signal (a pink-to-purple colored line) is observed when sufficient complex is formed. After sufficient time (such as 15 minutes) has passed, the multiple test line reading zone is viewed through the window of the device, and the results noted as shown in Table 1.

Example 3

Immunochemical Assay

[0076] This example describes a non-limiting embodiment of a method for determining the presence or absence in a sample of one or more members of a group of pathogens including at least one viral pathogen and at least one non-viral pathogen. In particular, this example describes a non-limiting embodiment of a method to simultaneously determine the presence or absence in a sample of viruses and a bacterium that are capable of causing similar disease symptoms in human subjects.

[0077] A lateral flow device, similar to devices described, for example, in U.S. Pat. No. 5,877,028, U.S. patent application Ser. Number 09/156,486, and U.S. patent application Ser. No. 09/518,165, and in U.S. patent application Ser. No. 09/397,110, was constructed for the determination of the presence or absence in a sample of influenza A virus, influenza B virus, and *Streptococcus pneumoniae*. Affinity-purified polyclonal antibodies specific to *Streptococcus pneumoniae* and two monoclonal antibodies specific respectively to influenza A and B viruses were immobilized in separate capture lines on nitrocellulose. Gold-labelled affinity-purified antibodies specific to each member of this group of pathogens were prepared by covalently coupling colloidal gold particles to the appropriate antibody, and the labelled antibodies temporarily immobilized on a conjugate pad as described above in Example 1.

[0078] Samples can consist of nasal, oral, oropharyngeal, or nasopharyngeal swabs, aspirates, washes, lavages, or discharges. In alternative embodiments, other materials (including, but not limited to, urine, blood, serum, plasma, sputum, tissue biopsies, cell extracts, environmental samples, and derivatives thereof) can also serve as suitable samples.

[0079] In one specific embodiment, the disease condition of interest that can potentially involve infection by at least member of the group consisting of influenza A virus, influenza B virus, and *Streptococcus pneumoniae* is acute otitis media. Acute otitis media can be caused by viral pathogens (including respiratory syncytial virus, influenza viruses, parainfluenza viruses, rhinoviruses, and adenoviruses) or by bacterial pathogens (including *Streptococcus pneumoniae*,

TABLE 1

Sample	Test Line		
	<i>Streptococcus pneumoniae</i>	<i>Legionella pneumoniae</i>	Influenza A
Negative urine	Negative	Negative	Negative
<i>Streptococcus pneumoniae</i> urine	Positive	Negative	Negative
<i>Legionella pneumoniae</i> urine	Negative	Positive	Negative
Influenza A in saline	Negative	Negative	Positive
Influenza A + <i>Streptococcus pneumoniae</i> urine	Positive	Negative	Positive
Influenza A + <i>Legionella pneumoniae</i> urine	Negative	Positive	Positive

Haemophilus influenzae, *Staphylococcus aureus*, and *Moraxella catarrhalis*, group A streptococci, *Streptococcus pyogenes*, *Pseudomonas aeruginosa*). Otitis media is often associated with respiratory viral infections, and some cases of otitis media may involve both viral and bacterial infections sequentially or concurrently (see, for example, Ruuskanen et al. (1989) *Pediatr. Infect. Dis. J.*, 8:94; Andrade et al. (1998) *Pediatrics*, 101:617; and Jones and Wilson (2002), "Otitis Media", on-line article at <http://www.emedicine.com/ped/topic1689.htm>, accessed 21 Jan. 2004, which are incorporated by reference in their entirety herein).

[0080] The assays for the different pathogens are run using one or more test strips similar to that described in Examples 1 and 2. In one embodiment, each pathogen is assayed for on a separate test strip. In an alternative embodiment, more than one pathogen is assayed for on a single test strip. In various embodiments, for example, influenza A and influenza B viruses may be assayed for on one test strip, and *Streptococcus pneumoniae* on a second test strip, or each pathogen may be assayed for on separate test strips, or all three pathogens may be assayed for using a single test strip using a single fluid path or more than one fluid path.

[0081] When testing for the etiologic agent responsible for otitis media, a suitable sample can be a nasal or nasopharyngeal swab inserted into the nares of a human subject. The swab is removed from the patient's nares, and contacted with a solution, such as a transport medium, to elute the sample. The sample is contacted to the test strip or strips, for example, by insertion of the swab into a device similar to that described in Example 2, closing the device to permit transfer of sample liquid to the bottom conductive pad (or pads) of the test strip (or test strips), thus initiating assay flow. The sample liquid then contacts the conjugate pad (or pads) and solubilizes the gold-labelled antibodies, allowing their movement onto the nitrocellulose membrane (or membranes), with excess fluid flowing into the absorbent pad (or pads). Antigen present in the sample is bound by the gold-labelled antibodies to form a complex, which is bound by the immobilized antibodies in the test lines. A visually detected signal (a pink-to-purple colored line) is observed when sufficient complex is formed. After sufficient time (such as 15 minutes) has passed, the test lines are viewed, and the results noted.

[0082] All headings are for the convenience of the reader and should not be used to limit the meaning of the text that follows the heading, unless so specified. Various changes and departures may be made to the present invention without departing from the spirit and scope thereof. Accordingly, it is not intended that the invention be limited to that specifically described in the specification or as illustrated in the drawings, but only as set forth in the claims.

What is claimed is:

1. A method of simultaneously determining the presence or absence in a sample of one or more members of a group of pathogens including at least one viral pathogen and at least one non-viral pathogen, said method comprising:

for each member of said group of pathogens, performing at least one assay for at least one epitope derived from said member,

wherein said at least one assay comprises:

- a) providing at least one binding agent capable of specifically binding to said at least one epitope derived from said member;
- b) contacting said at least one binding agent directly to said sample;
- c) allowing said at least one binding agent provided to specifically bind to and form a complex with said epitope; and
- d) detecting said complex, wherein said detection is positive if the concentration of said member in said sample is greater than or equal to a reference concentration, and said detection is negative if concentration of said member is less than said reference concentration.

2. The method of claim 1, wherein said sample is from a subject suspected of being diseased by at least one of said members.

3. The method of claim 2, wherein said positive detection is optionally quantitative.

4. The method of claim 2, wherein positive detection indicates that said subject is diseased by the member for which said positive detection was made.

5. The method of claim 2, wherein negative detection indicates that said subject is not diseased by the member for which said negative detection was made.

6. The method of claim 2, wherein said disease comprises a respiratory tract infection.

7. The method of claim 6, wherein said respiratory tract infection comprises pneumonia.

8. The method of claim 6, wherein said respiratory tract infection comprises influenza or an influenza-like illness.

9. The method of claim 6, wherein said respiratory tract infection comprises sinusitis.

10. The method of claim 6, wherein said respiratory tract infection comprises bronchitis.

11. The method of claim 6, wherein said respiratory tract infection comprises tonsillitis.

12. The method of claim 6, wherein said respiratory tract infection comprises pharyngitis.

13. The method of claim 6, wherein said respiratory tract infection comprises croup.

14. The method of claim 6, wherein said respiratory tract infection comprises bronchiolitis.

15. The method of claim 6, wherein said respiratory tract infection comprises chronic obstructive pulmonary disease.

16. The method of claim 2, wherein said respiratory tract infection comprises acute otitis media.

17. The method of claim 2, wherein said disease comprises conjunctivitis.

18. The method of claim 2, wherein said disease comprises meningitis.

19. The method of claim 2, wherein said disease comprises a central nervous system infection.

20. The method of claim 2, wherein said disease comprises a gastrointestinal infection.

21. The method of claim 2, wherein said disease comprises a bacterial superinfection.

22. The method of claim 2, wherein said disease comprises a viral superinfection.

23. The method of claim 1, wherein said at least one viral pathogen comprises at least one virus selected from the

group consisting of rhinovirus, influenza virus, human metapneumovirus, respiratory syncytial virus, adenovirus, parainfluenza virus, coronavirus, hantavirus, cytomegalovirus, coxsackie virus, herpes simplex virus, and echovirus.

24. The method of claim 1, wherein said at least one non-viral pathogen comprises at least one bacterial pathogen.

25. The method of claim 1, wherein said at least one non-viral pathogen comprises at least one eukaryotic pathogen.

26. The method of claim 1, wherein said at least one non-viral pathogen comprises at least one bacterial pathogen and at least one eukaryotic pathogen.

27. The method of claim 1, wherein said at least one viral pathogen is at least one selected from the group consisting of influenza virus A, influenza virus B, influenza virus C, human metapneumovirus, respiratory syncytial virus A, respiratory syncytial virus B, human parainfluenza virus type 1, human parainfluenza virus type 2, human parainfluenza virus type 3, rhinoviruses, coronaviruses, adenoviruses, hantaviruses, and cytomegaloviruses, and wherein said at least one non-viral pathogen is at least one selected from the group consisting of *Streptococcus pneumoniae*, *Chlamydia pneumoniae*, *Moraxella catarrhalis*, *Haemophilus influenzae*, *Haemophilus parainfluenzae*, a *Mycoplasma* species, *Mycoplasma pneumoniae*, a group A *streptococcus*, *Streptococcus pyogenes*, *Klebsiella pneumoniae*, a *Pseudomonas* species, a *Staphylococcus* species, *Staphylococcus aureus*, *Bordatella pertussis*, a *Neisseria* species, *Histoplasma capsulatum*, *Coccidioides immitis*, *Blastomyces dermatitidis*, *Paracoccidioides brasiliensis*, a *Candida* species, an *Aspergillus* species, a *Mucor* species, *Cryptococcus neoformans*, and *Pneumocystis carinii*.

28. The method of claim 1, wherein said at least one viral pathogen comprises at least one of influenza virus, respiratory syncytial virus, human metapneumovirus, parainfluenza virus, rhinovirus, and adenovirus, and said at least one non-viral pathogen comprises at least one of *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Haemophilus influenzae*, *Moraxella catarrhalis*, *Chlamydia pneumoniae*, *Haemophilus parainfluenzae*, a *Neisseria* species, and a group A *streptococcus*.

29. The method of claim 1, wherein said at least one viral pathogen is at least one selected from the group consisting of adenoviruses, herpes viruses, varicella-zoster viruses, enteroviruses, coxsackieviruses, molluscum contagiosum poxviruses, vaccinia poxviruses, influenza viruses, Epstein-Barr viruses, paramyxoviruses, and rubella viruses, and wherein said at least one non-viral pathogen is at least one selected from the group consisting of a group A *streptococcus*, *Streptococcus pyogenes*, *Streptococcus mitis*, *Streptococcus pneumoniae*, *Staphylococcus epidermidis*, *Staphylococcus aureus*, a *Corynebacterium* species, *Haemophilus* species, *Haemophilus influenzae*, *Neisseria* species such as *Neisseria meningitidis* and *Neisseria gonorrhoeae*, *Moraxella lacunata*, pseudomonads such as *Pseudomonas aeruginosa*, and *Chlamydia* species.

30. The method of claim 1, wherein said at least one viral pathogen is at least one selected from the group consisting of herpes viruses, varicella-zoster viruses, enteroviruses, polioviruses, arboviruses, mumps viruses, measles viruses, echoviruses, coxsackieviruses, human immunodeficiency viruses, and adenoviruses, and wherein said at least one non-viral pathogen is at least one selected from the group

consisting of *Haemophilus influenzae* type b, *Streptococcus pneumoniae*, *Neisseria meningitidis*, group B streptococci, *Streptococcus agalactiae*, *Streptococcus pyogenes*, non-group B streptococci, *Staphylococcus* species, *Listeria monocytogenes*, *Klebsiella pneumoniae*, *Serratia marcescens*, *Pseudomonas aeruginosa*, *Enterococcus faecium*, *Enterococcus faecalis*, *Proteus mirabilis*, *Escherichia coli*, *Mycobacterium tuberculosis*, *Legionella pneumophila*, and *Borrelia burgdorferi*.

31. The method of claim 1, wherein said at least one viral pathogen is at least one selected from the group consisting of enteroviruses, echoviruses, rotaviruses, caliciviruses, noroviruses, Norwalk-like viruses, Norwalk viruses, sapoviruses, astroviruses, and adenoviruses, and wherein said at least one non-viral pathogen is at least one selected from the group consisting of *Escherichia coli*, *E. coli* O157:H7, *Bacillus* species, *Staphylococcus aureus*, *Shigella* species, *Salmonella* species, *Campylobacter* species, *Clostridium difficile*, *Clostridium perfringens*, *Vibrio* species, *Listeria monocytogenes*, *Aeromonas* species, *Yersinia* species, *Plesiomonas* species, *Giardia lamblia*, *Entamoeba histolytica*, *Toxoplasma gondii*, and cryptosporidia.

32. The method of claim 1, wherein said at least one epitope is modified.

33. The method of claim 1, wherein said at least one binding agent comprises an antibody or antibody fragment.

34. The method of claim 1, wherein said at least one binding agent comprises a functional group or a detectable label.

35. The method of claim 1, wherein said at least one binding agent is further capable of binding to a mimotope that mimics said at least one epitope derived from said member.

36. The method of claim 1, wherein said at least one binding agent is used in more than one form.

37. The method of claim 36, wherein said assays comprise at least one sandwich immunoassay.

38. The method of claim 1, wherein said assays comprise at least one immunochromatographic assay.

39. The method of claim 38, wherein said at least one immunochromatographic assay comprises at least one sandwich assay.

40. The method of claim 1, wherein said assays are run in parallel.

41. The method of claim 1, wherein said assays are run sequentially.

42. The method of claim 1, wherein said assays are run coincidentally.

43. The method of claim 1, wherein said assays are run in a single path of fluid flow.

44. The method of claim 1, wherein said assays are run in multiple paths of fluid flow.

45. A kit for performing the method of claim 1.

46. The kit of claim 45, comprising at least one immunochromatographic device.

47. The kit of claim 45, wherein said assays are run in a single path of fluid flow.

48. The kit of claim 45, wherein said assays are run in multiple paths of fluid flow.

49. A kit for performing the method of claim 27.

50. The kit of claim 49, comprising at least one immunochromatographic device.

51. The kit of claim 49, wherein said assays are run in a single path of fluid flow.

- 52.** The kit of claim 49, wherein said assays are run in multiple paths of fluid flow.
- 53.** A kit for performing the method of claim 28.
- 54.** The kit of claim 53, comprising at least one immunochromatographic device.
- 55.** The kit of claim 53, wherein said assays are run in a single path of fluid flow.
- 56.** The kit of claim 53, wherein said assays are run in multiple paths of fluid flow.
- 57.** A kit for performing the method of claim 29.
- 58.** The kit of claim 57, comprising at least one immunochromatographic device.
- 59.** The kit of claim 57, wherein said assays are run in a single path of fluid flow.
- 60.** The kit of claim 57, wherein said assays are run in multiple paths of fluid flow.

- 61.** A kit for performing the method of claim 30.
- 62.** The kit of claim 61, comprising at least one immunochromatographic device.
- 63.** The kit of claim 61, wherein said assays are run in a single path of fluid flow.
- 64.** The kit of claim 61, wherein said assays are run in multiple paths of fluid flow.
- 65.** A kit for performing the method of claim 31.
- 66.** The kit of claim 65, comprising at least one immunochromatographic device.
- 67.** The kit of claim 65, wherein said assays are run in a single path of fluid flow.
- 68.** The kit of claim 65, wherein said assays are run in multiple paths of fluid flow.

* * * * *



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(54) FUNGAL ANTIGENS AND PROCESS FOR PRODUCING THE SAME

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435/6

(57)

ABSTRACT

There can be provided a fungal antigen which is an insoluble fraction obtainable from fungal cells of which cell wall has been substantially removed or at least partially removed; a process for producing the same; a nucleic acid encoding the fungal antigen; a biologic product containing the fungal antigen; a method of stimulating immunological responses by using the biologic product; a method of suppressing allergic reaction to fungi in a vertebrate; and a method for diagnosing a disease caused by fungi in a vertebrate.

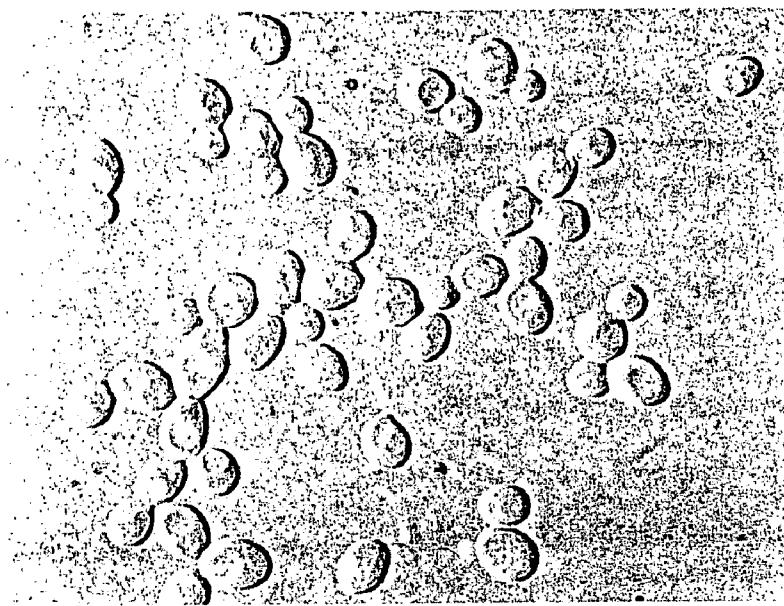


FIG.1A

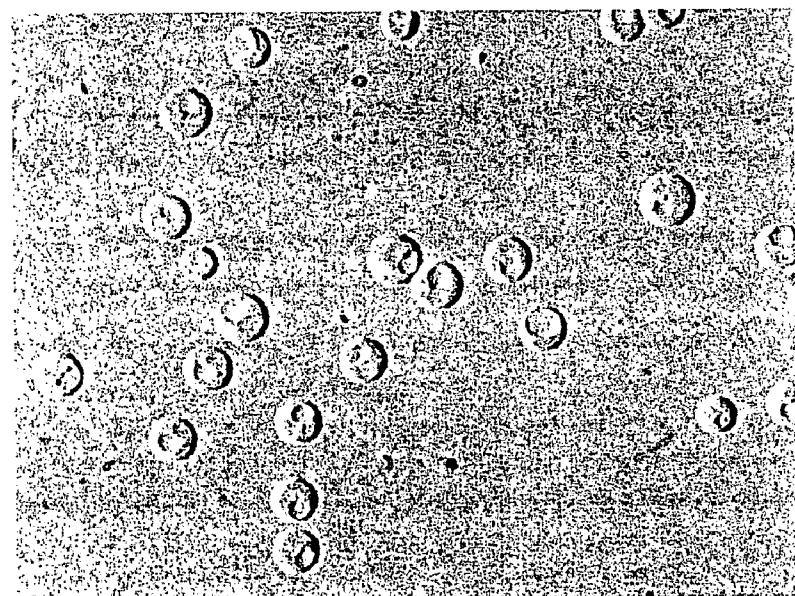


FIG.1B

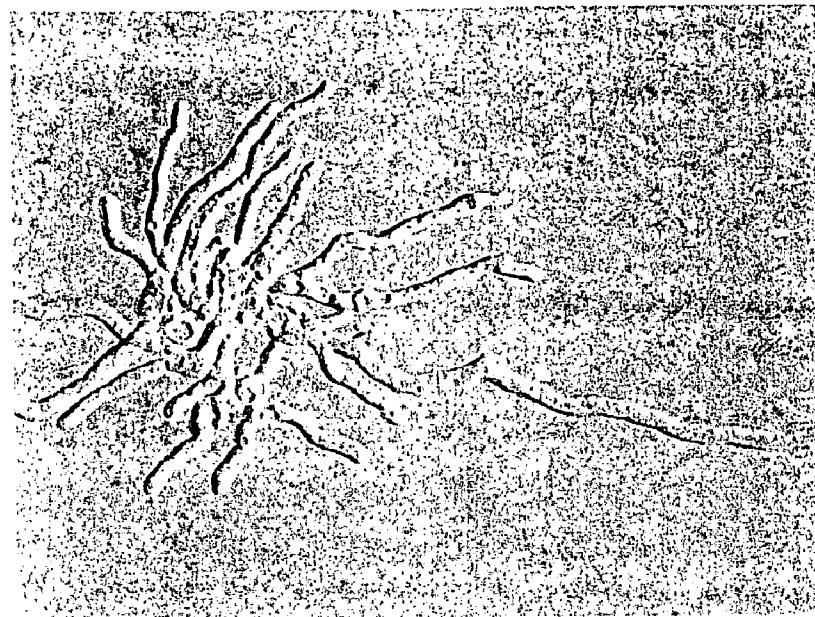


FIG.2A

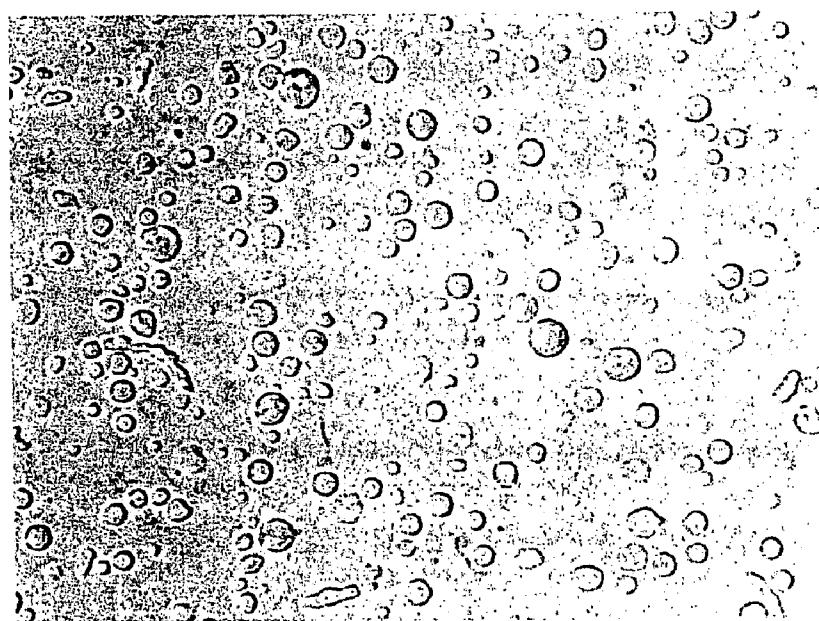


FIG.2B

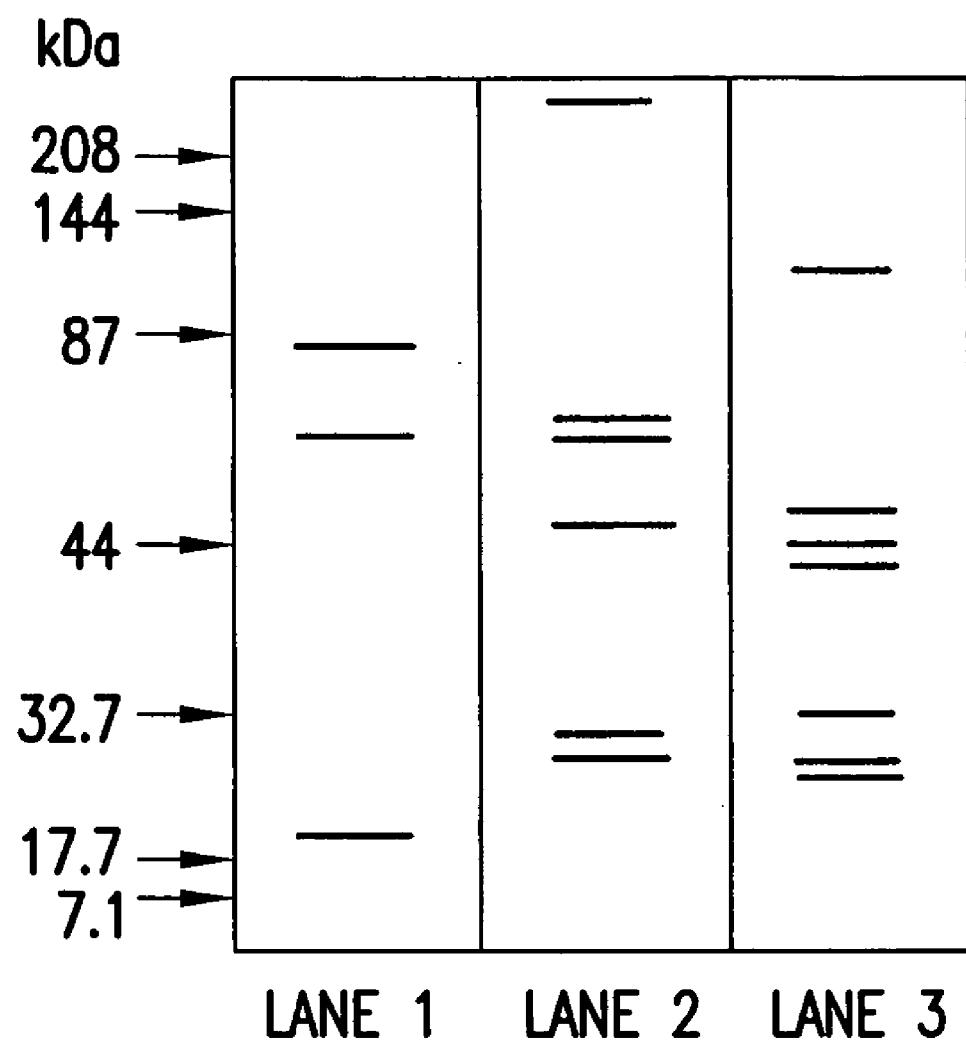


FIG.3

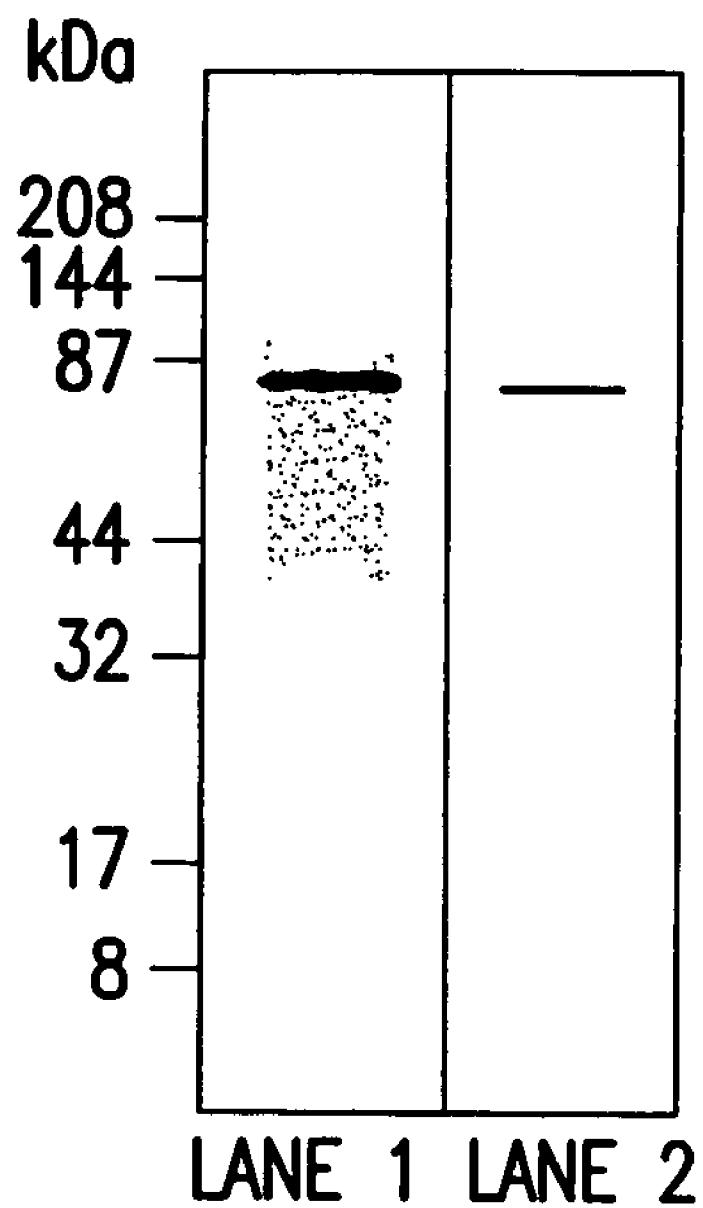


FIG.4

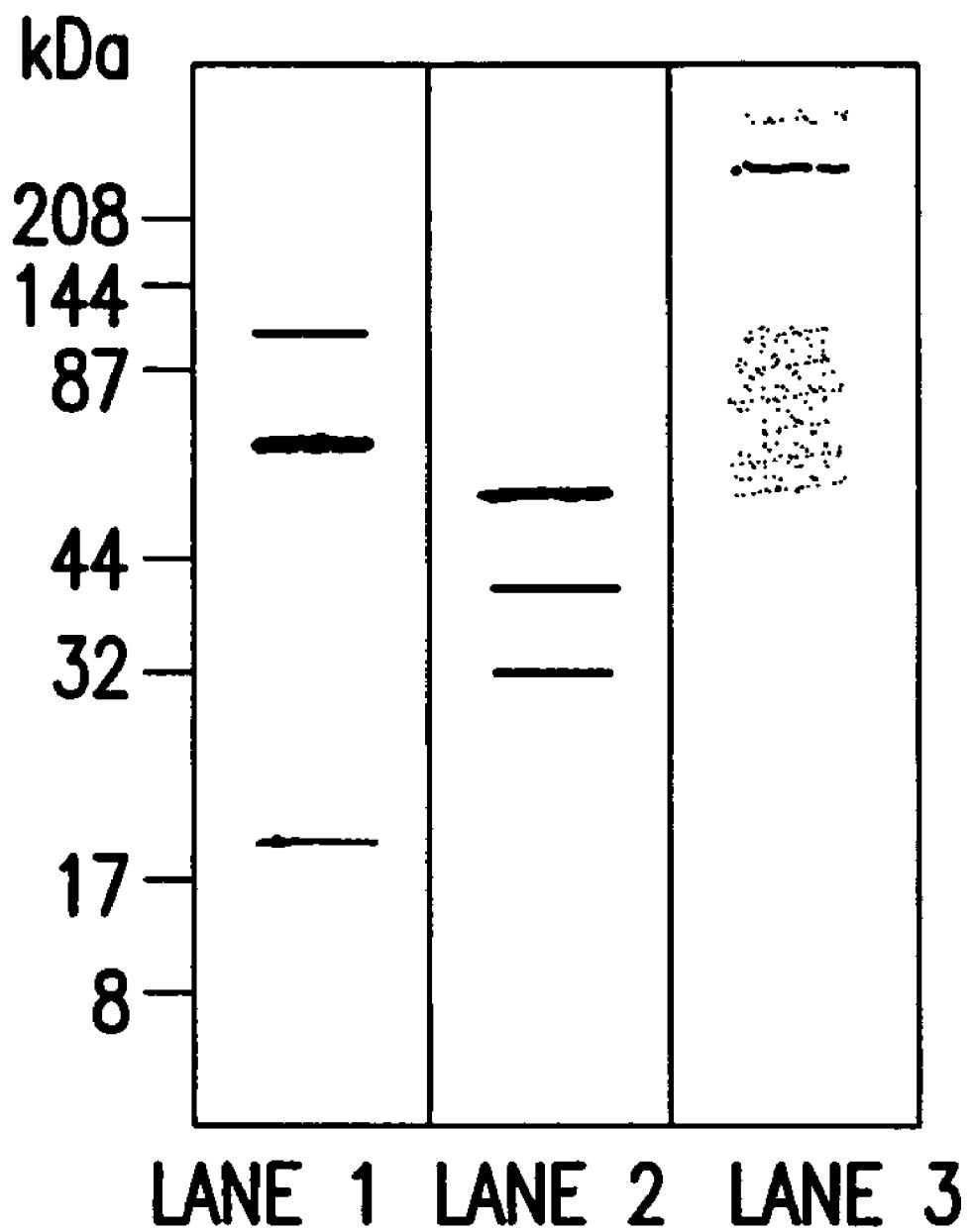


FIG.5

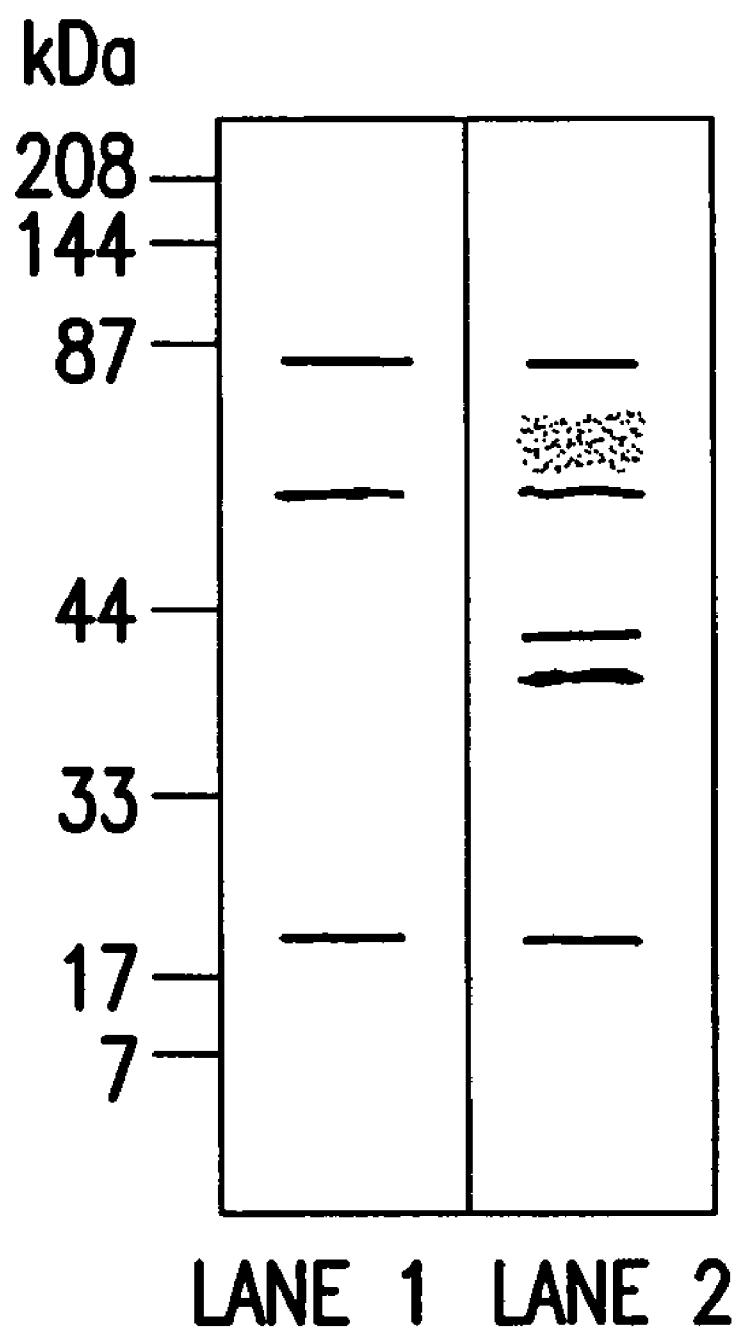


FIG.6

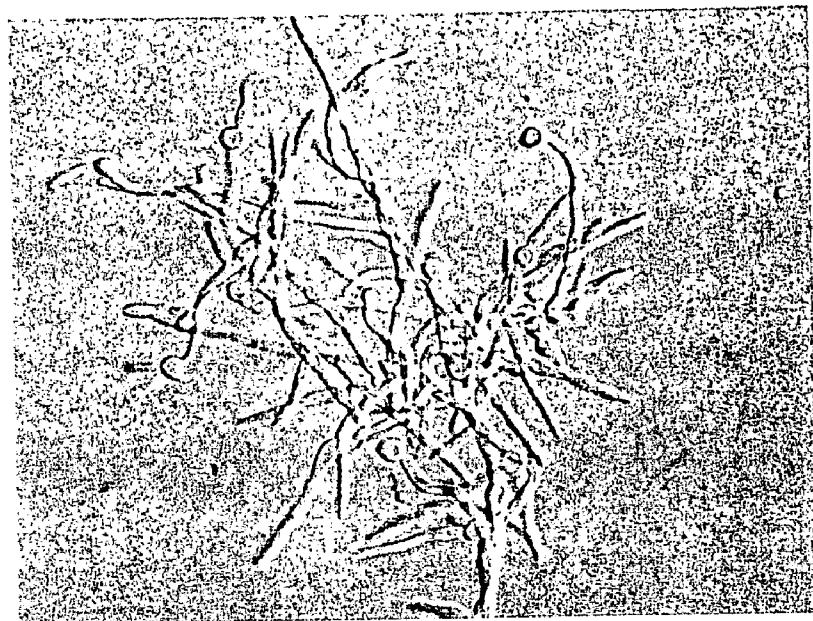


FIG.7A

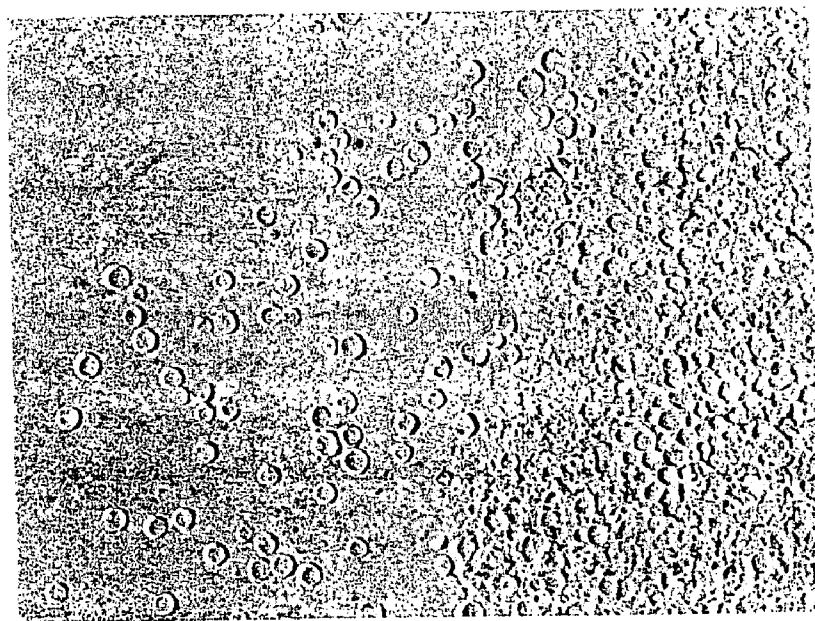


FIG.7B

FIG. 8

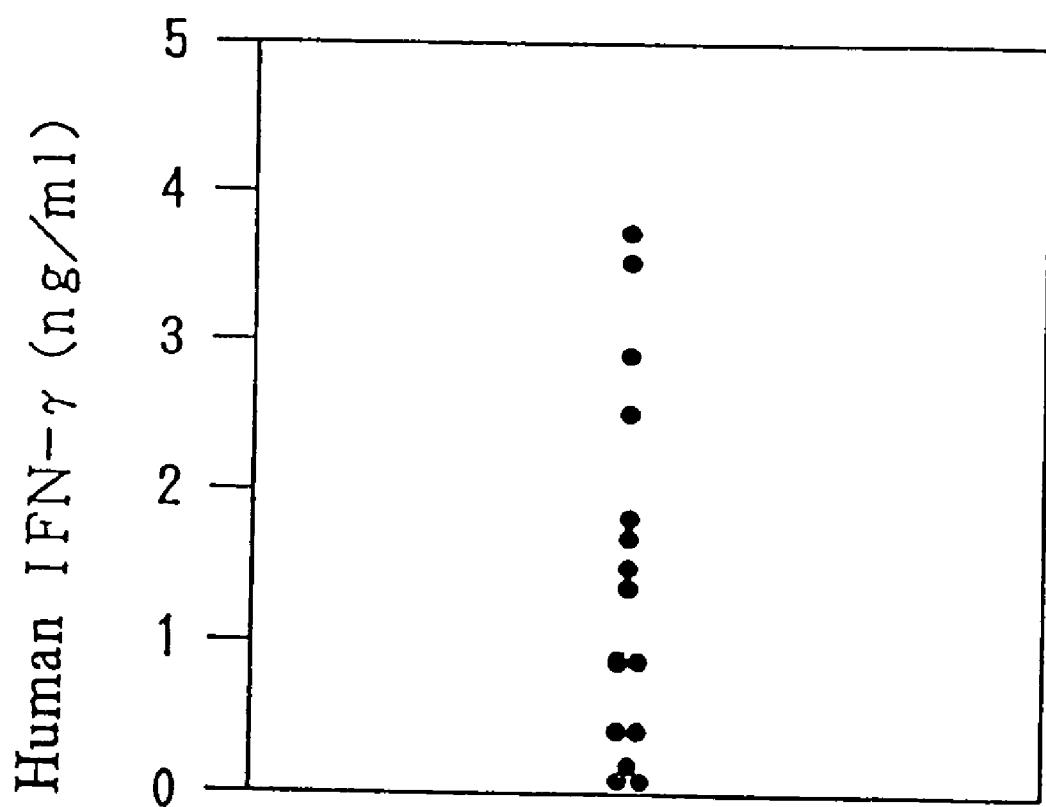
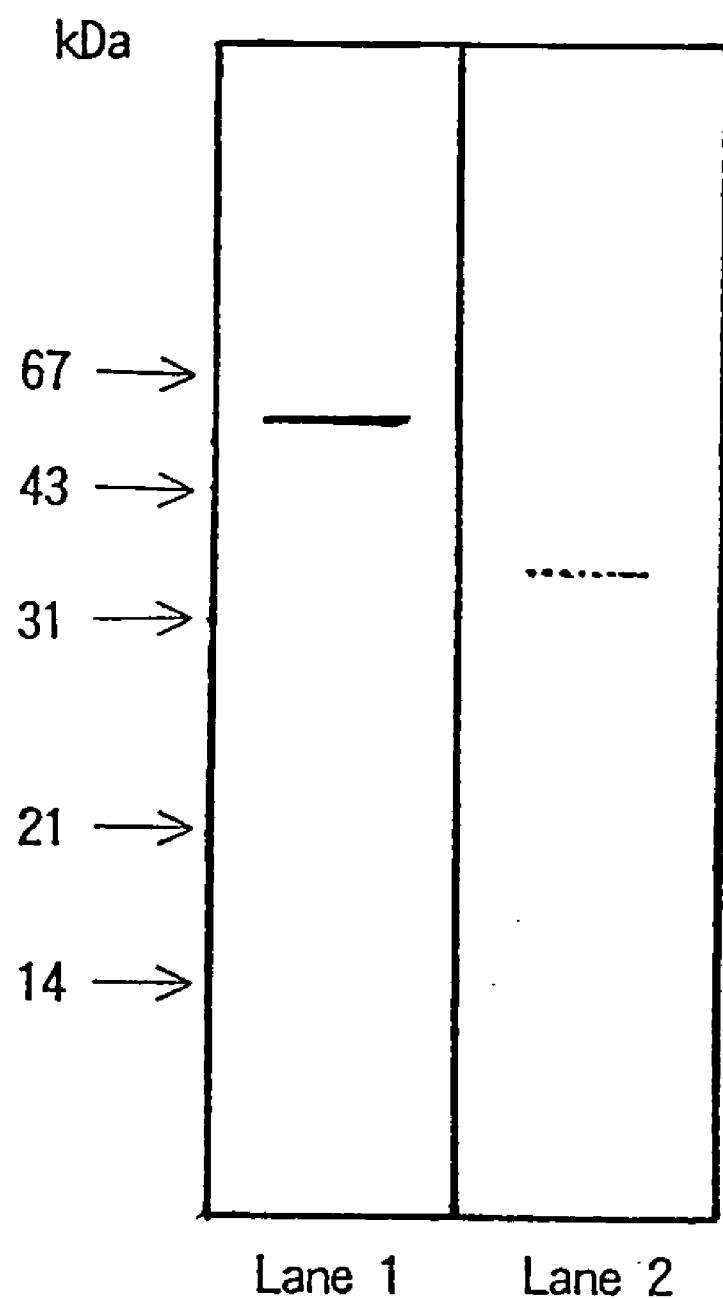


FIG.9



FUNGAL ANTIGENS AND PROCESS FOR PRODUCING THE SAME

[0001] This application is a divisional application of co-pending application Ser. No. 09/987,190, filed Nov. 13, 2001, which is a divisional application of co-pending Application No. 09/262,886, filed on Mar. 4, 1999, which issued as U.S. Pat. No. 6,333,164 on Dec. 25, 2001, which is a continuation-in-part application of PCT/JP97/03041, filed Aug. 29, 1997; and this application claims priority of Application No. 8-255400 filed in Japan on Sep. 4, 1996 and Application No. 9-99775 filed in Japan on Mar. 31, 1997 under 35 U.S.C. § 119, the entire contents of which are herein incorporated by reference.

BACKGROUND OF THE INVENTION

[0002] 1. Field of the Invention

[0003] The present invention relates to a fungal antigen effective for infectious diseases caused by fungi, which are pathogenic microorganisms, having cell wall, for prevention or treatment of allergoses, and for diagnosis of diseases caused by fungi, and a process for producing the same.

[0004] 2. Discussion of the Related Art

[0005] It has been known that fungi infect vertebrates such as humans and animals to cause all kinds of diseases. For example, superficial mycosis is caused in human skin, oral, or the like; systemic mycosis is caused in internal organs, brain, or the like, and similar infectious diseases are also caused to animals such as pets and domestic animals. Among them, *Candida*, such as *Candida albicans*, *Cryptococcus*, such as *Cryptococcus neoformans*, *Aspergillus*, such as *Aspergillus fumigatus*, *Pneumocystis carinii*, or the like have been known as major causative fungi which cause systemic mycosis by infecting humans. *Candida* which infects skin, oral, vagina, or the like, and *Dermatophytes* such as *Trichophyton mentagrophytes* and *Trichophyton rubrum* which infects skin of hands, feet, or the like have been taken for major causative fungi for superficial mycosis.

[0006] A lot of Dermophytes are fungi which cause infectious diseases to domestic animals, and the like, and it has been known that *Microsporum* such as *Microsporum canis* and *Microsporum gypseum* are such fungi other than *Trichophyton*, such as *Trichophyton verrucosum* mentioned above. In addition to these fungi, a wide variety of fungi occur in the living environment, and are assumed to infect humans and animals. Furthermore, recently, by the frequent use of a wide range of antibiotics, use of immunosuppressants, use of immunosuppressive anticancer agents, etc., patients administered with these drugs have become immunocompromised hosts, and opportunistic infection with fungi of low pathogenicity have been increased in normal individuals administered therewith. Also, AIDS patients suffer from frequent onset of thrush and complications of various mycoses. Patients on treatment with intravascular catheter indwelling, especially intravenous hyperalimentation (IVH), are likely to develop infectious diseases caused by fungi, especially with *Candida* owing to catheter.

[0007] On the other hand, allergoses, typically including asthma, atopic dermatitis, and allergic rhinitis, have been increasing dramatically, among which a very large number of allergoses are caused by fungi.

[0008] As for a lot of allergoses, because of sensitization with a causative antigen of its disease, an IgE antibody (reagin antibody) specific to the antigen as an allergen is produced in serum and tissue, so that the IgE antibody is bound to mast cells and basophil receptors. When re-exposed to the same antigen, the IgE bound to the cells is crosslinked with the antigen on the cell surface, thereby resulting in physiological effects of IgE-antigen interaction. These physiological effects are exhibited via a release of chemical mediators, such as histamine, serotonin, heparin, eosinophilic chemotactic factor, and various leukotrienes. These effects can be systemic or topical, depending on the route of an antigen entering the body and the pattern of IgE sedimentation on the mast cells or basophils.

[0009] The systemic symptoms include anaphylactic shock, which causes intravascular IgE-basophil response to the antigen. As a consequence, smooth muscle contraction and capillary dilation take place as major changes, thereby resulting in symptoms such as eruption, vomiting, diarrhea, and dyspnea. In more severe cases, it may lead to death. In addition, the topical symptoms generally develop on the epithelium surface at the site of an antigen entering the body as shown by reddening and papules. When bronchiolar smooth muscle contraction develops as a topical symptom, it is manifested as bronchial asthma.

[0010] As the causative strains for causing allergoses, there have been known *Penicillium*, *Candida*, *Aspergillus*, *Alternaria*, *Cladosporium*, *Malassezia*, *Botrytis*, *Mucor*, *Rhizopus*, *Aureobasidium*, *Fusarium*, *Trichoderma*, *Helminthosporium*, *Neurospora*, *Wallemia*, *Rhodotorula*, and *Trichophyton*.

[0011] As the therapy for fungal infections, a treatment with an antifungal agent is generally employed. A large number of drugs for superficial mycoses have been developed, and some excellent drugs for systemic infections are available. In terms of efficacy, toxicity, adverse reactions, etc., however, their effects are unsatisfactory. For example, amphotericin B that has long been used, causes various adverse reactions, including serious renal dysfunction. Although various azole antifungal agents, typically including fluconazole, have been developed, infections are highly likely to recur because their action is static. Also, resistant strains are emerging due to frequent use. As the resistant strains emerge, the cross-resistance takes place, because many of the antifungal agents presently in practical use possess similar action mechanisms, which can pose a major problem. In cases of superficial mycoses, various therapeutic drugs have been developed, but none can be said to be satisfactory, because it requires a long-term treatment period and recurrence is repeated. Therefore, a development of a further improved drug has been in demand. Moreover, since a treatment with topical preparations only would be unsatisfactory for some superficial mycoses, e.g., nail tinea, these superficial mycoses would require systemic medication such as griseofulvin. In this case, long-term administration would be necessitated, which can cause various adverse reactions by the drugs. Also, as in superficial mycoses and AIDS-related thrush, since repetitive infection is caused, there is a major problem in terms of costs, even if an effective antifungal agent is developed. As described above, a treatment with an antifungal agent has various problems.

[0012] The living body naturally possesses an ability to protect against infection by fighting against such foreign-

invading microorganisms. Vaccines utilize this ability. The prevention against infection with pathogenic bacteria has been carried out by vaccines and has been long used with fair efficacy. For such vaccines against bacterial infectious diseases, attenuated bacteria (*Mycobacterium tuberculosis*), killed bacteria (*Vibrio cholerae*), toxoids (*Corynebacterium diphtheriae*, *Clostridium tetani*), or purified antigens from capsular polysaccharides on cell surface (*Bordetella pertussis*, *Streptococcus pneumoniae*, influenza virus, *Neisseria meningitidis*) are employed as antigens. The vaccines provide an ability to protect against infection to the host by antibodies against antigenic molecules of the pathogen and by cellular immunity. It is considered that the antibodies serve to neutralize the toxic substances secreted by pathogens, and to prevent pathogens from invading host cells by binding to the cell surface molecules of the pathogen. In the cellular immunity, CD4+ cells and CD8+ T cells play a key role for recognizing the antigenic molecules of the pathogen and activating a protection reaction specific to the pathogen. Immunogenic substances, which are antigenic molecules possessed by the pathogens, have been isolated and identified, and some studies using these immunogens as sensitizing antigens (vaccines) have been made. In such cases, capsular polysaccharides, which are cell surface molecules as described above, are commonly used as immunogens.

[0013] An extremely large number and many kinds of fungi are present in the environment, and almost all vertebrates are sensitized with these fungi. Also, a large number of fungi are commonly present in the living bodies. The vertebrates are, therefore, generally provided with various immunological reactions for body protection against these fungi. Immunological reactions which have important roles against fungal infections show the phagocytosis and fungicidal actions of activated macrophages and polymorphonuclear leukocytes (PMN) and play a main role, and are also known to contribute to antibodies and cellular immunity. On their cell surface, fungi have a cell wall, comprising, as a main component, polysaccharides, such as mannan, glucan, and chitin, of which the content accounts for nearly 30% of the entire cell in some fungal cells [Klis, R. U. et al., Yeast, Vol. 10, 851-869, (1994)]. Of these cell wall components, mannan is the most antigenic. The mannan is a polysaccharide in the cell surface layer, and an antibody against the polysaccharide moiety is produced in large amounts. The cell wall glucans from fungi, typically including Zymosan, possess various biological activities, and are known to possess non-specific immunopotentiating actions. It is assumed that the cell wall components, including mannan on a cell surface of fungi, play an important role in causing infection as an adhesion molecule to the living body of cells.

[0014] Also, *Cryptococcus galactoxylomannan* [Devi, S. J. N. et al., *Infect. Immun.*, Vol. 59, 3700-3707 (1991)] and the *Candida albicans* adhesion factor phosphomannoprotein (WO 95/31998) have been reported to serve as vaccines, and antibodies against these antigenic molecules have been reported to possess protection activity against infection. Regarding the induction of immunological protection against infection with living or dead *Candida* cells, a large number of reports have been made [Segal, E. et al., *Critical Reviews in Microbiology*, Vol. 14, 229-271 (1987)]. In this case as well, it has been assumed that an immunological reaction mainly functions for body protection against the cell wall components which are the cell surface molecules.

[0015] Other vaccines against fungi include the ribosome vaccine [Segal, E., *Handbook of Applied Mycology, Volume 2: Immunizations against fungal diseases in man and animals., Humans, animals and insects*] has been tested for infectious diseases caused by fungi, typically including *Candida albicans* and *Trichophyton*, and studied on laboratory animals and partially on humans and domestic animals. Recently, there have been reported that enolase and stress protein HSP90 (Japanese Unexamined Patent Publication No. Hei 4-502257) can induce protective activity against infection.

[0016] However, it cannot be said that all of the above-mentioned antigenic molecules are confirmed to have satisfactory efficacy. Also, it is doubtful whether or not satisfactory efficacy can be obtained in highly diversified mammals by treatment with a single antigenic molecule.

[0017] On the other hand, therapies for allergoses include the use of antihistaminic drugs, steroid anti-inflammatory drugs, chemical mediator release suppressors, and the like. It should be noted, however, that the antihistamines have a risk of developing various adverse reactions, such as malaise, drowsiness, and vertigo, that the steroids have a risk of developing various adverse reactions, such as adrenal atrophy and dysfunction, and gastric ulcer, and that the chemical mediator release suppressors have a risk of also suppressing the action of chemical mediators involved in conditions other than the allergosis of interest. From this viewpoint, prevention method for reducing the chance of exposure to allergens specified by antigen diagnosis, and/or desensitization therapy using such causative allergens is considered to be an excellent therapy.

[0018] In allergoses, it is therefore necessary to first diagnose for identifying the causative antigen, and for this purpose, more than 100 kinds of commercially available allergen extracts, sometimes those prepared by the laboratory, are subjected to intradermal test for suspected antigen extracts. After a highly likely antigen is found, the antigen can be specified by determination of IgE antibody titer in sera, challenge test, or histamine release test using whole blood or lymphocytes.

[0019] As allergens by which allergic symptoms are provoked in humans, a large number of naturally occurring ones have been known. Commercially available food and other allergen extracts are supplied as crude extracts from natural allergens. Therefore, they are naturally agglomerates of many substances and contain a plurality of antigens. Recently, as a result of advances in separation and purification techniques and evaluation methods for allergen activity, antigenic proteins, which comprise the main body of allergens, are isolated and identified from a variety of food allergens.

[0020] Also, from each of allergens occurring in the environment, such as mites, *Cryptomeria japonica* pollen, and feline hair, antigenic proteins named as Der p I [Smith, W. A. et al., *Clin. Exp. Allergy*, Vol. 24, 220-228 (1994)], Cry j I [Sone, T. et al., *Biochem. Biophys. Res. Commun.*, Vol. 199, 619-625 (1994)], and Fel d I [Morgenstern, J. P. et al., *Proc. Natl. Acad. Sci. USA*, Vol. 88, 9690-9694 (1991)] have been isolated as major allergens. Furthermore, the genes encoding these allergen proteins have been isolated, so that pure allergen proteins can be prepared in large amounts by genetic engineering techniques.

[0021] In the meantime, efforts have been made to isolate allergens derived from fungi. Antigenic proteins have been isolated and identified from proteins existing in fungal cells. For example, alcohol dehydrogenase (Can a I) [Shen, H. D. et al., *Clin. Exp. Allergy*, Vol. 21, 675-681 (1991)] and enolase [Ishiguro, A. et al., *Infect. Immun.*, Vol. 60, 1550-1557 (1992)] have been isolated from *Candida albicans* and identified, and ribotoxin (Asp f Ia) [Mosor, M. et al., *J. Immunol.*, Vol. 149, 454-460 (1992)] have been isolated from *Aspergillus fumigatus* and identified, some of which have been known to act as allergens.

[0022] Generally, in the case of allergens from fungi, including *Candida* and *Aspergillus*, however, there are few cases where a single major allergen exists as an antigenic protein, but a plurality of antigenic proteins exist [Stewart, G. A. et al., *Clin. Exp. Allergy*, Vol. 26, 1020-1044 (1996)], in which different antigens by depending upon individuals, or a plurality of antigens for each individual, are recognized as allergens, to which the individuals react. In other words, even when the individuals are allergic to *Candida*, for instance, it is known that in many cases antigens to which each individual reacts are different antigens, and that each individual reacts to a plurality of antigens derived from *Candida*.

[0023] Presently commercially available diagnostic or therapeutic allergen extracts are for the most part simple extracts or hardly purified crude extracts, so that the included ingredients are substantially uncontrolled. The allergen extracts from fungi include those from *Candida*, *Aspergillus*, *Alternaria*, *Cladosporium*, *Malassezia*, *Penicillium*, and the like. However, the methods for production thereof differ from those for the allergen extracts from naturally occurring allergens in food or the environment described above. In other words, these extracts are not supplied as cultured cells of the causative fungus per se, but prepared from an extracellular product secreted in the culture broth as raw material, which can be considered as a side-product, obtained by subjecting a representative strain belonging to each genus to a long-term cultivation in a chemically defined medium containing a limited nutrient source. Therefore, the antigen obtainable by such production method is an autolysate of cells or an extracellular secretion, which presumably comprises, as a main component, cell wall polysaccharides typically including mannan and glucan. However, neither the contents of these antigens nor the kinds of other antigenic proteins have yet been clarified. In addition, sufficient care should be paid for its use, since their quality is diversified among manufacturers.

[0024] Cell wall polysaccharides richly contained in commercially available allergen extracts from fungi, especially mannan, serve as major allergens in some patients with allergy on one hand, and even normal individuals have large amounts of IgG and IgM against cell wall polysaccharides. In addition, mannan per se, especially neutral mannan, has been known to possess toxicity, including lethal action to the mouse [*Japanese Journal of Medical Mycology*, Vol. 36, 203-208 (1995)]. It has been also known that cell wall glucan possesses pathological actions, including induction of inflammation [Kogan, G. et al., *Biomedical and Biotechnological Advances in Industrial Polysaccharides*, 251-258 (1989)].

[0025] The use of mannan and other cell wall components, which are antigens, or fungal cells per se, as vaccines,

therefore, involves risks, such as causation of hypersensitivity. Also, in desensitization therapy etc. for allergoses, cell wall components do not always act as major allergens; therefore, when an allergen composition containing a cell wall component is used, its antigenicity is of concern, necessitating to be cautious when administering to humans. In this respect, presently available allergen extracts from fungi are completely unsatisfactory. Moreover, there are no known diagnostic and/or therapeutic pharmaceutical compositions in which a sufficient amount of an effective antigen is contained.

[0026] As described above, a development of novel therapeutic drugs of high efficacy and higher safety for mycoses is strongly desired, from the viewpoints of increasing incidence of mycoses, and further problems related to adverse reactions, development of resistant strains, medical costs, etc. in antifungal agents presently in use. The vaccines are advantageous over antifungal agents in many aspects, and if vaccines for such infectious diseases caused by fungi could be found, it would not only make it possible to prevent pain and weakening owing to being taken these infectious diseases, but also enable definite reduction of the dosage of drugs intended for the treatment of these infectious diseases. Furthermore, by avoiding the use of the drugs in such a way, selective pressure on pathogenic microorganisms due to overdoses of the antifungal agents is reduced, so that the prevalence of the resistant strains can be reduced. At present, however, no such highly effective vaccines have yet been found. Also, it is expected that sensitizing with a plurality of antigens has better induction of prevention against infection than sensitizing with a single antigen in the aspects of resistance and efficacy.

[0027] On the other hand, with the increase in the incidence of allergoses, numerous therapeutic or diagnostic allergen extracts have become commercially available, many of which effective ingredients, however, have not yet been clarified. As for fungi, although it remains unknown from which portions of the fungal cells the components are derived, from the methods for their production, it is assumed that its major component is polysaccharides derived from cell wall, clearly having a low content of antigenic components derived from intracellular components, and thus having a very uneven distribution in the antigenic component. For this reason, it is considered that satisfactory treatment or diagnosis cannot be carried out by using commercially available allergen extracts from fungi, and antigen extracts obtained by similar methods. Therefore, it is expected that allergen extracts having ingredients differing from those contained in conventional allergen extracts, and that the amounts of ingredients of those allergen extracts differing from those of conventional allergens exhibit high efficacy. Also, as for the present therapy of desensitization, which is considered effective for allergoses, it is necessary that an antigenic liquid is administered intradermally in small dosages at a time, once or twice a week, with increased dosage to a level maintained over a 3- to 4-month period, the administration of which is continued for 1 to 3 additional years. By the use of an antigen composition capable of easy volume increase and/or increased dosage, therefore, it is expected that an excellent therapeutic effect can be more easily obtained. Also, mammals typically including humans are generally diverse, and it is very likely that those recognized as antigens are different even if infected with, or

becomes allergic to, one kind of fungus. Antigens containing sufficient amounts of diversified antigenic components are, therefore, desirable.

[0028] Furthermore, it is diagnostically important to specify the causative antigen when choosing an effective therapy, whereby highly effective and safer treatments, such as desensitization therapy using the antigen, can be carried out. It is, therefore, preferable from these viewpoints to specify unknown antigens.

[0029] Accordingly, an object of the present invention is to provide a fungal antigen that can be used for effective, safer biologic products against diseases caused by such fungi, including, for instance, vaccine compositions, compositions for desensitization therapy, and diagnostic compositions. A further object of the present invention is to provide a method for producing the fungal antigen, and a nucleic acid encoding the fungal antigen.

[0030] These and other objects of the present invention will be apparent from the following description.

SUMMARY OF THE INVENTION

[0031] Since some of the cell wall components from fungi, which have conventionally been studied mainly as antigenic molecules, cause immunological reactions undesirable to living bodies, the present inventors have studied for substances that possess antigenicity, and activity as vaccines and/or allergens for components other than cell wall components, using protoplasts, as starting materials, obtained by removing the cell wall from fungal cells. As a result, the present inventors have clarified that insoluble fractions containing cytoplasmic membrane proteins and membrane proteins of cell organelle, obtained from protoplasts derived from fungi causative for infectious diseases, unexpectedly possess potent antigenicity. They have further clarified that even though the insoluble fractions substantially do not contain cell wall components, their activity as vaccines is at an equivalent level or higher than that of living cells. The present inventors have also clarified that a solubilized fraction obtainable from the insoluble fraction using a solubilizer, such as a surfactant, also possesses potent antigenicity and potent activity as vaccines.

[0032] Furthermore, the present inventors have clarified that since the product of the present invention can be obtained as a mixture of several kinds of antigens, it is expected to provide a broader range of immunological responses than a case of administration of a particular single antigenic component, and that in fact possesses more potent vaccine activity than any of antigenic components that have been conventionally studied. The present inventors have further clarified that the antigen acts to stimulate immunocytes, typically including lymphocytes, to possess an activity for releasing cytokines, such as IFN- γ from the cells. The cytokine-releasing cells include, for example, T lymphocytes, natural killer (NK) cells, and the like. On the other hand, the present inventors have clarified that the insoluble fraction obtainable from protoplasts derived from causative fungi of allergoses possesses potent antigenicity and sufficient activity as allergens. The present inventors have also clarified that the solubilized fraction obtainable from the insoluble fractions by using a solubilizer, such as a surfactant, also possesses potent antigenicity and sufficient activity as allergens. In addition, the present inventors have clarified

that the insoluble fraction obtainable from protoplasts derived from causative fungi of diseases and/or the solubilized fraction obtainable from the insoluble fraction possesses sufficient activity for diagnostic antigens. Further, the present inventors have succeeded in isolating a protein possessing antigenicity that has not conventionally been elucidated from the fractions. The present invention has been completed.

[0033] Specifically, the present invention is summarized as follows:

[1] a fungal antigen characterized in that the fungal antigen is an insoluble fraction obtainable from fungal cells of which cell wall has been substantially removed or at least partially removed;

[0034] [2] a fungal antigen comprising an antigenic protein having a vaccine activity or an allergen activity originated from *Candida albicans*, wherein the antigenic protein comprises the partial amino acid sequence as shown by SEQ ID NO: 1 in Sequence Listing and has a molecular weight of about 65,000 as determined by SDS-PAGE under reduced conditions;

[0035] [3] a fungal antigen comprising a peptide comprising an entire sequence of the amino acid sequence as shown by SEQ ID NO: 5 in Sequence Listing, or a partial sequence thereof, the peptide having a vaccine activity or an allergen activity;

[0036] [4] a fungal antigen comprising an antigenic protein having a vaccine activity or an allergen activity originated from *Candida albicans*, wherein the antigenic protein comprises the partial amino acid sequence as shown by SEQ ID NO: 2 in Sequence Listing and has a molecular weight of about 25,000 as determined by SDS-PAGE under reduced conditions;

[0037] [5] a fungal antigen comprising a peptide comprising an entire sequence of the amino acid sequence as shown by SEQ ID NO: 6 in Sequence Listing, or a partial sequence thereof, the peptide having a vaccine activity or an allergen activity;

[0038] [6] a fungal antigen comprising an antigenic protein having a vaccine activity or an allergen activity originated from *Candida albicans*, wherein the antigenic protein comprises the partial amino acid sequence as shown by SEQ ID NO: 3 in Sequence Listing and has a molecular weight of about 30,000 as determined by SDS-PAGE under reduced conditions;

[0039] [7] a fungal antigen comprising an antigenic protein having a vaccine activity or an allergen activity originated from *Candida albicans*, wherein the antigenic protein comprises the partial amino acid sequence as shown by SEQ ID NO: 4 in Sequence Listing and has a molecular weight of about 62,000 as determined by SDS-PAGE under reduced conditions;

[0040] [8] a fungal antigen comprising an antigenic protein having a vaccine activity or an allergen activity originated from *Candida albicans*, wherein the antigenic protein comprises the partial amino acid sequence as shown by SEQ ID NO: 14 in Sequence Listing and has a molecular weight of about 35,000 as determined by SDS-PAGE under reduced conditions;

[0041] [9] a fungal antigen comprising an antigenic protein having a vaccine activity or an allergen activity originated from *Candida albicans*, wherein the antigenic protein comprises the partial amino acid sequence as shown by SEQ ID NO: 15 in Sequence Listing and has a molecular weight of about 55,000 as determined by SDS-PAGE under reduced conditions;

[0042] [10] a process for producing a fungal antigen which is an insoluble fraction obtainable from fungal cells of which cell wall has been substantially removed or at least partially removed, characterized in that the process comprises the steps of:

[0043] (1) obtaining living fungal cells;

[0044] (2) obtaining fungal cells of which cell wall has been substantially removed or at least partially removed;

[0045] (3) bursting the fungal cells of which cell wall has been substantially removed or at least partially removed; and

[0046] (4) obtaining an insoluble fraction;

[11] a process for producing a fungal antigen which is a solubilized fraction extracted and separated from an insoluble fraction obtainable from fungal cells of which cell wall has been substantially removed or at least partially removed, characterized in that the process comprises the steps of:

[0047] (1) obtaining living fungal cells;

[0048] (2) obtaining fungal cells of which cell wall has been substantially removed or at least partially removed;

[0049] (3) bursting the fungal cells of which cell wall has been substantially removed or at least partially removed;

[0050] (4) obtaining an insoluble fraction; and

[0051] (5) extracting and separating a solubilized fraction from the insoluble fraction;

[12] a biologic product containing the fungal antigen of item [1] above, or a fungal antigen produced by the process of item [10] or [11] above;

[13] a cytokine releasing agent containing the fungal antigen of item [1] above, or a fungal antigen produced by the process of item [10] or [11] above;

[14] an allergen composition for preventing allergoses against fungi or exhibiting therapeutic effects therefor by administering to individuals, characterized in that the allergen composition contains the fungal antigen of item [1] above, or a fungal antigen produced by the process of item [10] or [11] above; and

[15] a diagnostic composition for a disease caused by fungi, characterized in that the diagnostic composition contains the fungal antigen of item [1] above, or a fungal antigen produced by the process of item [10] or [11] above.

BRIEF DESCRIPTION OF THE DRAWINGS

[0052] FIG. 1 is figures showing morphologies before and after cell wall removal from *Candida albicans* TIMM 1768 cells (yeast type), the figures being taken at a magnification of $\times 1,000$ using a differential interference microscope

(manufactured by NIKON Corporation), wherein A shows cells before cell wall removal, and B shows cells after cell wall removal.

[0053] FIG. 2 is figures showing morphologies before and after cell wall removal from *Aspergillus fumigatus* cells, the figures being taken at a magnification of $\times 400$ using a differential interference microscope (manufactured by NIKON Corporation), wherein A shows cells before cell wall removal, and B after cell wall removal.

[0054] FIG. 3 is a chart showing the presence of antibodies against proteins derived from *Candida albicans* insoluble fraction Ca-LSP contained in mouse anti-*Candida* serum (lane 1), rabbit anti-*Candida* serum (lane 2), and normal individual serum (lane 3).

[0055] FIG. 4 is a chart showing the presence of antibodies against a protein derived from *Aspergillus fumigatus* insoluble fraction Af-LSP (lane 1), and a protein derived from the *Cryptococcus neoformans* insoluble fraction Crn-LSP (lane 2), each being contained in mouse anti-*Aspergillus* serum.

[0056] FIG. 5 is a chart showing the presence of antibodies against a protein derived from *Candida albicans* insoluble fraction Ca-LSP (lane 1), a protein derived from the *Cryptococcus neoformans* insoluble fraction Crn-LSP (lane 2), and a protein derived from the *Aspergillus fumigatus* insoluble fraction Af-LSP (lane 3), each being contained in mouse anti-*Candida* serum.

[0057] FIG. 6 is a chart showing the presence of antibodies against a protein derived from yeast type *Candida albicans* insoluble fraction Ca-LSP (lane 1), and a protein derived from the mycelial *Candida albicans* insoluble fraction Ca-LSP-M (lane 2), each being contained in mouse anti-*Candida* serum.

[0058] FIG. 7 is figures showing morphologies before-hand after cell wall removal from mycelial *Candida albicans* cells, the figures being taken at a magnification of $\times 400$ using a differential interference microscope (manufactured by NIKON Corporation), wherein A shows cells before cell wall removal, and B shows cells after cell wall removal.

[0059] FIG. 8 is a graph showing the amount of human IFN- γ produced after 7 days from initiation of cultivation in an RPMI-1640 medium containing human peripheral blood mononucleated cells (PBMCs) supplemented with the Ca-LSP antigen liquid.

[0060] FIG. 9 is a chart showing the presence of antibodies against an antigenic protein derived from *Candida albicans* insoluble fraction Ca-LSP (lane 1, lane 2) contained in mouse anti-*Candida* serum.

DETAILED DESCRIPTION OF THE INVENTION

[0061] The present invention is hereinafter described in detail.

[0062] The fungal antigen of the present invention is characterized in that the fungal antigen is an insoluble fraction obtainable from fungal cells of which cell wall has been substantially removed or at least partially removed. Such fungal antigens can be, for example, used as biologic products. The fungal antigen of the present invention is

obtained from a causative fungus of an infectious disease or a causative fungus of an allergosis. The fungal antigen derived from a causative fungus of an infectious disease is capable of inducing immunity against infection in vertebrates, so that the fungal antigen can be suitably used particularly as a composition of vaccine. On the other hand, the fungal antigen derived from a causative fungus of an allergosis can be utilized to desensitize vertebrates, so that the fungal antigen can be suitably used to prevent and treat allergoses. Furthermore, such fungal antigens can suitably be used to diagnose diseases caused by fungi.

1. Fungal Cells

[0063] The fungi usable in the present invention are not particularly limited, and they include not only fungi possessing pathogenicity in vertebrates such as humans and animals, but also other fungi closely related thereto. Examples thereof include one or more fungi selected from the group consisting of fungi belonging to *Candida*, *Aspergillus*, *Cryptococcus*, *Mucor*, *Rhizopus*, *Absidia*, *Nocardia*, *Histoplasma*, *Blastomyces*, *Coccidioides*, *Trichophyton*, *Microsporum*, *Epidermophyton*, *Sporothrix*, Dematiaceous fungi, *Malassezia*, *Pneumocystis*, *Penicillium*, *Alternaria*, *Cladosporium*, *Botrytis*, *Aureobasidium*, *Fusarium*, *Trichoderma*, *Helminthosporium*, *Neurospora*, *Wallemia*, and *Rhodotorula*.

[0064] In the present invention, fungal infectious diseases in vertebrates include candidiasis, aspergillosis, cryptococcosis, mucormycosis, actinomycosis, histoplasmosis, blastomycosis, various skin mycoses, tinea versicolor, and *Pneumocystis carinii* pneumonia in humans. It is, therefore, preferable from the viewpoint of usefulness that the fungus usable in a vaccine composition in the present invention is a causative fungus of such a fungal infectious disease.

[0065] Concrete examples thereof include causative fungi of candidiasis such as *Candida albicans*, *C. tropicalis*, and *Candida glabrata*; causative fungi of aspergillosis such as *Aspergillus fumigatus* and *Aspergillus flavus*; causative fungi of cryptococcosis such as *Cryptococcus neoformans*; causative fungi of mucormycosis such as *Mucor* sp., *Absidia* sp., and *Rhizopus* sp.; causative fungi of actinomycosis such as *Nocardia asteroides*; causative fungi of other fungal infectious diseases in the internal organs such as *Trichosporon cutaneum*, *Rhodotorula glutinis*, *Geotrichum candidum*, *Pneumocystis carinii*, *Coccidioides immitis*, *Paracoccidioides brasiliensis*, *Histoplasma capsulatum*, and *Blastomyces dermatitidis*; *Trichophyton*, which is Dermatophytes, such as *Trichophyton mentagrophytes*, *Trichophyton rubrum*, and *Trichophyton verrucosum*; *Microsporum* such as *Microsporum canis*, *Microsporum gypseum*, and *Epidermophyton* sp.; *Phialophora* sp. and *Cladosporium* sp., which are Dematiaceous fungi; *Malassezia furfur*, which causes tinea versicolor; causative fungi for other skin mycoses such as *Sporothrix schenckii* and *Fonsecaea pedrosoi*, and the like.

[0066] The usable fungal strain is not particularly limited, as long as it is closely related to the causative fungus of the mycosis to be treated or prevented, and a strain possessing pathogenicity (e.g., lethal toxicity against mice) is desirable. Typical examples of the useful strains include *Candida albicans* ATCC 10231, TIMM 1768, and TIMM 0239 for candidiasis; *Aspergillus fumigatus* ATCC 28212, ATCC 42202, and TIMM 1776 for aspergillosis; and *Cryptococcus*

neoformans ATCC 24067, TIMM 0354, and capsule-deficient *Cryptococcus neoformans* TIMM 0357 for cryptococcosis. In addition, *Candida utilis*, yeasts of *Saccharomyces* such as *Saccharomyces cerevisiae*, yeasts of *Kluyveromyces* such as *Kluyveromyces marxianus* and *Kluyveromyces lactis* have been known to be closely related to *Candida albicans*, which are also usable in the present invention.

[0067] When used for the purpose of releasing a cytokine from cells, the fungal antigen is preferably derived from a normally colonizing fungus to which even normal individuals are immunologically sensitized, with a preference given to an antigen derived from *Candida albicans*.

[0068] On the other hand, when used to suppress an allergic reaction, the fungus usable for preparing the fungal antigen contained in the allergen composition of the present invention is preferably a causative fungus that provokes allergic symptoms in humans, from the viewpoint of its usefulness.

[0069] Concrete examples thereof include *Candida* such as *Candida albicans*, *Candida tropicalis*, *Candida glabrata*, and *Candida boidinii*; *Aspergillus* such as *Aspergillus fumigatus*, *Aspergillus restrictus*, and *Aspergillus versicolor*; *Trichophyton* such as *Trichophyton mentagrophytes*; *Malassezia* such as *Malassezia furfur*; *Mucor* such as *Mucor racemosus*; *Rhizopus* such as *Rhizopus oryzae*; *Penicillium* such as *Penicillium notatum*; *Alternaria* such as *Alternaria alternata* and *Alternaria kikuchiana*; *Cladosporium* such as *Cladosporium cladosporioides*, and *Cladosporium carionii*; *Botrytis* such as *Botrytis cinerea*; *Aureobasidium* such as *Aureobasidium pullulans*; *Fusarium* such as *Fusarium oxysporum*; *Trichoderma* such as *Trichoderma viridae*; *Helminthosporium* such as *Helminthosporium maydis*; *Neurospora* such as *Neurospora crassa*; *Wallemia* such as *Wallemia sebi*; *Rhodotorula* such as *Rhodotorula glutinis*, and the like.

[0070] The usable fungal strain is not particularly limited, as long as it is closely related to the causative fungus of the allergosis to be treated or prevented. Typical examples thereof include *Candida* such as *Candida albicans* ATCC 10231 and TIMM 1768, and *Candida boidinii* ATCC 18810 for preparing *Candida* antigens; *Aspergillus* such as *Aspergillus fumigatus* ATCC 28212 and TIMM 1776, and *Aspergillus restrictus* ATCC 16912 for preparing *Aspergillus* antigens; *Alternaria* such as *Alternaria alternata* IFO 31188 for preparing *Alternaria* antigens; *Malassezia* such as *Malassezia furfur* ATCC 14521 and TIMM 2782 for preparing *Malassezia* antigens; and the like.

[0071] In the present invention, in the case of a fungal antigen usable for diagnosing a disease caused by a fungus, the usable fungus is preferably the above-described fungi that causes the disease.

2. Fungal Antigens

[0072] The “fungal cells of which cell wall has been substantially removed” in the phrase “fungal cells of which cell wall has been substantially removed or at least partially removed,” as used in the present specification, refer to the protoplasts or protoplast-like cells of the fungal cells. The “fungal cells of which cell wall has been at least partially removed” refer to the spheroplasts or spheroplast-like cells of the fungal cells. Specifically, typical fungal cells of which cell wall has been substantially removed are the protoplasts

of the fungal cells, and typical fungal cells of which cell wall has been at least partially removed are the spheroplasts of the fungal cells. Accordingly, the phrase "insoluble fraction obtainable from fungal cells of which cell wall has been substantially removed or at least partially removed" means that the insoluble fraction is obtainable from the protoplasts, spheroplasts, or the like, of the fungal cells.

[0073] The phrase "the cell wall has been at least partially removed" means that cell wall-constituents, for example, mannan or glucan, are removed to an extent that the function of the cell wall such as morphological maintenance or osmotic pressure resistance to hypotonic solutions is lost, and that at the same time the cell wall is removed to an extent so as to at least not to cause any adverse effects of the cell wall component. In the present invention, it is preferable to use the fungal cells of which cell wall has been substantially removed. However, the fungal cells used may have cell wall components partially remaining therein, as long as the components derived from the cell wall do not give any adverse effects, such as hypersensitivity or lethality, on the living body, when administered to the living body. Specifically, the insoluble fraction contains relatively large intracellular structures, such as cell membranes, cell organelle (mitochondria, nuclei, lysosome, vacuoles, etc.), and cell organelle membranes; a protein bound to the cell membrane; and a protein bound to the cell organelle membrane. The insoluble fraction in the present invention needs not contain all the above-mentioned components, as long as it contains at least one of the components.

[0074] This insoluble fraction may further contain phospholipids, glycolipids, and other lipids, sugars, nucleic acids, etc. Moreover, in a case where the fungal cells of which cell wall partially remains therein are used, when the fungal antigen of the present invention is administered to the living body, it may contain components derived from the cell wall, as long as the components do not give any adverse effects, such as hypersensitivity or lethality, quantitatively or qualitatively on the living body. The amount of contamination with these antigenic components derived from the cell wall can, for example, be quantified by determining the inhibitory activity against an agglutination reaction using an antiserum against the cell wall component as described in Examples below.

[0075] The insoluble fraction in the present invention can be obtained, for example, by bursting the fungal cells of which cell wall has been substantially removed or at least partially removed. Further, a precipitate fraction obtainable by centrifuging the component thus obtained from bursting at about 100,000×g can also be used as the insoluble fraction.

[0076] Furthermore, the fungal antigen of the present invention may be a solubilized fraction extracted and separated from the insoluble fraction in the present invention. The solubilized fraction mainly contains antigenic soluble proteins. In addition, sugars and lipids may also be contained therein. The solubilized fraction can, for example, be sterilized by filtration in the purification step, thereby making it possible to prepare antigenic components, which are labile to sterilization procedures by heating or with organic solvents, with maintaining the activity in the solubilizing step. Such a solubilized fraction can be obtained by extraction and separation with a buffer containing a solubilizer, for example, a buffer containing a surfactant.

[0077] Furthermore, the fungal antigen of the present invention may be a fraction obtained by further purifying an insoluble fraction or solubilized fraction by a means of separation and purification appropriate for the purpose. For example, a fraction containing a molecule having binding ability to a sugar group-specific affinity medium is obtained by treating with the adsorbent a solubilized fraction from *Candida albicans* TIMM 1768 used as a starting material, and the fraction can also be used as the fungal antigen of the present invention. The sugar group-specific affinity medium includes, for example, immobilized concanavalin A (ConA) media. Because ConA binds to molecules containing α-D-mannopyranose, α-D-glucopyranose, or a sterically similar sugar residue thereof, components contained in the solubilized fraction can be further separated into some fractions on the basis of differences in the sugar residue contained in each component by using ConA-immobilized resin. For example, a ConA-binding fraction separable from the *Candida albicans* TIMM 1768 solubilized fraction (fraction having high content in proteins having ConA-binding sugar residues) exhibits sufficient protection activity against infection when administered to the mouse.

[0078] On the other hand, various fungal antigens of the present invention are present in fractions comprising molecules not having binding ability to sugar group-specific affinity media. In other words, the fungal antigen of the present invention also includes a fraction obtained as described above comprising molecules not having binding ability to sugar group-specific affinity media, and may include a fraction obtained by further purifying such a fraction. For example, by further subjecting a ConA-unbindable fraction derived from *Candida albicans* TIMM 1768 to ion exchange chromatography etc., purified fragments containing an antigenic protein having the partial amino acid sequence as shown by SEQ ID NO: 1 in Sequence Listing and a molecular weight of about 65,000 (SDS-PAGE, under reduced conditions); an antigenic protein having the partial amino acid sequence as shown by SEQ ID NO: 2 in Sequence Listing and a molecular weight of about 25,000 (SDS-PAGE, under reduced conditions); an antigenic protein having the partial amino acid sequence as shown by SEQ ID NO: 3 in Sequence Listing and a molecular weight of about 30,000 (SDS-PAGE, under reduced conditions); an antigenic protein having the partial amino acid sequence as shown by SEQ ID NO: 4 in Sequence Listing and a molecular weight of about 62,000 (SDS-PAGE, under reduced conditions); an antigenic protein having the partial amino acid sequence as shown by SEQ ID NO: 14 in Sequence Listing and a molecular weight of about 35,000 (SDS-PAGE, under reduced conditions); and an antigenic protein having the partial amino acid sequence as shown by SEQ ID NO: 15 in Sequence Listing and a molecular weight of about 55,000 (SDS-PAGE, under reduced conditions) can be obtained. The purified fraction or each isolated antigenic protein which is used as the fungal antigen of the present invention is useful in the therapy and diagnosis of diseases caused by fungi. These isolated antigenic proteins, in particular, are useful in identification of causative antigens etc. in diagnosis.

[0079] These antigenic proteins are derived from *Candida albicans* and possess vaccine activity against infectious diseases caused by *Candida albicans* or possess allergen activity useful in the prevention and therapy of allergic symptoms caused by *Candida albicans*. The term "vaccine

activity," as used in the present specification, means that the vaccine prepared by a conventional method using the fungal antigen of the present invention exhibits a pharmacological action effective as a vaccine. The term "allergen activity" means that an abnormally high value is obtained in an IgE antibody titer measurement test against the fungal antigen of the present invention by RAST etc. using a serum from a patient with allergosis, or a positive reaction is shown in a skin test using the fungal antigen of the present invention.

[0080] Furthermore, in the present invention, functional equivalents possessing properties immunologically equivalent to those of isolated antigenic proteins as described above are also encompassed in the scope of the fungal antigen of the present invention. For example, functional equivalents of various strains of *Candida albicans*, and fungi of *Candida* other than *Candida albicans*, are also encompassed in the present invention. More specifically, among the above-described six kinds of antigenic proteins, the antigenic protein having a molecular weight of about 65,000 has homology with the dihydrolipoamide dehydrogenase (DLDH) of *Saccharomyces cerevisiae* localized in mitochondria; the antigenic protein having a molecular weight of about 25,000 has homology with the superoxide dismutase (SOD) of *Saccharomyces cerevisiae* localized in mitochondria; the antigenic protein having a molecular weight of about 30,000 has homology with the citrate synthase of *Saccharomyces cerevisiae*; the antigenic protein having a molecular weight of about 62,000 has homology with the vacuolar aminopeptidase I of *Saccharomyces cerevisiae*; and the antigenic protein having a molecular weight of about 35,000 has homology with malate dehydrogenase of *Saccharomyces cerevisiae*. Antigens having equivalent immunological properties, such as vaccine activity and/or allergen activity, to malate dehydrogenase are also encompassed in the present invention. The antigenic protein having a molecular weight of about 55,000 has the partial amino acid sequence as shown by SEQ ID NO: 15 in Sequence Listing, and the antigenic protein was identified as catalase based on the fact that the above partial amino acid sequence is identical with the amino acid sequence of 2nd to 31st residues starting from the N-terminal of the protein consisting of 487 amino acids encoded by a catalase gene CAT1 [*"Infection Immunity,"* Vol. 66, 1953-1961 (1998)] which has been previously cloned. Incidentally, the reactivity of the protein encoded by CAT1 and anti-*Candida* serum has not been known, and the antigens having equivalent immunological properties, such as vaccine activity and/or allergen activity, to proteins encoded by CAT1 are also encompassed in the present invention.

[0081] The phrase "functional equivalent possessing immunologically equivalent properties," as used herein, is defined as a protein with substitution, insertion, deletion, or addition of one or more amino acids of which the immunological properties, such as vaccine activity and/or allergen activity, are equivalent to the above.

[0082] Also, an antigenic fragment can also be prepared based on an isolated antigenic protein. An antigenic fragment can, for example, be prepared by cleaving an isolated antigenic protein as the starting material by enzymatic digestion with a protease, such as lysyl endopeptidase or trypsin, or by chemical treatment with cyanogen bromide etc., and then isolating and purifying a fragment possessing the desired antigenicity by a known method for protein

purification. It is also possible to produce an antigenic fragment by chemical synthesis using peptide synthesis technology, on the basis of the information on the chemical structure of the antigenic fragments. The antigenic fragment of the present invention includes fragments of a fungi-derived antigenic protein that cause immune responses in mammals, especially in humans, including for instance, minimal level of IgE stimulation, IgE binding, and induction of IgG and IgM antibody production, or T cell responses, such as proliferation, and/or lymphokine secretion and/or T cell anergy induction.

[0083] The antigenicity of an antigenic fragment can also be evaluated by in vitro tests, such as RAST, ELISA, and histamine release tests, in addition to skin tests and intradermal tests in human volunteers.

[0084] Incidentally, for the purpose of increasing fungal antigen stability and/or increasing desired reactivity, i.e., enhancing the induction of individual protective immunity, attenuating allergic reactions, or inactivating enzymes, for therapeutic purposes, and enhancing specific antigen-antibody binding for diagnostic purposes, it is possible to modify an antigenic protein or antigenic fragment to a derivative thereof, or to bind it with polyethylene glycol (PEG) using the PEG method [Wie et al., *Int. Arch. Allergy Appl. Immunol.*, Vol. 64, 84-99 (1981)]. Protein modifications include pyridylethylation, reduction, alkylation, acylation, chemical coupling to appropriate carriers, mild formalin treatment, and guanidine hydrochloride treatment.

[0085] Alternatively, based on the information of a partial amino acid sequence for the above isolated antigenic protein nucleic acids encoding the antigen can be isolated by PCR and the like. An example thereof is described as follows:

[0086] First, cDNA library is prepared from cells expressing a desired antigenic protein. Next, PCR is carried out with genomic DNA for the cell expressing the antigenic protein as a template, by using an oligonucleotide usable for an amplification primer which is designed based on the nucleotide sequence of the nucleic acid which is deduced to encode a partial amino acid sequence of an antigenic protein; and a suitable oligonucleotide capable of forming an amplification primer pair with the above oligonucleotide for the above nucleic acid. A DNA encoding the desired antigenic protein can be selected from the cDNA library by hybridization using a DNA fragment obtained by the above PCR. For example, a DNA having the nucleotide sequence as shown by SEQ ID NO: 7 in Sequence Listing encoding a protein having the amino acid sequence as shown by SEQ ID NO: 5 can be isolated by the above method using the amino acid sequence information as described by SEQ ID NO: 1 in Sequence Listing, cDNA library of *Candida albicans* TIMM 1768, and genomic DNA of *Candida albicans* TIMM 1768.

[0087] In addition, nucleic acids encoding the antigenic protein can be isolated by RT-PCR using RNA from cells expressing the desired antigenic protein and amplification primers designed based on nucleotide sequences of a nucleic acid, the sequence being deduced to encode a partial amino acid sequence, and the like. For example, a DNA having the nucleotide sequence of SEQ ID NO: 8 in Sequence Listing encoding a protein having the amino acid sequence as shown by SEQ ID NO: 6 in Sequence Listing can be isolated by the above method using an amino acid sequence information as

described by SEQ ID NO: 2 in Sequence Listing and an RNA from *Candida albicans* TIMM 1768.

[0088] Incidentally, in the present invention, nucleic acids encoding a fungal antigen comprising a protein having the amino acid sequence as described by SEQ ID NO: 5 in Sequence Listing are not particularly limited to nucleic acids having the nucleotide sequence as shown by SEQ ID NO:7. Similarly, nucleic acids encoding a fungal antigen comprising a protein having amino acid sequence as shown by SEQ ID NO: 6 in Sequence Listing are not particularly limited to nucleic acids having the nucleotide sequence as shown by SEQ ID NO: 8 in Sequence Listing. Specifically, with regard to the codon designating an amino acid on a gene (triplet base combination), 1 to 6 kinds are known to exist for each kind of amino acids. Therefore, a large number of nucleic acids encoding an amino acid sequence can exist depending on the amino acid sequence. In nature, the nucleic acid is not stable, and it is not unusual that nucleic acid variations occur. A mutation on the nucleic acid may in some cases not cause a change of the amino acid sequence to be encoded (silent mutation). In this case, it can be said that different nucleic acids encoding the same amino acid sequence have been produced. Therefore, a possibility cannot be negated where even when a nucleic acid encoding a particular amino acid sequence is isolated, a variety of nucleic acids encoding the same amino acid sequence are produced with generation passage of the organisms containing the nucleic acids. Moreover, it is not difficult to artificially produce a variety of nucleic acids encoding the same amino acid sequence by means of various genetic engineering procedures.

[0089] For example, in the production of the protein by genetic engineering, when a codon used in the natural gene encoding the desired protein is low in usage in the host utilized, the amount of the protein expressed is sometimes low. In such a case, a high level expression of the desired protein is achieved by artificially converting the codon into another one of high usage in the host without changing the amino acid sequence encoded (for example, Japanese Examined Patent Publication No. Hei 7-102146). It is of course possible to artificially prepare a variety of genes encoding a particular amino acid sequence.

[0090] Furthermore, nucleic acids encoding the fungal antigen in the present invention encompass nucleic acids being capable of hybridizing to a nucleic acid comprising the entire sequence of the nucleotide sequence of SEQ ID NO: 5 or 6 in Sequence Listing, or a partial sequence thereof, and the peptide encoded by the above nucleic acid has a vaccine activity or an allergen activity equivalent to the fungal antigen of the present invention. As to the term "capable of hybridizing," the following conditions may be exemplified:

[0091] Specifically, a DNA-immobilized membrane is incubated with a probe at 50° C. for 12 to 20 hours in 6×SSC, wherein 1×SSC indicates 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0, containing 0.5% SDS, 0.1% bovine serum albumin (BSA), 0.1% polyvinyl pyrrolidone, 0.1% Ficol 400, and 0.01% denatured salmon sperm DNA. After termination of the incubation, the membrane is washed, initiating at 37° C. in 2×SSC containing 0.5% SDS, and changing the SSC concentration to 0.1×SSC and the SSC temperature to 50° C., until a signal from the immobilized DNA becomes distinguishable from the background.

[0092] When the above nucleic acid is used, an antigenic protein can be prepared by genetic engineering procedure as a recombinant protein in *Escherichia coli*, yeast, fungus, mammalian cells, or the like. In addition, an antigenic fragment of the above antigenic protein can be prepared by genetic engineering procedure by the use of a partial portion of the above nucleic acid.

[0093] When the above gene information can be obtained, a functional equivalent of the antigenic protein can be obtained by modifying a structure of the antigenic protein by a known method using mutagenesis at a particular site on the nucleic acid encoding an antigenic protein. For example, substitution, insertion, deletion or addition of amino acid residues can occur by substitution, insertion, deletion or addition of one or more of bases for nucleic acids encoding a protein. Specifically, the fungal antigens comprising a peptide resulting from at least one of deletion, addition, insertion or substitution of one or more of amino acid residues in the amino acid sequence comprising an amino acid sequence as shown by SEQ ID NO: 5 or SEQ ID NO: 6 in Sequence Listing or a partial portion thereof, and the peptide having the vaccine activity or the allergen activity, which is a mutant of the antigenic protein of the present invention and an example of a functional equivalent, are also included in the scope of the present invention. In addition, a mutant retaining to have the biological activity can be selected.

[0094] The gapped duplex method [Wilfried, K. et al., *Nucleic Acids Research*, Vol. 12, 24, 9441-9456, (1984)], the deletion method [Celeste, Y. P. et al., *Gene*, Vol. 33, 103-119, (1985)], the PCR method [*Gene*, Vol. 102, 67-70, (1991)], the uracil DNA method [Thomas, A. K. et al., *Methods in Enzymology*, Vol. 154, 367-382, (1987)] and the cassette mutation method [James, A. W. et al., *Gene*, Vol. 34, 315-323, (1985)] and the like are known as the methods for preparing the mutant.

[0095] The toxicity of the fungal antigen of the present invention (Ca-LSP in Example 1) is low, so that no abnormalities are observed even when intravenously administered to the mouse at 20 mg/kg.

3. Process for Producing Fungal Antigen

[0096] A process for producing fungal antigen which is an insoluble fraction obtainable from fungal cells of which cell wall has been substantially removed or at least partially removed include, for example, a process comprising the steps of:

[0097] (1) obtaining living fungal cells;

[0098] (2) obtaining fungal cells of which cell wall has been substantially removed or at least partially removed;

[0099] (3) bursting the fungal cells of which cell wall has been substantially removed or at least partially removed; and

[0100] (4) obtaining an insoluble fraction.

Step (1)

[0101] Step (1) comprises obtaining living fungal cells. More specifically, step (1) comprises culturing a fungus in a culture medium suitable for its growth, and obtaining fresh living fungal cells.

[0102] First, fungal cultivation can be carried out under temperature and other conditions in which fungi can grow in a nutrient medium containing carbon sources, nitrogen sources, and other nutrient sources suitable for each fungus. As the nutrient media usually usable for fungal cultivation, Sabouraud medium, Potato-Dextrose medium, Czapek-Dox medium, malt medium, yeast nitrogen base glucose chemically defined medium, and the like can be widely used, and serum and/or serum albumin may be added as necessary. Also, there are some fungi of which growth is suited in media supplemented with olive oil or the like, like *Malassezia furfur*. Although the culturing temperature is usually from about 15° to about 45° C., some fungi show morphological changes depending on the culturing temperature (many of which are known as dimorphic fungi), and an appropriate selection of a culturing temperature is necessitated. For instance, in the case of *Candida albicans*, for which preferably employable culturing temperature is in the range from 25° to 37° C., yeast-phase growth takes place at about 30° C. when cultured in usual media, whereas mycelial-phase growth is likely to take place around 37° C. For dimorphic fungi, culturing conditions may be altered according to the purpose, since changes also occur in cell wall components, and protein components, such as intracellular proteins, including membrane proteins. Many fungi aggregate or form lumps of cells, to give a nonuniform cell suspension, under ordinary culturing conditions, in which case the cell wall lytic enzyme etc. cannot sufficiently act on the fungus in the subsequent step. Therefore, in order to obtain a cell suspension as uniform as possible, the culturing method may be modified. In the case of *Aspergillus fumigatus*, for example, this problem can be solved by increasing the salt concentration by adding 0.5 to 1 M NaCl, or the like to a medium. Also, the fungus can be exemplified by the fungi described above.

Step (2)

[0103] Step (2) comprises obtaining fungal cells of which cell wall has been substantially removed or at least partially removed. Although the cell wall may be removed to an extent to at least show sensitivity to osmotic pressure, it is preferable that the cell wall is further removed to an extent of protoplast formation. Therefore, the fungal cells of which cell wall has been substantially removed or at least partially removed are preferably the protoplasts or spheroplasts of the fungal cells.

[0104] The fungal cells of which cell wall has been substantially removed or at least partially removed, can, for example, be obtained by allowing a cell wall lytic enzyme to act on the fungal cells, or by physically treating the fungal cells. The cell wall lytic enzyme treatment and the physical treatment may be used in combination.

[0105] There are various cell wall lytic enzymes known to date, commercial products including ZYMOLYASE (manufactured by Seikagaku Corporation), Lyticase (manufactured by Sigma), Yatalase (manufactured by Ozeki Corporation-Takara Shuzo Co., Ltd.), Chitinase (manufactured by Takara Shuzo Co., Ltd.), *Trichoderma* Lysing Enzyme (manufactured by Novo-Sigma), snail intestinal digestion enzyme β-glucuronidase (manufactured by Sigma), and Laminariase (manufactured by Sigma). These enzymes comprise lytic enzymes for various cell wall polysaccharides (chitin, β1,3-glucan, mannan, galactomannan, xyloglucan, etc.), many of which further contain proteases.

[0106] In order to lyse the cell wall of fungal cells and prepare naked cells sensitive to osmotic pressure, e.g., protoplasts, firstly fresh cells obtained by culturing are washed, and then suspended in a hypertonic buffer containing 0.8 to 1.5 M sorbitol, mannitol, or NaCl. A required amount of the cell wall lytic enzyme at temperature, buffer, and pH conditions suitable for the enzyme is acted on the suspension for 10 minutes to several hours to remove the cell wall. In this operation, the cell wall can be more completely removed by allowing to act a protease thereon in some cases. Some fungi do not necessitate protease action, in which case a protease inhibitor, such as PMSF or pepstatin, may be added.

[0107] The physical treatment can, for example, be carried out by suspending subject cells in a hypertonic buffer such as a 2.5 M sucrose solution to cause plasmolysis, and cutting off the cell wall with a knife.

Step (3)

[0108] Step (3) comprises bursting the fungal cells of which cell wall has been substantially removed or at least partially removed obtainable in step (2). Methods for cell bursting include, for example, ultrasonication, French press treatment, and hypotonic solution treatment utilizing differences in osmotic pressures. The bursting with hypotonic solution treatment can be carried out by sufficiently washing cells with a hypertonic solution, and then suspending the cells in a hypotonic solution, i.e., physiological saline or a buffer of low ionic strength (e.g., physiological saline in the case of *Candida albicans* TIMM 1768). The usable buffers include, for example, phosphate buffers and citrate buffers, each having a pH of 5 to 8. In order to recover the cell organelles as intact as possible, ionic strength can be selected. For example, in order to prepare mitochondria in a condition ensuring similar functions to that in the cells, cells of which cell wall has been substantially removed or at least partially removed are burst by treating the cells by means of ultrasonic, a Waring blender, a French press, or the like in a buffer containing 0.5 to 0.6 M sorbitol or 0.25 M sucrose to thereby obtain mitochondria in a state having similar functions to the cells.

Step (4)

[0109] Step (4) comprises obtaining an insoluble fraction.

[0110] The component obtainable by bursting obtained in step (3) is centrifuged or filtered to yield a precipitate or residue, which is taken as the insoluble fraction. The component obtained by bursting may be further finely disintegrated using ultrasonic or glass beads as occasion demands.

[0111] Although centrifugal conditions for obtaining the insoluble fraction are not particularly limited, it is preferable that centrifugation is carried out at about 100,000×g or less, more preferably 10,000×g or less, and that the centrifugation time is from 10 minutes to 3 hours.

[0112] Components recoverable as precipitates by centrifugation at 10,000×g or less are cytoplasmic membranes, and cell organelles, such as mitochondria, nuclei, lysosome, and vacuoles. Cytoplasmic membrane proteins and cell organelle membrane proteins can be obtained as precipitates in which the protein is bound to the membrane.

[0113] When centrifuged at 100,000×g for about one hour, ribosome is also recovered as a precipitate, which may be

contained in the insoluble fraction. Centrifugal conditions may be altered to separate individual cell organelles to some extent. For example, centrifugation at about 1,000 $\times g$ allows to separate nuclei. Also, the above-mentioned cell organelles can be separated and purified by density gradient centrifugation using sucrose etc. It is also possible to recover the insoluble fraction by filtration, and to classify it according to its particle size to some extent.

[0114] Because the insoluble fraction thus obtained is from the fungal cells of which cell wall has been substantially removed or at least partially removed, such as the protoplasts or spheroplasts of the fungal cells, the amount of cell wall components which can be contained in the insoluble fraction is low. For example, the amount of cell wall component contained in the insoluble fraction of the present invention can be quantified by utilizing an antigen-antibody reaction in which the cell wall component is taken as the antigen. More specifically, as described in Examples detailed below, when the fungal cells used are *Candida albicans* TMM 1768, for example, the serotype A mannan in the insoluble fraction can be quantified using serum factor No. 1 (manufactured by IATRON LABORATORIES, Inc.), which is an anti-*Candida* serum. The amount of serotype A mannan thus determined is preferably not greater than the detection limit (0.5 mg/ml).

[0115] The insoluble fraction obtainable as described above can also be washed and sterilized with an organic solvent, such as ethanol, isopropanol, phenol, or acetonitrile, or sterilized by heat treatment.

[0116] The insoluble fraction in the present invention can be obtained as described above. Also, in the present invention, a solubilized fraction obtainable by extracting and separating the insoluble fraction also serves as a fungal antigen. The solubilized fraction can, for example, be obtained by a process comprising the following steps:

[0117] (1) obtaining living fungal cells;

[0118] (2) obtaining fungal cells of which cell wall has been substantially removed or at least partially removed;

[0119] (3) bursting the fungal cells of which cell wall has been substantially removed or at least partially removed;

[0120] (4) obtaining an insoluble fraction; and

[0121] (5) extracting and separating a solubilized fraction from the insoluble fraction.

[0122] In the present invention, the solubilized fraction can be further separated and purified in step (6) by conventional means of separation and purification according to the purpose, as desired.

[0123] Of the above steps, steps (1) through (4) are the same as those for the process for producing an insoluble fraction. It should be noted, however, that although the cell wall component in the insoluble fraction usable in these steps is preferably removed to an extent that the insoluble fraction can be used clinically, this extent needs not always be the same level as the extent where the insoluble fraction per se is used as the fungal antigen. This is because the cell wall of fungal cells is rich in glucan, chitin, or the like, some of which components are insoluble, for instance, by surfactants and can be removed in the subsequent step comprising obtaining a solubilized fraction. Steps (5) and (6) will be hereinafter described.

Step (5)

[0124] Step (5) comprises extracting and separating a solubilized fraction from the insoluble fraction. For the extraction and separation, those generally used in methods for solubilization can be used. The solubilizers include, for example, salts, such as NaCl and KCl; chelating agents, such as EDTA; organic solvents, such as butanol; and buffers in which a protein denaturant, such as urea, is dissolved therein, it is preferable from the viewpoints of solubilized component stability and extraction efficiency that a buffer containing a surfactant is used. If satisfactory extraction effects cannot be obtained, the above-mentioned organic solvents and protein denaturants may be used in combination. Generally, a solubilized fraction can be obtained by suspending the insoluble fraction obtainable in step (4) in a buffer containing an appropriate solubilizer, such as a surfactant, for a given period of time, and then removing the insoluble components by centrifugation and/or filtration. The term "solubilized fraction," as used herein, is understood to include water-soluble components accompanying the insoluble fraction, for example, intraorganellar water-soluble components, and/or components solubilized by solubilizing treatment, including, for example, cytoplasmic membrane proteins and lipids. Also, when using a clinically usable surfactant, the solubilized fraction per se can be used as a fungal antigen without removing the surfactant.

[0125] The surfactant usable in solubilization of the membrane proteins etc. contained in the insoluble fraction usable in the present invention is preferably octylthioglucoside, Lubrol PX, Triton X-100, sodium lauryl sulfate (SDS), Nonidet P-40, and the like. The clinically usable surfactants include ionic (anionic, cationic, amphoteric) surfactants (e.g., alkyl sulfonates, benzalkonium chlorides, and the like) and nonionic surfactants (e.g., polyoxyethylene hydrogenated castor oils, polyoxyethylene sorbitol fatty acid esters, polyoxyethylene sorbitan fatty acid esters, polyoxyethylene glycerol fatty acid esters, polyethylene glycol fatty acid esters, polyoxyethylene alkyl phenyl ethers, and the like). The surfactant used in the present invention is preferably a nonionic surfactant. The polyoxyethylene hydrogenated castor oils include, for example, NIKKOL HCO-40, HCO-50, and HCO-60 (manufactured by Nikko Chemicals) and Uniox HC-40, HC-50, and HC-60 (manufactured by NOF Corporation).

[0126] The polyoxyethylene sorbitol fatty acid esters include, for example, NIKKOL GO-430, GO-440, GO-460, GL-1, Atlox 1045A, 1196, G-1045, and G-1441 (manufactured by Kao Atlas). The polyoxyethylene sorbitan fatty acid esters include TWEEN 20, TWEEN 40, TWEEN 60, TWEEN 80, EMASOL 1130, EMASOL 3130, NIKKOL TL-1010, TP-10, TS-10, and the like. The polyoxyethylene glycerol fatty acid esters include, for example, NIKKOL TMGS-15, TMGS-5, and the like. The polyoxyethylene glycol fatty acid esters include, for example, NIKKOL MYL-110, MYS-10, and the like. The polyoxyethylene alkyl phenyl ethers include NIKKOL NP-10, EMULGEN 810, and the like. Incidentally, in the case where surfactants such as SDS having a high protein solubilizing ability but limited for their clinical use are used, the antigenic components and the surfactant may be separated by a subsequent appropriate treatment as occasion demands.

[0127] From the viewpoint of sustaining antigenicity, etc., it is a matter of course to select an optimum kind of a

surfactant and an optimum concentration thereof for soluble components. Generally, the surfactant is effective as long as the concentration of the surfactant is equal to or higher than the level at which the surfactant forms a micelle when dissolved in an aqueous solvent, i.e., equal to or higher than the critical micellar concentration (hereinafter referred to as "CMC"). The surfactant is preferably used at concentrations of the CMC or higher and up to 10 times the CMC, with especially good action when solubilized at concentrations from the CMC to 5 times the CMC. The buffers include phosphate buffers and Tris-HCl buffers.

[0128] The solubilization is usually carried out by allowing the insoluble fraction to stand, or stirring the insoluble fraction, at a low temperature of about 4° C. for one hour to overnight. In this operation, a protease inhibitor may be added. The solubilized fraction can, for example, be obtained as a supernatant of the centrifuged solubilization treatment liquid at about 100,000×g for about one hour, or as a filtrate of the filtered solubilization treatment liquid. It is also possible to remove the solubilizer used for solubilization by dialyzing the supernatant or filtrate against a solubilizer-free buffer or a buffer containing a clinically usable surfactant; or adding an organic solvent such as ethanol or acetone, allowing to make the protein insoluble and form sedimentation, and collecting the sedimentation by centrifugation, or the like. The solubilized fraction may also be washed and sterilized with an organic solvent, such as ethanol, isopropanol, phenol, or acetonitrile, or sterilized by heat treatment.

[0129] In addition, when the solubilized fraction is dialyzed against a solubilizer-free buffer, a portion of hydrophobic components, including lipids, is obtained as precipitates. These precipitate components and solution components are all encompassed in the scope of the solubilized fraction in the present specification.

[0130] In the present invention, as step (6), the solubilized fraction may be further purified by conventional means of separation and purification according to its purpose, including, for instance, means of separation and purification based on differences in component affinity, charged states, molecular weights, hydrophobicity, and the like as desired. For example, the solubilized fraction can be purified by fractionation based on differences in the sugar residues contained in the glycoprotein with a sugar group-specific affinity medium. The sugar group-specific affinity media include, for example, immobilized lectin media. In particular, preference is given to ConA-bound resins for the separation of a component having a ConA-binding sugar residue (α -D-glucose residue and α -D-mannose residue of which C-3, C-4, and C-6 hydroxyl groups are unsubstituted), e.g., a glycoprotein, which can be found in many of fungi, rich in ConA-binding mannose residues. For purification, it is desirable to use a buffer according to its purpose, and a surfactant, an organic solvent, and the like may be also added. The degree of purification may be increased using an ion exchange resin or gel filtration carrier.

[0131] Also, in the present invention, the fungal antigen of the present invention can easily be produced by general genetic engineering techniques using a nucleic acid encoding the fungal antigen of the present invention described above.

4. Biologic Products

[0132] The biologic product of the present invention contains the fungal antigen described above as an active ingredient. A biologic product is a vaccine or similar preparation derived from a pathogenic microorganism of an infectious disease, and used to diagnose, prevent or treat a disease or a disorder. In the present invention, the fungus is used as the starting material therefor. Besides, the biologic product containing therapeutic sera or the like obtainable by using the antigen of the present invention is also included. Among them, the fungal antigen of the present invention, which contains a large number of kinds of fungal proteins, is capable of inducing acquired immune in vertebrates, so that it can particularly preferably be used in a vaccine composition. In other words, the vaccine preparation of the present invention, having protective immunity against infection or therapeutic effects against a mycotic infectious disease in vertebrates, contains the fungal antigen described above as an active ingredient. The fungal antigen contained in the biologic product or vaccine composition of the present invention as an active ingredient can, for example, be obtained by the production method described above. Incidentally, in the present specification, a vaccine composition is simply referred to as a vaccine in some cases.

[0133] When the fungal antigen of the present invention is used as a vaccine composition, in order to get more potent humoral and/or cellular immunity, it is preferable to administer the fungal antigen in the form of preparation of a suspension or solution containing an adjuvant as described below. Although the adjuvant is usually administered together with the antigen, the adjuvant may be administered before or after antigen administration. The adjuvants suitable for vaccination for mammals include complete or incomplete Freund's adjuvant; gels made of inorganic substances such as aluminum hydroxide and alum; surfactants, such as lysolecithin, dimethyloctadecyl ammonium bromide and lysolecithin; polyanions, such as dextran sulfate and poly-IC; peptides, such as muramyl dipeptide and tuftsin; Monophosphoryl Lipid A (MPL) manufactured by Ribi; TiterMax, manufactured by CytRx; cholera toxin (CT); B subunit of CT; heat-labile toxin (LT), without being limited thereto. The antigen can also be administered by incorporating it in a liposome or other microcarriers. As a matter of course, antigens of some different fungi can also be used in admixture, whereby protective immunity against a plurality of mycotic infectious diseases is induced. The vaccine composition of the present invention may be used in combination with antifungal agents, such as fluconazole and amphotericin B, and β -lactam antibiotics and other various antibacterial antimicrobial agents. The vaccine composition of the present invention exhibits an additively or geometrically enhanced effectiveness when used in combination with an antifungal agent.

[0134] Vertebrates are fish, amphibians, reptiles, birds, humans, and mammals except humans, which produce antibodies in reaction with antigens, so that all vertebrates are capable of reacting with vaccines. Although vaccines are generally applied to mammals, such as humans or domestic animals, vertebrates, e.g., fish cultured for commercial purposes, are encompassed in the scope of the present invention, as long as they possess the above-described properties.

[0135] As the route of administration, the fungal antigen of the present invention may be administered orally, trans-

mucosally (e.g., nasally, intravaginally), percutaneously (subcutaneously or intracutaneously), or intravenously. Representative initial doses are 0.001 to mg/kg body weight as an amount of protein, and depending upon to the degree of prevention or therapy required the dose can be increased, or the number of administration can be increased.

[0136] When an insoluble fraction or a solubilized fraction derived therefrom, which is the fungal antigen of the present invention, is administered, potent cellular immunity and/or humoral immunity can be induced, whereby fungal infection can be prevented or treated. The protective effects and therapeutic effects can be induced not only against the fungus of interest for protection or therapy but also against other fungi though with some insufficiency. This is presumably due to the fact that commonness of antigens among fungi and/or activation of the immune system induce release of superoxide anions, nitric oxide, and various cytokines, which possess a broad spectrum of antimicrobial activity.

[0137] In addition, the present invention provides 1) a pharmaceutical composition for inducing protective immunity against fungi or exhibiting therapeutic effects by administering to individuals, characterized in that the pharmaceutical composition contains the fungal antigen described above, or a fungal antigen produced by the process described above; 2) a vaccine composition for inducing protective immunity against fungi or exhibiting therapeutic effects by administering to individuals, characterized in that the vaccine composition contains the fungal antigen described above, or a fungal antigen produced by the process described above; 3) a method of stimulating immune responses against fungi in a vertebrate, comprising the step of administering the above vaccine composition; and 4) a method of stimulating immune responses against fungi in a vertebrate, wherein proliferation of fungi used in the preparation of the vaccine composition and/or fungal strains closely related thereto is suppressed by the immune responses in a vertebrate to which the vaccine composition is administered, to prevent or treat diseases caused by the fungi.

[0138] The fungal antigen of the present invention can be used in the form of a biologic product, such as a cytokine releasing agent, and an allergen composition usable for desensitization therapy for allergoses and other purposes, as well as the above-described vaccine composition. Further, the fungal antigen of the present invention can also be used for in vivo diagnosis and/or laboratory diagnosis for determination of past history of infection by skin reactions, allergosis diagnosis by scratch tests, and for other purposes. Preparations used for laboratory diagnosis include, for example, immunological diagnostic agents, such as micro-titer reagents, latex agglutination reagents, immunonephelometric reagents, and enzyme immunoassay reagents.

[0139] When used to an individual, the cytokine releasing agent of the present invention can be used in the form of a lyophilized powder or an appropriate salt solution or suspension, or a suspension or solution containing the above-described adjuvant. The cytokine releasing agent can also be used as a therapeutic agent for a disease on which the released cytokine is effective. For example, when the cytokine released is IFN- γ , the cytokine releasing agent can be used for a therapeutic agents for cancers, bacterial infectious diseases, and allergoses.

[0140] As the route of administration, it may be administered percutaneously (subcutaneously or intracutaneously),

nebulized via intrapulmonary, administered transmucosally (e.g., via nose, eye, vagina, or the like), orally, subglossally, or intravenously. For example, a representative dose for treating cancer is 0.02 μ g to 1 mg/kg per administration in the case of humans, and depending upon to the diseases treated and purposes required, the dose can be increased, or the number of administration can be increased. For example, the dose can be increased to a level of about 100 mg/kg per administration.

[0141] When the allergen composition of the present invention is administered to a patient for the purpose of preventing or treating allergosis, the allergen composition can be used in the form of an appropriate salt solution or suspension, and may be supplemented with polyethylene glycol or phenol. Further, it can also be administered as the suspension or solution containing an adjuvant usable for making vaccine preparations for mammals as described above. The adjuvant can be usually administered together with an antigen, and it may be given before or after antigen administration. The antigen can also be administered by incorporating it in a liposome or other microcarriers. As a matter of course, an insoluble fraction or solubilized fraction thereof can be mixed with similar fractions from some different fungi, or also mixed with commercially available fungal allergen extracts, various allergen extracts, such as those of house dusts and *Cryptomeria japonica*, and/or with purified allergens. By the use of the mixture, desensitization immunity against a plurality of allergens can be induced in patients with allergoses sensitive to a plurality of allergens.

[0142] As the route of administration, it may be administered percutaneously (subcutaneously or intracutaneously), nebulized via intrapulmonary, administered transmucosally (e.g., via nose, eye, vagina, or the like), orally, subglossally, or intravenously. A representative initial dose for treating depends upon the route of administration, and is, for example, 0.2 ng to 0.1 mg/kg per administration, and depending upon the degree of prevention and therapy required the dose can be increased, or the number of administration can be increased.

[0143] In addition, the present invention provides 1) an allergen composition for preventing allergoses against fungi or exhibiting therapeutic effects by administering to individuals, characterized in that the allergen composition contains the fungal antigen described above, or a fungal antigen produced by the process described above; 2) a method of suppressing allergic reaction to fungi in a vertebrate, comprising the step of administering the allergen composition; and 3) a method of suppressing allergic reaction to fungi in a vertebrate, wherein allergoses caused by fungi used in the preparation of the allergen composition and/or fungal strains closely related thereto are prevented or treated by the immune responses in a vertebrate to which the allergen composition is administered.

[0144] When the fungal antigen of the present invention is used in an individual for the purpose of in vivo diagnosis, e.g., in inhalation challenging test, skin test, or nasal or eye mucosal test, it can be used in the form of a lyophilized powder or an appropriate salt solution or suspension, and polyethylene glycol and/or phenol may be added thereto. For patch tests, it is possible to use a solution of the above-mentioned antigenic component in a mixture of white petrolatum as a base material supplemented with a surfactant, such as sodium lauryl sulfate.

[0145] The fungal antigen of the present invention can also be used for laboratory diagnoses, e.g., diagnostic methods based on antigen-antibody reactions, such as agglutination, precipitation reaction, and neutralization reaction; diagnostic methods using labeled antibody; histamine release test; lymphocyte transformation test; and leukocyte migration inhibition test. For example, when used as an antigen for IgE antibody titer, the above-described antigen component can be used by immobilizing it on a solid phase, such as a paper disc, cellulose sponge, or microplate.

[0146] The present invention also provides 1) a diagnostic composition for a disease caused by fungi, characterized in that the diagnostic composition contains the fungal antigen described above, or a fungal antigen produced by the process described above; and 2) a method for diagnosing a disease caused by fungi in a vertebrate, comprising using the diagnostic composition above.

[0147] Vertebrates which are subjects in the present invention are fish, amphibians, reptiles, birds, humans, and mammals except humans, which produce antibodies in reaction with antigens, so that all vertebrates are capable of reacting with antigens. Although the fungal antigens of the present invention are generally applied to mammals, such as humans or domestic animals, vertebrates, e.g., fish cultured for commercial purposes, are encompassed in the scope of the present invention, as long as they possess the above-described properties.

EXAMPLES

[0148] The present invention will be described concretely by the working examples, without intending to limit the scope of the present invention to these examples.

Example 1

Preparation of Cell Fraction and Insoluble Fraction of *Candida albicans* Cells

[0149] 1) Preparation of protoplast cells: A platinum loopful of *Candida albicans* TIMM 1768 in Sabouraud agar slant culture was inoculated to an YPD medium (1% by weight yeast extract, 2% by weight polypeptone, 2% by weight glucose) in a test tube. After shaking culture at 30° C. for 24 hours, a portion of the culture was transferred to the YPD medium in an Erlenmeyer flask and subjected to shaking culture overnight at 35° C. The culture obtained was centrifuged at 2,000×g for 10 minutes to harvest the cells. The cells obtained were of an yeast phase. The cells were washed once with sterile water, and then washed once with an SSB solution (50 mM phosphate buffer, pH 7.5, containing 0.8 M sorbitol). After the cells were again suspended in an appropriate volume of the SSB solution, an SSB solution containing 100 mM EDTA in a volume of one-eighth that of the above SSB solution, and an appropriate volume of 2-mercaptoethanol were added thereto, followed by gentle shaking. Subsequently, to this suspension was added ZYMOL-YASE 20T (manufactured by Seikagaku Corporation) to make up a final concentration of 0.3 mg/ml, followed by gentle shaking at 35° C. for one hour. Further, *Trichoderma* Lysing Enzyme (manufactured by Sigma) was added to make up a final concentration of 1 mg/ml, followed by gentle shaking at 35° C. for one hour. The suspension obtained was centrifuged at 2,000×g for 10 minutes to

harvest the protoplast cells. The cells were sufficiently washed with the SSB solution and subjected to cell fractionation.

[0150] 2) Subcellular fractionation from protoplast cells and preparation of antigen solutions: To the protoplast cells obtained as described above was added sterile physiological saline to make up a cell density of about 4×10^9 cells/ml, followed by sufficient stirring, after which the mixture was allowed to stand on ice for 10 minutes. After having confirmed that the protoplast cells were burst, the mixture was centrifuged at 10,000×g for 30 minutes, and the precipitate obtained was taken as an insoluble fraction (hereinafter referred to as "Ca-LSP"). The centrifugal supernatant was further centrifuged at 100,000×g for 60 minutes. The precipitate obtained was taken as a ribosome fraction (hereinafter referred to as "HSP"), and the centrifugal supernatant as a soluble fraction (hereinafter referred to as "HSS," wherein HSP90 and enolase were contained in this fraction). After suspending the Ca-LSP in the physiological saline again, the Ca-LSP was subjected to ultrasonic treatment, and then sterilized in a boiling water bath for five minutes, to yield an LSP antigen solution containing a membrane protein, and the like. The HSP was also suspended in the physiological saline to make up an appropriate protein concentration, and this suspension was taken as an antigen solution. The HSS was also assayed for protein concentration, and an appropriate volume was taken as an antigen. The Ca-LSP antigen solution obtained by treating the cells obtained from a 2-liter culture as described above had a protein concentration of 2.3 mg/ml, wherein the amount of protein was quantified by using bicinchoninic acid (BCA) reagent with BSA as a standard).

[0151] 3) Confirmation for extent of cell wall removal of fungal cells: The extent of cell wall removal was confirmed by microscopic observation of cell morphology, by counting the number of living cells after bursting in physiological saline, and by a quantitation based on inhibition of agglutination with a serum factor by the antigen. In the case of *Candida albicans* or *Aspergillus fumigatus* cells, for example, when the cell wall was removed by the above-described method, marked changes in morphologies took place (**FIGS. 1 and 2**). Also, the protoplast cells prepared by the above-described method were burst in physiological saline, and living cells contained therein accounted for less than 1%. When 100 µl of the Ca-LSP antigen solution prepared above was spread over the YPD agar medium and cultured at 30° C. for four days, no *Candida albicans* cell colonies appeared, demonstrating that the living cells were not present in the Ca-LSP antigen solution. No colonies appeared from the HSP antigen solution or the HSS antigen solution.

[0152] On the other hand, serum factor No. 1 (manufactured by IATRON LABORATORIES, Inc.), an anti-*Candida* serum, causes to agglutinate cells of *Candida albicans* TIMM 1768 (serotype A). With inhibitory activity to this agglutination, the remaining amount of the cell wall components contained in the insoluble fraction was quantified as the amount of the cell wall mannan, a constituent component. The comparative control for cell wall mannan used was the Allergen Scratch Extract "Torii"*Candida* (manufactured by Torii Pharmaceutical Co., Ltd.), a commercially available *Candida* allergen extract.

[0153] As positive controls, serotype A mannan purified by the method of Kobayashi et al. [Kobayashi, H. et al., *Arch. Biochem. Biophys.* Vol. 272, 364-375 (1989)] from *Candida albicans* J-1012 strain (serotype A) was used in solutions at various concentrations. Although the commercially available *Candida* allergen extract (protein concentration: about 0.4 mg/ml) contained 4.5 mg/ml of serotype A *Candida albicans* cell wall mannan (hereinafter simply referred to as "serotype A mannan"), Ca-LSP (protein concentration: about 2.3 mg/ml) did not inhibit its agglutination, which clarified that the content of the serotype A mannan in the antigenic component of the present invention was not more than the detection limit by the method of 0.5 mg/ml. In other words, the fungal antigen of the present invention was found to have a high protein content and a cell wall mannan content of not more than the detection limit according to the method described above. Thus, it was shown to be clearly different from the conventional allergen extract.

[0154] The Ca-LSP antigen solution obtained was assayed for neutral sugar, lipid, and nucleic acid contents, and a portion thereof was taken and lyophilized, and then weighed. As a result, about 130 mg of the lyophilized residue (23 mg protein, 2 mg neutral sugars, 8 mg lipids, 90 mg NaCl as calculated, small amounts of nucleic acids and water as other components) was contained in 10 ml of the Ca-LSP antigen solution.

Example 2

Preparation of Insoluble Fraction of *Aspergillus fumigatus*

[0155] 1) Preparation of insoluble fraction of *Aspergillus fumigatus* (Af-LSP) (1): Physiological saline containing 0.1% by weight of Tween 80 was added to a Sabouraud dextrose agar slant culture of *Aspergillus fumigatus* TIMM 1776 to prepare a spore suspension. A portion of the suspension was transferred to a Potato-Dextrose medium (manufactured by Difco) in an Erlenmeyer flask and subjected to shaking culture overnight at 30° C. The obtained culture was filtered with a glass filter to harvest mycelium. The mycelium was suspended in 10 mM phosphate buffer, pH 6.0, containing 0.8 M NaCl, and Yatalase (manufactured by Takara Shuzo Co., Ltd.) was added thereto to make up a final concentration of 10 mg/ml, followed by gentle shaking at 30° C. for four hours. The suspension obtained was filtered with a glass filter to harvest the protoplast cells.

[0156] The cells were washed twice with 0.8 M NaCl. Thereafter, to the protoplast cells obtained was added sterile physiological saline to make up a cell density of 1×10^8 cells/ml to be burst. An insoluble fraction was harvested by centrifuging the solution at 10,000×g for 30 minutes. After suspending the insoluble fraction in the physiological saline again, the insoluble fraction was subjected to ultrasonic treatment, and then sterilized in a boiling water bath for five minutes, to yield an insoluble fraction of *Aspergillus fumigatus* Af-LSP, the antigen solution No. 1 (protein concentration: about 0.9 mg/ml).

[0157] 2) Preparation of insoluble fraction of *Aspergillus fumigatus* (Af-LSP) (2): A portion of a spore suspension prepared in the same manner as in the above item 1) was transferred to a Potato-Dextrose medium (manufactured by Difco) containing 0.8 M NaCl in an Erlenmeyer flask and

subjected to shaking culture overnight at 30° C. The turbidity of the culture was of the same level as that of item 1). The obtained culture was filtered with a glass filter to harvest mycelium. The mycelium was suspended in 10 mM phosphate buffer, pH 6.0, containing 0.8 M NaCl. To the suspension were added Yatalase (final concentration: 10 mg/ml), *Trichoderma* Lysing Enzyme (final concentration: 3 mg/ml), and ZYMOLYASE 20T (final concentration: 1 mg/ml), followed by gentle shaking at 30° C. for two hours. The cell suspension obtained was filtered with a glass filter, and the protoplast cells were harvested from the filtrate. The number of the protoplast cells was counted, and as a result, it was found that the count of the protoplast cells was about twice that of the same volume of culture obtained in item 1) above. Therefore, it was clarified that the yield of protoplast cells was improved by the use of this culture method. The cells were washed twice with 0.8 M NaCl, and the obtained protoplast cells were treated in the same manner as in item 1) above, to yield an insoluble fraction of *Aspergillus fumigatus* Af-LSP, the antigen solution No. 2.

Example 3

Preparation of Insoluble Fraction of *Cryptococcus neoformans* (Crn-LSP)

[0158] A platinum loopful of *Cryptococcus neoformans* TIMM 0354 in Sabouraud dextrose agar slant culture was inoculated to the YPD medium in an Erlenmeyer flask, followed by shaking culture at 30° C. overnight. The culture obtained was centrifuged to harvest the cells. The cells were washed once with sterile water, and then suspended in 100 mM citrate buffer, pH 5.8, containing 1 M sorbitol and 100 mM EDTA. *Trichoderma* Lysing Enzyme was added thereto to make up a final concentration of 5 mg/ml, followed by gentle shaking at 37° C. for one hour. The suspension obtained was centrifuged at 2,000×g for 10 minutes to harvest the protoplast cells. After the cells were washed with the above hypertonic buffer, sterile physiological saline was added to suspend the protoplast cells to make up a concentration of 1×10^8 cell/ml to be burst. The suspension was centrifuged at 10,000×g for 30 minutes to harvest an insoluble fraction. After suspending the insoluble fraction in the physiological saline again, the insoluble fraction was subjected to ultrasonic treatment, sterilized in a boiling water bath for five minutes, and then centrifuging at 10,000×g for 30 minutes, to yield an insoluble fraction. The insoluble fraction was taken as an insoluble fraction of *Cryptococcus neoformans*, Crn-LSP antigen solution (protein concentration: about 2.9 mg/ml).

Example 4

Preparation of Solubilized Fraction from *Candida albicans* Insoluble Fraction Ca-LSP

[0159] To 100 ml of the Ca-LSP antigen solution obtained in Example 1 (protein concentration: 2.3 mg/ml) was added 100 ml of a 40 mM bis-Tris buffer (pH 6.5) containing 100 mM octylthioglucoside. After stirring the mixture overnight at 4° C., the mixture was centrifuged at 100,000×g for one hour, to yield 200 ml of a solution of a 50 mM octylthioglucoside-solubilized fraction (Ca-LSP-S) as the supernatant (protein concentration: 0.4 mg/ml). A 100 ml portion of this solution was concentrated by ultrafiltration (cutoff molecular weight: 10,000), and the concentrate was then

dialyzed against phosphate-buffered physiological saline to remove the octylthioglucoside. This dialyzate was further filtered using a membrane filter with a pore size of 0.22 µm to yield 20 ml of a solution of a surfactant-removed solubilized fraction (Ca-LSP-SD) (protein concentration: 1.3 mg/ml).

Example 5

Fractionation of *Candida albicans* Solubilized Fraction (Ca-LSP-S) Using ConA Column

[0160] The remaining 100 ml of Ca-LSP-S obtained in Example 4 was concentrated by ultrafiltration (protein concentration: 3 mg/ml), and 1.5 times by volume of 20 mM bis-Tris buffer (pH 6.5) was then added to make up a final octylglucoside concentration of 20 mM. To the solution obtained was added NaCl to make up a final concentration of 0.25 M, and further were added CaCl₂ and MnCl₂ to make up a final concentration of 1 mM. Next, this mixture was then applied to a column of ConA Sepharose 4B (Pharmacia-LKB), previously equilibrated with buffer A (20 mM bis-Tris, 20 mM octylthioglucoside, 0.25 M NaCl, 1 mM CaCl₂, 1 mM MnCl₂ (pH 6.5)). The non-adsorbed components were washed with buffer A. The effluent fraction and the washed fraction obtained were combined and taken as the ConA column non-adsorbed fraction. Next, the ConA column adsorbed components were then eluted with buffer A containing 0.25 M methyl-D-glucose, and the eluate taken as the ConA column eluted fraction. The ConA column non-adsorbed fraction and the ConA column eluted fraction obtained were concentrated by ultrafiltration (cutoff molecular weight: 10,000), and the concentrates obtained were referred to as "Ca-ConA-Pass" and "Ca-ConA-Elute," respectively.

Example 6

Production of Vaccine Preparations

[0161] 1) Production of Water-in-Oil (Incomplete Freund's Adjuvant) Preparation

[0162] A necessary volume of each of the above-described antigen solutions derived from various LSPs (Ca-LSP etc.), which are insoluble fractions, surfactant-removed solubilized fractions (Ca-LSP-SD etc.) derived from LSP, and ConA column eluted fraction (Ca-ConA-Elute) were taken, and sufficiently mixed with an equal volume of an incomplete Freund's adjuvant (hereinafter referred to as "IFA") (manufactured by Difco) to yield a water-in-oil vaccine preparation.

[0163] 2) Production of Alum Preparation

[0164] A necessary volume of each of the above-described antigen solutions derived from various LSPs, which are insoluble fractions, or surfactant-removed solubilized fractions (Ca-LSP-SD etc.) derived from LSP described above was taken, and an equal volume of alum (manufactured by Pierce) was added dropwise thereto with stirring. After adding the entire content, the mixture was additionally stirred for 30 minutes to yield a vaccine preparation.

Example 7

Comparison of Vaccine Activity of Insoluble Fraction Ca-LSP Derived from *Candida albicans* with HSP and HSS Antigen Solutions and Comparison with Living Cell Vaccine

[0165] 1) Comparison of vaccine activity of Ca-LSP, HSP and HSS antigen solutions: Each of the Ca-LSP, HSP and HSS antigen solutions obtained in Example 1 was diluted with physiological saline to make up a protein concentration of 400 µg/ml. According to Example 6, an equal volume of IFA was added to each dilution to yield a vaccine preparation, which was then subcutaneously inoculated to C57BL/6 mice (six weeks of age, female, five animals per group) at 0.1 ml per animal to immunize the mice. The group in which physiological saline was given in place of the antigen solution was used for control. One week later, the same volume was again subcutaneously inoculated. Specifically, the dose per animal is 20 µg protein/administration for all antigens. One week after second immunization, all immunized mice were intravenously infected with 2.5×10^5 cells of *Candida albicans* TIMM 1768 cultured in a Sabouraud dextrose liquid medium. After infection, the mice were observed for survival for 30 days.

[0166] The results are shown in Table 1. The insoluble fraction Ca-LSP exhibited more potent vaccine activity than the ribosome fraction (HSP) and the soluble fraction (HSS).

TABLE 1

Group Administered with	Mean Survival ± SD Days	Number of Surviving Mice after 30 Days/ Number of Mice Used
Physiological Saline	5.8 ± 1.6	0/5
Ca-LSP	>28.8 ± 2.7	4/5
HSP	7.6 ± 3.4	0/5
HSS	7.4 ± 0.9	0/5

[0167] 2) Comparison of vaccine activity of *Candida albicans* insoluble fraction Ca-LSP with living cell vaccine: The concentrations of the Ca-LSP antigen solutions were adjusted to make up a dosage of Ca-LSP of 0.2 µg protein/administration, 2 µg protein/administration, or 20 µg protein/administration. Thereafter, a vaccine preparation was obtained according to Example 6, which was then subcutaneously inoculated to C57BL/6 mice (five animals per group) twice at a one-week interval in the same manner as in item 1) of Example 7 to immunize the mice. In addition, *Candida albicans* TIMM 1768 was subjected to shaking culture overnight in a Sabouraud dextrose medium, and the cells were harvested by centrifugation. The cells were washed with physiological saline, and the cells obtained were suspended in physiological saline to make up a cell density of 1×10^6 cells/ml, 1×10^7 cells/ml, or 1×10^8 cells/ml. To each of suspension was added an equal volume of IFA and mixed, and thereafter subcutaneously inoculated at 0.1 ml per mouse to immunize the mice. One week later, the living *Candida* cells as prepared in the same manner as above were subcutaneously inoculated in the same cell number for each mouse. Specifically, the dosage per mouse is 5×10^4 cells/administration, 5×10^5 cells/administration, or

5×10^6 cells/administration. For control, a mixture of physiological saline and IFA was administered by subcutaneously inoculating twice in a one-week interval. After one week of second immunization, all immunized mice were intravenously infected with 2.5×10^5 cells of *Candida albicans* TIMM 1768 cultured in a Sabouraud dextrose medium. After infection, the mice were observed for survival for 30 days.

[0168] The results are shown in Table 2. Ca-LSP exhibited more potent protective activity against infection even at a dosage of 2 μg protein/administration, and exhibited superior protective activity against infection than the immunity of the living cells.

TABLE 2

Group Administered with	Dosage at One Time	Mean Survival \pm SD Days	Number of Surviving Mice after 30 Days/ Number of Mice Used
Physiological Saline	—	4.0 \pm 1.4	0/5
Ca-LSP	0.2*	9.6 \pm 2.5	0/5
	2	>27.6 \pm 4.3	2/5
	20	>30.0 \pm 0.0	5/5
Living Cells	5×10^4	16.8 \pm 6.3	0/5
	5×10^5	19.6 \pm 9.0	0/5
	5×10^6	>20.8 \pm 10.1	5/5

* μg protein.

Example 8

Protective Activity Against Infection of Surfactant-Removed Solubilized Fraction Derived from *Candida albicans* Insoluble Fraction Ca-LSP

[0169] After the surfactant-removed solubilized fraction (Ca-LSP-SD) derived from Ca-LSP prepared in Example 4 was diluted to a concentration such that a dose is adjusted to 20 μg protein/administration, a vaccine preparation was produced therefrom according to Example 6. The vaccine preparation was then subcutaneously inoculated to C57BL/6 mice (five animals per group) twice at a one-week interval in the same manner as in item 1) of Example 7 to immunize the mice. For control, a preparation of *Candida albicans* insoluble fraction Ca-LSP with IFA, and a mixture of physiological saline and IFA were administered in the same manner for immunization. One week after immunization, mice were intravenously infected with 2.5×10^5 cells of *Candida albicans* TIMM 1768. After infection, the mice were observed for survival for 30 days. The results are shown in Table 3. The solubilized fraction LSP exhibited protective activity against infection of the same level as that of the insoluble fraction LSP.

TABLE 3

Group Administered with	Mean Survival \pm SD Days	Number of Surviving Mice after 30 Days/ Number of Mice Used
Physiological Saline	6.6 \pm 2.9	0/5
Ca-LSP	>26.8 \pm 4.7	2/5
Ca-LSP-SD	>24.6 \pm 5.1	2/5

Example 9

Protective Activity Against Infection of Ca-ConA-Elute Derived from *Candida albicans* Insoluble Fraction Ca-LSP

[0170] Ca-ConA-Elute obtained in Example 5, the fraction containing high content of a glycoprotein having ConA-binding oligomannose, was diluted with physiological saline to make up a protein concentration of 4 $\mu\text{g}/\text{ml}$. According to Example 6, an equal volume of IFA was added to the dilution to prepare a vaccine preparation, which was then administered to C57BL/6 mice (six weeks of age, female, five animals per group) in the same manner as in item 1) of Example 7 to confirm the protective action against infection with *Candida albicans* TIMM 1768. The results are shown in Table 4. The Ca-ConA-Elute exhibited sufficient protective activity against infection when the dosage is 0.2 μg protein/administration.

TABLE 4

Group Administered with	Mean Survival \pm SD Days	Number of Surviving Mice after 30 Days/ Number of Mice Used
Physiological Saline	4.0 \pm 1.4	0/5
Ca-ConA-Elute	>20.8 \pm 12.3	2/5

Example 10

Vaccine Action of *Candida albicans* Insoluble Fraction Ca-LSP in Various Mouse Candidiasis Systemic Infection Models

[0171] 1) Protection against infection in vaccinated mice in immunocompetent state: After diluting to a concentration such that a dose of Ca-LSP as prepared in Example 1 is adjusted to 20 μg protein/administration, a vaccine preparation was produced according to Example 6. The vaccine preparation was subcutaneously administered to C57BL/6 mice (five animals per group) twice at a one-week interval to immunize the mice in the same manner as in item 1) of Example 7. For control, a mixture of physiological saline and IFA was administered in the same manner as above for immunization. After second immunization, each immunized mouse was subjected to intraperitoneal administration of 200 mg/kg cyclophosphamide on the third day to give an immunosuppressed state. Four days later, the mice were intravenously infected with 5×10^4 cells of *Candida albicans*

TIMM 1768. After infection, the mice were observed for survival for 30 days. The results are shown in Table 5. Even when the immune response is decreased by cyclophosphamide, the Ca-LSP-immunized group had sufficient protective action against infection.

TABLE 5

Group Administered with	Mean Survival ± SD Days	Number of Surviving Mice after 30 Days/ Number of Mice Used
Physiological Saline	1.6 ± 1.3	0/5
Ca-LSP	>27.6 ± 5.4	4/5

[0172] 2) Persistence of protection by vaccination with Ca-LSP: After diluting to a concentration such that a dose of Ca-LSP as prepared in Example 1 is adjusted to 20 µg protein/administration, a vaccine preparation was produced according to Example 6. The vaccine preparation was subcutaneously administered to C57BL/6 mice (five animals per group) twice at a one-week interval to immunize the mice in the same manner as in item 1) of Example 7. After second immunization, each mouse was intravenously infected on the thirty-fourth day with 1×10^5 cells of *Candida albicans* TIMM 1768. After infection, the mice were killed on the twelfth day, and both kidneys were aseptically excised. To the kidneys was added 6 ml of physiological saline, and a homogenate was obtained using a homogenizer. The homogenate was diluted with physiological saline ($\times 1$, $\times 10$, $\times 100$). A 100 µl portion of each dilution was spread over a Sabouraud dextrose agar medium and cultured at 30° C. for one day, and the colonies appeared were counted. The results are shown in Table 6. It was evident from the results that immunization with Ca-LSP resulted in a decrease of viable cell numbers in kidneys, with protective immunity against infection lasting even at the thirty-fourth day after immunization.

TABLE 6

Group Administered with	Colony Forming Units ($\times 10^3$)*
Physiological Saline	9100, 1400, 2800, 1600, —**
Ca-LSP	130, 26, 0, 0, 0

*Number of cells forming colonies, in homogenates (6 ml) of both kidneys of each five mice.
**Died before killing.

Example 11

Infection with *Candida albicans* TIMM 0239

[0173] After diluting to a concentration such that a dose of Ca-LSP as prepared in Example 1 was adjusted to 20 µg protein/administration, a vaccine preparation was produced according to Example 6. The vaccine preparation was subcutaneously administered to C57BL/6 mice (five animals per group) twice at a one-week interval in the same manner as in item 1) of Example 7 to immunize the mice. Also, those in which physiological saline was used in place of Ca-LSP were used for control. One week after immunization, each mouse (five animals per group) was intravenously infected

with 5×10^5 or 1×10^6 cells of *Candida albicans* TIMM 0239, a strain differing from *Candida albicans* TIMM 1768, used for preparation of the immunized antigen. After infection, the mice were observed for survival for 30 days. The results are shown in Table 7. It is evident from the results that when immunized with the LSP derived from a strain of *Candida albicans*, protective immunity against infection to other *Candida albicans* strains is also induced.

TABLE 7

Count of Infected Cells (Cells)	Group Administered with	Mean Survival ± SD Days	Number of Surviving Mice after 30 Days/ Number of Mice Used
5×10^5	Physiological Saline	8.6 ± 8.7	0/5
5×10^5	Ca-LSP	>30.0 ± 0.0	5/5
1×10^6	Physiological Saline	2.0 ± 0.7	0/5
1×10^6	Ca-LSP	>24.0 ± 9.2	2/5

Example 12

Specific Delayed-Type Hypersensitivity (DTH) Reaction to Ca-LSP of Mice Immunized with Living *Candida albicans* Cells

[0174] In the same manner as in item 2) of Example 7, C57BL/6 mice (five animals per group) were subcutaneously immunized with 5×10^4 , 5×10^5 , or 5×10^6 living cells twice at a one-week interval. Also, a Ca-LSP preparation with IFA was subcutaneously administered to C57BL/6 mice (five animals per group) such that doses were adjusted to 0.2, 2, and 20 µg protein/administration twice at a one-week interval to immunize the mice. On the sixth day after immunization, 50 µl of a Ca-LSP antigen solution was subcutaneously administered at a concentration of 200 µg protein/ml to the footpads of each mouse. Twenty-four hours later, footpad swelling was measured.

[0175] The results are shown in Table 8. It was evident from these results that the cellular immunity to Ca-LSP was established in individual mice sensitized with living cells in which a DTH reaction for recognizing Ca-LSP as an antigen is induced, i.e., in mice acquiring protective immunity against infection. Also, in the Ca-LSP-immunized mice, potent cellular immunity to Ca-LSP has been induced.

TABLE 8

Group Administered with	Dosage per Administration	Swelling of Footpad ± SD ($\times 10^{-2}$ mm)
Physiological Saline	—	14.2 ± 9.5
Living Cells	5×10^4 cells	123.0 ± 34.5
	5×10^5 cells	114.8 ± 21.2
	5×10^6 cells	144.0 ± 17.1
Ca-LSP	0.2*	85.0 ± 16.6
	2	109.2 ± 26.5
	20	120.4 ± 18.6

*µg protein.

Example 13

Specific Proliferation of Splenic Lymphocytes from Mice Immunized with *Candida albicans* Cells in Response to *Candida albicans* Ca-LSP

[0176] From BALB/c mice immunized with 5×10^6 living cells in the same manner as in item 2) of Example 7, spleens were excised on the fifteenth day after final immunization, and homogenized in an RPMI-1640 medium to yield a cell suspension. To this suspension was added an RPMI-1640 medium, and this suspension was washed and centrifuged, after which the cells were re-suspended in an RPMI-1640 medium supplemented with 10^6 fetal calf serum (FCS). This cell suspension was applied on a nylon wool column and cultured at 37°C . for one hour, followed by elution with the 10% FCS-supplemented RPMI-1640 medium, to yield a T cell-rich fraction. The cells were harvested by centrifugation, and suspended in the 10% FCS supplemented RPMI-1640 medium to make up a cell density of 1×10^7 cells/ml. After a 100 μl aliquot of an appropriately diluted Ca-LSP antigen solution was poured into each well of a 96-well microplate, the cell suspension was added at 100 μl per well. Two days after cultivation at 37°C . in 5% CO_2 , ^3H -thymidine (0.5 $\mu\text{Ci}/\text{well}$) was added thereto. After 18 hours of cultivation, the cells were recovered and assayed for the amount of ^3H -thymidine uptake.

[0177] The results are shown in Table 9. The splenocytes derived from immunized mice exhibited dose-dependent proliferation to Ca-LSP.

TABLE 9

Ca-LSP Concentration**	^3H -Thymidine Uptake (cpm) \pm SD	Stimulation Index (SI)*
0	2477 \pm 219	1.0
0.05	2882 \pm 334	1.2
0.5	14357 \pm 2771	5.8
5	41736 \pm 2326	16.9

$$* \text{SI} = \frac{[\text{Amount of } ^3\text{H}-\text{thymidine Uptake with Adding Ca-LSP (cpm)}]}{[\text{Amount of } ^3\text{H}-\text{thymidine Uptake without Adding Ca-LSP (cpm)}]}$$

** μg protein.

Example 14

Antibodies Against Proteins Derived from *Candida albicans* Insoluble Fraction Ca-LSP in Blood from Mammals Immunized or Sensitized with Living *Candida albicans* Cells

[0178] 1) Antibodies against Ca-LSP-derived proteins in blood from mice immunized with living *Candida albicans* cells: From BALB/c mice immunized with 5×10^6 living cells in the same manner as in item 2) of Example 7, an anti-*Candida* serum was prepared. Next, a sample buffer for SDS electrophoresis was then added to Ca-LSP, followed by treatment in a boiling water bath for three minutes and subsequently centrifuged. The supernatant was subjected to 12.5% SDS-PAGE. After electrophoresis, the supernatant was blotted onto a PVDF membrane and subjected to blocking overnight with Block Ace. Thereafter, the PVDF membrane was reacted with a 50-fold dilution of the anti-

serum, and then with a rat anti-mouse IgG antibody as a secondary antibody to detect antigen proteins. As a result, as shown in FIG. 3 (lane 1), IgG antibodies against some proteins contained in Ca-LSP were induced in the serum from immunized mice acquiring protective immunity against infection. The protein of which molecular weight is near 65,000 is the protein described in Example 15.

[0179] 2) Antibodies against Ca-LSP contained in rabbit anti-*Candida* serum: A commercially available rabbit anti-*Candida* serum (purchased from Dainippon Pharmaceutical) was used as a primary antibody and a goat anti-rabbit IgG antibody as a secondary antibody. The proteins contained in Ca-LSP were separated by SDS-PAGE, blotted onto a PVDF membrane, and subjected to Western blotting to detect an antigenic protein in the same manner as in item 1) of Example 14. As a result, as shown in FIG. 3 (lane 2), antibodies against some of proteins contained in Ca-LSP were contained in the rabbit anti-*Candida* serum. In other words, it was clarified that components of Ca-LSP acted as antigens in the rabbit as well. The protein detected near 65 kD is the same as that described in Example 15.

[0180] 3) Antibodies against Ca-LSP in human blood: *Candida albicans* is a fungus normally colonizing in humans, and it has been known that almost all humans are sensitized with *Candida albicans* cells. In view of this, in order to evaluate whether or not antibodies against proteins derived from Ca-LSP are present in normal individual blood, proteins derived from Ca-LSP were subjected to Western blotting to detect an antigenic protein in the same manner as in item 1) of Example 14 by using a normal individual serum as a primary antibody and a goat anti-human IgG antibody as a secondary antibody. As shown in FIG. 3 (lane 3), IgG antibodies against some proteins contained in Ca-LSP were detected in the normal individual serum, so that it was clarified that proteins contained in Ca-LSP act as antigens in humans as well.

Example 15

Purification of Antigenic Proteins from *Candida albicans* Solubilized Fraction (Ca-LSP-S) (1)

[0181] 1) Isolation of proteins: Ca-ConA-Pass as obtained in Example 5 was applied to a MonoQ column (manufactured by Pharmacia-LKB), previously equilibrated with buffer B (20 mM bis-Tris, 20 mM octylthioglucoside, 1 mM CaCl_2 , 1 mM MnCl_2 (pH 6.5)). After column washing with buffer B, elution was carried out on a linear gradient of 0-0.8 M NaCl in buffer B. The fraction obtained was subjected to immunoblotting under the same conditions as in item 1) of Example 14. Fractions containing proteins positive for some of mouse anti-*Candida* sera were collected and dialyzed against buffer B.

[0182] The dialyzate obtained was applied to hydroxyapatite (manufactured by Mitsui Toastu Chemicals, Inc.), previously equilibrated with buffer B. After washing with buffer B, elution was carried out on a linear gradient of 0-0.5 M NaCl in buffer B. The fraction eluted was again subjected to immunoblotting under the same conditions as in item 1) of Example 14. A protein having a molecular weight of about 65,000 (SDS-PAGE, under reduced conditions) showing strong binding to the mouse anti-*Candida* serum, and a protein having a molecular weight about 25,000 (SDS-

PAGE, under reduced conditions) showing weak binding to the anti-*Candida* serum were isolated.

[0183] The N-terminal amino acid sequences of the two proteins obtained were determined by using the L-500 amino acid analyzer (manufactured by Hitachi Ltd.), and it was anticipated that each had amino acid sequences as shown by SEQ ID NO: 1 in Sequence Listing and SEQ ID NO: 2 in Sequence Listing. Based on information obtained, the amino acid sequence was subjected to homology search to known proteins, and it was found that the protein having a molecular weight of about 65,000 (SDS-PAGE, under reduced conditions) had homology with dihydrolipoamide dehydrogenase (DLDH) of *Saccharomyces cerevisiae* localized in mitochondria, and that the protein having a molecular weight of about 25,000 (SDS-PAGE, under reduced conditions) had homology with superoxide dismutase (SOD) of *Saccharomyces cerevisiae* localized in mitochondria, both of which were deduced to be proteins derived from mitochondria.

[0184] Separately, the fractions obtained by fractionation of Ca-ConA-Pass through the MonoQ column were assayed for proliferation inductive activity for splenic lymphocytes from immunized mice in the same manner as in Example 13, together with protein separation by SDS-PAGE and analysis by silver staining. The fraction eluted near 0.12 M NaCl from the MonoQ column chromatography of Ca-ConA-Pass was collected, again applied to the MonoQ column, and eluted on a linear density gradient of 0-0.24 M NaCl in buffer B. From the eluted fraction obtained, a protein having a molecular weight of about 30,000 (SDS-PAGE, under reduced conditions) could be isolated.

[0185] Similarly, the fraction eluted near 0.64 M NaCl from the MonoQ column was again applied to the MonoQ column chromatography of Ca-ConA-Pass and eluted on a linear density gradient of 0.24-0.8 M NaCl in buffer B. From the eluted fraction obtained, a protein having a molecular weight of about 62,000 (SDS-PAGE, under reduced conditions) could be isolated. These proteins were clearly shown to promote ³H-thymidine uptake to splenic lymphocytes of mouse immunized with living fungi prepared in the same manner as in Example 13 at a final protein concentration of 5 µg/ml, demonstrating lymphocyte proliferation inducing activity, though their binding to the mouse anti-*Candida* serum described in item 1) of Example 14 was extremely low.

[0186] The N-terminal amino acid sequences of the two proteins obtained were determined by using the L-500 amino acid analyzer (manufactured by Hitachi Ltd.), and it was anticipated that each had amino acid sequences as shown by SEQ ID NO: 3 in Sequence Listing and SEQ ID NO: 4 in Sequence Listing. Based on the information obtained, the amino acid sequence was subjected to homology search to known proteins, and it was found that the protein having a molecular weight of about 30,000 had homology with citrate synthase of *Saccharomyces cerevisiae*, and the protein having a molecular weight of about 62,000 had homology with vacuolar aminopeptidase I of *Saccharomyces cerevisiae*.

[0187] 2) Antigenicity test for isolated proteins: The four proteins isolated above (the protein having a molecular weight of about 65,000; the protein having a molecular weight of about 25,000; the protein having a molecular

weight of about 30,000; and the protein having a molecular weight of about 62,000) were assayed for the amount of ³H-thymidine uptake by splenic lymphocytes derived from mice immunized with living fungi in the same manner as in Example 13. As a result, all proteins exhibited lymphocyte proliferation inducing activity at a protein level of 5 µg/ml per assay.

[0188] Furthermore, the antigenic proteins described above were subcutaneously administered in the same manner as in Example 12 to the footpads of the mice immunized with living *Candida albicans* cells to test whether or not a DTH reaction was induced. As a result of the test for the four antigenic proteins described above, all of these proteins gave significant footpad swelling when administered at 5 µg/administration.

[0189] It was clarified from the above results that the four proteins isolated were all recognized by individuals acquiring protective immunity against infection.

Example 16

Acquisition of Protective Immunity Against Infection by Transfer of Splenocyte Derived from Mice Immunized with *Candida albicans* Insoluble Fraction Ca-LSP

[0190] A preparation of Ca-LSP mixed with IFA was subcutaneously administered to BALB/c mice (five animals per group) at 0.1 ml per animal twice at a one-week interval in the same manner as in item 1) of Example 7 to immunize the mice. The dose is 20 µg protein/administration. For control, a mixture of physiological saline and IFA was administered in the same manner as above for immunization. One week after second immunization, spleens were excised from five immunized mice and homogenized in an RPMI-1640 medium to yield a cell suspension (about 8×10⁷ cells/0.5 ml), and the 0.5 ml portion was transferred into C.B.-17/scid mice (five animals per group). One day later, each mouse was intravenously infected with 5×10⁴ cells of *Candida albicans* TIMM 1768. Furthermore, for control, normal (without splenocyte transfer) C.B.-17/scid mice (five animals) were intravenously infected with the same number of cells of *Candida albicans* TIMM 1768. After infection, the mice were killed on the fifth day, and both kidneys were aseptically excised and homogenized with adding 6 ml of physiological saline to yield a homogenate. The resulting homogenate was diluted (x1, x10, x100) with physiological saline, and thereafter, a 100 µl portion of each dilution was spread over Sabouraud dextrose agar medium and cultured at 30° C. for one day. The number of the colonies formed were counted. The results were shown in Table 10.

TABLE 10

Transfer of Splenocytes from Mice	Colony Forming Units ($\times 10^2$)*	Ave. Colony Forming Units ± SD ($\times 10^{-2}$ cells)
None (Normal)	239, 119, 151, 119, 110	148 ± 54
Physiological Saline	21, 61, 85, 155, 172	99 ± 64
Ca-LSP	9, 17, 2, 49, 182	52 ± 75

*Number of cells forming colonies contained in homogenates (6 ml) of both kidneys of each five mice.

[0191] By transferring splenocytes derived from mice immunized with Ca-LSP, the viable cell numbers in kidneys significantly ($p < 0.05$) decreased, as compared with normal mice. In other words, it was clarified that adoptive transfer of immunity with splenocytes of mice immunized with Ca-LSP could be carried out.

Example 17

Vaccine Activity of *Aspergillus fumigatus* Insoluble Fraction Af-LSP

[0192] A vaccine preparation, produced according to Example 6 using the Af-LSP antigen solution 1 prepared in item 1) of Example 2, was subcutaneously administered to C57BL/6 mice at 2 or 20 µg protein/administration twice at a one-week interval to immunize the mice. For control, a mixture of physiological saline and IFA was administered in the same manner as above for immunization. After immunization, each mouse was intravenously infected on the eighth day with 2×10^6 spores of *Aspergillus fumigatus* TIMM 1776. After infection, the mice were observed for survival for 30 days.

[0193] The results are shown in Table 11. Prominent protective immunity against infection was observed after two administrations of 20 µg protein/administration, and significant prolongation of survival days could be seen even at 2 µg protein/administration. In other words, it was also shown that Af-LSP could be used as a vaccine.

TABLE 11

Group Administered with	Dosage per Administration	Mean Survival ± SD Days	Number of Surviving Mice after 30 Days/ Number of Mice Used
Physiological Saline	—	5.3 ± 0.5	0/6
Af-LSP	2*	9.6 ± 4.8	0/5
	20	>11.7 ± 6.5	2/6

*µg protein.

Example 18

Antibodies Against Proteins Derived from *Aspergillus fumigatus* Insoluble Fraction Af-LSP in Blood from Mice Administered with Living *Aspergillus fumigatus* Cells

[0194] A suspension of *Aspergillus fumigatus* TIMM 1776 spores (1×10^8 spores/ml) was mixed with an equal volume of complete Freund's adjuvant, and 0.1 ml of the resulting mixture was subcutaneously administered to BALB/c mice twice at a one-week interval to immunize the mice. One week after immunization, blood was collected to obtain an anti-*Aspergillus* serum.

[0195] After the Af-LSP antigen solution 1 prepared in item 1) of Example 2 was separated by SDS-PAGE, the separated components were blotted onto a PVDF membrane to detect antigenic proteins by Western blotting in the same manner as in item 1) of Example 14 using the anti-*Aspergillus* serum as a primary antibody and a rabbit anti-mouse IgG

antibody as a secondary antibody. As a result, as shown in FIG. 4 (lane 1), antibodies against proteins in Af-LSP were contained. In other words, the proteins contained in Af-LSP are recognized as antigens by the living body suffering from *Aspergillus* infection. Therefore, as described in Example 17, it appears that specific protective immunity can be performed by inducing immunity to Af-LSP.

Example 19

Cross Reactivity Among Insoluble Fractions Derived from Fungi)

[0196] 1) Cross reactivity of anti-*Candida* serum or anti-*Aspergillus* serum to proteins derived from other kinds of fungi: Af-LSP antigen solution 1 and Crn-LSP were separated by SDS-PAGE, and each was blotted onto a PVDF membrane to detect antigenic proteins by Western blotting in the same manner as in item 1) of Example 14 using an anti-*Candida* serum [item 1) of Example 14] as a primary antibody and a rabbit anti-mouse IgG antibody as a secondary antibody. As a result, as shown in FIG. 5, the anti-*Candida* serum exhibited cross reactivity to proteins derived from Crn-LSP (lane 2). Also observed was weak cross reactivity to Af-LSP derived from *Aspergillus* (lane 3). Incidentally, lane 1 shows an example where an insoluble fraction, Ca-LSP, was used.

[0197] Also, after separation of Crn-LSP by SDS-PAGE, the separated components were blotted onto a PVDF membrane to detect antigenic proteins by Western blotting in the same manner as in item 1) of Example 14 using an anti-*Aspergillus* serum (Example 18) as a primary antibody and a rabbit anti-mouse IgG antibody as a secondary antibody. The anti-*Aspergillus* serum exhibited weak but detectable cross reactivity to a protein contained in Crn-LSP derived from *Cryptococcus* (FIG. 4, lane 2).

[0198] 2) Induction of specific cellular immunity and cellular immunity against Af-LSP in Ca-LSP-immunized mice: A preparation of Ca-LSP mixed with IFA and a preparation of Af-LSP antigen solution No. 1 mixed with IFA were subcutaneously administered to C57BL/6 mice twice at a one-week interval at 0.2 µg protein/administration, 2 µg protein/administration, or 20 µg protein/administration to immunize the mice. For control, immunization was carried out with a mixture of physiological saline and IFA in the same manner as above. After immunization, Af-LSP was subcutaneously administered on the sixth day to the footpads of each mouse (five animals per group) at 20 µg protein/50 µl. Twenty-four hours later, the footpad swelling was measured. The results are shown in Table 12. From the finding that the swelling was greater in the Af-LSP-immunized group, it was clarified that a DTH reaction with considerable selectivity to Af-LSP was induced. On the other hand, a significant DTH reaction to Af-LSP occurred in the group immunized with Ca-LSP at 20 µg protein, which clarified that the cellular immunity involving a cross reaction was induced. In other words, the presence of a protein showing a cross reaction with different fungi was demonstrated. Therefore, it appears that infection with different fungi by the use of a single kind of LSP can be protected (see Example 20).

TABLE 12

Group Administered with	Dosage per Administration	Swelling of Foodpad ± SD ($\times 10^{-2}$ mm)
Physiological Saline	—	6.8 ± 5.8
Af-LSP	0.2*	21.0 ± 16.1
	2	42.6 ± 18.8
	20	90.0 ± 37.1
Ca-LSP	0.2*	7.4 ± 3.4
	2	5.2 ± 5.7
	20	28.0 ± 10.9

*μg protein.

Example 20

Vaccine Activity of *Candida albicans* Insoluble Fraction Ca-LSP in Mouse Aspergillosis Infection Model

[0199] A preparation of Ca-LSP mixed with IFA was subcutaneously administered to C57BL/6 mice twice at a one-week interval at 20 μg protein/administration to immunize the mice. For control, immunization was carried out with a mixture of physiological saline and IFA in the same manner as above. After immunization, mice were intravenously infected on the eighth day with 2×10^6 spores of *Aspergillus fumigatus* TIMM 1776. After infection, the mice were observed for survival for 30 days. The results are shown in Table 13. It was demonstrated that protective immunity against infection to *Aspergillus* infection can be induced by immunizing with Ca-LSP.

TABLE 13

Group Administered with	Mean Survival ± SD Days	Number of Surviving Mice after 30 Days/ Number of Mice Used
Physiological Saline	6.4 ± 0.9	0/6
Ca-LSP	>22.6 ± 9.0	2/5

Example 21

Preparation of *Candida albicans* Mycelial Cells and Preparation of Insoluble Fraction Ca-LSP-M Derived from Mycelial Cells

[0200] In the same manner as in Example 1, a portion of a culture obtained by subjecting *Candida albicans* TIMM 1768 to shaking culture in the YPD medium at 30° C. for 24 hours was inoculated to an RPMI-1640 medium supplemented with 10% FCS in an Erlenmeyer flask, and subjected to shaking culture at 37° C. for four hours, to yield *Candida albicans* mycelial cells. The culture was filtered with a glass filter, and after recovery, the cells were washed with the SSB solution, and then re-suspended in the SSB solution. The suspension was then treated with ZYMOLYASE, a *Trichoderma* lysing enzyme in the same manner as in Example 1. In order to separate mycelial cells with protoplast cells, the suspension was filtered by a glass filter, and the filtrate was recovered. The filtrate obtained was centrifuged at 1,000×g for 5 minutes to harvest protoplast cells. These cells were washed with the SSB solution, after which sterile physiological saline was added. After being sufficiently stirred, the mixture was allowed to stand on ice for 10 minutes. After having confirmed of bursting of the protoplast cells, the mixture was centrifuged at 10,000×g for 30 minutes, and the precipitate obtained was taken as the insoluble fraction derived from mycelial cells (hereinafter referred to as "Ca-LSP-M").

for 5 minutes to harvest protoplast cells. These cells were washed with the SSB solution, after which sterile physiological saline was added. After being sufficiently stirred, the mixture was allowed to stand on ice for 10 minutes. After having confirmed of bursting of the protoplast cells, the mixture was centrifuged at 10,000×g for 30 minutes, and the precipitate obtained was taken as the insoluble fraction derived from mycelial cells (hereinafter referred to as "Ca-LSP-M").

[0201] After suspending in physiological saline, Ca-LSP-M was subjected to ultrasonic treatment, and then sterilized in a boiling water bath for 5 minutes, to yield 2 ml of a Ca-LSP-M antigen solution containing membrane proteins etc. (protein concentration: 1.2 mg/ml) from 100 ml of the cell culture. After Ca-LSP-M and control Ca-LSP (both containing about 4 μg of protein) were separated by SDS-PAGE, each was blotted onto a PVDF membrane to detect antigenic proteins by Western blotting in the same manner as in item 1 of Example 14 using an anti-*Candida* serum [item 1) of Example 14] as a primary antibody and a rat anti-mouse IgG antibody as a secondary antibody. As a result, as shown in FIG. 6 (lane 2), IgG antibodies induced against some proteins contained in Ca-LSP-M were detected in the anti-*Candida* serum, with bands distinguishable from that of Ca-LSP of yeast phase cells on lane 1 of FIG. 3 (FIG. 6, lane 1). In addition, the amount of antibodies appeared to be greater. The morphological changes in the *Candida albicans* mycelial cells used in this Example before and after cell wall removal treatment are shown in FIG. 7.

Example 22

Diagnosis by Human Skin Test

[0202] The physiological saline solution of Ca-LSP obtained in Example 1 (protein concentration: 2.3 mg/ml) was diluted with physiological saline to make up a protein concentration of 1.0 mg/ml, after which it was further diluted 100-folds and 1,000 folds. The skin test was performed as follows. Patch Star (manufactured by Torii Pharmaceutical Co., Ltd.), previously impregnated with 20 μl of each dilution, was attached on the arm skin of four volunteers for two days, and then the Patch Star was removed. The skin reaction for erythema and papules was observed one hour later. The judgment was made according to the criteria of the International Contact Dermatitis Research Group (ICDRG). Of the four volunteers, two with allergic predisposition showed clear erythema, and one showed slight erythema. Accordingly, the fungal antigen of the present invention was shown to be effective in the diagnosis utilizing the DTH reactions in individuals.

Example 23

Determination of IgE Antibody Titer in Human Plasma

[0203] Paper discs were activated with cyanogen bromide, and the antigen (a solution prepared by diluting Ca-LSP-S obtained in Example 4 to make up 100 μg/ml protein concentration) was coupled to the paper discs according to the method of Miyamoto et al. [Miyamoto et al., *Allergy*, Vol. 22, 584-594 (1973)]. The IgE antibody titer in human plasma was determined as described below. One paper disc coupled with the antigen as prepared above and 50 μl of

human serum were added to a polystyrene tube, and allowed to stand at room temperature for three hours. Next, the paper disc was washed three times with physiological saline containing 0.2% Tween 20, after which 50 µl of ¹²⁵I-labeled anti-human IgE antibody in the RAST-RIA kit (manufactured by Pharmacia) was added, and the plate was kept standing at room temperature for 16 hours.

[0204] After the disc was further washed three times with the above washing solution, radioactivity was determined using a gamma counter. At the same time, the IgE antibody titer was calculated from a standard curve prepared with a control reagent of the RAST-RIA kit. Of the allergic patients, twenty-four positive patients for skin test with a commercially available diagnostic intracutaneous allergen extract (manufactured by Torii Pharmaceutical Co., Ltd.) were subjected to measurement for IgE antibody titration against Ca-LSP-S, and as a result, 15 showed positive responses (positive being defined as 0.35 PRU/ml or higher). Therefore, the positive rate to Ca-LSP-S was high in allergic patients, which clarified that the fungal antigen of the present invention consisting of an insoluble fraction is effective in the detection of IgE antibodies.

Example 24

Cytokine Production from Human Peripheral Blood Mononucleated Cells (PBMCs) by Ca-LSP

[0205] PBMCs were obtained by leukopheresis from normal individuals, followed by collecting the leukocyte fraction, and further separation processes described below. Specifically, the fraction was about 2-fold diluted with an RPMI-1640 medium, then overlaid on a centrifugation separation medium of Ficoll-Paque (manufactured by Pharmacia) and centrifuged at 500×g and room temperature for 20 minutes. The intermediary PBMC layer was recovered by pipetting, washed, and suspended in a solution consisting of 90% fetal bovine serum (FCS, manufactured by Intergen) and 10% dimethyl sulfoxide (manufactured by Sigma) for preservations in liquid nitrogen. The treatment of PBMC with Ca-LSP was carried out as described below.

[0206] After being lysed, the above PBMC sample in storage was suspended in an RPMI-1640 medium supplemented with human AB serum (manufactured by Irvine Scientific) to make up a final concentration of 5% (v/v). This suspension was diluted to a cell density of 1.5×10^6 cells/ml and dispensed into wells of a 24-well microplate at 1 ml per well. Next, a Ca-LSP antigen solution, prepared by diluting the solution of Ca-LSP in physiological saline obtained in Example 1 (protein concentration: 2.3 mg/ml) was added at 50 µl/well to be 5 µg protein/well, followed by cultivation at 37° C. in 5% CO₂. On the seventh day after cultivation initiation, the culture supernatant was collected and assayed for IFN-γ content using a human IFN-γ ELISA kit (manufactured by Amersham LIFE SCIENCE). This measurement was carried out according to the protocol of the manufacturer. Here, the detection limit of the kit is 0.002 ng/ml.

[0207] The IFN-γ contents on the seventh day are shown in FIG. 8. Human PBMCs produced IFN-γ in response to Ca-LSP. Incidentally, the amount of IFN-γ produced in the 15 samples was in the range 1.435±1.210 (mean±SD) ng/ml.

[0208] For control, physiological saline at 50 µl/well was added in place of the Ca-LSP antigen solution, and IFN-γ

contents were determined in the same manner as above. The amount of IFN-γ detected in these wells was not more than the detection limit of the kit.

Example 25

Preparation of Reagent for Intradermal Test and Titration Reagent for Diagnosis of Fungal Allergy

[0209] A Ca-LSP antigen solution prepared in Example 1 is dried and collected as a powder to be used as a reagent for intradermal test to fungal allergy and as a titration reagent for the diagnosis of the fungal allergy. The reagent for intradermal test is prepared by 1,000-fold dilution to make up a 1 mg/ml protein concentration using as a solvent a 0.9% physiological saline supplemented with 0.5% phenol. The titration reagent for the diagnosis of the fungal allergy is prepared by using the dilution of a stock solution as a titration reagent for histamine release, which is dissolved in Hank's buffer in a 1 mg/ml protein concentration.

Example 26

Preparation of Antigenic Agent for Desensitization Therapy

[0210] A Ca-LSP antigen solution prepared in Example 1 is dried and collected as a powder to be used as a therapeutic agent for desensitization to fungal allergen. The active component as allergen is dissolved in a 0.9% physiological saline supplemented with 0.5% phenol at a concentration of 1 mg/ml to give a stock solution of an antigen for desensitization therapy.

Example 27

Isolation of Nucleic Acid Encoding *Candida albicans* Antigenic Protein

[0211] 1) Isolation of DNA encoding a protein having a molecular weight of about 65,000: In order to isolate a nucleic acid encoding a protein having a molecular weight of about 65,000 (hereinafter referred to as 65k protein) which was isolated in item 1) of Example 15, firstly, a cDNA library for *Candida albicans* TIMM 1768 was prepared.

[0212] In order to extract and purify a total RNA from fungal cells, the above fungi were first cultured in 200 ml of the YPD medium at 35° C. Thereafter, the resulting cells were recovered by centrifugation at 2000×g for 5 minutes, and then washed once with distilled water. The obtained cells were rapidly frozen by liquid nitrogen. Thereafter, the frozen cells were disrupted to a powdery state with a mortar. A total RNA was recovered and isolated from the resulting disrupted cells by using RNA extraction kit manufactured by Pharmacia. poly(A)⁺ RNA was prepared from the above total RNA by using Oligotex-dT 30<Super> (manufactured by Takara Shuzo Co., Ltd.). Next, cDNA was prepared from 5 µg of the poly(A)⁺ RNA by using Takara cDNA synthesis kit (manufactured by Takara Shuzo Co., Ltd.). After ligation of the synthesized cDNA with a lambda phage vector µSCREEN-1 (manufactured by Novagen), a cDNA library was constructed by carrying out in vitro packaging by phage maker system, Phage Pack Extract (manufactured by Novagen).

[0213] It was deduced that the 65k protein is DLDH homolog from *Saccharomyces cerevisiae* based on the analy-

sis for amino acid sequence in item 1) of Example 15. An oligonucleotide DL2 having a nucleotide sequence complementary to the nucleotide sequence which was deduced to encode an amino acid sequence, a highly conserved amino acid sequence in DLDHs from other organisms, and an oligonucleotide having a nucleotide sequence which was deduced to encode a partial sequence of the amino acid sequence of SEQ ID NO: 1 in Sequence Listing was synthesized and purified to be used as primers for PCR. The nucleotide sequence for DL1 is shown by SEQ ID NO: 9 in Sequence Listing, and the nucleotide sequence for DL2 is shown by SEQ ID NO: 10 in Sequence Listing. Genomic DNA was extracted and purified from *Candida albicans* TIMM1768 by the method of P. Philippse et al [Methods in Enzymology, 194, 169-175 (1991)], in order to use it as a template for PCR. PCR was carried out using the purified genomic DNA as a template and DL1 and DL2 as primers. The reaction conditions for PCR were 30 cycles of temperature shifts consisting of 94° C. for 1 minute, 55° C. for 1.5 minutes, and 72° C. for 2 minutes. As a result, a DNA having a length of about 1 kbp was amplified. After cloning of the above DNA into pUC118 vector (manufactured by Takara Shuzo Co., Ltd.), its nucleotide sequence was determined. The nucleotide sequence of the amplified DNA was as shown by SEQ ID NO: 13 in Sequence Listing. In addition, the amino acid sequence deduced to be encoded by the above nucleotide sequence had an amino acid sequence identical to that of the determined N-terminal of the 65k protein. Therefore, it was obvious that the amplified DNA fragment obtained was a partial portion of a DNA encoding the 65k protein.

[0214] Next, in order to obtain the whole cDNA encoding the 65k protein, screening of the cDNA library was carried out using the above amplified DNA fragment as a probe. The cDNA library obtained as described above was inoculated to a host *Escherichia coli* ER1647, mixed with top agarose (LB medium containing 0.7% agarose), and the mixture was overlayed on an LB plate, and then cultured at 37° C. overnight to form plaques. The resulting plaques were transferred to nylon membrane (Hybond-N, manufactured by Amersham), and thereafter, plaque hybridization was carried out. The above PCR fragment with 1 kb was labelled by a random primer DNA labelling kit (manufactured by Takara Shuzo Co, Ltd.) and [α -³²P] dCTP and used as the probe for hybridization. As a result of screening of 1.6×10^5 plaques, a large number of phage clones hybridized with the probe. Twenty-eight clones out of the hybridized clones which exhibited strong signals were further analyzed. Automatic subcloning in *Escherichia Coli* gave *Escherichia coli* clones harboring plasmids which resulted from automatic subcloning of a region containing cDNAs from these phages. Plasmids were purified from the above *Escherichia coli*, and then the length of cDNAs and patterns for DNA bands resulting from restriction endonuclease digestion of the cDNAs were evaluated. Thereafter, a cDNA which is considered to contain the 65k protein gene was selected, and then the nucleotide sequence thereof was determined. The DNA nucleotide sequence was shown by SEQ ID NO: 7. It was deduced that the 65k protein was the protein having the amino acid sequence as shown by SEQ ID NO: 5 in Sequence Listing.

[0215] 2) Isolation of DNA encoding an antigenic protein having a molecular weight of about 25,000: In order to isolate a DNA encoding a protein having a molecular weight

of about 25,000 (hereinafter, referred to 25K protein) which was isolated in item 1) of Example 15, firstly, oligonucleotides SO1 and SO2 which were respectively deduced to encode partial portions of the amino acid sequence of SEQ ID NO: 2 in Sequence Listing were synthesized, purified, and used as primers for PCR. The nucleotide sequence of SO1 is shown by SEQ ID NO: 11 in Sequence Listing, and the nucleotide sequence of SO2 is shown by SEQ ID NO: 12 in Sequence Listing. Next, RT-PCR was carried out using Takara RNA LA PCR kit (AMV) Ver. 1.1 (manufactured by Takara Shuzo Co., Ltd.) by the use of 0.5 μ g of the isolated poly(A)⁺ RNA. Specifically, cDNA was synthesized from 0.5 μ g of poly(A)⁺ RNA by a reaction of AMV reverse transcriptase (at 45° C., for 30 minutes) using oligo(dT)20-M4 adaptor primer. PCR was carried out using the above cDNA as a template and SO1 primer and M13M4 primer (manufactured by Takara Shuzo Co., Ltd.) as primers under condition of 35 cycles of temperature shifts consisting of 94° C. for 0.5 minute, 55° C. for 2 minutes, and 72° C. for 2 minutes. A second PCR (nested PCR) was also carried out using the resulting reaction mixture for PCR as a template. SO2 primer and M13M4 primer were used as primers in this reaction. As a result of PCR, a DNA with a length of about 700 bp was amplified. After cloning of the above amplified DNA to pUC118 vector, a nucleotide sequence was determined. The determined nucleotide sequence is shown by SEQ ID NO: 8 in Sequence Listing. The amino acid sequence which is deduced to be encoded by the above nucleotide sequence is shown by SEQ ID NO: 6 in Sequence Listing. N-terminal portion thereof was identical to the amino acid sequence determined from the 25k protein. It was clear that the PCR fragment was a DNA encoding the 25k protein having homology with SOD.

Example 28

Purification of Antigenic Proteins from *Candida albicans* Solubilized Fraction (Ca-LSP-S) (2)

[0216] Another cultivated *Candida albicans* cells were used to prepare Ca-ConA-Pass in the same manner as in Example 5. The resulting concentrate was purified in the same manner as in Example 15 to search for a novel antigenic protein. As a result, novel proteins having a molecular weight of about 55,000 (SDS-PAGE, under reduced conditions) showing strong binding to the mouse anti-*Candida* serum, and having a molecular weight about 35,000 (SDS-PAGE, under reduced conditions) showing weak binding to the anti-*Candida* serum were isolated. The protein having a molecular weight of about 55,000 had the partial amino acid sequence as shown by SEQ ID NO: 15. The protein having a molecular weight of about 55,000 was identified as catalase from the findings that this partial amino acid sequence had an identical sequence with a partial amino acid sequence of 2nd to 31st residues starting from the N-terminal of the protein encoding a catalase gene CAT1 of *Candida albicans*, and that both proteins had no contradictions in terms of their molecular weights. The protein having a molecular weight about 35,000 had the partial amino acid sequence as shown by SEQ ID NO: 14, and this sequence had homology with malate dehydrogenase of *Saccharomyces cerevisiae*.

Example 29

Production of Antigen Preparation for Nasal Administration

[0217] Equal volumes of an aqueous solution of cholera toxin B subunit, prepared to make up a concentration of 1 mg/ml of cholera toxin B subunit (manufactured by Sigma) in distilled water for injection (manufactured by Otsuka Pharmaceutical Co., Ltd.), and the antigen solution Ca-LSP (protein concentration: 1 mg/ml) obtained in Example 1 were mixed to prepare an antigen preparation for nasal administration.

Example 30

Protection Against Infection with Antigen Preparation for Nasal Administration

[0218] The antigen preparation produced in Example 29 was nasally administered in 20 μ l portions to each of C57BL/6 mice under anesthesia. After 1, 3 and 5 weeks after administration, the same volume of the antigen preparation was again nasally administered to the mice. This was referred to "group immunized with Ca-LSP." Incidentally, as a control, each of the preparations obtained by using a physiological saline solution of ovalbumin prepared to make up a concentration of 1 mg/ml, or a physiological saline, in place of the Ca-LSP used in Example 29 was similarly administered, and each was referred to "group immunized with ovalbumin" or "group immunized with physiological saline." On the eighth day after the final preparation administration, blood was drawn from each group of mice, and the anti Ca-LSP antibody titer in sera was measured. As a result, an increase of the antibody titer was observed in the mice of the group immunized with Ca-LSP. In addition, on the sixteenth day after the final preparation administration, a Ca-LSP antigen solution was subcutaneously administered at a concentration of 10 μ g protein/50 μ l to the footpads of each group of mice. Twenty-four hours later, footpad swelling was measured. As a result, prominent DTH reaction was observed in the group of mice immunized with Ca-LSP.

[0219] On the tenth day from the final preparation administration, the mice in each group were intravenously inoculated with 0.5 ml of a cell suspension of *Candida albicans* TIMM 0136 (2×10^5 cells/ml). One week after the cell administration, the mice were killed, and both kidneys were aseptically excised and homogenized with adding 6 ml of physiological saline to yield a homogenate. The resulting homogenate was diluted twice or twenty times with physiological saline, and thereafter, a 100 μ l portion of each dilution was spread over Sabouraud dextrose agar plate medium and cultured at 30° C. for two days. The number of the colonies formed were counted. The results were shown in Table 14.

TABLE 14

Group Administered with	Ave. Colony Forming Units \pm SD (\log_{10})
Ovalbumin	4.14 \pm 0.61
Ca-LSP	2.85 \pm 0.79
Physiological Saline	4.00 \pm 0.44

[0220] It was found that the cell numbers in kidneys significantly decreased in group administered with Ca-LSP.

[0221] It was clarified from the above results that mice administered with the preparation produced in Example 29 acquired protective immunity against infection to *Candida albicans*.

Example 31

Preparation of *Candida* Antigen

[0222] To 100 ml of the Ca-LSP antigen solution obtained in Example 1 at a concentration of 2.3 mg protein/ml was added 25 ml of a 5% aqueous sodium lauryl sulfate (SDS) solution. After stirring the liquid mixture at 4° C. overnight, the centrifugation was carried out at 100,000xg for one hour, and an SDS solubilized product was obtained as a supernatant. To the solubilized product was added the four-folds amount of ethanol under ice-cooling with stirring. The insoluble precipitated *Candida* antigen was collected by centrifugation. The composition of the resulting precipitate was analyzed, and the contents other than water were as follows. Protein 80% by weight, sugar 10% by weight, and nucleic acids 10% by weight, differing from the Ca-LSP antigen solution in that substantially no lipids were contained.

Example 32

Suppression Action of Tumor Metastasis with *Candida* Antigenic Protein

[0223] The Ca-LSP antigen solution obtained in Example 1 and IFA were used to prepare a vaccine preparation according to the method described in item 1) of Example 6. The C57BL/6 mice was subcutaneously inoculated with 20 μ g protein of the vaccine preparation twice in a one-week interval. For control, a mixture of physiological saline and IFA was inoculated in the same manner as above. One week after the final administration, 0.2 ml of a suspension of B16BL6 melanoma cells at a cell density of 2.5×10^5 cells/ml was inoculated to each group of mice via tail vein. A suspension of physiological saline of *Candida* antigen produced in Example 31 at a concentration of 50 μ g protein/ml was intravenously inoculated in an amount of 0.2 ml per mouse to a half of each group of mice three times: One day before the melanoma cell inoculation, one day later, and three days later. To the remaining half of each group of mice, a suspension of physiological saline of *Candida* antigen produced in Example 31 at a concentration of 1.25 mg protein/ml was orally administered in an amount of 0.2 ml per mouse for five consecutive days starting from one day before the melanoma inoculation. On the fourteenth day after the melanoma inoculation, the mice were killed, and both lungs were aseptically excised, and the number of formed metastatic nodules was counted. The results thereof are shown in Table 15. It is clear from the table that the metastasis of the tumor is prominently suppressed by intravenously inoculating the *Candida* antigen, and that the metastasis of the tumor can be also suppressed by orally administering the antigen with somewhat less effectiveness than that intravenously inoculated.

Table 15
[0224]

TABLE 15

Group Administered with	Method of Administration	Number of Formed Metastatic Nodules ± SD in Lung
Ca-LSP	Intravenous Administration	24 ± 17
Physiological Saline	Intravenous Administration	150 ± 45
Ca-LSP	Oral Administration	123 ± 39
Physiological Saline	Oral Administration	159 ± 39

Example 33

Antigenic Cross-Reactivity Between Yeasts of *Kluyveromyces* sp. and *Candida albicans*

[0225] Each of *Kluyveromyces* (*K.*) *marxianus* IFO 1735, *K. lactis* IFO 1903 and *C. albicans* TIMM 1768 was cultured in the YPD medium. Each of the resulting living cell suspensions was mixed with IFA in a 1:1 ratio by volume to make up a final cell density of 5×10^7 cells/ml. Separately, a liquid mixture was prepared by mixing physiological saline with IFA in a 1:1 ratio by volume. 0.1 ml each of the four separate liquid mixtures prepared above was subcutaneously inoculated to C57BL/6 mice twice in a one-week interval. After one week from the final administration of the suspension, 50 µl of the Ca-LSP antigen solution obtained in Example 1, in which the concentration of protein was 200 µg/ml, was subcutaneously administered to the footpads of each group of mice. Twenty-four hours later, footpad swelling was measured. As shown in Table 16, prominent DTH reaction was also observed for mice administered with either *K. marxianus* or *K. lactis*, with slight weaker reaction as compared to the mice immunized with *C. albicans*.

[0226] One week after the final administration, the mice in each group were intravenously infected with 0.5 ml of a cell suspension of *Candida albicans* TIMM 0136 to make up a cell density of 2×10^5 cells/ml. One week after the infection, the mice were killed, and both kidneys were aseptically excised and homogenized with adding 6 ml of physiological saline to yield a homogenate. The resulting homogenate was diluted twice or twenty times with physiological saline, and thereafter, a 100 µl portion of each dilution was spread over Sabouraud dextrose agar plate medium and cultured at 30°

C. for two days. The number of the colonies formed were counted. The results were shown in Table 16.

TABLE 16

Group Administered with	Swelling of Footpad ± SD ($\times 10^{-2}$ mm)	Ave. Colony Forming Units ± SD (\log_{10})
<i>K. marxianus</i>	26.0 ± 6.0	3.01 ± 0.714
<i>K. lactis</i>	32.6 ± 11.0	3.54 ± 1.25
<i>C. albicans</i>	93.4 ± 31.4	1.78 ± 0.00
Physiological Saline	0.3 ± 11.9	3.99 ± 0.56

[0227] It was found that the cell numbers in kidneys significantly decreased in the mice immunized with *K. marxianus*, with slightly less in its degree as compared with the group of mice immunized with Ca-LSP. Therefore, it was clarified from the above results that the mice acquired protective immunity against *C. albicans* infection. Also, it was found that the cell numbers in kidneys decreased in the mice immunized with *K. lactis*.

INDUSTRIAL APPLICABILITY

[0228] The fungal antigen of the present invention can be used as biologic products, such as vaccines, compositions used in desensitization therapy for allergies, the cytokine releasing agents, and diagnostics for diseases, which are highly effective against infectious diseases caused by fungi. In other words, when compared in its vaccine effects, the fungal antigen of the present invention has the same level of effects as that immunized with the living cells, remarkably superior as compared with conventional fungal antigens. Also, even in the aspect of safety, in the fungal antigen of the present invention, the content of the cell wall components is low besides the facts that no living cells are contained and toxicity is low. Therefore, when used as vaccines or preparations for desensitization therapy, adverse reactions caused by cell wall components such as mannan and glucan can be suppressed, so that the immune reactions which are advantageous to individuals can be strengthened. Also, the fungal antigen shows high sensitivity in the examination of the allergies.

[0229] The present invention being thus described, it will be obvious that the same may be varied in many ways. Such variations are not to be regarded as a departure from the spirit and scope of the invention, and all such modifications as would be obvious to one skilled in the art are intended to be included within the scope of the following claims.

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Xaa Ile
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<223> OTHER INFORMATION: any Xaa = any amino acid, unknown or other

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<213> ORGANISM: Candida albicans

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Phe Val Arg Tyr Ala Ser Thr Lys Lys Tyr Asp Val Val Val Ile Gly
20 25 30

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Gly Gly Pro Gly Gly Tyr Val Ala Ala Ile Lys Ala Ala Gln Leu Gly
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Leu Asn Thr Ala Cys Ile Glu Lys Arg Gly Ala Leu Gly Gly Thr Cys
 50 55 60

Leu Asn Val Gly Cys Ile Pro Ser Lys Ser Leu Leu Asn Asn Ser His
 65 70 75 80

Leu Leu His Gln Ile Gln His Glu Ala Lys Glu Arg Gly Ile Ser Ile
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Gln Gly Glu Val Gly Val Asp Phe Pro Lys Leu Met Ala Ala Lys Glu
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Lys Ala Val Lys Gln Leu Thr Gly Gly Ile Glu Met Leu Phe Lys Lys
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Asn Lys Val Asp Tyr Leu Lys Gly Ala Gly Ser Phe Val Asn Glu Lys
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Thr Val Lys Val Thr Pro Ile Asp Gly Ser Glu Ala Gln Glu Val Glu
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Ala Asp His Ile Ile Val Ala Thr Gly Ser Glu Pro Thr Pro Phe Pro
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Gly Ile Glu Ile Asp Glu Glu Arg Ile Val Thr Ser Thr Gly Ile Leu
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Ser Leu Lys Glu Val Pro Glu Arg Leu Ala Ile Ile Gly Gly Gly Ile
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Ile Gly Leu Glu Met Ala Ser Val Tyr Ala Arg Leu Gly Ser Lys Val
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Thr Val Ile Glu Phe Gln Asn Ala Ile Gly Ala Gly Met Asp Ala Glu
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Tyr Ala Asn Ile Pro Ser Val Met Tyr Thr His Pro Glu Val Ala Trp
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Ala Val Glu Ala Lys Ser Lys Gly Glu Val Lys Lys Leu Val Ala Leu	35	40
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Gln Lys Ala Ile Asn Phe Asn Gly Gly Tyr Leu Asn His Cys Leu	50	55
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Trp Trp Lys Asn Leu Ala Pro Val Ser His Gly Gly Gln Pro Pro	65	70
	75	80

Ser Glu Asp Ser Lys Leu Gly Lys Gln Ile Val Lys Gln Phe Gly Ser	85	90
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Leu Asp Lys Leu Ile Glu Ile Thr Asn Gly Lys Leu Ala Gly Ile Gln	100	105
	110	

Gly Ser Gly Trp Ala Phe Ile Val Lys Asn Lys Ala Asn Gly Asp Thr	115	120
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	140	

Leu Val Pro Leu Ile Ala Ile Asp Ala Trp Lys His Ala Tyr Tyr Leu	145	150
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gaagatgtca aatccggtaa aaaatctgac	cttgaagccg atgtcttggt ggttgcatt	960
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gaagttaaaa aattgggtgc cttacaaaaa	gccatcaatt tcaacggtgg tgggttacctc	180
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gaagctgaaa gaagatttga attttaagtt	actggacaaa agtcaagtac atatttaat	600
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caaggtgaag ttggcggtga tttccaaaaa ttgatggctg ccaaggaaaa agccgtcaaa 240
caattgaccg gtggatttga aatgttgtc aaaaagaaca aggttgacta cttgaaagga 300
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944

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1				5			10				15				
Ala	Thr	Gln	Arg	Val	Gly	Gln	His	Gly	Pro	Leu	Leu	Leu	Gln		
		20				25					30				

What is claimed:

1. An isolated nucleic acid encoding a fungal antigen which is an antigenic protein having a vaccine activity or an allergen activity originating from *Candida albicans*, wherein said antigenic protein comprises the amino acid sequence as shown by SEQ ID NO:5 in Sequence Listing.

2. The nucleic acid according to claim 1, wherein said nucleic acid comprises an entire sequence of the nucleotide sequence as shown by SEQ ID NO:7 in Sequence Listing.

3. An isolated nucleic acid encoding a fungal antigen which originates from the genus *Candida* and has a vaccine activity or an allergen activity, wherein said nucleic acid hybridizes to the complete complement of a nucleic acid of the nucleotide sequence as shown in SEQ ID NO:7 in Sequence Listing in 6×SSC containing 0.5% SDS, 0.1% bovine serum albumin (BSA), 0.1% polyvinyl pyrrolidone, 0.1% Ficoll 400, and 0.01% denatured salmon sperm DNA at 50° C.; followed by washing initially at 37° C. in 2×SSC containing 0.5% SDS and changing the SSC concentration to 0.1×SSC and the SSC temperature to 50° C.

4. An isolated nucleic acid encoding a fungal antigen which is an antigen comprising an antigenic protein having a vaccine activity or an allergen activity originating from *Candida albicans*, wherein said antigenic protein comprises the amino acid sequence as shown by SEQ ID NO:6 in Sequence Listing.

5. The nucleic acid according to claim 4, wherein said nucleic acid comprises an entire sequence of the nucleotide sequence as shown by SEQ ID NO:8 in Sequence Listing.

6. An isolated nucleic acid encoding a fungal antigen which originates from the genus *Candida* and has a vaccine activity or an allergen activity, wherein said nucleic acid hybridizes to the complete complement of a nucleic acid of the nucleotide sequence as shown in SEQ ID NO:8 in Sequence Listing in 6×SSC containing 0.5% SDS, 0.1% bovine serum albumin (BSA), 0.1% polyvinyl pyrrolidone, 0.1% Ficoll 400, and 0.01% denatured salmon sperm DNA at 50° C.; followed by washing initially at 37° C. in 2×SSC containing 0.5% SDS and changing the SSC concentration to 0.1×SSC and the SSC temperature to 50° C.

7. A process for producing a fungal antigen which is an insoluble fraction obtainable from fungal cells of which cell wall has been substantially removed or at least partially removed, characterized in that said process comprises the steps of:

- (1) obtaining living fungal cells;
- (2) obtaining fungal cells of which cell wall has been substantially removed or at least partially removed;
- (3) bursting the fungal cells of which cell wall has been substantially removed or at least partially removed; and
- (4) obtaining an insoluble fraction.

8. A process for producing a fungal antigen which is a solubilized fraction extracted and separated from an insoluble fraction obtainable from fungal cells of which cell wall has been substantially removed or at least partially removed, characterized in that said process comprises the steps of:

- (1) obtaining living fungal cells;
- (2) obtaining fungal cells of which cell wall has been substantially removed or at least partially removed;
- (3) bursting the fungal cells of which cell wall has been substantially removed or at least partially removed;
- (4) obtaining an insoluble fraction; and
- (5) extracting and separating a solubilized fraction from the insoluble fraction.

9. The process according to claim 8, further comprising the step of purifying the resulting solubilized fraction by the use of a sugar group-specific affinity medium.

10. The process according to claim 8, wherein said solubilized fraction contains a soluble protein.

11. The process according to claim 8, wherein said step of extracting and separating a solubilized fraction from the insoluble fraction includes a step of solubilizing the insoluble fraction with a buffer containing a surfactant.

12. The process according to claim 9, wherein said sugar group-specific affinity medium is an immobilized concanavalin A medium.

13. The process according to claim 7, wherein said fungal cells of which cell wall has been substantially removed or at least partially removed are obtained by enzymatic lysis treatment of the cell wall and/or physical treatment of the cell wall.

14. The process according to claim 7, wherein said fungal cells of which cell wall has been substantially removed or at least partially removed are protoplasts or spheroplasts of the fungal cells.

15. The process according to claim 7, wherein said insoluble fraction is obtained by subjecting components obtained by bursting the fungal cells of which cell wall has been substantially removed or at least partially removed to centrifugation treatment under conditions of about 100,000×g.

16. A method of stimulating immunological responses against fungi in a vertebrate, comprising the step of administering a vaccine composition for inducing protective immunity against fungi or exhibiting therapeutic effects therefor by administering to individuals, characterized in that said vaccine composition contains the fungal antigen of claim 1 or claim 4 or a fungal antigen produced by the process of claim 7 or claim 8.

17. The method according to claim 16, wherein proliferation of fungi used in the preparation of the vaccine composition and/or fungal strains closely related thereto is

suppressed by the immunological responses in a vertebrate to which the vaccine composition is administered, to thereby prevent or treat diseases caused by the fungi and/or the fungal strains closely related thereto.

18. A method of suppressing allergic reaction to fungi in a vertebrate, comprising the step of administering an allergen composition for preventing allergoses against fungi or exhibiting therapeutic effects therefor by administering to individuals, characterized in that the allergen composition contains the fungal antigen of claims 1 or claim 4, or a fungal antigen produced by the process of claim 7 or claim 8.

19. The method according to claim 18, wherein allergoses caused by fungi used in the preparation of the allergen

composition and/or fungal strains closely related thereto are prevented or treated by the immunological responses in a vertebrate to which the allergen composition is administered.

20. A method for diagnosing a disease caused by fungi in a vertebrate, comprising using the diagnostic composition for a disease caused by fungi, characterized in that said diagnostic composition contains the fungal antigen of claim 1 or claim 4, or a fungal antigen produced by the process of claim 7 or claim 8.

* * * * *



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(19) United States

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Arumugham et al. (43) Pub. Date: Jun. 14, 2007(54) IMMUNOGENIC PEPTIDE CARRIER
CONJUGATES AND METHODS OF
PRODUCING SAME(76) Inventors: **Rasappa G. Arumugham**, Chapel Hill,
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(21) Appl. No.: 10/583,464

(22) PCT Filed: Dec. 17, 2004

(86) PCT No.: PCT/US04/42701

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(2), (4) Date: Jan. 16, 2007**Related U.S. Application Data**(60) Provisional application No. 60/530,480, filed on Dec.
17, 2003.**Publication Classification**

(51) Int. Cl.

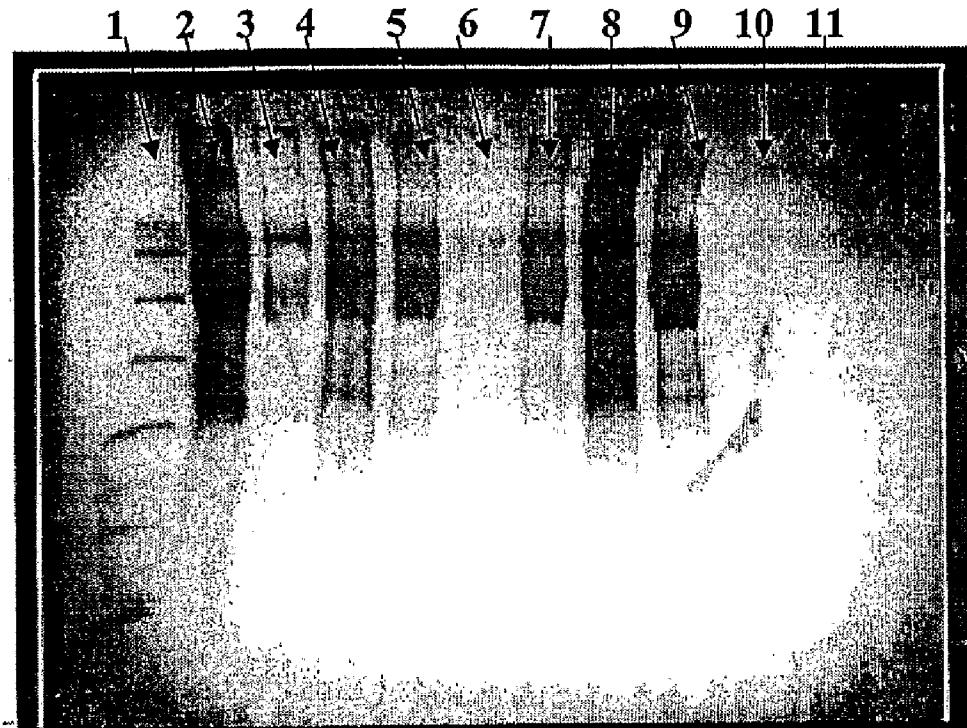
C12Q 1/68 (2006.01)
C12P 21/06 (2006.01)

(52) U.S. Cl. 435/69.1; 435/6

(57)

ABSTRACT

The present invention is directed to methods of producing conjugates of peptide immunogens with protein/polypeptide carrier molecules, which are useful as immunogens, wherein peptide immunogens are conjugated to protein carriers via activated functional groups on amino acid residues of the carrier or of the optionally attached linker molecule, and wherein any unconjugated reactive functional groups on amino acid residues are inactivated via capping, thus retaining the immunological functionality of the carrier molecule, but reducing the propensity for undesirable reactions that could render the conjugate less safe or effective. Furthermore, the invention also relates to such immunogenic products and immunogenic compositions containing such immunogenic products made by such methods.



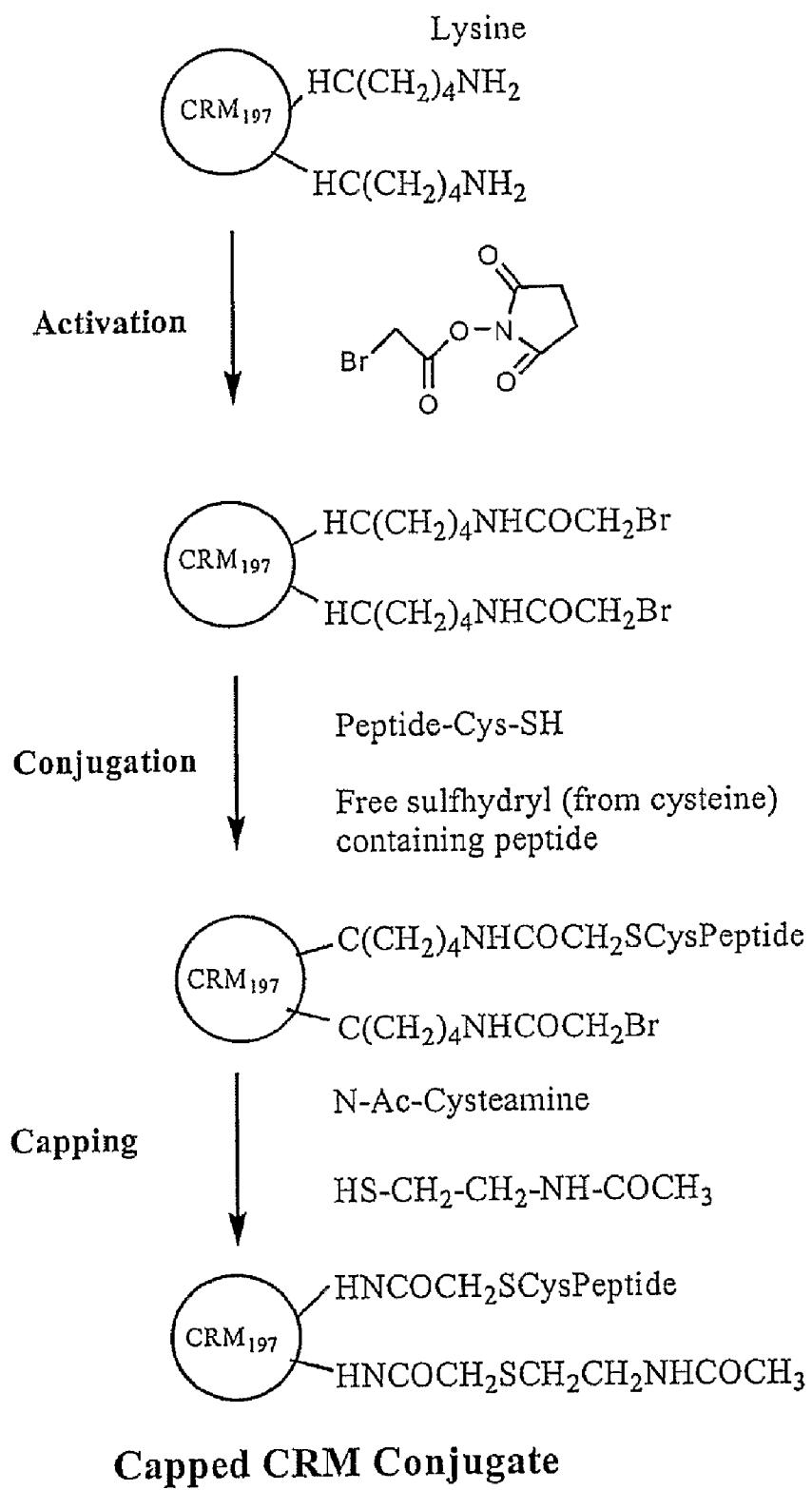


Figure 1

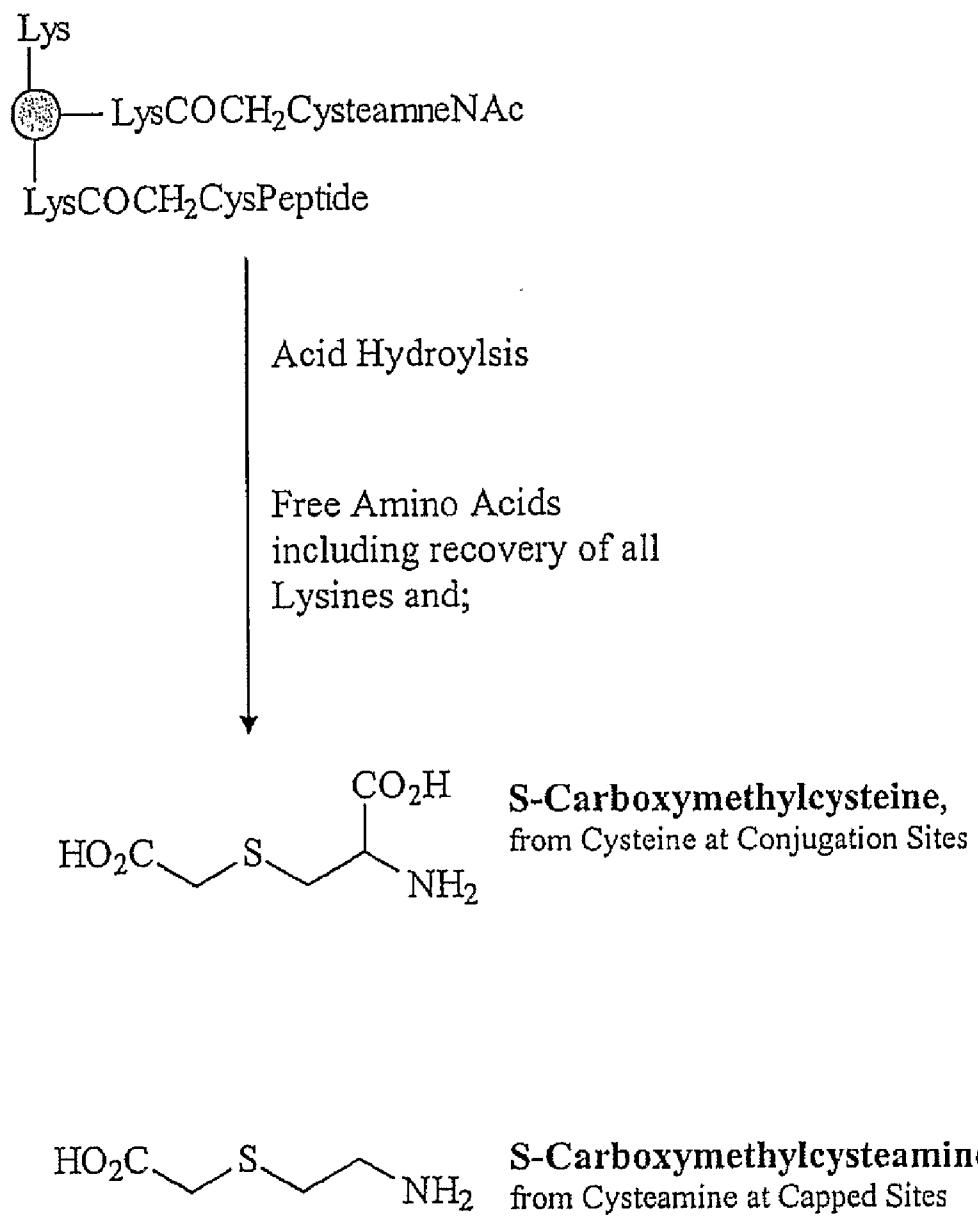


Figure 2

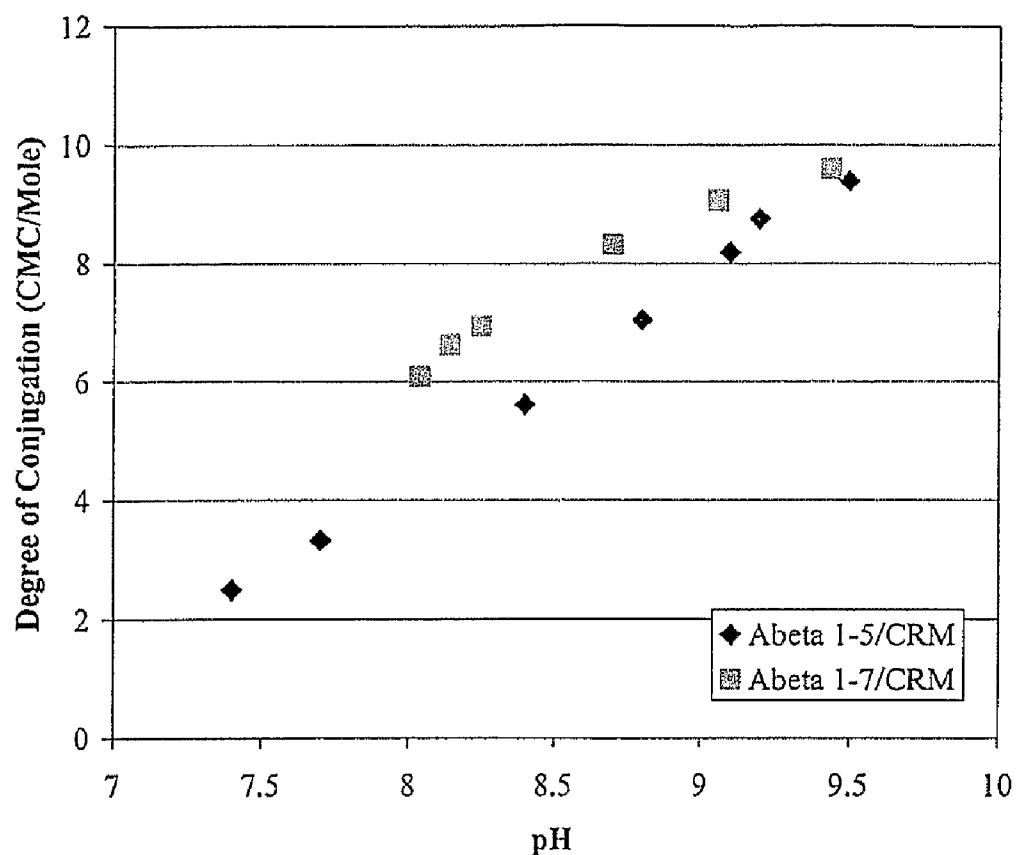


Figure 3

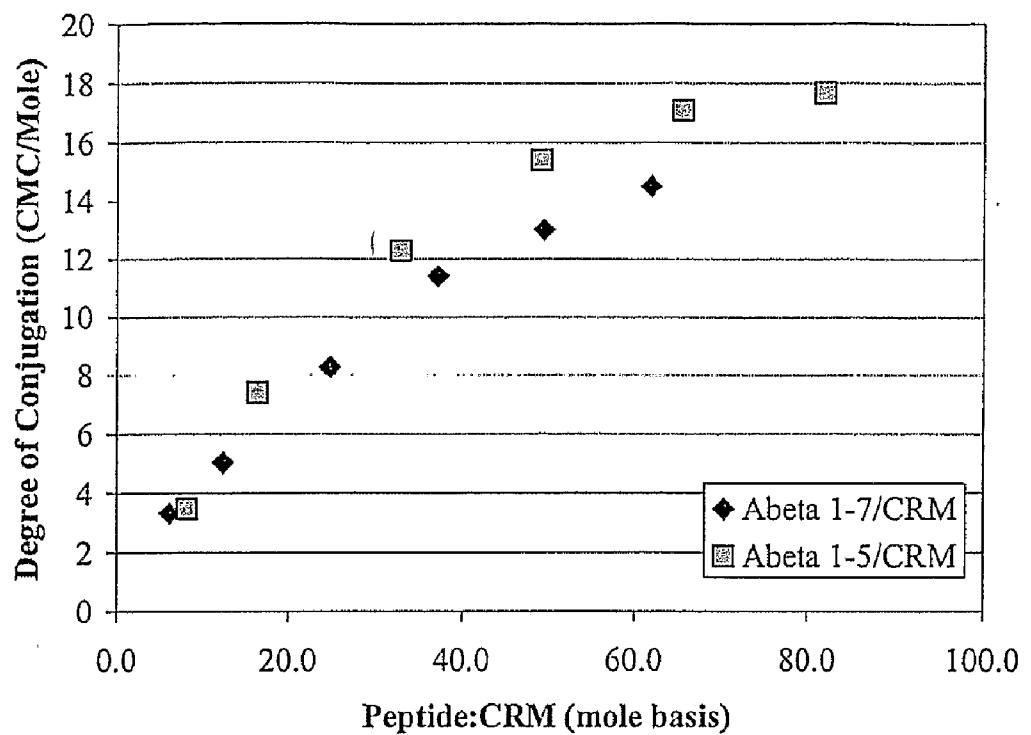


Figure 4

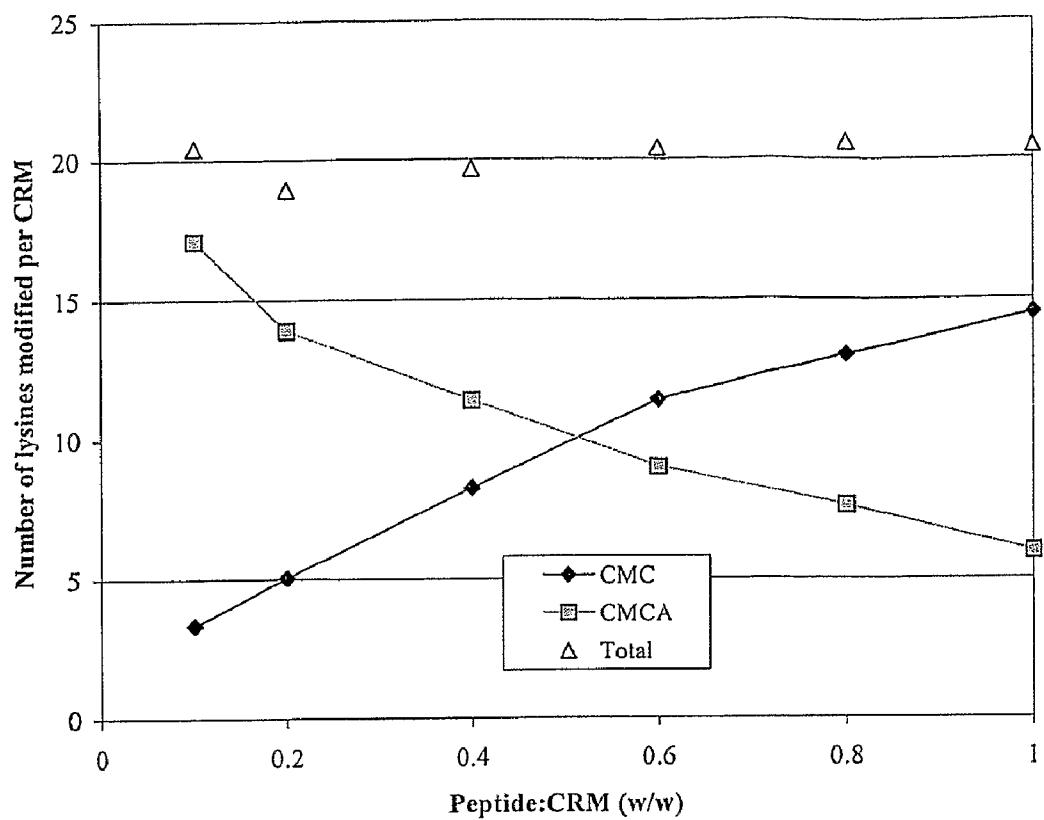


Figure 5

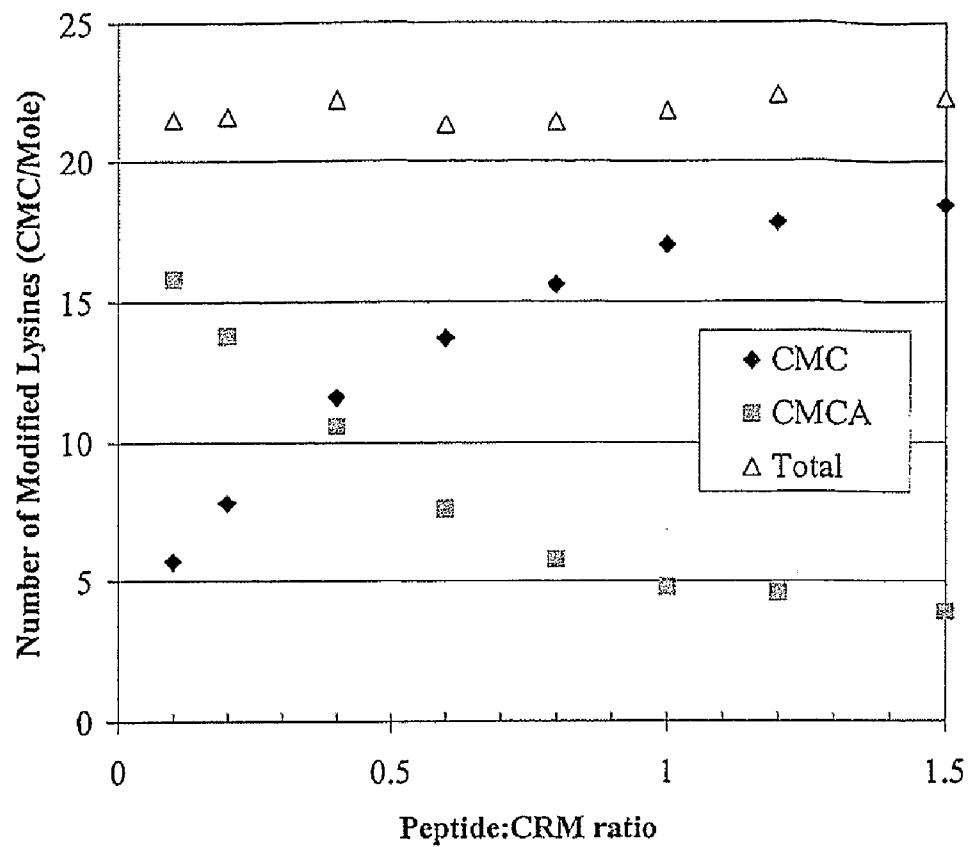


Figure 6

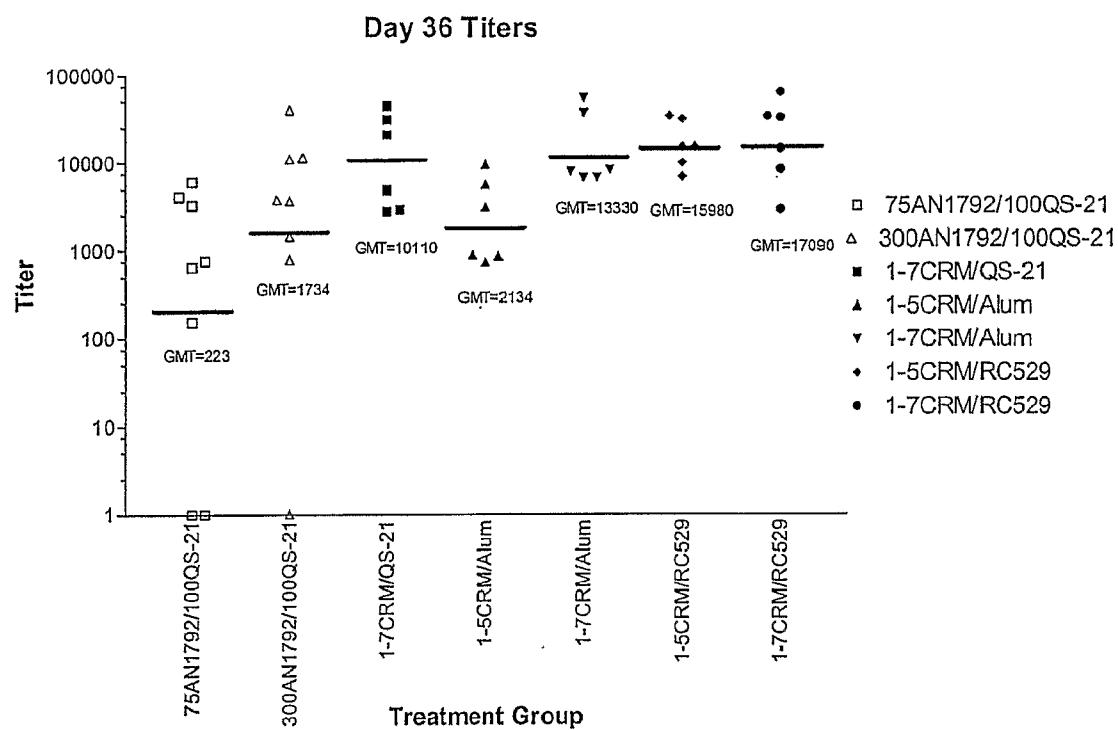


Figure 7

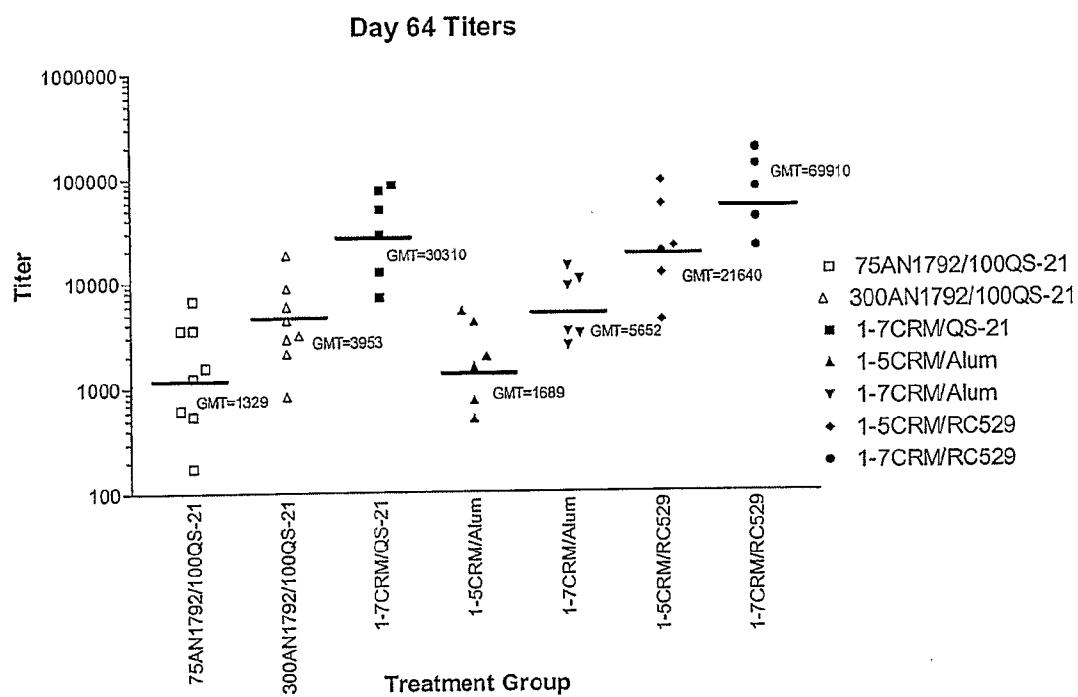


Figure 8

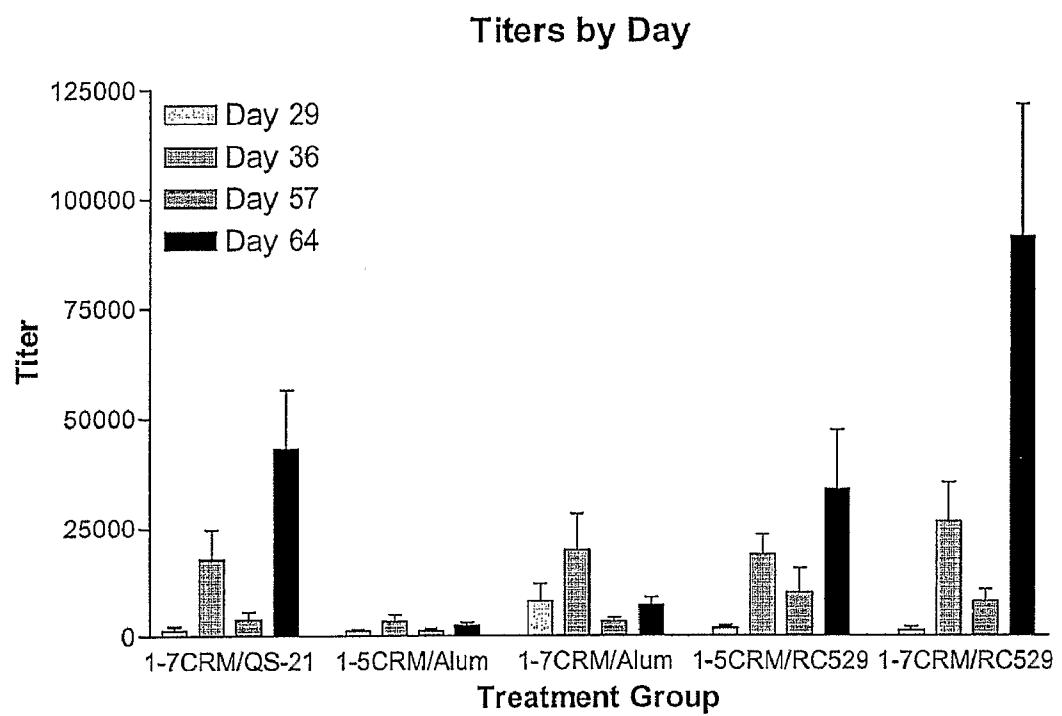


Figure 9

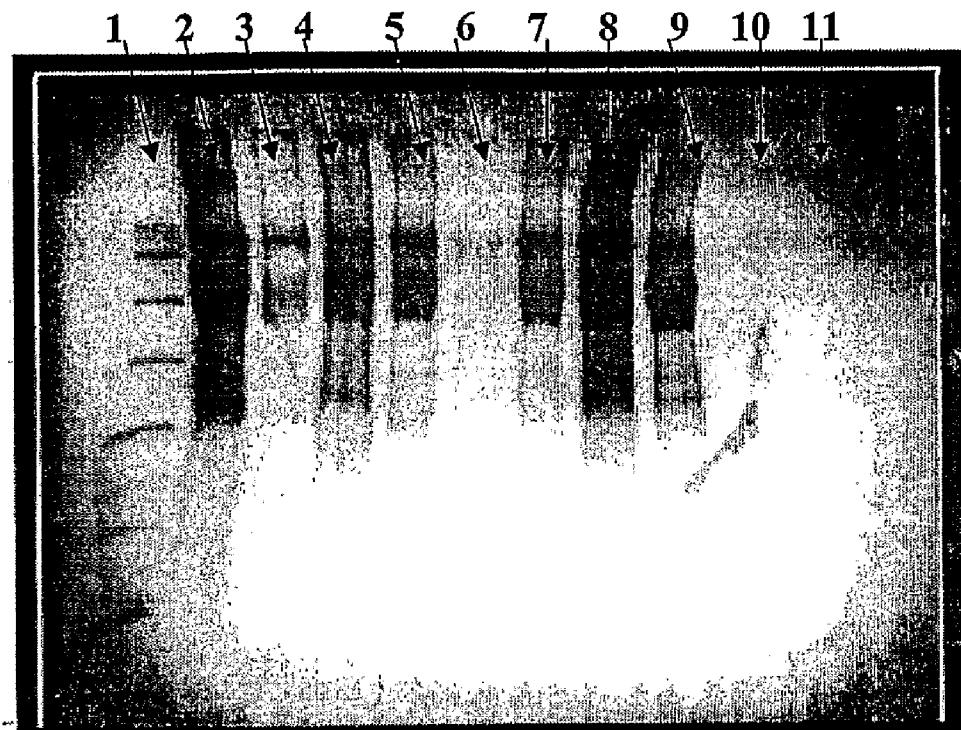


Figure 10

IMMUNOGENIC PEPTIDE CARRIER CONJUGATES AND METHODS OF PRODUCING SAME

CROSS-REFERENCES TO RELATED APPLICATIONS

[0001] This application claims the benefit under 35 U.S.C. § 119(e) of U.S. Provisional Patent Application Ser. No. 60/530,480, filed Dec. 17, 2003, which is incorporated herein by reference in its entirety for all purposes.

BACKGROUND OF THE INVENTION

[0002] The essence of adaptive immunity is the ability of an organism to react to the presence of foreign substances and produce components (antibodies and cells) capable of specifically interacting with and protecting the host from their invasion. An “antigen” or “immunogen” is a substance that is able to elicit this type of immune response and also is capable of interacting with the sensitized cells and antibodies that are manufactured against it.

[0003] Antigens or immunogens are usually macromolecules that contain distinct antigenic sites or “epitopes” that are recognized and interact with the various components of the immune system. They can exist as individual molecules composed of synthetic organic chemicals, proteins, lipoproteins, glycoproteins, RNA, DNA, or polysaccharides, or they may be parts of cellular structures (bacteria or fungi) or viruses (Harlow and Lane 1988a,b,c; Male et al., 1987).

[0004] Small molecules like short peptides, although normally able to interact with the products of an immune response, often cannot cause a response on their own. These peptide immunogens or “haptens” as they are also called, are actually incomplete antigens, and, although not able by themselves to cause immunogenicity or to elicit antibody production, can be made immunogenic by coupling them to a suitable carrier. Carriers typically are protein antigens of higher molecular weight that are able to cause an immunological response when administered *in vivo*.

[0005] In an immune response, antibodies are produced and secreted by the B-lymphocytes in conjunction with the T-helper (T_H) cells. In the majority of hapten-carrier systems, the B cells produce antibodies that are specific for both the hapten and the carrier. In these cases, the T lymphocytes will have specific binding domains on the carrier, but will not recognize the hapten alone. In a kind of synergism, the B and T cells cooperate to induce a hapten-specific antibody response. After such an immune response has taken place, if the host is subsequently challenged with only the hapten, usually it will respond by producing hapten-specific antibodies from memory cells formed after the initial immunization.

[0006] Synthetic haptens mimicking some critical epitopic structures on larger macromolecules are often conjugated to carriers to create an immune response to the larger “parent” molecule. For instance, short peptide segments can be synthesized from the known sequence of a protein and coupled to a carrier to induce immunogenicity toward the native protein. This type of synthetic approach to the immunogen production has become the basis of much of the current research into the creation of vaccines. However, in many instances, merely creating a B-cell response by using

synthetic peptide-carrier conjugates, however well designed, will not always guarantee complete protective immunity toward an intact antigen. The immune response generated by a short peptide epitope from a larger viral particle or bacterial cell may only be sufficient to generate memory at the B cell level. In these cases it is generally now accepted that a cytotoxic T-cell response is a more important indicator of protective immunity. Designing peptide immunogens with the proper epitopic binding sites for both B-cell and T-cell recognition is one of the most challenging research areas in immunology today.

[0007] The approach to increasing immunogenicity of small or poorly immunogenic molecules by conjugating these molecules to large “carrier” molecules has been utilized successfully for decades (see, e.g., Goebel et al. (1939) *J. Exp. Med.* 69: 53). For example, many immunogenic compositions have been described in which purified capsular polymers have been conjugated to carrier proteins to create more effective immunogenic compositions by exploiting this “carrier effect.” Schneerson et al. (1984) *Infect. Immun.* 45: 582-591). Conjugation has also been shown to bypass the poor antibody response usually observed in infants when immunized with a free polysaccharide (Anderson et al. (1985) *J. Pediatr.* 107: 346; Insel et al. (1986) *J. Exp. Med.* 158: 294).

[0008] Hapten-carrier conjugates have been successfully generated using various cross-linking/coupling reagents such as homobifunctional, heterobifunctional, or zero-length cross linkers. Many such methods are currently available for coupling of saccharides, proteins, and peptides to peptide carriers. Most methods create amine, amide, urethane, isothiourea, or disulfide bonds, or in some cases thioethers. A disadvantage to the use of coupling reagents, which introduce reactive sites into the side chains of reactive amino acid molecules on carrier and/or hapten molecules, is that the reactive sites if not neutralized are free to react with any unwanted molecule either *in vitro* (thus adversely affecting the functionality or stability of the conjugate(s)) or *in vivo* (thus posing a potential risk of adverse events in persons or animals immunized with the preparations). Such excess reactive sites can be reacted or “capped”, so as to inactivate these sites, utilizing various known chemical reactions, but these reactions may be otherwise disruptive to the functionality of the conjugates. This may be particularly problematic when attempting to create a conjugate by introducing the reactive sites into the carrier molecule, as its larger size and more complex structure (relative to the hapten) may render it more vulnerable to the disruptive effects of chemical treatment. In fact, no examples are known of methods whereby a conjugate is made by first activating the carrier, then reacting with the hapten in a conjugation reaction, and finally “capping” the remaining reactive sites, while preserving the ability of the resulting conjugate to function as an immunogenic composition having the desired properties of the “carrier effect”.

BRIEF SUMMARY OF THE INVENTION

[0009] The present invention is directed to methods of producing an immunogenic conjugate of a peptide immunogen with a protein/polypeptide carrier, wherein the peptide immunogen is conjugated to the carrier via derivatized functional groups of amino acid residues of the carrier such as lysine residues, and wherein any unconjugated, deriva-

tized functional groups of the amino acid residues are inactivated via capping to block them from reacting with other molecules, including proteins/polypeptides thereby preserving the functionality of the carrier, such that it retains its ability to elicit the desired immune responses against the peptide immunogen that would otherwise not occur without a carrier. Furthermore, the invention also relates to conjugates produced by the above methods, and to immunogenic compositions containing such conjugates.

[0010] In one embodiment, the invention is directed to a first method for conjugating a peptide immunogen via a reactive group of an amino acid residue of the peptide immunogen to a protein/polypeptide carrier having one or more functional groups, the method comprising the steps of: (a) derivatizing one or more of the functional groups of the protein/polypeptide carrier to generate a derivatized molecule with reactive sites; (b) reacting the derivatized protein/polypeptide carrier of step (a) with a reactive group of an amino acid residue of the peptide immunogen under reaction conditions such that the peptide immunogen is conjugated to the derivatized protein/polypeptide carrier via the functional groups; and (c) further reacting the conjugate with a capping reagent to inactivate free, reactive functional groups on the activated protein/polypeptide carrier, thereby preserving the functionality of the carrier against the peptide immunogen that would otherwise not occur without a carrier.

[0011] In one embodiment, the protein/polypeptide carrier is selected from the group consisting of human serum albumin, keyhole limpet hemocyanin, immunoglobulin molecules, thyroglobulin, ovalbumin, influenza hemagglutinin, PAN-DR binding peptide (PADRE polypeptide), malaria circumsporozoite (CS) protein, hepatitis B surface antigen (HB_SAg₁₉₋₂₈), Heat Shock Protein (HSP) 65, Bacillus Calmette-Guerin (BCG), cholera toxin, cholera toxin mutants with reduced toxicity, diphtheria toxin, CRM₁₉₇ protein that is cross-reactive with diphtheria toxin, recombinant Streptococcal C5a peptidase, *Streptococcus pyogenes* ORF1224, *Streptococcus pyogenes* ORF1664, *Streptococcus pyogenes* ORF 2452, *Chlamydia pneumoniae* ORF T367, *Chlamydia pneumoniae* ORF T858, Tetanus toxoid, HIV gp120 T1, microbial surface components recognizing adhesive matrix molecules (MSCRAMMS), growth factor/hormone, cytokines and chemokines.

[0012] In another embodiment, the protein/polypeptide carrier contains a T-cell epitope.

[0013] In yet another embodiment, the protein/polypeptide carrier is a bacterial toxoid such as a tetanus toxoid, cholera toxin or cholera toxin mutant as described above. In a preferred embodiment, the protein/polypeptide carrier is CRM₁₉₇.

[0014] In still yet another embodiment, the protein/polypeptide carrier may be an influenza hemagglutinin, a PADRE polypeptide, a malaria CS protein, a Hepatitis B surface antigen (HSB_SAg₁₉₋₂₈), a heat shock protein 65 (HSP 65), or a polypeptide from *Mycobacterium tuberculosis* (BCG).

[0015] In a preferred embodiment, the protein/polypeptide carrier is selected from Streptococcal rC5a peptidase, *Streptococcus pyogenes* ORF 1224, *Streptococcus pyogenes* ORF1664 or *Streptococcus pyogenes* ORF2452, *Chlamydia pneumoniae* ORF T367, and *Chlamydia pneumoniae* ORF T858.

[0016] In one embodiment, protein/polypeptide carrier is a growth factor or hormone, which stimulates or enhances immune response and is selected from the group consisting of IL-1, IL-2, γ -interferon, IL-10, GM-CSF, MIP-1 α , MIP-1 β , and RANTES.

[0017] In one embodiment, the peptide immunogen is selected from a bacterial protein, a viral protein, and a eukaryotic protein.

[0018] In another embodiment, the peptide immunogen is derived from a bacterial protein antigen from *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Neisseria meningitidis*, *Neisseria gonorrhoeae*, *Haemophilus influenzae*, *Escherichia coli*, *Klebsiella enterobacter*, *Listeria monocytogenes*, *Vibrio cholerae*, *Clostridium perfringens*, *Clostridium botulinum*, *Pseudomonas* species, *Salmonella typhimurium*, *Borrelia burgdorferi*, *Shigella flexneri*, *Shigella boydii*, *Shigella dysenteriae*, *Alloioococcus otitidis* and Group B streptococci.

[0019] In another embodiment, the peptide immunogen is derived from a protein antigen from a virus selected from the group consisting of human immunodeficiency virus (HIV), herpes simplex virus (HSV), human papilloma virus (HPV), parainfluenza virus (PIV), vesicular stomatitis virus (VSV), respiratory syncytial virus (RSV), Epstein-Barr virus (EBV), coronavirus, vaccinia virus, rotavirus, rabies virus, hepatitis C virus (HCV) and hepatitis B virus (HBV).

[0020] In yet another embodiment, the peptide immunogen is derived from a protein antigen from a fungus selected from a *Candida* species, a *Cryptococcus* species, a *Coccidioides* species, a *Histoplasma* species, and an *Aspergillus* species.

[0021] In another embodiment, the peptide immunogen is derived from a protein antigen from a parasite selected from a *Plasmodium*, a *Trypanosome*, a *Schistosome*, and a *Leishmania*.

[0022] In another embodiment, the peptide immunogen is derived from a protein antigen from a eukaryote. In a preferred embodiment, the eukaryote is a human.

[0023] In yet another preferred embodiment, the peptide immunogen from the human is derived from a malignant tumor. In a more preferred embodiment, the peptide immunogen is from a tumor antigen from a renal cell carcinoma, a breast carcinoma, a melanoma and a prostate carcinoma. In another preferred embodiment, the peptide antigen is derived from the tumor antigen, carcinoembryonic antigen (CEA).

[0024] In one aspect, the invention provides a peptide immunogen comprising A β peptide or fragments of A β or analogs thereof eliciting an immunogenic response against certain epitopes within A β . Immunogenic peptides of the invention include immunogenic heterologous peptides. In some immunogenic peptides, an A β fragment is linked to a carrier to form an immunogenic heterologous peptide, and then this heterologous peptide is linked to a carrier using a method of the present invention to form a conjugate.

[0025] In another aspect of the invention, the peptide immunogen is a polypeptide comprising an N-terminal segment of at least residues 1-5 of A β , the first residue of A β being the N-terminal residue of the polypeptide, wherein the polypeptide is free of a C-terminal segment of A β . In yet

another aspect of the invention, the peptide immunogen is a polypeptide comprising an N-terminal segment of A β , the segment beginning at residue 1-3 of A β and ending at residues 7-11 of A β . In some aspects of the invention, the peptide immunogen is an agent that induces an immunogenic response against an N-terminal segment of A β , the segment beginning at residue 1-3 of A β and ending at residues 7-11 of A β without inducing an immunogenic response against an epitope within residues 12-43 of A β 43. In another aspect of the invention, the peptide immunogen is a heterologous polypeptide comprising a segment of A β linked to a heterologous amino acid sequence that induces a helper T-cell response against the heterologous amino acid sequence and thereby a B-cell response against the N-terminal segment.

[0026] In some peptide immunogens, the N-terminal segment of A β is linked at its C-terminus to a heterologous polypeptide. In some peptide immunogens, the N-terminal segment of A β is linked at its N-terminus to a heterologous polypeptide. In some peptide immunogens, the N-terminal segment of A β is linked at its N and C termini to first and second heterologous polypeptides. In some peptide immunogens, the N-terminal segment of A β is linked at its N terminus to a heterologous polypeptide, and at its C-terminus to at least one additional copy of the N-terminal segment. In some peptide immunogens, the polypeptide comprises from N-terminus to C-terminus, the N-terminal segment of A β , a plurality of additional copies of the N-terminal segment, and the heterologous amino acid segment.

[0027] In some of the above peptide immunogens, the polypeptide further comprises at least one additional copy of the N-terminal segment. In some of the above peptide immunogens, the fragment is free of at least the 5 C-terminal amino acids in A β 43.

[0028] In some aspects of the above peptide immunogens, the fragment comprises up to 10 contiguous amino acids from A β .

[0029] In another aspect, the invention provides a peptide immunogen comprising A β peptide or fragments of A β or analogs thereof eliciting an immunogenic response against certain epitopes within A β may be in a configuration referred to as a multiple antigenic peptide (MAP) configuration.

[0030] In some of the above aspects of the invention, the peptide immunogen from the N-terminal half of A β . In some aspects of the invention, the peptide immunogen is an A β fragment selected from the group consisting of A β 1-3, 1-4, 1-5, 1-6, 1-7, 1-10, 1-11, 1-12, 1-16, 3-6, and 3-7. In some of the above aspects of the invention, the peptide immunogen is from the internal region of A β . In some aspects of the invention, the peptide immunogen is an A β fragment selected from the group consisting of A β 13-28, 15-24, 17-28, and 25-35. In some of the above aspects of the invention, the peptide immunogen from the C-terminal end of A β . In some aspects of the invention, the peptide immunogen is an A β fragment selected from the group consisting of A β 33-42, 35-40, and 35-42. In some aspects of the invention, the peptide immunogen is an A β fragment selected from the group consisting of A β 1-3, 1-4, 1-5, 1-6, 1-7, 1-10, 1-11, 1-12, 1-16, 1-28, 3-6, 3-7, 13-28, 15-24, 17-28, 25-35, 33-42, 35-40, and 35-42. In some aspects of the invention, the peptide immunogen is an A β fragment

selected from the group consisting of A β 1-5, A β 1-7, A β 1-9, and A β 1-12. In some aspects of the invention, the peptide immunogen is an A β fragment selected from the group consisting of A β 1-5-L, A β 1-7-L, A β -9-L, and A β 1-12-L, where L is a linker. In some aspects of the invention, the peptide immunogen is an A β fragment selected from the group consisting of A β 1-5-L-C, A β 1-7-L-C, A β 1-9-L-C, and A β 1-12-L-C, where C is a cysteine amino acid residue.

[0031] In some aspects of the invention, the peptide immunogen is an A β fragment selected from the group consisting of A β 16-22, A β 16-23, A β 17-23, A β 17-24, A β 18-24, and A β 18-25. In some aspects of the invention, the peptide immunogen is an A β fragment selected from the group consisting of A β 16-22-C, A β 16-23-C, A β 17-23-C, A β 17-24-C, A β 18-24-C, and A β 18-25-C, where C is a cysteine amino acid residue. In other aspects of the invention, the peptide immunogen is an A β fragment selected from the group consisting of C-A β 16-22, C-A β 16-23, C-A β 17-23, C-A β 17-24, C-A β 18-24, and C-A β 18-25, where C is a cysteine amino acid residue.

[0032] In some of the above peptide immunogens, the heterologous polypeptide is selected from the group consisting of peptides having a T-cell epitope, a B-cell epitope and combinations thereof.

[0033] In one embodiment, the functional group of one or more amino acid molecules of the protein/polypeptide carrier or of the optionally attached polypeptide linker is derivatized using a cross-linking reagent. In another embodiment, the derivatizing reagent is a zero-length cross-linking reagent. In another embodiment, the derivatizing reagent is a homobifunctional cross-linking reagent. In yet another embodiment, the derivatizing reagent is a heterobifunctional cross-linking reagent.

[0034] In a preferred embodiment, the heterobifunctional reagent is a reagent that reacts with a primary or a ϵ -amine functional group of one or more amino acid molecules of the protein/polypeptide carrier and a pendant thiol group of one or more amino acid molecules of the peptide immunogen. In one embodiment, the heterobifunctional reagent is N-succinimidyl bromoacetate.

[0035] In another embodiment, the primary or ϵ -amine functional group is lysine. In yet another embodiment, the derivatization of the primary or ϵ -amine functional group of the lysine of the protein/polypeptide carrier with N-succinimidyl bromoacetate results in the bromoacetylation of the primary or ϵ -amine residues on lysine molecules on the protein/polypeptide carrier. In a more preferred embodiment, the pendant thiol group is a cysteine residue of the peptide immunogen, which may be localized at the amino-terminus of the peptide immunogen, at the carboxy-terminus of the peptide immunogen or internally in the peptide immunogen.

[0036] In another embodiment, the pendant thiol group is generated by a thiolating reagent such as N-acetyl homocysteinethio lactone, Traut's reagent (2-iminothiolane) SATA (N-Succinimidyl S-acetylthioacetate), SMPT (4-Succinimidyl oxycarbonyl-methyl2-pyridyl dithio toluene), Sulfo LC SPDP (Sulfo Succinimidyl pyridyl dithio propionamido hexanoate), SPDP (Succinimidyl pyridyl dithio propionate). In a preferred embodiment, the capping reagent that is used to inactivate free reactive, functional groups on the activated

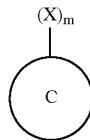
protein/polypeptide carrier is selected from the reagent group consisting of cysteamine, N-acetylcysteamine, and ethanolamine.

[0037] In a particularly preferred embodiment, the capping reagent that is used to inactivate free reactive functional groups on the activated protein/polypeptide carrier is selected from the reagent group consisting of sodium hydroxide, sodium carbonate, ammonium bicarbonate and ammonia.

[0038] In one embodiment, the reactive group of the amino acid residue of the peptide immunogen is a free sulphydryl group.

[0039] In another embodiment, one or more of the functional groups are on a linker, which is optionally attached to the protein/polypeptide carrier. In a preferred embodiment, the linker is a peptide linker. In a more preferred embodiment, the peptide linker is polylysine.

[0040] In another embodiment, the invention is directed to a second method for conjugating a peptide immunogen of a protein/polypeptide with a protein/polypeptide carrier having the structure:

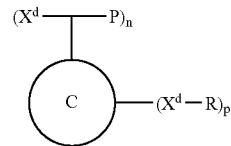


wherein,

[0041] C is a protein/polypeptide carrier and X is a derivatizable functional group of an amino acid residue on the protein/polypeptide carrier or optionally of an amino acid residue of a peptide linker covalently attached to the protein/polypeptide carrier, and wherein m is an integer greater than 0, but less than or equal to 85, the method comprising the steps of:

[0042] (a) derivatizing one or more of the functional groups of the protein/polypeptide carrier or of the optionally attached linker molecule to generate a derivatized molecule with reactive sites;

[0043] (b) reacting the derivatized protein/polypeptide carrier of step (a) with a reactive group of an amino acid residue of the peptide immunogen to form a covalently coupled peptide immunogen-protein/polypeptide carrier conjugate; and (c) further reacting the said conjugate with a capping reagent to inactivate the free reactive functional groups on the activated protein/polypeptide carrier, such that the capped groups are not free to react with other molecules, including proteins/polypeptides thereby preserving the functionality of the carrier, such that it retains its ability to elicit the desired immune responses against the peptide immunogen that would otherwise not occur without a carrier so as to generate a capped peptide immunogen-protein/polypeptide carrier conjugate having the formula:



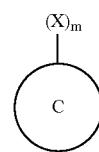
wherein,

[0044] C is the protein/polypeptide carrier and X^d is a derivatized functional group of an amino acid residue of the protein/polypeptide carrier or optionally of an amino acid residue of a peptide linker covalently attached to the protein/polypeptide carrier, and, wherein,

[0045] P is the peptide immunogen molecule covalently attached to the derivatized functional group on the amino acid residue on the protein carrier or optionally on an amino acid residue on a peptide linker covalently attached to a protein/polypeptide carrier, R is a capping molecule covalently attached to the derivatized functional group on an amino acid residue on the protein/polypeptide carrier or optionally on an amino acid residue on a peptide linker covalently attached to a protein/polypeptide carrier, n is an integer greater than 0, but less than or equal to 85, and p is an integer greater than 0, but less than 85.

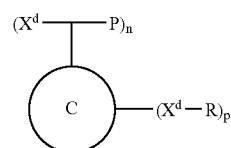
[0046] The detailed embodiments for the first method described above are also applicable to the conjugates just described prepared by the second method.

[0047] In one embodiment, the invention is directed to peptide immunogen-protein/polypeptide carrier conjugates wherein the protein/polypeptide carrier has the formula:



wherein,

[0048] C is a protein/polypeptide carrier and X is a derivatizable functional group of an amino acid residue on the protein/polypeptide carrier or optionally of an amino acid residue of a peptide linker covalently attached to the protein/polypeptide carrier, and, wherein, m is an integer greater than 0, but less than or equal to 85, and wherein the capped peptide immunogen-protein/polypeptide carrier conjugate has the formula:

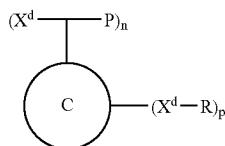


wherein,

[0049] C is the protein/polypeptide carrier and X^d is a derivatized functional group of an amino acid residue of the protein/polypeptide carrier or optionally of an amino acid residue of a peptide linker covalently attached to the protein/polypeptide carrier, and, wherein, P is the peptide immunogen molecule covalently attached to the derivatized functional group of the amino acid residue of the protein carrier or optionally of an amino acid residue of a peptide linker covalently attached to a protein/polypeptide carrier, R is a capping molecule covalently attached to the derivatized functional group of an amino acid residue of the protein/polypeptide carrier or optionally of an amino acid residue of a peptide linker covalently attached to a protein/polypeptide carrier, thereby preserving the functionality of the carrier, such that it retains its ability to elicit the desired immune responses against the peptide immunogen that would otherwise not occur without a carrier, n is an integer greater than 0, but less than or equal to 85, and p is an integer greater than 0, but less than 85.

[0050] The detailed embodiments for the first and second methods described above are also applicable to the conjugates just described.

[0051] In another embodiment, the invention is directed to peptide immunogen-protein/polypeptide carrier conjugates generated according to the second method of the invention and having the formula:



wherein,

[0052] C is the protein/polypeptide carrier and X^d is a derivatized functional group of an amino acid residue of the protein/polypeptide carrier or optionally of an amino acid residue of a peptide linker covalently attached to the protein/polypeptide carrier, and, wherein, P is the peptide immunogen molecule covalently attached to the derivatized functional group of the amino acid residue of the protein carrier or optionally of an amino acid residue of a peptide linker covalently attached to a protein/polypeptide carrier, R is a capping molecule covalently attached to the derivatized functional group of an amino acid residue of the protein/polypeptide carrier or optionally of an amino acid residue of a peptide linker covalently attached to a protein/polypeptide carrier, thereby preserving the functionality of the carrier, such that it retains its ability to elicit the desired immune responses against the peptide immunogen that would otherwise not occur without a carrier, n is an integer greater than 0, but less than or equal to 85, and p is an integer greater than 0, but less than 85.

[0053] The detailed embodiments for the second method described above are also applicable to the conjugates generated by the second method, as just described.

[0054] In another embodiment, the invention is directed to immunogenic compositions comprising a conjugate of a

peptide immunogen with a protein/polypeptide carrier generated by the second method of the invention, together with one or more pharmaceutically acceptable excipients, diluents, and adjuvants.

[0055] The detailed embodiments for the second method and the conjugates generated thereby described above are also applicable to immunogenic compositions containing those conjugates as just described.

[0056] In another embodiment, the invention is directed to a method for inducing an immune response in a mammalian subject, which comprises administering an effective amount of an immunogenic composition of the present invention to the subject.

[0057] The detailed embodiments applicable to the immunogenic composition containing the conjugates of the present invention are also applicable to the embodiment of the invention directed to the method of use of these immunogenic compositions.

BRIEF DESCRIPTION OF THE DRAWINGS

[0058] FIG. 1: Flow chart depicting the process chemistry used for conjugation of A β peptide fragments to protein/polypeptide carrier CRM₁₉₇ to form the A β /CRM₁₉₇ conjugate.

[0059] FIG. 2: Flow chart depicting acid hydrolysis chemistry used for quantitative determination of S-carboxymethylcysteine and S-carboxymethylcysteamine as evaluation of the degree of conjugation of peptide immunogen-protein/polypeptide conjugates such as the A β /CRM₁₉₇ conjugate.

[0060] FIG. 3: This figure depicts the pH dependence of the A β peptide/CRM conjugation reaction.

[0061] FIG. 4: This figure depicts the dependence of A β -peptide/CRM conjugation on peptide: CRM ratio.

[0062] FIG. 5: Verification of capping process for A β 1-7/CRM conjugation. The pH of the reaction was 9.15. Reaction time with peptide was 16 hrs, capping with N-acetylcysteamine was 8 hrs.

[0063] FIG. 6: Conjugation and capping with various peptide: CRM ratios with peptide. The pH of the reaction was 9.0. Reaction time with peptide was 16 hrs, capping with N-acetylcysteamine was 8 hrs.

[0064] FIG. 7: Day 36 titers of primate sera following immunization of primates with A β peptide conjugates with various adjuvants.

[0065] FIG. 8: Day 64 titers of primate sera following immunization of primates with A β -peptide conjugates with various adjuvants.

[0066] FIG. 9: Primate titers by day and treatment group. Primates were immunized with A β 1-7 or A β 1-5 CRM₁₉₇ conjugates with alum or 529 as adjuvants and titers of anti-A β antibodies were measured at day 29, 36, 57 and 54.

[0067] FIG. 10: Peptide-protein conjugates were characterized using SDS-PAGE Western blot analysis with a tris-tricine precast gel. The lanes are: marker (lane 1); L-28375 24/01 (lane 2); L-28375 24/02 (lane 3); L-28375 24/03 (lane 4); L-28375 24/04 (lane 5); L-18375 24/05 (lane 6); L-28375 24/06 (lane 7) L-28375 24/07 (lane 8); L-28375 24/08 (lane 9); L-28375 24/09 (Mock) (lane 10); and, BrAcCRM₁₉₇ (lane 11).

BRIEF DESCRIPTION OF SEQUENCES

SEQ ID NO: Sequence	Description
1 DAEFR-C	A β 1-5-C
2 DABFRHD-C	A β 1-7-C
3 DAEFRHDSG-C	A β 1-9-C
4 DAEFRHDSGYEV-C	A β 1-12-C
5 DAEFR-GAGA-C	A β 1-5-L-C
6 DAEFRHD-GAGA-C	A β 1-7-L-C
7 DAEFRHDSG-GAGA-C	A β 1-9-L-C
8 DAEFRHDSGYEV-GAGA-C	A β 1-12-L-C
9 VEYGSDKRFEAD-C	A β 12-1-C
10 GAGA	Linker peptide
11 PKYVKQNTLKLAT	Influenza Hemagglutinin: HA ₃₀₇₋₃₁₉
12 AKXVAAWTLKAAA	PAN-DR Peptide (PADRE peptide)
13 EKKIAKMEKASSVFNV	Malaria CS: T3 epitope
14 FELLTRILTI	Hepatitis B surface antigen: HB _s Ag ₁₉₋₂₈
15 DQSIGDLIAIEAMDVKVGNEG	Heat Shock Protein 65: hsp65 ₁₅₃₋₁₇₁
16 QVHFQPLPPAVVKL	Bacillus Calmette-Guerin (BCG)
17 QYIKANSKFIGITEL	Tetanus toxoid: TT ₈₃₀₋₈₄₄
18 FNNFTVSFWLRVPKVSASHLE	Tetanus toxoid: TT ₉₄₇₋₉₆₇
19 KQIINNMWQEVGKAMY	HIV gp120 T1
20 DAEFRHD-QYIKANSKFIGITEL-C- FNNFTVSFWLRVPKVSASHLE- DAEFRHD	A β ₁₋₇ /TT ₈₃₀₋₈₄₄ /C/TT ₉₄₇₋₉₆₇ /A β ₁₋₇
21 DAEFRHDSGYEVHHQKLVFFAEDVGSN KGAIIGLMVGVVIA	A β ₁₋₄₂
22 DAEFRHDQYIKANSKFIGITEL	AN90549: A β ₁₋₇ /TT ₈₃₀₋₈₄₄ (used in a MAP4 configuration)
23 DAEFRHDFNNFTVSFWLRVPKVSASHLE	AN90550: A β ₁₋₇ /TT ₉₄₇₋₉₆₇ (used in a MAP4 configuration)
24 DAEFRHD- QYIKANSKFIGITELFNNFTVSFWLRVPK VSASHLE	AN90542: A β ₁₋₇ /TT ₈₃₀₋₈₄₄ + TT ₉₄₇₋₉₆₇ (used in a linear configuration)
25 EFRHDSG-QYIKANSKFIGITEL	AN90576: A β ₃₋₉ /TT ₈₃₀₋₈₄₄ (used in a MAP4 configuration)
26 AKXVAAWTLKAAA-DAEFRHD	AN90562: A β ₁₋₇ /PADRE
27 DAEFRHD-DAEFRHDD- AEFRHDAKXVAAWTLKAAA	AN90543: A β ₁₋₇ × 3/PADRE
28 AKXVAAWTLKAAA-DAEFRHD- DAEFRHD-DAEFRHD	PADRE/A β ₁₋₇ × 3
29 DAEFRHD-AKXVAAWTLKAAA	A β ₁₋₇ × 3/PADRE

-continued

BRIEF DESCRIPTION OF SEQUENCES

SEQ ID NO: Sequence	Description
30 DAEFRHD-ISQAVHAAHAEINEAGR	$\text{A}\beta_{1-7}$ /albumin fragment
31 FRHDSGY-ISQAVHAAHAEINEAGR	$\text{A}\beta_{4-10}$ / albumin fragment
32 EFRHDSG-ISQAVHAAHAEINEAGR	$\text{A}\beta_{3-9}$ / albumin fragment
33 PKYVKQNTLKLAT-DAEFRHD- DAEFRHD-DAEFRHD	$\text{HA}_{307-319}/\text{A}\beta_{1-7} \times 3$
34 DAEFRHD-PKYVKQNTLKLAT- DAEFRHD	$\text{A}\beta_{1-7}/\text{HA}_{307-319}/\text{A}\beta_{1-7}$
35 DAEFRHD-DAEFRHD-DAEFRHD- PKYVKQNTLKLAT	$\text{A}\beta_{1-7} \times 3/\text{HA}_{307-319}$
36 DAEFRHD-DAEFRHD- PKYVKQNTLKLAT	$\text{A}\beta_{1-7} \times 2/\text{HA}_{307-319}$
37 DAEFRHD-PKYVKQNTLKLAT- EKKIAKMEKASSVFNV- QYIKANSKFIGITEL- FNNFTVSWLRLVPKVSASHLE- DAEFRHD	$\text{A}\beta_{1-7}/\text{HA}_{307-319}/\text{Malaria CS}/$ $\text{TT}_{830-844}/\text{TT}_{947-967}/\text{A}\beta_{1-7}$
38 DAEFRHD-DAEFRHD-DAEFRHD- QYIKANSKFIGITEL-C- FNNFTVSWLRLVPKVSASHLE	$\text{A}\beta_{1-7} \times 3/\text{TT}_{830-844}/\text{C}/\text{TT}_{947-967}$
39 DAEFRHD-QYIKANSKFIGITEL-C- FNNFTVSWLRLVPKVSASHLE	$\text{A}\beta_{1-7}/\text{TT}_{830-844}/\text{C}/\text{TT}_{947-967}$
40 GADDVVDSKSFSVFMENFSSYHGTPGY VDSIQKG1QKPKSGTQGMYDDDWKEFY STDNKYDAAGYSVDNENPLSGKAGGVV KVTVPGTLKVLALKVVDNAETIKKELGLS LTEPLMEQVGTEEFIKRGFDGASRVVLS LPFAEGSSSVEYINNWEQAKALSVELEIN FETRGKRGQDAMYEYMAQACAGNVR RSVGSSLSCINLDWDVIRDKTKTKIESLK EHGPIKKNMSESPNKTSEEKAQKQYLEE FHQTALEHPELS ELKTVTGTNPVFAGAN YAAWAVNVVAQVIDSETADNLKETTAAL SILPGIGSVMGIAADGAVHHNTTEIVAQSI ALSSLMVAQAIPLVGELVDIGFAAYNFV ESTINLFQVNVHSYNRPAYSPGHKTQPL HDGYAVSWNTVEDSIIIRTGFQGESGHDI KITAENTPLPIAGVLLPTIPGKLDVNKS THISVNGRKIRMRCRAIDGDVTFCRPKSP VYVGNGVHANLHVAFHRSSSEKIHSNEI SSDSIGVLGYQKTVVDHTKVNSKLSLFFEI KS	CRM β_{197}
41 ISQAVHAAHAEINEAGR	Albumin fragment
42 DAEFGHDSGFEVRHQKLVFFAEDVGSN KGAIIGLMVGGVVIA	Murine $\text{A}\beta_{1-42}$
43 VFFAEDVG-C	$\text{A}\beta_{18-25}-\text{C}$
44 LVFFAEDV-C	$\text{A}\beta_{17-24}-\text{C}$
45 KLVFFAED-C	$\text{A}\beta_{16-23}-\text{C}$
46 C-VFFAEDVG	$\text{C}-\text{A}\beta_{18-25}$
47 C-LVFFAEDV	$\text{C}-\text{A}\beta_{17-24}$
48 C-KLVFFAED	$\text{C}-\text{A}\beta_{16-23}$
49 VFFAEDV-C	$\text{A}\beta_{18-24}-\text{C}$

-continued

BRIEF DESCRIPTION OF SEQUENCES

SEQ ID NO: Sequence	Description
50 LVFFAED-C	A β ₁₇₋₂₃ -C
51 KLVFFAE-C	A β ₁₆₋₂₂ -C
52 C-VFFAEDV	C-A β ₁₈₋₂₄
53 C-LVFFAED	C-A β ₁₇₋₂₃
54 C-KLVFFAE	C-A β ₁₆₋₂₂

DETAILED DESCRIPTION OF THE INVENTION

[0068] The present invention is directed to methods of generating peptide immunogen-carrier conjugates wherein the unreacted active functional groups on the carrier which are generated during activation are inactivated by using capping reagents such as N-Acetylcysteamine in order to prevent them from reacting further. The present invention is also directed to capped carrier-peptide immunogen conjugates generated by those methods and to immunogenic compositions comprising said conjugates.

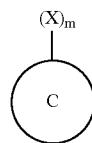
[0069] The approach of increasing immunogenicity of small or poorly immunogenic molecules, such as saccharides, through conjugation has been utilized successfully for decades (see, e.g., Goebel et al. (1939) *J. Exp. Med.* 69: 53), and many immunogenic compositions have been described in which purified capsular polymers have been conjugated to carrier proteins to create more effective immunogenic compositions by exploiting this "carrier effect". For example, Schneerson et al. (*J. Exp. Med.* 152: 361-376, 1980), describe *Haemophilus influenzae* b polysaccharide protein conjugates that confer immunity to invasive diseases caused by that microorganism. Conjugates of PRP (polyribosylribitol phosphate, a capsular polymer of *H. influenzae* b) have been shown to be more effective than immunogenic compositions based on the polysaccharide alone (Chu et al., (1983) *Infect. Immun.* 40: 245; Schneerson et al. (1984), *Infect. Immun.* 45: 582-591). Conjugation has also been shown to bypass the poor antibody response usually observed in infants when immunized with a free polysaccharide (Anderson et al. (1985) *J. Pediatr.* 107: 346; Insel et al. (1986) *J. Exp. Med.* 158: 294).

[0070] A further advantage of using as the protein carrier a bacterial toxin or toxoid against which routine immunization of humans (e.g., tetanus or diphtheria) is a standard practice is that a desired immunity to the toxin or toxoid is induced along with immunity against the pathogens associated with the capsular polymer.

[0071] Antigenic determinant/hapten-carrier conjugates also are being used to produce highly specific monoclonal antibodies that can recognize discrete chemical epitopes on the coupled hapten. The resulting monoclonals often are used to investigate the epitopic structure and interactions between native proteins. In many cases, the antigenic deter-

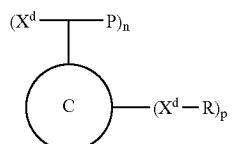
minants/haptens used to generate these monoclonals are small peptide segments representing crucial antigenic sites on the surface of larger proteins. The criteria for a successful carrier to be used in generating an antigenic determinant/hapten-carrier conjugate are the potential for immunogenicity, the presence of suitable functional groups for conjugation with an antigenic determinant/hapten, reasonable solubility properties even after derivatization and lack of toxicity in vivo.

[0072] These criteria are met by the conjugates generated by the methods of the instant invention. The conjugates may be any stable peptide immunogen-carrier conjugates generated using the conjugation process described herein. The conjugates are generated using a process of the instant invention wherein a protein/polypeptide carrier having the following structure:



is covalently attached to a protein/polypeptide carrier, wherein,

[0073] C is a protein/polypeptide carrier and X is a derivatizable functional group on an amino acid residue on the protein/polypeptide carrier or optionally on an amino acid residue on a peptide linker covalently attached to the protein/polypeptide carrier, and wherein m is an integer greater than 0, but less than or equal to 85, is covalently attached to a peptide immunogen and wherein the peptide immunogen/protein/polypeptide carrier conjugate has the following formula, is represented by the following formula:



wherein,

[0074] C is the protein/polypeptide carrier and X^d is a derivatized functional group on an amino acid residue on the protein/polypeptide carrier or optionally on an amino acid residue on a peptide linker covalently attached to the protein/polypeptide carrier, P is a peptide immunogen covalently attached to the derivatized functional group on the amino acid residue on the protein/polypeptide carrier or optionally on an amino acid residue on a peptide linker covalently attached to a protein/polypeptide carrier, R is a capping molecule covalently attached to the derivatized functional group on an amino acid residue on the protein/polypeptide carrier or optionally on an amino acid residue on a peptide linker covalently attached to a protein/polypeptide carrier thereby preserving the functionality of the carrier, such that it retains its ability to elicit the desired

are not limited to, albumin (including human serum albumin), keyhole limpet hemocyanin, immunoglobulin molecules, thyroglobulin, ovalbumin, tetanus toxoid, or a toxoid from other pathogenic bacteria having reduced toxicity, including mutants, such as diphtheria, *E. coli*, cholera, or *H. pylori*, or an attenuated toxin derivative. One such carrier is the CRM₁₉₇ protein (SEQ ID NO.:40) that is cross-reactive with diphtheria toxin.

[0076] Other carriers include T-cell epitopes that bind to multiple MHC alleles, e.g., at least 75% of all human MHC alleles. Such carriers are sometimes known in the art as “universal T-cell epitopes.” Exemplary carriers with universal T-cell epitopes include:

Influenza Hemagglutinin: HA ₃₀₇₋₃₁₉	PKYVKQNTLKLAT (SEQ. ID NO. 11)
PAN-DR Peptide (PADRE peptide)	AKXVAAWTLKAAA (SEQ. ID NO. 12)
Malaria CS: T3 epitope	EKKIAKMEKASSVFNV (SEQ. ID NO. 13)
Hepatitis B surface antigen: HB _S Ag ₁₉₋₂₈	FELLTRILTI (SEQ. ID NO. 14)
Heat Shock Protein 65: hsp65 ₁₅₃₋₁₇₁	QSIGDLIAEAMDKVGNEG (SEQ. ID NO. 15)
Bacillus Calmette-Guerin (BCG)	QVHFQPLPPAVVKL (SEQ. ID NO. 16)
Tetanus toxoid: TT ₈₃₀₋₈₄₄	QYIKANSKFIGITEL (SEQ. ID NO. 17)
Tetanus toxoid: TT ₉₄₇₋₉₆₇	NNFTVFSFWLRVPKVSASHLE (SEQ. ID NO. 18)
HIV gp ₁₂₀ T1:	KQIINMWQEVGKAMY (SEQ. ID NO. 19)
CRM ₁₉₇	See the Brief Description of the Sequences (SEQ ID NO. :40)
Albumin fragment	ISQAVHAAHAEINEAGR (SEQ ID NO. 41)

immune responses against the peptide immunogen that would otherwise not occur without a carrier, n is an integer greater than 0, but less than or equal to 85, and p is an integer greater than 0, but less than 85.

Selection Of Carriers

[0075] Some peptide immunogens contain the appropriate epitope for inducing an immune response, but are too small to be immunogenic. In this situation, the peptide immunogens are linked to a suitable carrier to help elicit an immune response. In the above schematic representation of the peptide immunogens-carrier conjugate generated by a process of the present invention, C is a protein/polypeptide carrier to which peptide immunogens are conjugated directly via derivatized functional groups on amino acid residues on the carrier themselves or indirectly via derivatized functional groups on peptide linkers covalently attached to the carriers. Suitable protein/polypeptide carriers include, but

[0077] Other carriers for stimulating or enhancing an immune response and to which a peptide immunogen or a hapten can be conjugated include cytokines such as IL-1, IL-1 α and β peptides, IL-2, γ INF, IL-10, GM-CSF, and chemokines, such as MIP 1 α and β and RANTES. Immunogenic peptides can also be linked to proteins/peptide carriers that enhance transport across tissues, as described in O’Mahony, WO 97/17163 and WO 97/17614, which are hereby incorporated by reference in their entirety for all purposes.

[0078] Still further carriers include recombinant Streptococcal C5a peptidase, *Streptococcus pyogenes* ORFs 1224, 1664 and 2452, *Chlamydia pneumoniae* ORFs T367 and T858, growth factors, and hormones.

[0079] In one preferred embodiment of the present invention, the carrier protein is CRM₁₉₇, a non-toxic mutant of diphtheria toxin with one amino acid change in its primary

sequence. The glycine present at the amino acid position 52 of the molecule is replaced with a glutamic acid due to a single nucleic acid codon change. Due to this change, the protein lacks ADP-ribosyl transferase activity and becomes non-toxic. It has a molecular weight of 58,408 Da. CRM₁₉₇ is produced in large quantities by recombinant expression in accordance with U.S. Pat. No. 5,614,382, which is hereby incorporated by reference. Conjugations of saccharides as well as peptides to CRM₁₉₇ are carried out by linking through the ε-amino groups of lysine residues. It has been well established through several commercial products that CRM₁₉₇ is an excellent and safe carrier for B-cell epitopes.

Immunogenic Peptides

[0080] As used herein, the term "peptide immunogen" or "hapter" is any protein or subunit structure/fragment/analog derived therefrom that can elicit, facilitate, or be induced to produce an immune response on administration to a mammal. In particular, the term is used to refer to a polypeptide antigenic determinant from any source (bacteria, virus or eukaryote), which may be coupled to a carrier using a method disclosed herein. Such polypeptide immunogen/antigenic determinants may be of viral, bacterial or eukaryotic cell origin.

[0081] Peptide immunogens from a bacterial cell include those derived from bacterial cell surface or secreted proteins, which can be used in protein-based immunogenic compositions. Exemplary bacterial strains include *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Neisseria meningitidis*, *Haemophilus influenzae*, *Klebsiella* spp., *Pseudomonas* spp., *Salmonella* spp., *Shigella* spp., *Alloioococcus otiditis*, and Group B *streptococci*.

[0082] Exemplary peptide immunogens from viruses include those derived from human immunodeficiency virus (HIV), herpes simplex virus (HSV), human papilloma virus (HPV), parainfluenza virus (PIV), vesicular stomatitis virus (VSV), respiratory syncytial virus (RSV), Epstein-Barr virus (EBV), coronavirus, vaccinia virus, rotavirus, rabies virus, hepatitis C virus (HCV) and hepatitis B virus (HBV) to name a few.

[0083] Exemplary fugal peptide immunogens include those derived from *Candida albicans*, *Cryptococcus neoformans*, *Coccidioides* spp., *Histoplasma* spp., and *Aspergillus* spp. Parasitic antigens include those derived from *Plasmodium* spp., *Trypanosoma* spp., *Schistosoma* spp., *Leishmania* spp. and the like.

[0084] Exemplary eukaryotic peptide immunogens that can be conjugated to a carrier for use as an immunotherapy in the prevention, treatment, prophylaxis or amelioration of various human diseases include those associated with tumor cells, those derived from Aβ, a peptide of 39-43 amino acids, preferably 42 amino acids, which is the principal component of characteristic plaques of Alzheimer's disease (AD) (see U.S. Pat. No. 4,666,829; Glenner & Wong (1984) *Biochem. Biophys. Res. Commun.* 120: 1131, Hardy (1984) *TINS* 20: 1131; Hardy (1977) *TINS* 20: 154), those derived from amyloid peptides of amylin, a polypeptide material produced by pancreatic islet cells that has been implicated in type II diabetes, peptides derived from low density lipoprotein gene products, which have been implicated in atherosclerosis and antigenic peptides derived from inflammatory cytokines and growth factors such as inter-

leukin 6 (IL-6), tumor necrosis factor α (TNF-α) and GDF-8. Such eukaryotic peptide immunogens may include either T-cell (CTL) or B-cell epitope.

[0085] A "CTL epitope" is one derived from selected epitopic regions of potential target antigens, such as tumor associated antigens, including, but not limited to, renal cell carcinoma, breast cancer, carcinoembryonic antigens, melanoma (MAGE) antigens, and prostate cancer specific antigens such as prostate specific membrane antigen (PSMA) and prostate stem cell antigen (PSCA), hepatitis C antigens,

[0086] Aβ, also known as β-amyloid peptide, or A4 peptide (see U.S. Pat. No. 4,666,829; Glenner & Wong, *Biochem. Biophys. Res. Commun.*, 120, 1131 (1984)), is a peptide of 39-43 amino acids, which is the principal component of characteristic plaques of Alzheimer's disease. Aβ is generated by processing of a larger protein APP by two enzymes, termed β and γ secretases (see Hardy, *TINS* 20, 154 (1997)). Known mutations in APP associated with Alzheimer's disease occur proximate to the site of β or γ secretase, or within Aβ. For example, position 717 is proximate to the site of γ-secretase cleavage of APP in its processing to Aβ, and positions 670/671 are proximate to the site of β-secretase cleavage. It is believed that the mutations cause AD by interacting with the cleavage reactions by which Aβ is formed so as to increase the amount of the 42/43 amino acid form of Aβ generated.

[0087] Aβ has the unusual property that it can fix and activate both classical and alternate complement cascades. In particular, it binds to Clq and ultimately to C3bi. This association facilitates binding to macrophages leading to activation of B cells. In addition, C3bi breaks down further and then binds to CR2 on B cells in a T cell dependent manner leading to a 10,000-fold increase in activation of these cells. This mechanism causes Aβ to generate an immune response in excess of that of other antigens.

[0088] Aβ has several natural occurring forms. The human forms of Aβ are referred to as Aβ39, Aβ40, Aβ41, Aβ42 and Aβ43. The sequences of these peptides and their relationship to the APP precursor are illustrated by FIG. 1 of Hardy et al., *TINS* 20, 155-158 (1997). For example, Aβ42 has the sequence:

(SEQ ID NO. 21)
 $\text{H}_2\text{N-Asp-Ala-Glu-Phe-Arg-His-Asp-Ser-Gly-Tyr-Glu-}$
 $\text{Val-His-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-}$
 $\text{Val-Gly-Ser-Asn-Lys-Gly-Ala-Ile-Ile-Gly-Leu-Met-}$
 $\text{Val-Gly-GLy-Val-Val-Ile-Ala-OH.}$

Aβ41, Aβ40 and Aβ39 differ from Aβ42 by the omission of Ala, Ala-Ile, and Ala-Ile-Val respectively from the C-terminal end. Aβ43 differs from Aβ42 by the presence of a threonine residue at the C-terminus.

[0089] Peptide immunogens which are fragments of Aβ are advantageous relative to the intact molecule for use in the present methods for several reasons. First, because only certain epitopes within Aβ induce a useful immunogenic response for treatment of Alzheimer's disease, an equal dosage of mass of a fragment containing such epitopes provides a greater molar concentration of the useful immu-

nogenic epitopes than a dosage of intact A β . Second, certain peptide immunogens of A β generate an immunogenic response against amyloid deposits without generating a significant immunogenic response against APP protein from which A β derives. Third, peptide immunogens of A β are simpler to manufacture than intact A β due to their shorter size. Fourth, peptide immunogens of A β do not aggregate in the same manner as intact A β , simplifying preparation of conjugates with carriers.

[0090] Some peptide immunogens of A β have a sequence of at least 2, 3, 5, 6, 10, or 20 contiguous amino acids from a natural peptide. Some peptide immunogens have no more than 10, 9, 8, 7, 5 or 3 contiguous residues from A β . In a preferred embodiment, peptide immunogens from the N-terminal half of A β are used for preparing conjugates. Preferred peptide immunogens include A β 1-5, 1-6, 1-7, 1-10, 1-11, 3-7, 1-3, and 1-4. The designation A β 1-5 for example, indicates an N-terminal fragment including residues 1-5 of A β . A β fragments beginning at the N-terminus and ending at a residue within residues 7-11 of A β are particularly preferred. The fragment A β 1-12 can also be used but is less preferred. In some methods, the fragment is an N-terminal fragment other than A β 1-10. Other preferred fragments include A β 13-28, 15-24, 1-28, 25-35, 35-40, 35-42 and other internal fragments and C-terminus fragments.

[0091] Some A β peptides of the invention are immunogenic peptides that on administration to a human patient or animal generate antibodies that specifically bind to one or more epitopes between residues 16 and 25 of A β . Preferred fragments include A β 16-22, 16-23, 17-23, 17-24, 18-24, and 18-25. Antibodies specifically binding to epitopes between residues 16 and 25 specifically bind to soluble A β without binding to plaques of A β . These types of antibody can specifically bind to soluble A β in the circulation of a patient or animal model without specifically binding to plaques of A β deposits in the brain of the patient or model. The specific binding of antibodies to soluble A β inhibits the A β from being incorporated into plaques thus either inhibiting development of the plaques in a patient or inhibiting a further increase in the size or frequency of plaques if such plaques have already developed before treatment is administered.

[0092] Preferably, the fragment of A β administered lacks an epitope that would generate a T-cell response to the fragment. Generally, T-cell epitopes are greater than 10 contiguous amino acids. Therefore, preferred fragments of A β are of size 5-10 or preferably 7-10 contiguous amino acids or most preferably 7 contiguous amino acids; i.e., sufficient length to generate an antibody response without generating a T-cell response. Absence of T-cell epitopes is preferred because these epitopes are not needed for immunogenic activity of fragments, and may cause an undesired inflammatory response in a subset of patients (Anderson et al., (2002) *J. Immunol.* 168, 3697-3701; Senior (2002) *Lancet Neurol.* 1, 3).

[0093] Fragment A β 15-25 and subfragments of 7-8 contiguous amino acids thereof are preferred because these peptides consistently generate a high immunogenic response to A β peptide. These fragments include A β 16-22, A β 16-23, A β 16-24, A β 17-23, A β 17-24, A β 18-24, and A β 18-25. Particularly preferred A β 15-25 subfragments are 7 contiguous amino acids in length. The designation A β 15-21 for example, indicates a fragment including residues 15-21 of

A β and lacking other residues of A β , and preferably 7-10 contiguous amino acids. These fragments can generate an antibody response that includes end-specific antibodies.

[0094] Peptide immunogens of A β s require screening for activity in clearing or preventing amyloid deposits (see WO 00/72880, which is incorporated herein in its entirety for all purposes). Administration of N-terminal fragments of A β induces the production of antibodies that recognize A β deposits in vivo and in vitro. Fragments lacking at least one, and sometimes at least 5 or 10 C-terminal amino acids present in naturally occurring forms of A β are used in some methods. For example, a fragment lacking 5 amino acids from the C-terminal end of AP43 includes the first 38 amino acids from the N-terminal end of A β .

[0095] Unless otherwise indicated, reference to A β includes the natural human amino acid sequences indicated above as well as analogs including allelic, species and induced variants. Analogs typically differ from naturally occurring peptides at one, two or a few positions, often by virtue of conservative substitutions. Analogs typically exhibit at least 80 or 90% sequence identity with natural peptides. Some analogs also include unnatural amino acids or modifications of N- or C-terminal amino acids at one, two, or a few positions. For example, the natural aspartic acid residue at position 1 and/or 7 of A β can be replaced with iso-aspartic acid.

[0096] Examples of unnatural amino acids are D, alpha, alpha-disubstituted amino acids, N-alkyl amino acids, lactic acid, 4-hydroxyproline, gamma-carboxyglutamate, epsilon-N,N,N-trimethyllysine, epsilon-N-acetyllysine, O-phosphoserine, N-acetylserine, N-formylmethionine, 3-methylhistidine, 5-hydroxylysine, omega-N-methylarginine, beta-alanine, ornithine, norleucine, norvaline, hydroxyproline, thyroxine, gamma-amino butyric acid, homoserine, citrulline, and isoaspartic acid. Immunogenic peptides also include analogs of A β and fragments thereof. Some therapeutic agents of the invention are all-D peptides, e.g., all-D A β , all-D A β fragment, or analogs of all-D A β or all-D A β fragment. Fragments and analogs can be screened for prophylactic or therapeutic efficacy in transgenic animal models in comparison with untreated or placebo controls as described in WO 00/72880.

[0097] Peptide immunogens also include longer polypeptides that include, for example, an immunogenic of A β peptide, together with other amino acids. For example, preferred immunogenic peptides include fusion proteins comprising a segment of A β linked to a heterologous amino acid sequence that induces a helper T-cell response against the heterologous amino acid sequence and thereby a B-cell response against the A β segment. Such polypeptides can be screened for prophylactic or therapeutic efficacy in animal models in comparison with untreated or placebo controls as described in WO 00/72880.

[0098] The A β peptide, analog, immunogenic fragment or other polypeptide can be administered in disaggregated or aggregated form. Disaggregated A β or fragments thereof means monomeric peptide units. Disaggregated A β or fragments thereof are generally soluble, and are capable of self-aggregating to form soluble oligomers, protofibrils and ADDLs. Oligomers of A β and fragments thereof are usually soluble and exist predominantly as alpha-helices or random coils. Aggregated A β or fragments thereof means oligomers

of A β or fragments thereof that have associate into insoluble beta-sheet assemblies. Aggregated A β or fragments thereof also means fibrillar polymers. Fibrils are usually insoluble. Some antibodies bind either soluble A β or fragments thereof or aggregated A β or fragments thereof. Some antibodies bind both soluble A β or fragments thereof and aggregated A β or fragments thereof.

[0099] Immunogenic peptides also include multimers of monomeric immunogenic peptides. Immunogenic peptides other than A β peptides should induce an immunogenic response against one or more of the preferred fragments of A β listed above (e.g., A β 1-3, 1-7, 1-10, and 3-7).

[0100] Immunogenic peptides of the present invention are linked to a carrier using a method of the present invention to form a conjugate. The immunogenic peptide can be linked at its amino terminus, its carboxyl terminus, or both to a carrier to form a conjugate. Optionally, multiple repeats of the immunogenic peptide can be present in the conjugate.

[0101] An N-terminal fragment of A β can be linked at its C-terminus to a carrier peptide to form a conjugate. In such conjugates, the N-terminal residue of the fragment of A β constitutes the N-terminal residue of the conjugate. Accordingly, such conjugates are effective in inducing antibodies that bind to an epitope that requires the N-terminal residue of A β to be in free form. Some immunogenic peptides of the invention comprise a plurality of repeats of an N-terminal segment of A β linked at the C-terminus to one or more copy of a carrier peptide to form a conjugate. The N-terminal fragment of A β incorporated into such conjugates sometimes begins at A β 1-3 and ends at A β 7-11. A β 1-7, 1-3, 1-4, 1-5, and 3-7 are preferred N-terminal fragment of A β . Some conjugates comprise different N-terminal segments of A β in tandem. For example, a conjugate can comprise A β 1-7 followed by A β 1-3 linked to a carrier.

[0102] In some conjugates, an N-terminal segment of A β is linked at its N-terminal end to a carrier peptide. The same variety of N-terminal segments of A β can be used as with C-terminal linkage. Some conjugates comprise a carrier peptide linked to the N-terminus of an N-terminal segment of A β , which is in turn linked to one or more additional N-terminal segments of A β in tandem. Preferably, such immunogenic A β fragments, once conjugated to an appropriate carrier, induce an immunogenic response that is specifically directed to the A β fragment without being directed to other fragments of A β .

[0103] Immunogenic peptides of the invention include immunogenic heterologous peptides. In some immunogenic peptides, an A β fragment is linked to a carrier to form an immunogenic heterologous peptide. This heterologous peptide is linked to a carrier using a method of the present invention to form a conjugate. Some of these immunogenic heterologous peptides comprise fragments of A β linked to tetanus toxoid epitopes such as described in U.S. Pat. No. 5,196,512, EP 378,881 and EP 427,347. Optionally, an immunogenic peptide can be linked to one or multiple copies of a carrier, for example, at both the N and C termini of the carrier to form an immunogenic heterologous peptide. Other of these immunogenic heterologous peptides comprise fragments of A β linked to carrier peptides described in U.S. Pat. No. 5,736,142. For example, an immunogenic heterologous peptide can comprise A β 1-7 followed by A β

1-3 followed by a carrier. Examples of such immunogenic heterologous peptides include:

A β 1-7/Tetanus toxoid 830-844 + 947-967 in a linear configuration
(SEQ ID NO. :24)

DAEFRHD-QYIKANSKFIGITELFNNFTVSFWLRVPKVSASHLE

Peptides described in U.S. PAT. NO. 5,736,142
(all in linear configurations):
PADRE/A β 1-7:
(SEQ ID NO. :26)

AKXVAAWTLKAAA-DAEFRHD
(SEQ ID NO. :26)

A β 1-7 × 3/PADRE:
(SEQ ID NO. :27)
DAEFRHD-DAEFRHD-DAEFRHD-AKXVAAWTLKAAA

PADRE/A β 1-7 × 3:
(SEQ ID NO. :28)
AKXVAAWTLKAAA-DAEFRHD-DAEFRHD-DAEFRHD

A β 1-7/PADRE:
(SEQ ID NO. :29)
DAEFRHD-AKXVAAWTLKAAA

A β 1-7/albumin fragment:
(SEQ ID NO. :30)
DAEFRHD-ISQAVHAAHAEINEAGR

A β 4-10/albumin fragment:
(SEQ ID NO. :31)
FRHDSGY-ISQAVHAAHAEINEAGR

A β 3-9/albumin fragment:
(SEQ ID NO. :32)
EFRHDSG-ISQAVHAAHAEINEAGR

HA₃₀₇₋₃₁₉/A β 1-7 × 3:
(SEQ ID NO. :33)
PKYVKQNTLKLAT-DAEFRHD-DAEFRHD-DAEFRHD

A β 1-7/HA₃₀₇₋₃₁₉/A β 1-7:
(SEQ ID NO. :34)
DAEFRHD-PKYVKQNTLKLAT-DAEFRHD

A β 1-7 × 3/HA₃₀₇₋₃₁₉:
(SEQ ID NO. :35)
DAEFRHD-DAEFRHD-DAEFRHD-PKYVKQNTLKLAT

A β 1-7 × 2/HA₃₀₇₋₃₁₉:
(SEQ ID NO. :36)
DAEFRHD-DAEFRHD-PKYVKQNTLKLAT

A β 1-7/HA₃₀₇₋₃₁₉/Malaria CS/TT₈₃₀₋₈₄₄/TT₉₄₇₋₉₆₇/
A β 1-7
(SEQ ID NO. :37)
DAEFRHD-PKYVKQNTLKLAT-EKKIAKMEKASSVFNV-QYIKANSKFIG
ITEL-FNNFTVSFWLRVPKVSASHLE-DAFFRHD

A β 1-7 × 3/TT₈₃₀₋₈₄₄/C/TT₉₄₇₋₉₆₇
(SEQ ID NO. :38)
DAEFRHD-DAEFRD-DAEFRHD-QYIKANSKFIGITEL-C-
FNNFTVSFWLRVPKVSASHLE

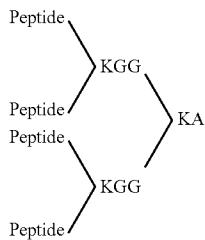
A β 1-7/TT₈₃₀₋₈₄₄/C/TT₉₄₇₋₉₆₇
(SEQ ID NO. :39)
DAEFRHD-QYIKANSKFIGITELCFNNFTVSFWLRVPKVSASHLE

A β 1-7/TT₈₃₀₋₈₄₄/C/TT₉₄₇₋₉₆₇/A β 1-7
(SEQ ID NO. :20)
DAEFRHD-QYIKANSKFIGITEL-C-FNNFTVSFWLRVPKVSASHLE-DA
EFRHD

[0104] Some immunogenic heterologous peptides comprise a multimer of immunogenic peptides represented by the formula 2^x, in which x is an integer from 1-5. Preferably

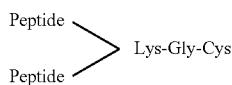
x is 1, 2 or 3, with 2 being most preferred. When x is two, such a multimer has four immunogenic peptides linked in a preferred configuration referred to as MAP4 (see US 5,229,490). Such immunogenic peptides are then linked to a carrier using a method of the present invention to form a conjugate.

[0105] The MAP4 configuration is shown below, where branched structures are produced by initiating peptide synthesis at both the N-terminal and side chain amines of lysine. Depending upon the number of times lysine is incorporated into the sequence and allowed to branch, the resulting structure will present multiple N-termini. In this example, four identical N-termini have been produced on the branched lysine-containing core. Such multiplicity greatly enhances the responsiveness of cognate B cells.



[0106] Examples of such immunogenic heterologous peptides include:

A β 1–7/Tetanus toxoid 830–844 in a MAP4 configuration:
DAEFRHD-QYIKANSKFIGITEL (SEQ ID NO.: 22)
A β 1–7/Tetanus toxoid 947–967 in a MAP4 configuration:
DAEFRHD-FNNFTVSPWLRVPKVSAHLE (SEQ ID NO.: 23)
A β 3–9/Tetanus toxoid 830–844 in a MAP4 configuration:
EFRHDSG-QYIKANSKFIGITEL (SEQ ID NO.: 25)
DAEFRHD-QYIKANSKFIGITEL on a 2 branched resin



[0107] The A β peptide, analog, active fragment or other polypeptide can be administered in associated or multimeric form or in dissociated form. Therapeutic agents also include multimers of monomeric immunogenic agents. Agents other than A β peptides should induce an immunogenic response against one or more of the preferred fragments of A β listed above (e.g., 1-10, 1-7, 1-3, and 3-7), and can also be conjugated to a carrier using a method of the present invention. Preferably, such agents, once conjugated to an appropriate carrier, induce an immunogenic response that is specifically directed to one of these fragments without being directed to other fragments of A β . To facilitate the conjugation of an peptide immunogen with a carrier, additional amino acids can be added to the termini of the antigenic determinants. The additional residues can also be used for modifying the physical or chemical properties of the peptide immunogen. Amino acids such as tyrosine, cysteine, lysine, glutamic or aspartic acid, or the like, can be introduced at the C- or N-terminus of the peptide immunogen. Additionally, peptide ulcers containing amino acids such as glycine and alanine can also be introduced. In addition, the antigenic determinants can differ from the natural sequence by being

modified by terminal NH₂-group acylation, e.g., by alkanoyl (C1-C20) or thioglycolyl acetylation, terminal-carboxy amidation, e.g., ammonia, methylamine, etc. In some instances these modifications may provide sites for linking to a support or other molecule.

[0108] The peptide immunogens used to generate conjugates of the present invention using a process disclosed herein can be combined via linkage to form polymers (multimers), or can be formulated in a composition without linkage, as an admixture. Where a peptide is linked to an identical peptide, thereby forming a homopolymer, a plurality of repeating epitopic units are presented. For example, multiple antigen peptide (MAP) technology is used to construct polymers containing both CTL and/or antibody peptides and peptides. When the peptides differ, e.g., a cocktail representing different viral subtypes, different epitopes within a subtype, different HLA restriction specificities, or peptides which contain T-helper epitopes, heteropolymers with repeating units are provided. In addition to covalent linkages, noncovalent linkages capable of forming intermolecular and intrastructural bonds are also contemplated.

[0109] Such peptide immunogens and their analogs are synthesized by solid phase peptide synthesis or recombinant expression, or are obtained from natural sources. Automatic peptide synthesizers are commercially available from numerous suppliers, such as Applied Biosystems, Foster City, Calif.

[0110] Recombinant expression can be in bacteria (such as *E. coli*), yeast, insect cells or mammalian cells. Procedures for recombinant expression are described by Sambrook et al., *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Press, NY, 2nd ed., 1989). Some immunogenic peptides are also available commercially (e.g., American Peptides Company, Inc., Sunnyvale, Calif., and California Peptide Research, Inc., Napa, Calif.).

[0111] Random libraries of peptides or other compounds can also be screened for suitability as a peptide immunogen. Combinatorial libraries can be produced for many types of compounds that can be synthesized in a step-by-step fashion. Such compounds include polypeptides, beta-turn mimetics, hormones, oligomeric N-substituted glycines, and oligocarbamates and the like. Large combinatorial libraries of the compounds can be constructed by the encoded synthetic libraries (ESL) method described in WO 95/12608, WO 93/06121, WO 94/08051, WO 95/35503 and WO 95/30642 (each of which is incorporated by reference for all purposes). Peptide libraries can also be generated by phage display methods (see, e.g., Devlin, WO 91/18980).

Derivatization and Conjugation of an Immunogenic Peptide to a Protein Carrier

[0112] The site of attachment of a peptide immunogen to a protein/polypeptide carrier, and the nature of the cross-linking agent that is used to attach a peptide immunogen to the carrier are both important to the specificity of the resultant antibody generated against it. For proper recognition, the peptide immunogen must be coupled to the carrier with the appropriate orientation. For an antibody to recognize subsequently the free peptide immunogens without carrier, the peptide immunogen-protein/polypeptide carrier conjugate must present the peptide immunogens in an exposed and accessible form. Optimal orientation is often

achieved by directing the cross-linking reaction to specific sites on the peptide immunogens. One way to achieve this with a peptide immunogen is by attaching a terminal cysteine residue during peptide synthesis. This provides a sulphydryl group on one end of the peptide for conjugation to the carrier. Cross-linking through this group provides attachment of the peptide immunogen only at one end, thereby ensuring consistent orientation.

[0113] In peptide immunogen-carrier conjugation, the goal is not to maintain the native state or stability of the carrier, but to present the hapten in the best possible way to the immune system. In reaching this goal, the choice of conjugation chemistry may control the resultant titer, affinity, and specificity of the antibodies generated against the hapten. It may be important in some cases to choose a cross-linking agent containing a spacer and long enough to present the antigen in an unrestricted fashion. It also may be important to control the density of the peptide immunogen on the surface of the carrier. Too little peptide immunogen substitution may result in little or no response. A peptide immunogen density too high actually may cause immunological suppression and decrease the response. In addition, the cross-linker itself may generate an undesired immune response. These issues need to be taken into consideration in selecting not only the appropriate cross-linking reagents, but also the appropriate ratios of protein/polypeptide carrier and peptide immunogen.

[0114] A variety of means of attaching the protein/peptide carriers to the peptide immunogens are possible. Ionic interactions are possible through the termini or through the ϵ -amino group of lysine. Hydrogen bonding between the side groups of the residues and the peptide immunogen are also possible. Finally, conformation interactions between the protein/peptide carriers and the immunogenic peptide may give rise to a stable attachment.

[0115] Peptide immunogens-carrier conjugates have been successfully generated using various cross-linking reagents such as zero-length, homobifunctional or heterobifunctional cross linkers. The smallest available reagent systems for bioconjugation are the so-called zero-length cross-linkers. These compounds mediate the conjugation of two molecules by forming a bond containing no additional atoms. Thus, one atom of a molecule is spacer. In many conjugation schemes, the final complex is bound together by virtue of chemical components that add foreign structures to the substances being cross-linked. In some applications, the presence of these intervening linkers may be detrimental to the intended use. For instance, in the preparation of peptide immunogen-carrier conjugates the complex is formed with the intention of generating an immune response to the attached hapten. Occasionally, a portion of the antibodies produced by this response will have specificity for the cross-linking agent used in the conjugation procedure. Zero-length cross-linking agents eliminate the potential for this type of cross-reactivity by mediating a direct linkage between two substances.

[0116] Homobifunctional reagents, which were the first cross-linking reagents used for modification and conjugation of macromolecules, consisted of bireactive compounds containing the same functional group at both ends (Hartman and Wold, 1966). These reagents could tie one protein to another by covalently reacting with the same common groups on both molecules. Thus, the lysine 6-amines or N-terminal

amines of one protein could be cross-linked to the same functional groups on a second protein simply by mixing the two together in the presence of the homobifunctional reagent.

[0117] Heterobifunctional conjugation reagents contain two different reactive groups that can couple to two different functional targets on proteins and other macromolecules. For example, one part of a cross-linker may contain an amine-reactive group, while another portion may consist of a sulphydryl-reactive group. The result is the ability to direct the cross-linking reaction to selected parts of target molecules, thus garnering better control over the conjugation process.

[0118] Heterobifunctional reagents are used to cross-link proteins and other molecules in a two-or three-step process that limits the degree of polymerization often obtained using homobifunctional cross-linkers.

[0119] Many methods are currently available for coupling of peptide immunogens to protein/polypeptide carriers using zero-length, homobifunctional or heterobifunctional crosslinkers. Most methods create amine, amide, urethane, isothiourea, or disulfide bonds, or in some cases thioethers. The more general method of coupling proteins or peptides to peptides utilizes bifunctional crosslinking reagents. These are small spacer molecules having active groups at each end. The spacer molecules can have identical or different active groups at each end. The most common active functionalities, coupling groups, and bonds formed are:

- [0120] 1. Aldehyde-amino \rightarrow secondary amine
- [0121] 2. Maleimido-sulphydryl \rightarrow thioether
- [0122] 3. Succinimidio-amino \rightarrow amide
- [0123] 4. Imidate esters-amino \rightarrow amide
- [0124] 5. Phenyl azides-amino \rightarrow phenyl amine
- [0125] 6. Acyl halide-sulphydryl \rightarrow thioether
- [0126] 7. Pyridyldisulfides-sulphydryl \rightarrow disulfide
- [0127] 8. Isothiocyanate-amino \rightarrow isothiourea.

[0128] The reactivity of a given carrier protein, in terms of its ability to be modified by a cross-linking agent such that it can be conjugated to a peptide immunogen, is determined by its amino acid composition and the sequence location of the individual amino acids in the three dimensional structure of the molecule, as well as by the amino acid composition of the peptide immunogen.

[0129] In the case of linkers ("L") between protein/peptide carriers and other peptides (e.g., a protein/peptide carriers and an peptide immunogen), the spacers are typically selected from Ala, Gly, or other neutral spacers of nonpolar amino acids or neutral polar amino acids. In certain embodiments the neutral spacer is Ala. It will be understood that the optionally present spacer need not be comprised of the same residues and thus may be a hetero- or homo-oligomer. Exemplary spacers include homo-oligomers of Ala. When present, the spacer will usually be at least one or two residues, more usually three to six residues. In other embodiments the protein/polypeptide carrier is conjugated to an peptide immunogen, preferably with the protein/peptide carrier positioned at the amino terminus. The peptide may be joined by a neutral linker, such as Ala-Ala-Ala or the like,

and preferably further contain a lipid residue such palmitic acid or the like which is attached to alpha and epsilon amino groups of a Lys residue ((PAM)₂Lys), which is attached to the amino terminus of the peptide conjugate, typically via Ser-Ser linkage or the like.

[0130] In some aspects of the invention, the peptide immunogen is an A β fragment selected from the group consisting of A β 1-5-L, A β 1-7-L, A β 1-9-L, and A β 1-12-L. In some aspects of the invention the linker is GAGA (SEQ ID NO: 10).

[0131] To facilitate the conjugation of a peptide immunogen with a carrier, additional amino acids can be added to the termini of the antigenic determinants. The additional residues can also be used for modifying the physical or chemical properties of the peptide immunogen. Amino acids such as tyrosine, cysteine, lysine, glutamic or aspartic acid, or the like, can be introduced at the C- or N-terminus of the peptide immunogen. Additionally, peptide linkers containing amino acids such as glycine and alanine can also be introduced. In addition, the antigenic determinants can differ from the natural sequence by being modified by terminal NH₂-group acylation, e.g., by alkanoyl (C₁-C₂₀) or thioglycolyl acetylation, terminal-carboxy amidation, e.g., ammonia, methylvamine, etc. In some instances these modifications may provide sites for linking to a support or other molecule.

[0132] In some aspects of the invention, the peptide immunogen is an A β fragment selected from the group consisting of A β 1-5-C, A β 1-7-C, A β 1-9-C, and A β 1-12-C, where C is a cysteine amino acid residue. In some aspects of the invention, the peptide immunogen is an A β fragment selected from the group consisting of A β 1-5-L-C, A β 1-7-L-C, A β 1-9-L-C, and A β 1-12-L-C.

[0133] The peptide immunogen is linked to the protein/peptide carrier either directly or via a linker either at the amino or carboxy terminus of the peptide immunogen. The amino terminus of either the peptide immunogen or the protein/peptide carrier may be acylated. In addition, the peptide immunogen -protein/peptide carrier conjugate may be linked to certain alkanyol (C₁-C₂₀) lipids via one or more linking residues such as Gly, Gly-Gly, Ser, Ser-Ser as described below. Other useful lipid moieties include cholesterol, fatty acids, and the like.

[0134] Peptide immunogens can be linked to a carrier by chemical crosslinking. Techniques for linking an immunogen to a carrier include the formation of disulfide linkages using N-succinimidyl-3-(2-pyridyl-thio) propionate (SPDP) (Carlsson, J et al. (1978) *Biochem J.* 173: 723,) and succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC) (if the peptide lacks a sulphydryl group, this can be provided by addition of a cysteine residue to the hapten). These reagents create a disulfide linkage between themselves and peptide cysteine resides on one protein and an amide linkage through the 6-amino on a lysine, or other free amino group in other amino acids. A variety of such disulfide/amide-forming agents are described in *Immune. Rev.* 62: 85 (1982). Other bifunctional coupling agents form a thioether rather than a disulfide linkage. The thioether forming agents include reactive ester of 6-maleimidocapric acid, 2-bromoacetic acid, and 2-iodoacetic acid, 4-(N-maleimidomethyl) cyclohexane-1-carboxylic acid. The carboxyl groups can be activated by combining them with succinimide or 1-hydroxyl-2-nitro-4-sulfonic acid, sodium salt.

[0135] Most frequently, lysine residues are the most abundant amino acid residues found on carrier proteins, and these residues are modified using cross-linking reagents to generate nucleophilic sites that are then coupled to a hapten. This coupling is achieved via any of the hydrophilic side chains on the hapten molecules that are chemically active. These include the guanidyl group of arginine, the (-carboxyl groups of glutamate and aspartic acid, the sulphydryl group of cysteine, and the ϵ -amino group of lysine, to name a few. Modification of proteins such that they can now be coupled to other moieties is achieved using crosslinking reagents, which react with any of the side chains on the protein carrier or hapten molecules.

[0136] In one aspect of the present invention, the carrier protein with or without a linker molecule is functionalized (derivatized) with a reagent that introduces reactive sites into the carrier protein molecule that are amenable to further modification to introduce nucleophilic groups. In one embodiment, the carrier is reacted with a haloacetylating reagent, which preferentially reacts with a number of functional groups on amino acid residues of proteins such as the sulphydryl group of cysteine, the primary ϵ -amine group of lysine residue, the α terminal of α -amines, the thioether of methionine and both imidazoyl side chain nitrogens of histidine (Gurd, 1967). In a preferred embodiment, the primary ϵ -amine groups on lysine residues of the carrier protein are derivatized with b N-hydroxysuccinimidyl bromoacetate to generate a bromoacetylated carrier. Conjugation of peptide immunogen and the activated protein carrier was carried out by slowly adding the activated carrier to the solution containing the peptide immunogen.

[0137] By using the process of this invention, the peptide immunogens discussed in section B, above, may be conjugated to any of the carriers discussed in section A, above. The conjugates resulting from the process of this invention are used as immunogens for the generation of antibodies against A β for use in passive/active immunotherapy. Furthermore, A β or an A β fragment linked to a carrier can be administered to a laboratory animal in the production of monoclonal antibodies to A β .

[0138] In one aspect of the invention, the conjugate is a conjugate selected from the group consisting of A β 1-7-CRM₁₉₇, (A β 1-7 \times 3)-CRM₁₉₇, and (A β 1-7 \times 5)-CRM₁₉₇. In one aspect of the invention, the conjugate is a conjugate selected from the group consisting of CRM₁₉₇-A β 1-5, CRM₁₉₇-A β 1-7, CRM₁₉₇-A β 1-9, and CRM₁₉₇-A β 1-12. In another aspect of the invention, the conjugate is a conjugate selected from the group consisting of A β 1-5-C-CRM₁₉₇, A β 1-7-C-CRM₁₉₇, A β 1-9-C-CRM₁₉₇, and A β 1-12-C-CRM₁₉₇, A β 16-23-C-CRM₁₉₇, A β 17-24-C-CRM₁₉₇, A β 18-25-C-CRM₁₉₇, CRM₁₉₇-C-A β 16-23, CRM₁₉₇-C-A β 17-24, CRM₁₉₇-C-A β 18-25, A β 16-22-C-CRM₁₉₇, A β 17-23-C-CRM₁₉₇, A β 18-24-C-CRM₁₉₇, CRM₁₉₇-C-A β 16-22, CRM₁₉₇-C-A β 17-23, and CRM₁₉₇-C-A β 18-24. In yet another aspect of the invention, the conjugate is a conjugate selected from the group consisting of selected from the group consisting of A β 1-5-L-C-CRM₁₉₇, A β 1-7-L-C-CRM₁₉₇, A β 1-9-L-C-CRM₁₉₇, and A β 1-12-L-C-CRM₁₉₇.

Capping

[0139] A disadvantage to the use of coupling reagents, which introduce reactive sites into the side chains of reactive amino acid molecules on carrier and/or hapten molecules, is

that the reactive sites if not neutralized are free to react with any unwanted molecule either in vitro or in vivo. In the process of the present invention, capping of the unreacted functional groups is accomplished by reaction of the conjugates with pendant reactive groups with reagents which inactivate/cap the reactive groups. Exemplary inactivating/capping reagents for use with the conjugation process of the present invention include cysteamine, N-acetylcysteamine, and ethanolamine. Alternatively, capping is accomplished by reaction with ammonia or ammonium bicarbonate, either of which converts the haloacetyl groups to aminoacetyl groups. Capping is also accomplished at alkaline pH (9.0-9.8) using sodium hydroxide or sodium carbonate, which converts the haloacetyl groups to hydroxyacetyl groups. One potential advantage of converting the haloacetyl groups to aminoacetyl or hydroxyacetyl groups, as opposed to the reaction with cysteamine derivatives, ethanolamine etc., is the introduction of relatively smaller size chemical functionalities, by reaction with ammonia or hydroxide/carbonate. The resulting capped functional groups, e.g. aminoacetyl or hydroxyacetyl, provide relatively less perturbation in the carrier protein portion of the conjugate. The capped peptide immunogen-carrier protein is purified as necessary using known methods, such as chromatography (gel filtration, ion exchange, hydrophobic interaction or affinity), dialysis, ultrafiltration-diafiltration, selective precipitation using ammonium sulfate or alcohol, and the like.

Immunogenic Conjugates and Compositions

[0140] The capped peptide immunogen-carrier protein conjugates are administered in an immunogenic composition to mammals, particularly humans, for prophylactic and/or therapeutic purposes. The conjugates of the present invention are used to elicit and/or enhance immune responses against immunogens. For instance, CTL-carrier conjugates are used to treat and/or prevent viral infection, amyloidogenic diseases, cancer etc. Alternatively, polypeptide immunogen-carrier conjugates, which induce antibody responses, are also used. Examples of diseases, which can be treated using the conjugates of the present invention, include various bacterial infections, viral infections, fungal infections, parasitic infections and cancer.

[0141] In therapeutic applications, a conjugate of the present invention is administered to an individual already suffering from an amyloidogenic disease such as Alzheimer's disease, cancer, or infected with a pathogenic microorganism. Those in the incubation phase or the acute phase of the disease may be treated with the conjugate of the present invention separately or in conjunction with other treatments, as appropriate.

[0142] In therapeutic applications, an immunogenic composition of the present invention is administered to a patient in an amount sufficient to elicit an effective CTL response or humoral response to the microorganism, amyloid plaque or to a tumor antigen expressed on a cancer cell, and to cure, or at least partially arrest disease progression, symptoms and/or complications. An amount adequate to accomplish this is defined as "therapeutically effective dose." Amounts effective for this use will depend in part on the peptide composition, the manner of administration, the stage and severity of the disease being treated, the weight and general state of health of the patient, and the judgment of the prescribing physician.

[0143] Therapeutically effective amounts of the immunogenic compositions of the present invention generally range for the initial immunization for therapeutic or prophylactic administration, from about 0.1 µg to about 10,000 µg of peptide for a 70 kg patient, usually from about 0.1 to about 8000 µg, preferably between about 0.1 to about 5000 µg, and most preferably between 0.1 to about 1,000 µg. These doses are followed by boosting dosages of from about 0.1 µg to about 1000 µg of peptide pursuant to a boosting regimen over weeks to months depending upon the patient's response and condition by measuring specific immune responses.

[0144] Further, the present invention is used prophylactically to prevent and/or ameliorate bacterial infections, viral infections, fungal infections, parasitic infections, amyloidogenic disease, or cancer. Effective amounts are as described above. Additionally, one of ordinary skill in the art would also know how to adjust or modify prophylactic treatments, as appropriate, for example by boosting and adjusting dosages and dosing regimes.

[0145] Therapeutic administration may begin at the first sign of the disease or the detection or surgical removal of tumors or shortly after diagnosis in the case of acute infection. This is followed by boosting doses until the disease progression is halted or reversed or the symptoms are substantially abated and for a period thereafter. In chronic infection, initial high doses followed by boosting doses may be required.

[0146] Treatment of an infected individual with the compositions of the invention may hasten resolution of the infection in acutely infected individuals. For those individuals susceptible (or predisposed) to developing chronic infection the compositions are particularly useful in methods for preventing the evolution from acute to chronic infection. Where the susceptible individuals are identified prior to or during infection, for instance, as described herein, the composition can be targeted to them, minimizing need for administration to a larger population.

[0147] The conjugates of the present invention are also used for the treatment of chronic infection and to stimulate the immune system to eliminate virus-infected cells in individuals with latent infections. It is important to provide an amount of the immunogenic composition of the present invention in a formulation and mode of administration sufficient to effectively elicit and/or enhance an immune response. Thus, for treatment of chronic infection, a representative dose is in the range from about 0.1 µg to about 10,000 µg of peptide, usually from about 0.1 to about 8000 µg, preferably between about 0.1 to about 5000 µg, and most preferably between 0.1 to about 1,000 µg for a 70 kg patient per dose. Immunizing doses followed by boosting doses at established intervals, e.g., from one to four weeks, may be required, possibly for a prolonged period of time to effectively immunize an individual. In the case of chronic infection, administration should continue until at least clinical symptoms or laboratory tests indicate that the viral infection has been eliminated or substantially abated and for a period thereafter.

[0148] Immunogenic compositions of the present invention for therapeutic or prophylactic treatment can be administered by parenteral, topical, intravenous, oral, subcutaneous, intra-arterial, intra-cranial, intra-peritoneal, intra-nasal or intramuscular means for prophylactic and/or therapeutic

treatment. One typical route of administration of an immunogenic agent is subcutaneous, although other routes can be equally effective. Another common route is intra-muscular injection. This type of injection is most typically performed in the arm or leg muscles. In some methods, agents are injected directly into a particular tissue where deposits have accumulated, for example intra-cranial injection. Intra-muscular injection or intravenous infusion is preferred for administration of antibody. In some methods, particular therapeutic antibodies are injected directly into the cranium. Because of the ease of administration, the immunogenic compositions of the invention are particularly suitable for oral administration. The invention further provides immunogenic compositions for parenteral administration, which comprise a solution of the peptides or conjugates, dissolved or suspended in an acceptable carrier, preferably an aqueous carrier.

[0149] A variety of diluents, excipients and buffers may be used, e.g., water, buffered water, phosphate buffered saline, 0.3% glycine, hyaluronic acid and the like. These compositions may be sterilized by conventional, well-known sterilization techniques, or may be sterile filtered. The resulting aqueous solutions may be packaged for use as is, or lyophilized, the lyophilized preparation being combined with a sterile solution prior to administration. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as buffering agents, tonicity adjusting agents, wetting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, sorbitan monolaurate, triethanolamine oleate, etc.

[0150] For solid compositions, conventional nontoxic solid carriers may be used. These may include, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, talcum, cellulose, glucose, sucrose, magnesium carbonate, and the like. For oral administration, a pharmaceutically acceptable nontoxic composition is formed by incorporating any of the normally employed excipients, such as those carriers previously listed, and generally 10-95% of active ingredient, that is, one or more conjugates of the invention, and more preferably at a concentration of 25-75%.

[0151] The concentration of immunogenic compositions of the present invention in the pharmaceutical formulations can vary widely, i.e., from less than about 0.1%, usually at or at least about 2% to as much as 20% to 50% or more by weight, and will be selected primarily by fluid volumes, viscosities, etc., in accordance with the particular mode of administration selected.

[0152] The conjugates of the present invention may also be administered via liposomes, which serve to target the conjugates to a particular tissue, such as lymphoid tissue, or targeted selectively to infected cells, as well as increase the half-life of the peptide composition. Liposomes include emulsions, foams, micelles, insoluble monolayers, liquid crystals, phospholipid dispersions, lamellar layers and the like. In these preparations the composition to be delivered is incorporated as part of a liposome, alone or in conjunction with a molecule, which binds to, for example, a receptor prevalent among lymphoid cells. These molecules would include monoclonal antibodies, which bind to the CD45 antigen, or with other therapeutic or immunogenic compo-

sitions. Thus, liposomes filled with a desired composition of the present invention can be directed to the site of lymphoid cells, where the liposomes then deliver the selected therapeutic/immunogenic peptide compositions. Liposomes for use in the invention are formed from standard vesicle-forming lipids, which generally include neutral and negatively charged phospholipids and a sterol, such as cholesterol. The selection of lipids is generally guided by consideration of liposome size, acid lability and stability of the liposomes in the blood stream. A variety of methods are available for preparing liposomes, as described in, e.g., Szoka, et al., *Ann. Rev. Biophys. Bioeng.* 9:467 (1980), U.S. Pat. Nos. 4,235,871, 4,501,728, 4,837,028, and 5,019,369, incorporated herein by reference.

[0153] For targeting to the immune cells, a ligand to be incorporated into the liposome can include antibodies or fragments thereof specific for cell surface determinants of the desired immune system cells. A liposome suspension containing a composition of the present invention may be administered intravenously, locally, topically, etc. in a dose which varies according to, inter alia, the manner of administration, the composition being delivered, and the stage of the disease being treated.

[0154] For aerosol administration, the compositions of the present invention are preferably supplied in finely divided form along with a surfactant and propellant. Typical percentages of the composition are 0.01-20% by weight, preferably 1-10%. The surfactant must, of course, be nontoxic, and preferably soluble in the propellant. Representative of such agents are the esters or partial esters of fatty acids containing from 6 to 22 carbon atoms, such as caproic, octanoic, lauric, palmitic, stearic, linoleic, linolenic, olesteric and oleic acids with an aliphatic polyhydric alcohol or its cyclic anhydride. Mixed esters, such as mixed or natural glycerides may be employed. The surfactant may constitute 0.1- 20% by weight of the composition, preferably 0.25-5%. The balance of the composition is ordinarily propellant. A carrier can also be included, if desired, as with lecithin for intranasal delivery.

[0155] The compositions of this invention may also be used to make monoclonal antibodies. Such antibodies may be useful as potential diagnostic or therapeutic agents.

[0156] The compositions of the present invention may also find use as diagnostic reagents. For example, a composition of the invention may be used to determine the susceptibility of a particular individual to a treatment regimen, which employs the polypeptide immunogens, and thus may be helpful in modifying an existing treatment protocol or in determining a prognosis for an affected individual. In addition, the compositions of the present invention may also be used to predict which individuals will be at substantial risk for developing chronic infection.

[0157] Conjugates of the present invention can optionally be administered in combination with other agents that are at least partly effective in treatment and/or amelioration of a disease and/or its symptoms. In the case of Alzheimer's and Down's syndrome, in which amyloid deposits occur in the brain, the conjugates of the invention can be administered in conjunction with other agents that increase passage of the agents of the invention across the blood-brain barrier.

[0158] The immunogenic composition typically contains an adjuvant. An adjuvant is a substance that enhances the

immune response when administered together with an immunogen or antigen. A number of cytokines or lymphokines have been shown to have immune modulating activity, and thus may be used as adjuvants, including, but not limited to, the interleukins 1- α , 1- β , 2, 4, 5, 6, 7, 8, 10, 12 (see, e.g., U.S. Pat. No. 5,723,127), 13, 14, 15, 16, 17 and 18 (and its mutant forms), the interferons- α , β and γ , granulocyte-macrophage colony stimulating factor (see, e.g., U.S. Pat. No. 5,078,996) macrophage colony stimulating factor, granulocyte colony stimulating factor, GSF, and the tumor necrosis factor α and β . Still other adjuvants useful in this invention include a chemokine, including without limitation, MCP-1, MIP-1 α , MIP-1 β , and RANTES. Adhesion molecules, such as a selectin, e.g., L-selectin, P-selectin and E-selectin may also be useful as adjuvants. Still other useful adjuvants include, without limitation, a mucin-like molecule, e.g., CD34, GlyCAM-1 and MadCAM-1, a member of the integrin family such as LFA-1, VLA-1, Mac-1 and p150.95, a member of the immunoglobulin super family such as PECAM, ICAMs, e.g., ICAM-1, ICAM-2 and ICAM-3, CD2 and LFA-3, co-stimulatory molecules such as CD40 and CD40L, growth factors including vascular growth factor, nerve growth factor, fibroblast growth factor, epidermal growth factor, B7.2, PDGF, BL-1, and vascular endothelial growth factor, receptor molecules including Fas, TNF receptor, Flt, Apo-1, p55, WSL-1, DR3, TRAMP, Apo-3, AIR, LARD, NGRF, DR4, DR5, KILLER, TRAIL-R2, TRICK<2>, and DR6. Still another adjuvant molecule includes Caspase (ICE). See, also International Patent Publication Nos. WO98/17799 and WO99/43839, which are incorporated herein by reference in their entirety for all purposes.

[0159] Suitable adjuvants used to enhance an immune response include, without limitation, MPL™ (3-O-deacylated monophosphoryl lipid A; Corixa, Hamilton, Mont.), which is described in U.S. Pat. No. 4,912,094, which is hereby incorporated by reference for all purposes. Also suitable for use as adjuvants are synthetic lipid A analogs or aminoalkyl glucosamine phosphate compounds (AGP), or derivatives or analogs thereof, which are available from Corixa (Hamilton, Mont.), and which are described in U.S. Pat. No. 6,113,918, which is hereby incorporated by reference. One such AGP is 2-[(R) -3-Tetradecanoyloxytetradecanoylamino] ethyl 2-Deoxy-4-O-phosphono-3-O-[(S) -3-tetradecanoyloxytetradecanoyl]-2-[(R) -3-tetradecanoyloxytetradecanoyl-amino]-b-D-glycopyranoside, which is known as 529 (also known as RC529; Corixa). This 529 adjuvant is formulated as an aqueous form (529 AF) or as a stable emulsion (529 SE).

[0160] Still other adjuvants include mineral oil and water emulsions, calcium salts such as calcium phosphate, aluminum salts (alum), such as aluminum hydroxide, aluminum phosphate, etc., Amphigen, Avridine, L121/squalene, D-lactide-polylactide/glycoside, pluronic acids, polyols, muramyl dipeptide, killed *Bordetella*, saponins, such as Stimulon™ QS-21 (Antigelics, Framingham, Mass.), described in U.S. Pat. No. 5,057,540, which is hereby incorporated by reference, and particles generated therefrom such as ISCOMS (immunostimulating complexes), *Mycobacterium tuberculosis*, bacterial lipopolysaccharides, synthetic polynucleotides such as oligonucleotides containing a CpG motif (U.S. Pat. No. 6,207,646, which is hereby incorporated by reference), a pertussis toxin (PT), or an *E. coli* heat-labile toxin (LT), particularly LT-K63, LT-R72, PT-K9/G129; see,

e.g., International Patent Publication Nos. WO 93/13302 and WO 92/19265, which are/ incorporated herein by reference for all purposes.

[0161] Also useful as adjuvants are cholera toxins and mutants thereof, including those described in published International Patent Application number WO 00/18434 (wherein the glutamic acid at amino acid position 29 is replaced by another amino acid (other than aspartic acid, preferably a histidine). Similar CT toxins or mutants are described in published International Patent Application number WO 02/098368 (wherein the isoleucine at amino acid position 16 is replaced by another amino acid, either alone or in combination with the replacement of the serine at amino acid position 68 by another amino acid; and/or wherein the valine at amino acid position 72 is replaced by another amino acid). Other CT toxins are described in published International Patent Application number WO 02/098369 (wherein the arginine at amino acid position 25 is replaced by another amino acid; and/or an amino acid is inserted at amino acid position 49; and/or two amino acids are inserted at amino acid position 35 and 36).

[0162] It is to be understood that reference throughout this specification to any theory to explain the results described is not to limit the scope of the invention. Independent of the method by which the invention functions, the results and advantages described herein may be achieved by reference to the following examples of the invention.

[0163] It will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the appended claims. All publications, patents and patent applications mentioned in this specification are incorporated by reference in their entirety for all purposes to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference.

EXAMPLE 1

Conjugation of CRM₁₉₇ To A β Peptide

[0164] Conjugation of haptens/antigenic peptides was carried out by reacting activated carrier CRM₁₉₇, which has thirty-nine lysine residues, to a hapten/antigenic peptide having a pendant thiol-group using the method described below (FIG. 1). All the A β peptides contained a cysteine residue at the carboxy terminus to facilitate the conjugation of these peptides through the cysteinyl sulphydryl group to the carrier protein. These peptides were produced by solid phase synthesis.

I. Activation

[0165] Free amino groups of CRM₁₉₇ were bromoacetylated by reaction with an excess of bromoacetic acid N-hydroxysuccinimide ester (Sigma Chemical Co., St. Louis, Mo.) (Bernatowicz and Matsueda, 1986). To an ice-cold solution of CRM₁₉₇ (~15 mg), 10% (v/v) 1.0 M NaHCO₃ (pH 8.4) was added. Bromoacetic acid N-hydroxysuccinimide ester, equal in weight to that of CRM₁₉₇ used, was dissolved in 200 μ L dimethylformamide (DMF), added slowly to the CRM₁₉₇, and gently mixed at room temperature in the dark for 1 hour. The resulting bromoacetylated (activated) protein was purified by passage through a desalt-

ing (P6-DG) column using PBS/1 mM EDTA (pH 7.0) as the eluent. Following purification, the fractions corresponding

reactive amino residues on the carrier was then subsequently verified using mass spectrometry.

Conjugate	Immunogenic Peptide	
A β 1-5-C-CRM ₁₉₇	DAEFR-C	(SEQ. ID. NO. :1)
A β 1-7-C-CRM ₁₉₇	DAEFRHD-C	(SEQ. ID NO. :2)
A β 1-9-C-CRM ₁₉₇	DAEFRHDSG-C	(SEQ ID NO :3)
A β 1-12-C-CRM ₁₉₇	DAEFRHDSGYEV-C	(SEQ ID NO :4)
A β 1-5-L-C-CRM ₁₉₇	DAEFR-GAGA-C	(SEQ ID NO. :5)
A β 1-7-L-C-CRM ₁₉₇	DAEFRHD-GAGA-C	(SEQ ID NO. :6)
A β 1-9-L-C-CRM ₁₉₇	DAEFRHDSG-GAGA-C	(SEQ ID NO. :7)
A β 1-12-L-C-CRM ₁₉₇	DAEFRHDSGYEV-GAGA-C	(SEQ ID NO. :8)
A β 12-1-C-CRM ₁₉₇ (-VE CONTROL)	VEYGSDHRFEAD-C	(SEQ ID NO. :9)

L = linker (GAGA) (SEQ ID NO.: 10)

to activated CRM₁₉₇ were pooled and the protein concentration was estimated by BCA protein assay. The protein amino groups, both before and after treatment with bromoacetic acid N-hydroxysuccinimide ester, were reacted with 2,4,6-trinitrobenzenesulfonic acid (TNBSA), which served as an indicator of bromoacetylation (Means et al., 1972).

II. Conjugation

[0166] Prior to conjugation, the peptides were reacted with 5,5'-dithio-bis(2-nitrobenzoic acid) [Ellman's reagent] to verify the content of free-SH groups (between 62-88% reduced). For the first four A β peptides (amino acids 1-7 without linker, amino acids 1-12 with GAGAC linker, amino acids 1-9 with GAGAC linker, and amino acids 1-7 with GAGAC linker), approximately 8.0-10.0 mg of peptide was dissolved in sterile distilled water to an approximate concentration of 20 mg/ml. The peptide was slowly added to cold activated CRM₁₉₇ in a 1:1 ratio (w/w) and the pH was adjusted to approximately 7.0-7.2 with the addition of 20-36 μ l of 1 N NaOH. The resulting material was gently mixed overnight at 4° C. in the dark followed by dialysis in the dark against two 1L changes of PBS, pH 7.2. For the next four A β peptides (amino acids 1-5 without linker, amino acids 1-9 without linker, amino acids 1-12 without linker, and amino acids 1-5 with linker), reaction with Ellman's reagent was used to verify the free —SH groups. CRM₁₉₇ was bromoacetylated, purified, and reacted with TNBSA as previously described. The pH of each peptide was adjusted to 7.0 with the addition of 0.1 M NaPO₄ (pH 8.5) at 2.2 \times the volume of the dissolved peptide. The peptide was slowly added to cold activated CRM₁₉₇ in a 1:1 ratio and allowed to react overnight at 4° C. in the dark. The resulting material was dialyzed. A final control peptide (1-12 mer in reverse orientation) was conjugated to CRM₁₉₇ as described above with the following modification. Rather than adjusting the pH of the peptide to 7.0, the pH of the activated CRM₁₉₇ was adjusted to approximately 7.5 with the addition of 20% (v/v) 0.5 M NaPO₄ (pH 8.0). Each conjugate, after dialysis, was transferred into a sterile 15 mL polypropylene tube, wrapped in aluminum foil, and stored at 4° C. Activation of the

EXAMPLE 2

Preparation of A β Peptide-CRM₁₉₇ Conjugate and Purification by Ultrafiltration Bromoacetylation of CRM₁₉₇

[0167] CRM₁₉₇ (100 mg) in 0.01 M sodium phosphate buffer, 0.9% NaCl, pH 7.0, was reacted with bromoacetic acid N-hydroxysuccinimide ester (dissolved to 20 mg/mL in DMSO) at a 1:1 weight ratio under an argon atmosphere. The reaction was titrated as needed to maintain the pH at 7.0. The mixture was stirred in dark for 1.5 hours at room temperature. The reaction mixture was 1.2 μ m filtered into the retentate reservoir of a UF/DF system (Millipore Lab-scale TFF, Billerica, Mass.). Purification was done using a 10K or 30K UF membrane by diafiltration (30-fold) against 0.01 M sodium phosphate buffer/0.9% NaCl, pH 7.0. The bromoacetylated CRM₁₉₇ was filtered by passing through a 0.2 μ m filter. The degree of bromoacetylation was determined by reacting the activated CRM₁₉₇ with cysteine, followed by amino acid analysis and quantitation of the resulting carboxymethylcysteine (CMC).

Conjugation of A β Peptide and Bromoacetylated CRM₁₉₇ and Capping with N-Acetylcycteamine

[0168] Bromoacetylated CRM₁₉₇ (50 mg) was transferred to a reaction vessel. To the stirred solution, maintained at 2-8° C., was added 1 M sodium carbonate/bicarbonate. Titration was performed to achieve a target pH of 9.0, under argon atmosphere. Separately, 50 mg of A β peptide was weighed out and dissolved in water for injection (WFI) to 20 mg/mL. To this solution was added 1 M sodium carbonate/bicarbonate until pH 9.0 was attained. The peptide solution was added to the bromoacetylated CRM₁₉₇ solution, and the mixture was stirred at 2-8° C. for 14-18 hours. The remaining bromoacetyl groups were capped with a 20-fold molar excess of N-acetylcycteamine for 3-6 hours at 2-8° C.

[0169] The reaction mixture was filtered through 1.2 μ m filter into the retentate reservoir of a UF/DF system (Millipore XL), and the conjugate was purified at room tempera-

ture by 30-fold diafiltration on a 10K or 30K MWCO membrane (Millipore) by diafiltering against 0.01 M sodium phosphate buffer/0.9% NaCl, pH 7.0. The retentate was collected and 0.21 µm filtered and analyzed for protein content (Lowry or Micro-BCA colorimetric assay), by SDS-PAGE, by amino acid analysis, and for immunogenicity in mice.

EXAMPLE 3

Conversion by Capping of the Unreacted Bromoacetyl Groups to Aminoacetyl Groups

[0170] Bromoacetylated CRM₁₉₇ (50 mg), prepared as described above in Example 2, was transferred to a reaction vessel. To the stirred solution, maintained at 2-8° C., was added 1M sodium carbonate/bicarbonate. Titration was performed to achieve a target pH of 9.0, under argon atmosphere. Separately, 50 mg of Aβ peptide was weighed out and dissolved in WFI to 20 mg/mL. To this solution was added 1 M sodium carbonate/bicarbonate until pH 9.0 was attained. The peptide solution was added to the bromoacetylated CRM₁₉₇ solution, and the mixture was stirred at 2-8° C. for 14-18 hours. The remaining bromoacetyl groups were capped using 8% ammonium bicarbonate solution for 4 hours at 2-8° C.

[0171] The reaction mixture was 1.2 µm filtered into the retentate reservoir of a UF/DF system (Millipore XL), and the conjugate was purified at room temperature by 30-fold diafiltration on a 10K or 30K MWCO membrane by diafiltering vs 0.01 M sodium phosphate buffer/0.9% NaCl, pH 7.0. The retentate was collected and 0.2 µm filtered and analyzed for protein content (Lowry or Micro-BCA colorimetric assay), by SDS-PAGE, by amino acid analysis, and for immunogenicity in mice.

EXAMPLE 4

Quantitative Determination of S-Carboxymethylcysteine and S-Carboxymethylcysteamine as Evaluation of Degree of Conjugation and Capping of Peptide Immunogen-Protein/Polypeptide Conjugates

[0172] Acid hydrolysis of protein-peptide conjugates generated using bromoacetyl activation chemistry resulted in the formation of acid stable S-carboxymethylcysteine (CMC) from the cysteines at the conjugated sites and the formation of acid stable S-carboxymethylcysteamine (CMCA) from the cysteamine at the capped sites (FIG. 2). All of the conjugated and capped lysines were converted back to lysine and detected as such. All other amino acids were hydrolyzed back to free amino acids except for tryptophan and cysteine, which were destroyed by the hydrolysis conditions. Asparagine and glutamine were converted to aspartic acid and glutamic acid respectively.

[0173] Conjugate samples were diluted with deionized water to a total protein concentration of less than 1 mg/mL. Two 10 microgram aliquots of each conjugate were dried and resuspended in 100 µL of 6N HCl [Pierce], 5 µL of melted phenol [Sigma-Aldrich], and 1 µL of 2-mercaptoethanol [Sigma-Aldrich]. The samples were then incubated under vacuum (100 mT) at 110° C. for 22 hours. The resulting hydrolysates were dried, resuspended in 250 µL of Beckman Na-S sodium citrate sample dilution buffer (pH

2.2) [Beckman Instruments, Inc., Fullerton, Calif., and filtered using Whatman 0.2 µm nylon syringe tip filters and 1 mL syringes.

[0174] Each sample was then loaded into a Beckman 6300 amino acid analyzer sample loop and placed in the analyzer. The amino acids of each hydrolyzed sample and control were separated using ion exchange chromatography followed by reaction with Beckman Ninhydrin NinRX solution at 135° C. The derivatized amino acids were then detected in the visible range at 570 nm and 440 nm (see Table 1). A standard set of amino acids [Pierce Amino Acid Standard H] containing 500 picomoles of each amino acid was run along with the samples and controls for each set of analysis. S-carboxymethylcysteine [Sigma-Aldrich] was added to the standard.

TABLE 1

Retention Times for Amino Acids Using Gradient Program 1 on the Beckman 6300 Amino Acid Analyzer			
Retention Time (min.)	Amino Acid		Wavelength used for Detection
8.3	Carboxymethylcysteine	CMC	570
9.6	Aspartic Acid & Asparagine	Asx	570
11.3	Threonine	Thr	570
12.2	Serine	Ser	570
15.8	Glutamic Acid & Glutamine	Glx	570 & 440
18.5	Proline	Pro	440
21.8	Glycine	Gly	570
23.3	Alanine	Ala	570
29.0	Valine	Val	570
32.8	Methionine	Met	570
35.5	Isoleucine	Ile	570
36.8	Leucine	Leu	570
40.5	Tyrosine	Tyr	570
42.3	Phenylalanine	Phe	570
45.4	Carboxymethylcysteamine	CMCA	570
48.8	Histidine	His	570
53.6	Lysine	Lys	570
70.8	Arginine	Arg	570

[0175] The areas of each standard peak were used as a quantitative equivalence for proportional evaluation of each sample. Proline was determined from 440 nm and was converted to an equivalence in 570 nm using Glutamic acid, the closest amino acid.

[0176] Each of these picomole values was converted to a molar ratio of amino acid residues using a comparison of picomoles of lysine to the theoretical lysine value present in the protein. Lysine was chosen for this evaluation based on its covalent attachment to Cysteine and Cysteamine and the expected similar hydrolysis. The resulting numbers of moles of amino acids were then compared to the amino acid composition of the protein and reported along with the values for CMC and CMCA. The CMC value was used directly for evaluation of the degree of conjugation and the CMCA value was used directly for evaluation of the degree of capping.

EXAMPLE 5

Characterization and Optimization of Aβ-CRM₁₉₇ Peptide Conjugates

[0177] To verify conjugation, all peptide-CRM₁₉₇ conjugates were analyzed by amino acid analysis and matrix-

assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry. For each conjugate, the moles of peptide conjugated to each mole CRM₁₉₇ was determined by amino acid analysis (number of S-carboxymethylcysteine residues) and MALDI-TOF mass spectrometry. The values determined by each method were generally in agreement.

I. Size Exclusion Chromatography:

[0178] Batch concentrate samples were removed from storage and allowed to warm to room temperature. The A β peptide conjugate sample was gently mixed to insure a homogeneous preparation. The A β peptide conjugate sample was spun in an Eppendorf micro-centrifuge to remove any particulates. The supernatant was withdrawn for TosoHaas TSK-Gel G3000SW chromatography (TosoHaas, Stuttgart, Germany). A TosoHaas TSIK-Gel G3000SW column was connected to a HPLC system and the pressure limit was set to 1.4 MPa. The column was equilibrated with at least 30 mL of PBS (10 mM sodium phosphate, 150 mM NaCl, pH 7.2 \pm 0.1) at a flow rate of 0.75 mL/min. The A β peptide conjugate sample was loaded onto the TosoHaas TSK-Gel G3000SW column using the following parameters:

[0179] Concentration of A β peptide conjugate sample: 1.5 \pm 1.0 mg/mL

[0180] Flow rate: 0.75 mL/min

[0181] Sample Volume: 0.1 mL

[0182] Run Time: 30 minutes

[0183] The absorbance was monitored at both 280 nm and 210 nm. For long term storage, the TosoHaas TSK-Gel G3000SW column was equilibrated with at least 50 mL of 20% ethanol at a flow rate of 0.5-1.0 mL/min.

II. PAGE (Polyacrylamide Gel Electrophoresis):

[0184] The activated (bromoacetylated) CRM₁₉₇ and the A β peptide-CRM₁₉₇ conjugates were examined by SDS-Gels using a NuPAGE Bis-Tris Electrophoresis (Novex, Frankfurt, Germany) with a neutral pH, pre-cast polyacrylamide mini-gel system and NuPAGE MES SDS Running Buffer. An 8 μ g aliquot of each activated CRM or conjugate was mixed with reducing sample buffer and heated at 100° C. for 5 minutes. The conjugates and molecular weight (MW) standards (Invitrogen, Carlsbad, Calif.) were loaded on a 10% (w/v, acrylamide) NuPage gel (Novex) based upon a Bis-Tris-HCl buffered system and run on MES SDS Running Buffer-PAGE (Laemmli). Following SDS-PAGE, the gel was stained with Pierce Gel Code Blue (Pierce, Rockford, Ill.). A β peptide-CRM₁₉₇ conjugate was represented by a major band around 66 kDa, above the band of native CRM and a dimer band around 120 kDa, along with minor multimer bands (data not shown).

III. MALDI-TOF Mass Spectrometry Analysis of Peptide-CRM₁₉₇ Conjugates:

[0185] Mass spectrometry was used for immediate approximation of the degree of conjugation. Suitable aliquots of activated CRM₁₉₇ and conjugate samples were analyzed by MALDI-TOF mass spectrometry using 3,5-dimethoxy-4-hydroxy-cinnamic acid (sinapinic acid) as the

matrix. The molecular weight of activated CRM₁₉₇ determined by MALDI-TOF mass spectrometry (Finnigan MAT Lasermat 2000 Mass Spectrometer, Ringoes, N.Y.) was found to be centered around 60.5 kDa and for conjugates varied from 65 kDa to 74 kDa depending on the degree of conjugation (data not shown). Up to 22 of the lysines (~50%) in CRM₁₉₇ were found to be modified at 1:1 ratio.

IV. Optimization Experiments:

[0186] The degree of activation and conjugation are a function of reagent:protein ratio, temperature of the reaction and pH of the reaction buffer. Some examples are given below to illustrate the optimal conjugation conditions carried out to identify the optimal pH conditions in order to have reproducible process control parameters for conjugation reactions. Results (FIG. 3) showed that the conjugation reaction to A β 5mer (DAEFRC)(SEQ ID NO: 1) as well as A β 7mer (DAEFRHDC)(SEQ ID NO:2) is pH dependent and yields a higher degree of modification/conjugation when the pH of the reaction condition is increased. Using the TFA salt of 5mer and 7mer peptides, the degree of conjugation was evaluated at pH 9.0 with varying amounts of peptide load (FIG. 4). It is evident from these results that peptide conjugates with a defined number of peptide copies per CRM molecule can be generated by varying the peptide/activated CRM ratio during the conjugation process. Similar experiments were done using acetate salt of A β 7mer peptide.

[0187] For the A β 1-7/CRM conjugation, the capping process was evaluated by comparing the moles of CMCA per CRM to the moles of CMC per CRM. Since the total of the CMC and CMCA was constant for each peptide:CRM ratio tested, the capping process was presumed to be complete (FIG. 5). The total modification in the conjugate stayed between 19 and 21, comparable to the number of lysines bromoacetylated (FIG. 5). These experiments were done with TFA as the counterion for the peptide. The A β 1-7/CRM conjugation was repeated using the acetate salt of the peptide rather than the TFA salt, and these data are shown in FIG. 5 and 6. The capping process appeared to go to completion, with the total of the CMC and CMCA for each point staying between 20 and 22. The conditions for the A β -CRM conjugation reaction have been optimized at pH 9.0, with the degree of conjugation controlled by the peptide to CRM ratio in the reaction. By varying the ratio from 0.1 to 1.5, the degree of conjugation can be varied (FIG. 6).

[0188] The degree of activation and conjugation are a function of reagent:protein ratio, temperature of the reaction and pH of the reaction buffer. The degree of modification (conjugation) for each conjugate was calculated by subtracting the mass value of activated CRM₁₉₇ from the mass value of each conjugate and dividing by the mass of the peptide used to prepare the conjugate. The degree of modification (conjugation) for all of the conjugates is described in the Table 2.

[0189] The degree of conjugation was also compared to the values determined by the estimated amount of S-carboxymethylcysteine residues formed per mole of CRM₁₉₇ (also shown in Table 2).

TABLE 2

Sample	Da	Degree of conjugation	Degree of conjugation
	(From Mass Spectrometry)	(From Mass Spectrometry)	(From CMC-Amino Acid Analysis)
CRM ₁₉₇	58,408	—	—
BrAc-CRM	60,752	19	—
A β 1-7/CRM	74,463	14	15
A β 1-7/CRM	72,375	12	14
A β 1-5/CRM	75,425	20	21
A β 1-5/CRM	71,690	15	18

EXAMPLE 6

Immunogenicity Studies of A β Peptide Conjugates

[0190] Peptides spanning N-terminal residues 1-5, 1-7, 1-9, and 1-12 of A β (with and without the linker sequence GAGAC) and a peptide corresponding to the N-terminus of A β in reverse sequence from amino acid twelve to amino acid one (1-12mer in reverse sequence), each conjugated to CRM₁₉₇, were used to immunize mice along with an unconjugated A β 1-12mer peptide in a formulation with STIMULON™ QS-21. Each group of mice was immunized subcutaneously with a dose of either 30 μ g or 5 μ g of one of the samples formulated with 20 μ g of the adjuvant STIMULON™ QS-21, at the beginning of the study (week 0) and subsequently at weeks 3 and 6. The study protocol is illustrated in Table 3.

[0191] As shown in Table 3, peptides spanning N-terminal residues 1-5, 1-7, 1-9, and 1-12 of A β (with and without the linker sequence GAGAC) and a peptide corresponding to the N-terminus of A β in reverse sequence from amino acid

twelve to amino acid one (1-12mer in reverse) conjugated to CRM₁₉₇ were used to immunize mice along with unconjugated A β 1-12mer peptide in a formulation with QS-21. Each group of mice was vaccinated subcutaneously with a dose of either 30 μ g or 5 μ g of one of the samples formulated with 20 μ g of the adjuvant QS-21, at the beginning of the study (week 0) and subsequently at weeks 3 and 6. Swiss Webster mice were used for the entire study with 5 mice in each group. Injection volume=100 μ l; B=Bleed; V=vaccinate; E=exsanguine.

[0192] Anti-A β titers were measured by ELISA against A β and CRM₁₉₇ as described below. Briefly, Costar 96 well plates (#3591) were coated overnight at room temperature with 2 μ g/mL A β 1-42 in sterile carbonate/bicarbonate buffer, pH 9.6. Plates were emptied and blocked for two hours at room temperature with 200 μ l/well of 0.05% BSA in 1 \times PBS/0.05% Tween 20. Blocked plates were emptied and washed with a plate washer containing TBS, 0.1% Brij-35 (without azide) wash buffer. All primary antisera were serially diluted with 0.05% BSA in 1 \times PBS containing 0.05% Tween 20/0.02% Azide and 100 μ L of each dilution was then transferred to the appropriate plate wells and incubated at room temperature for 2 hours. Plates were then emptied/washed as described above. Alkaline phosphatase conjugated goat anti-mouse IgG secondary antibody from Southern Biotech (city, state) was diluted 1:1000 with 0.05% BSA in PBS containing 0.05% Tween 20/0.02% Azide and 100 μ L was added to each well and incubated at room temperature for 1 hour. Plates were then emptied/washed as described above and finally incubated at room temperature for 1 hour with 100 μ L/well of a 1 mg/mL solution of p-nitrophenyl phosphate substrate prepared in diethanolamine/MgCl₂, pH 9.8. The color development was stopped with the addition of 50 μ L/well of 3 N NaOH. Plates were read at 405 nM with a 690 nM reference. Endpoint titers were calculated at an O.D. of 0.1 AU.

TABLE 3

Mouse Immunization Study Protocol

Group Code	Description	Dose (μ g)	Wk 0	Wk 3	Wk 6	Wk 8	Wk 13	Wk 16
AE488	CRM/1-7 w/o linker	30	B, V	B, V	B, V	B	B	E
AE489	CRM/1-12 with linker	30	B, V	B, V	B, V	B	B	E
AE490	CRM/1-9 with linker	30	B, V	B, V	B, V	B	B	E
AE491	CRM/1-7 with linker	30	B, V	B, V	B, V	B	B	E
AE492	CRM/1-5 w/o linker	30	B, V	B, V	B, V	B	B	E
AE493	CRM/1-9 w/o linker	30	B, V	B, V	B, V	B	B	E
AE494	CRM/1-12 w/o linker	30	B, V	B, V	B, V	B	B	E
AE495	CRM/1-5 with linker	30	B, V	B, V	B, V	B	B	E
AE496	CRM/1-7 w/o linker	5	B, V	B, V	B, V	B	B	E
AE497	CRM/1-12 with linker	5	B, V	B, V	B, V	B	B	E
AE498	CRM/1-9 with linker	5	B, V	B, V	B, V	B	B	E
AE499	CRM/1-7 with linker	5	B, V	B, V	B, V	B	B	E

TABLE 3-continued

<u>Mouse Immunization Study Protocol</u>								
Group Code	Description	Dose (μg)	Wk 0	Wk 3	Wk 6	Wk 8	Wk 13	Wk 16
AE500	CRM/1-5 w/o linker	5	B, V	B, V	B, V	B	B	E
AE501	CRM/1-9 w/o linker	5	B, V	B, V	B, V	B	B	E
AE502	CRM/1-12 w/o linker	5	B, V	B, V	B, V	B	B	E
AE503	CRM/1-5 with linker	5	B, V	B, V	B, V	B	B	E
AE504	CRM ₁₉₇ C1-6151	30	B, V	B, V	B, V	B	B	E
AE505	CRM ₁₉₇ C1-6151	5	B, V	B, V	B, V	B	B	E
AE506	CRM/12-1 mer	30	B, V	B, V	B, V	B	B	E
AE507	CRM/12-1 mer	5	B, V	B, V	B, V	B	B	E
AE508	1-12 mer peptide	30	B, V	B, V	B, V	B	B	E
AE509	1-12 mer peptide	5	B, V	B, V	B, V	B	B	E
AE510	Ab	30	B, V	B, V	B, V	B	B	E
AE511	Ab	5	B, V	B, V	B, V	B	B	E

CRM₁₉₇ ELISA

[0193] Greiner 96 well plates (#650011) were coated at 37° C. for 90 minutes with 5.0 μg/mL (100 μL/well) of CRM₁₉₇ in sterile carbonate/bicarbonate buffer, pH 9.6. Plates were emptied and washed with a plate washer containing 1× TBS, 0.1% Brij-35 wash buffer. All primary antisera were serially diluted with 1× PBS containing 0.3% Tween 20/EDTA and 100 μL of each dilution was then transferred to the appropriate plate wells and incubated at 37° C. for 1 hour. The plates were then emptied/washed as described above. Alkaline phosphatase conjugated goat anti-mouse IgG secondary antibody from Southern Biotech was diluted 1:1000 with 1× PBS containing 0.05% Tween 20/0.02% Azide and 100 μL was added to each well and incubated at 37° C. for 1 hour. Plates were then emptied/washed as described above and finally incubated at room temperature for 1 hour with 100 μL/well of a 1 mg/mL solution of p-nitrophenyl phosphate substrate prepared in diethanolamine/MgCl₂, pH 9.8. The development was stopped with the addition of 50 μL/well of 3 N NaOH. Plates were read at 405 nM with a 690 nM reference. Endpoint titers were calculated at an O.D. of 0.1 AU.

[0194] Tables 4-6 illustrate end point ELISA titers against Aβ. Following primary immunization, all eight conjugates (excluding the negative control) induced measurable anti-Aβ IgG immune responses. However, the 30 kg dose, but not the 5 μg dose, of Aβ gave a positive response at week 3 following primary immunization. Among all the conjugates, it appears that Aβ 1-7 peptide conjugated without linker elicited as good as or better response than other conjugates studied. At 5 μg dose, Aβ 1-5C did better at weeks 8-16. Aβ 1-7C was best at 30 kg dose. Analysis of antibody titers after second and third immunization with either 5 or 30 μg dose indicate that the maximal immune response to Aβ for most of the conjugates was seen after the second immunization. At least in mice, the third immunization did not appear to enhance the immune response. Aβ peptide however, needed three immunizations with the 30 μg dose to reach maximal

immune response against the peptide (Table 5). In terms of antibody decay over an extended period of time, the antibody level from the groups immunized with conjugates was reduced by 2 to 3 fold as compared to the highest level within that group. Individual samples from weeks 6 and 8 were analyzed to calculate GMTs against Aβ for each of the group (Table 6) to see if any conjugate group was substantially better than the others. Statistical analysis of week 6 titers from Aβ1-5C, Aβ1-7C and Aβ 1-9C conjugates indicated that the Aβ 1-7 conjugate induced a significantly higher titer. It is also evident from this experiment that the linker sequence GAGAC did not contribute to enhancing the immune response to the peptide.

TABLE 4

Table 4. Weeks 0, 3, 6, 8, 13, and 16 ELISA endpoint titers against Aβ using antisera from 5 μg dose of peptide conjugates spanning lengths of the N-terminus of Amyloid Aβ peptide, Ref: Elan hyperimmune polyclonal #592 = 3,073,307. Endpoint at O.D. 0.1 AU. Swiss Webster mice were immunized SC-N with 5 μg of above antigens formulated with 20 μg STIMULON™ QS-21 at weeks 0, 3, and 6.

Group	Week 3	Week 6	Week 8	Week 13	Week 16
1-5C	<100	14,960	687,691	882,012	625,208
1-7C	<100	51,253	1,280,181	860,463	520,060
1-9C	<100	18,615	1,008,872	622,325	348,967
1-12C	<100	615	132,009	390,624	166,162
1-5LC	<100	4,999	458,075	454,631	237,573
1-7LC	<100	17,693	849,170	842,402	446,089
1-9LC	<100	18,544	1,465,115	1,180,347	571,127
1-12LC	<100	12,664	908,360	598,867	368,101
CRM ₁₉₇	<100	<100	<100	<100	<100
1-42	<100	<100	<100	<100	<100
1-12	<100	<100	<100	<100	<100
12-1C	<100	<100	<100	<100	<100

[0195]

TABLE 5

Table 5. Weeks 0, 3, 6, 8, 13, and 16 ELISA endpoint titers against A β using antisera from 30 μ g dose of peptide conjugates spanning varying lengths of the N-terminus of Amyloid A β peptide.
 Ref: Elan hyperimmune polyclonal #592 = 3,073,307.
 Endpoint at O.D. 0.1 AU. Swiss Webster mice were immunized SC-N with 30 μ g of above antigens formulated with 20 μ g STIMULON™ QS-21 at weeks 0, 3, and 6.

Group	Week 0	Week 3	Week 6	Week 8	Week 13	Week 16
1-5C	<100	18,150	590,355	332,832	204,645	176,159
1-7C	<100	100,672	1,840,741	647,470	592,638	779,072
1-9C	<100	18,520	1,184,696	713,494	363,459	327,065
1-12C	<100	7,837	1,325,725	1,126,389	681,268	577,604
1-5LC	<100	16,347	469,191	184,077	177,358	164,680
1-7LC	<100	47,866	971,229	462,200	463,466	529,726
1-9LC	<100	59,002	921,544	787,273	405,023	500,468
1-12LC	<100	27,348	697,150	483,320	284,800	397,816
CRM ₁₉₇	<100	<100	<100	<100	<100	<100
1-42	<100	160	3,327	109,718	48,646	27,901
1-12	<100	<100	<100	<100	<100	<100
12-1C	<100	<100	<100	<100	<100	<100

[0196]

TABLE 6

Table 6. Weeks 6 and 8 ELISA endpoint GMTs against A β using antisera from 30 μ g dose of peptide conjugates spanning varying lengths of the N-terminus of Amyloid-A β . Ref: Elan Hyperimmune Polyclonal #592 = 3,073,307. Endpoint at O.D. 0.1 AU. Swiss Webster mice were immunized SC-N with 30 μ g of above antigens formulated with 20 μ g STIMULON™ QS-21 at weeks 0, 3, and 6

Group	Week 6	Week 8
1-5C	237,668 ^a	61,671 ^b
1-7C	1,866,702 ^a	881,146 ^b
1-9C	963,323 ^a	595,414 ^b
1-12C	940,260	955,470
1-5LC	395,553	141,084
1-7LC	516,921	394,521
1-9LC	826,773	562,458
1-12LC	544,768	376,952
1-42	365	4,565

^aStatistical analysis of week 6 titers from 1-5C, 1-7C, and 1-9C using Tukey-Kramer show a statistical difference between 1-5C vs 1-7C only, whereas, analysis using Student's T-test shows a statistical difference between 1-5C vs 1-7C and 1-5C vs 1-9C.

^bStatistical analysis of week 8 titers from 1-5C, 1-7C, and 1-9C does not show a statistical difference among the three groups. However, there appears to be a trend that may indicate a difference between 1-5C vs 1-7C.

PDAPP Mouse Brain Tissue Staining

[0197] The PDAPP brain tissue staining assay provides an indication of the functionality of the A β peptide conjugates and/or A β 1-42 antisera. Serum samples from individual mouse groups were separately analyzed for their ability to recognize PDAPP mouse brain tissue plaques containing amyloid peptide. The results are shown in Table 7A and 7B. With the exception of the A β 5mer conjugate antisera, there was a dose-related response in recognizing the plaques. Independent of the linker, 30kg conjugate-induced antisera had better reactivity patterns as compared to that of 5 μ g conjugate antisera. However, with the A β 5mer conjugate antisera, there seems be similar or better reactivity for the 5 μ g group. Comparing all these results, it is concluded that

conjugates made from A β 1-5mer through A β 1-9mer are sufficient in eliciting plaques recognizing immune response in mice and the presence of linker is not essential. The following conclusions can be drawn from this study: (a) All of the peptide conjugates induced high titered antisera against the carrier protein CRM₁₉₇ to equal or slightly higher levels as compared to the unconjugated CRM₁₉₇ control (not shown). (b) The conjugates with the GAGAC linker did not enhance immunogenicity or functionality compared to conjugates without the linker. (c) The immunogenicity data and PDAPP brain tissue staining (an initial indication of functional antibody) show that the A β 1-5mer and A β 1-7mer conjugates appeared to be the preferred immunogens for further development.

TABLE 7A

Vaccine	Without Linker		With Linker		
	Animal #	PDAPP Staining	Vaccine	Animal #	PDAPP Staining
CRM/A β 1-5	1	+ (no diffuse)	CRM/A β 1-5	1	-
	2	++/+++		2	-
	3	++/+++		3	\pm
	4	++		4	\pm
	5	++		5	\pm
CRM/A β 1-7	1	++	CRM/A β 1-7	1	+
	2	++		2	++
	3	++		3	++
	4	++		4	+
	5	++		5	++
CRM/A β 1-9	1	+	CRM/A β 1-9	1	++
	2	+/++		2	++
	3	\pm		3	+
	4	\pm		4	+
	5	\pm		5	+
CRM/A β 1-12	1	-	CRM/A β 1-12	1	+
	2	?		2	+
	3	\pm		3	++

TABLE 7A-continued

PDAPP mouse brain tissue staining. 5 µg Dose					
	Without Linker		With Linker		
Vaccine	Animal #	PDAPP Staining	Vaccine	Animal #	PDAPP Staining
CRM/A β 12-1mer	4	-	A β 42	4	-
	5	\pm		5	\pm
	1	-		1	-
	2	-		2	-
	3	\pm		3	-
	4	-		4	-
	5	\pm		5	-

All antiserum diluted 1:1000 for staining procedure.

[0198]

TABLE 7B

PDAPP mouse brain tissue staining. 30 µg Dose					
	Without Linker		With Linker		
Vaccine	Animal #	PDAPP Staining	Vaccine	Animal #	PDAPP Staining
CRM/A β 1-5	1	-	CRM/A β 1-5	1	+
	2	++/++		2	-
	3	-		3	-
	4	\pm		4	\pm
	5	++		5	-
CRM/A β 1-7	1	++/++	CRM/A β 1-7	1	+
	2	++		2	\pm /+
	3	++		3	++/++
	4	++		4	\pm /+
	5	++/+++		5	++/++
CRM/A β 1-9	1	++/+++	CRM/A β 1-9	1	++/++
	2	++		2	++
	3	++		3	++
	4	+		4	\pm
	5	+		5	++/++
CRM/A β 1-12	1	-	CRM/A β 1-	1	++/++
	2	++/++	12	2	+
	3	++/++		3	-
	4	\pm		4	++/++
	5	\pm		5	+
CRM/A β 12-1mer	1	-	A β 42	1	\pm
	2	-		2	-
	3	-		3	-
	4	-		4	-
	5	-		5	-

All antiserum diluted 1:1000 for staining procedure.

EXAMPLE 7

Immunogenicity Studies in Monkeys

[0199] Groups of 6 monkeys received 30 µg of 7mer conjugate (total conjugate) adjuvanted with either STIMULON™ QS-21, alum or RC529 SE formulation at days 0, 29 and 58. Additional groups included were 30 µg 5mer conjugate with either alum ($Al(OH)_3$) or RC529 SE, 75 and 300 µg of A β with STIMULON™ QS-21 as positive controls.

Positive controls were immunized every two weeks. At day 36 and 64 the anti-A β antibody titers were determined (FIGS. 7-9). On day 36, 7mer/CRM conjugates with STIMULON™ QS-21, Alum and RC529 SE elicited GMT titers of 10110, 13330 and 17090 respectively (FIG. 7). In contrast, A β 1-42 plus STIMULON™ QS-21 elicited GMTs of 223 and 1734 at 75 and 300 µg dose levels, respectively. The A β 5mer conjugate elicited a titer of 2134 with alum and 15980 with RC529 SE. On day 64, i.e. after 3 doses of conjugates with either STIMULON™ QS21 or RC-529 SE induced substantially higher titers than post second dose (GMTs 69910 for 7 mer/RC-529 SE; 21640 for A β 5mer/RC-529 SE and 30310 for A β 7mer/STIMULON™ QS-21) (FIG. 8). Conjugates with alum elicited reduced titers at post third immunization compared to post second immunization. It appears that the A β 7mer conjugate elicited a better response as compared to the A β 5mer conjugate. In monkeys, adjuvanting A β 7mer conjugate with RC-529 SE or STIMULON™ QS-21 elicited the highest response (FIG. 9). The response to the A β 7mer conjugate with alum was moderate and similar to that of 300 µg A β 1-42 with STIMULON™ QS-21.

[0200] Several conclusions can be drawn from the current example. First, both conjugates are very immunogenic in primate species. Second, the presence of adjuvants in the immunization formulation significantly influences the immune response. Third, except for the aluminum adjuvant, RC-529 SE and STIMULON™ QS-21 enhance the immune response after each dose of immunization at least up to three doses (FIGS. 9). Overall, A β 7mer conjugate induced higher antibody response in the presence of 529, followed by STIMULON™ QS-21 (see FIG. 9).

EXAMPLE 8

Preparation of Multiple Antigenic Peptide (MAP) Conjugates and their Immunogenicity Study

[0201] Several methods are available for generating multiple antigenic sites on the carriers. In the previous examples, each antigenic site is separately conjugated to the carrier by defined conjugation and capping chemistries. In this example, multiple antigenic sites are constructed by solid phase synthesis of tandem repeats of A β 1-7 mer. Alternatively these tandem repeats can be coupled with T-cell epitopes with or without linking through a lysine core as described elsewhere. These multiple antigenic peptides were synthesized with an additional cysteinyl residue for conjugation to the carrier protein. Peptides containing one repeat unit (1-7), three repeat units (1-7)₃ and five repeat units (1-7)₅ with an additional cysteinyl residue at the carboxyl end were synthesized. These peptides were covalently attached to bromoacetylated CRM overnight through their C-terminal cysteine residues. The reaction was carried out at pH 9.0-9.2 with peptide:CRM ratios added as outlined in Table 8. Bromoacetyl groups, which did not react with peptide, were capped with N-acetylcysteamine. These lots represent conjugates containing one single copy, three tandem copies, and five tandem copies of the A β 1-7 peptide conjugated to CRM, respectively. Table 8 briefly outlines the properties of the samples.

TABLE 8

<u>Multiple Antigenic Peptide (MAP) Conjugate Samples</u>		
Conjugate	Peptide: CRM (w/w)	pH of reaction
Ab(1-7) ₁ /CRM	0.37	8.99
Ab(1-7) ₃ /CRM	1.02	8.95
Ab(1-7) ₅ /CRM	1.67	9.17

[0202] Peptide load (the average number of A β 1-7 peptides per carrier) and capping numbers (Table 9) are the numbers of unique amino acids (CMC or CMCA) per carrier as determined by amino acid analysis. The CMC and CMCA values were referenced to lysine.

TABLE 9

<u>Degree of Conjugation and Capping of Each Conjugate</u>		
CONJUGATE	Peptide Load (CMC)	Capping (CMCA)
A β (1-7) ₁ /CRM	12.5	11.7
A β (1-7) ₃ /CRM	10.4	15.2
A β (1-7) ₅ /CRM	9.8	15.9

[0203] Swiss-Webster mice (10 per group) were immunized subcutaneously with 1 or 0.1 μ g A β /CRM conjugated peptide. Half of the mice were immunized with the composition formulated with 100 μ g of the adjuvant Al(OH)₃, and half were immunized without adjuvant. Immunizations were scheduled at weeks 0 and 3. Bleeds were scheduled for weeks 0, 3, and 6. Serum samples were analyzed for antibody response against A β 1-42 mer peptide. The results are shown in Table 10.

TABLE 10

<u>Anti-Aβ Endpoint Titers for Multiple Antigenic Peptide (MAP) Conjugates</u>					
Group Code	Sample Description	Adjuvant	Wk 0 Pool	Wk 3 GMT	Wk 6 GMT
AG332	1 μ g A β (1-7) ₁ /CRM	Al(OH) ₃	<100	18,096	100,279
AG333	1 μ g A β (1-7) ₃ /CRM	Al(OH) ₃	<100	44,911	420,235
AG334	1 μ g A β (1-7) ₅ /CRM	Al(OH) ₃	<100	27,032	394,488
AG335	0.1 μ g A β (1-7) ₁ /CRM	Al(OH) ₃	<100	19,350	66,834
AG336	0.1 μ g A β (1-7) ₃ /CRM	Al(OH) ₃	<100	13,307	208,272
AG337	0.1 μ g A β (1-7) ₅ /CRM	Al(OH) ₃	<100	1,196	22,665
AG338	1 μ g A β (1-7) ₁ /CRM	None	<100	5,273	370,980
AG339	1 μ g A β (1-7) ₃ /CRM	None	<100	9,299	541,093
AG340	1 μ g A β (1-7) ₅ /CRM	None	<100	3,100	185,272
AG341	0.1 μ g A β (1-7) ₁ /CRM	None	<100	340	25,839
AG342	0.1 μ g A β (1-7) ₃ /CRM	None	<100	128	5,553
AG343	0.1 μ g A β (1-7) ₅ /CRM	None	<100	668	2,098

[0204] All conjugates induced anti-As 1-42 antibody titer after primary immunization and the levels were substantially increased after the booster dose. In the absence of aluminum adjuvant, the differences in dose response were evident both at week 3 and week 6 bleeds. The higher dose elicited high-titered antibody response. Aluminum adjuvant elicited substantially higher antibody response at week 3 at both dose levels (0.1 and 1 μ g) as compared to the unadjuvanted groups. After secondary immunization, conjugates given at 1 μ g dose elicited 5 to 10 fold increase in antibody levels. At this dose level peptide conjugates with 3 and 5 repeats

induced higher antibody response than a single repeat containing conjugate. The titers against the CRM carrier were also determined, and these are listed in Table 11.

TABLE 11

<u>Anti-CRM Endpoint Titers for Multiple Antigenic Peptide (MAP) Conjugates</u>					
Group Code	Sample Description	Adjuvant	Wk 0 Pool	Wk 3 GMT	Wk 6 GMT
AG332	1 μ g A β (1-7) ₁ /CRM	Al(OH) ₃	<50	10,531	114,602
AG333	1 μ g A β (1-7) ₃ /CRM	Al(OH) ₃	<50	4,274	83,065
AG334	1 μ g A β (1-7) ₅ /CRM	Al(OH) ₃	<50	1,680	49,320
AG335	0.1 μ g A β (1-7) ₁ /CRM	Al(OH) ₃	<50	1,114	13,231
AG336	0.1 μ g A β (1-7) ₃ /CRM	Al(OH) ₃	<50	197	1,484
AG337	0.1 μ g A β (1-7) ₅ /CRM	Al(OH) ₃	<50	65	222
AG338	1 μ g A β (1-7) ₁ /CRM	None	<50	35	309
AG339	1 μ g A β (1-7) ₃ /CRM	None	<50	29	1,085
AG340	1 μ g A β (1-7) ₅ /CRM	None	<50	29	542
AG341	0.1 μ g A β (1-7) ₁ /CRM	None	<50	25	55
AG342	0.1 μ g A β (1-7) ₃ /CRM	None	<50	25	34
AG343	0.1 μ g A β (1-7) ₅ /CRM	None	<50	29	ND

Animals were immunized at weeks 0 and 3 and bled at weeks 0, 3, and 6.

Adjuvant: 100 μ g Al(OH)₃ or none.

ND = Not Determined.

[0205] Data in Table 11 indicates that the unadjuvanted groups induced very low levels of anti-CRM antibody response at both 1 μ g as well as 0.1 μ g dose levels even after two immunizations. However conjugates with aluminum hydroxide adjuvant induced substantial levels of anti-CRM antibody response at 1 μ g dose and much lower response at 0.1 μ g dose. In the presence of the adjuvant, CRM titers were highest for the single repeat conjugate, intermediate for the triple repeat conjugate, and lowest for the quintuple repeat conjugate. This is as expected, since the CRM dose per peptide dose is lowest for A β (1-7)₅/CRM, and highest for A β (1-7)₁/CRM. The differences were only statistically significant at week 6 for the 0.1 μ g dose.

[0206] The objective of the current invention is to elicit high titered immunogenic response against the antigenic hapten and not necessarily against the carrier protein. Under certain circumstances it is desirable to elicit optimal immune response against the hapten antigenic determinant with least or no immune response against the carrier protein. For such applications, conjugates with tandem repeats of multiple antigenic determinants with unadjuvanted formulation will serve the need.

EXAMPLE 9

Preparation of A β -Peptide Conjugates with Various Carrier Proteins and their Immunogenicity

[0207] This example compares the immunogenicity of conjugates using six different carrier proteins. The acetate salt of A β 1-7 was added to bromoacetylated carriers in a 1:1 ratio by weight at pH 9. All conjugates except A β 1-7/rC5ap were capped with N-acetylcycteamine. All of the alternative carriers are recombinant bacterial proteins, including CRM (diphtheria toxin), recombinant C5a peptidase (rC5ap; cloned from *Streptococcus agalactiae*, includes D130A and S512A mutations), ORFs 1224, 1664, 2452 (all cloned from *Streptococcus pyogenes*), and T367, T858 (each cloned from *Chlamydia pneumoniae*). A summary of the carriers used is found in Table 12. The degree of conjugation and capping of each A β 1-7 conjugate to these carriers are presented in Table 13.

[0208] This study showed that the recombinant C5a peptidase conjugate induced higher titers against A β than most of the other carriers tested, including CRM. This difference was statistically significant for week 6 titers of groups that received aluminum hydroxide. In addition, the A β 1-7/T858 conjugate was significantly more immunogenic than most other conjugates in the absence of adjuvant. The only conjugate that performed poorly relative to the CRM control conjugate was A β 1-7/T367, a conjugate that also did not react with an A β specific monoclonal antibody by Western blot. This study confirms that numerous other carriers can be successfully used to immunize against the A β peptide.

TABLE 12

<u>List of Carriers and Conjugate Properties</u>		
CARRIER PROTEIN	MW of carrier (Da)	# of lysines
CRM	58,408	39
rC5ap	108,560	85
ORF1224	30,950	18
ORF1664	31,270	38
ORF2452	31,790	29
T367	49,700	29
T858	37,190	23

[0209]

TABLE 13

<u>Degree Of Conjugation and Capping of Each Conjugate</u>		
CONJUGATE	Peptide load (CMC)	Capping (CMCA)
A β 1-7/rC5ap	25.9	—
A β 1-7/ORF1224	12.8	5.7
A β 1-7/ORF1664	13.4	10.8
A β 1-7/ORF2452	12.03	10.5

TABLE 13-continued

<u>Degree Of Conjugation and Capping of Each Conjugate</u>		
CONJUGATE	Peptide load (CMC)	Capping (CMCA)
A β 1-7/T367	13.2	8.2
A β 1-7/T858	5.2	1.7

Conjugation results: Peptide load (the average number of A β 1-7 peptides per carrier) and capping number are the numbers of unique amino acids (CMC or CMCA) per carrier as determined by amino acid analysis. The CMC and CMCA values were referenced to lysine.

Immunization Results

[0210] The geometric mean titer for each group in this study is listed in Table 14. At week 3, regardless of the presence of adjuvant, A β 1-7/rC5ap induced significantly higher anti-A β titers than the corresponding conjugates prepared with *Streptococcus pyogenes* ORFs 1224, 1664, 2452, or *Chlamydia pneumoniae* ORFs T367 and T858. At week 3 in the absence of adjuvant, A β 1-7/rC5ap was also more immunogenic than all other conjugates except A β 1-7/T858. The T858 conjugate without Al(OH)₃ induced higher titers than the ORF1224, ORF1664, ORF2452, and CRM conjugates without adjuvant. The only conjugate that was significantly less immunogenic than A β 1-7/CRM was A β 1-7/T367 ($p<0.00002$). The T367 carrier performed poorly with or without adjuvant at both weeks 3 and 6. At week 6, the rC5ap conjugate with aluminum hydroxide was more immunogenic ($p<0.04$) than all the other conjugates except A β 1-7/ORF2452. In the absence of adjuvant, both A β 1-7/rC5ap and A β 1-7/T858 induced significantly higher titers than the ORF1224, ORF1664, or T367 conjugates. A β 1-7/CRM without aluminum hydroxide induced higher titers than either A β 1-7/ORF1664 or A β 1-7/T367.

TABLE 14

<u>Anti-Aβ1-42 Endpoint Titers.</u>					
GROUP CODE	SAMPLE DESCRIPTION	ADJUVANT	WK 0 POOL	WK 3 GMT	WK 6 GMT
AG344	5 μ g A β 1-7/CRM	Al(OH) ₃	<100	21,404	54,157
AG345	5 μ g A β 1-7/rC5ap	Al(OH) ₃	<100	61,967	402,972
AG346	5 μ g A β 1-7/ORF1224	Al(OH) ₃	<100	10,711	30,084
AG347	5 μ g A β 1-7/ORF1664	Al(OH) ₃	<100	7,188	43,226
AG348	5 μ g A β 1-7/ORF2452	Al(OH) ₃	<100	11,437	109,091
AG349	5 μ g A β 1-7/T367	Al(OH) ₃	<100	321	5,139
AG350	5 μ g A β 1-7/T858	None	<100	16,656	33,328
AG351	5 μ g A β 1-7/CRM	None	<100	2,615	119,488
AG352	5 μ g A β 1-7/rC5ap	None	<100	11,858	279,113
AG353	5 μ g A β 1-7/ORF1224	None	<100	1,674	18,719
AG354	5 μ g A β 1-7/ORF1664	None	<100	119	9,832
AG355	5 μ g A β 1-7/ORF2452	None	<100	2,493	76,038
AG356	5 μ g A β 1-7/T367	None	<100	50	620
AG357	5 μ g A β 1-7/T858	None	<100	28,820	275,202

Animals were immunized at weeks 0 and 3 and bled at weeks 0, 3, and 6. Dose is based on the total amount of conjugate. Adjuvant: 100 μ g Al(OH)₃ or none.

EXAMPLE 10

Preparation of Additional A_β Peptide-Protein Conjugates

I. Activation

[0211] Thawed CRM₁₉₇ (8 mL, 59.84 mg, at 7.48 mg/mL) was dissolved in 0.1 M borate buffer (pH 9, 3.968 mL) to bring the concentration to 5 mg/mL. The solution was cooled in an ice bath to 0-5° C. Bromoacetic acid N-hydroxysuccinimide (59.9 mg) (Aldrich-Sigma) was dissolved in DMF (100 µL) (Aldrich-Sigma) and added dropwise, to the solution of CRM₁₉₇. Upon addition of the bromoacetic acid N-hydroxysuccinimide, a precipitate was observed. When the pH was checked, it decreased to pH 6. The pH of the reaction mixture was brought back to pH 9 by adding more 0.1 M borate buffer. Reaction mixture was then stirred at 4° C. for 1 hr, with gentle swirling. The mixture was purified and concentrated using YM-10 centriprep centrifugal concentration and repurified on Sephadex G-25 using 10 mM borate as the eluent. Fractions positive to Bradford Reagent were pooled and concentrated using centriprep YM-10. The degree of bromoacetylation was determined by Bradford assay (linear). The concentration was found to be 5.36 mg/mL (Yielded 30 mg). The final concentration was then adjusted to be 5 mg/mL and was stored in the freezer in 5% sucrose until further use.

II. Conjugation

[0212] For each conjugation, thawed bromoacetylated CRM₁₉₇ was used. Peptides were dissolved in borate buffer (2.5 mg in 125 mL of 0.1 M borate buffer). Slight insolubility was observed with A_β peptides KLVFFAEDC (SEQ ID NO:45), CLVFFAEDV (SEQ ID NO:47), CKLVFFAED (SEQ ID NO:48), and LVFFAEDC (SEQ ID NO:50). Bromoacetylated CRM₁₉₇ (5 mg/mL) was treated with the peptide solutions/suspensions. The ratio of the peptide and protein in the mixture was 1:2. Turbidity was observed in the conjugate mixtures with peptides KLVFFAEDC (SEQ ID NO:45), CLVFFAEDV (SEQ ID NO:47), CKLVFFAED (SEQ ID NO:48), and KLVFFAEDC (SEQ ID NO:45). The mixtures were then checked for pH (pH 9) and incubated at 4° C. overnight with slow swirling. Final concentrations of the mixtures were made to 3 mg/mL before incubation. The turbidity of the conjugate mixtures with peptides CLVFFAEDV (SEQ ID NO:47) and LVFFAEDC (SEQ ID NO:50) disappeared after incubation. However, KLVFFAEDC (SEQ ID NO:45) and CKLVFFAED (SEQ ID NO:48) were still slightly turbid. Soluble mock protein conjugate was also prepared with cysteamine at a ratio of 1: 1 (w/w). Synthesized peptides were obtained from BIOSOURCE with about 95% purity. Octamers:

Octamers:	
LVFFAEDVC	(SEQ ID NO: 44)
KLVFFAEDC	(SEQ ID NO: 45)
VFFAEDVGC	(SEQ ID NO: 43)
CLVFFAEDV	(SEQ ID NO: 47)
CKLVFFAED	(SEQ ID NO: 48)

CVFFAEDVG	-continued (SEQ ID NO: 46)
Heptamers:	
VFFAEDVC	(SEQ ID NO: 49)

LVFFAEDC (SEQ ID NO: 50)

III. Capping Unreacted Lysine Groups on Protein:

[0213] The unreacted lysines were capped with N-acetyl-cysteamine (CMCA; Aldrich-Sigma) at a ratio of 1/1 (w/w) for 4 hr at 4° C. while swirling in the dark. The unreacted peptides and capping reagents were removed from the conjugates by dialysis using Slide-A-Lyzer cassette (M_w cut off 10,000) (Pierce) against PBS buffer (2 L) overnight (13 hr). Buffer exchange and dialysis was done twice (2×14 hr). Slight insolubility was observed in the conjugates with peptides KLVFFAEDC (SEQ ID NO:45) and CKLVFFAED (SEQ ID NO:48). All conjugates were then stored in the refrigerator at 4° C. in a preservative.

IV. Characterization of the Protein Carrier:

[0214] MALDI-TOF MS was used to determine the mass of bromoacetylated CRM₁₉₇ and the mass of the mock conjugate N-acetylcysteamine-CRM₁₉₇. Based on the masses of the CRM₁₉₇ and bromoacetylated CRM₁₉₇, 11 lysine residues were modified.

[0215] (59941.46-58590.29)/122=11

Where; Mw of CRM₁₉₇ is 58624.29

[0216] Mw of bromoacetylated CRM₁₉₇ is 59941.46

[0217] Mw of bromoacetate is 122

[0218] The degree of bromoacetylation was more than 28%. (The total number of lysines in CRM₁₉₇ was 39). From these 11 modified lysine residues, 10 were coupled with cysteamine. The coupling efficiency was 90%.

[0219] (61143-59941)/119=10

Where; Mw of bromoacetylated CRM₁₉₇ is 59941.46

[0220] Mw of mock conjugate is 61143

[0221] Mw of the N-acetylcysteamine is 119

(10/11)×100=90

V. Characterization of the Peptide-Protein Conjugates by SDS-PAGE Western Blot Analysis with Tris-Tricine Precast Gel:

[0222] The protein-peptide conjugates were analyzed by Western blot. The lanes are: marker (lane 1); L-28375 24/01 (lane 2); L-28375 24/02 (lane 3); L-28375 24/03 (lane 4); L-28375 24/04 (lane 5); L-28375 24/05 (lane 6); L-28375 24/06 (lane 7) L-28375 24/07 (lane 8); L-28375 24/08 (lane 9); L-28375 24/09 (Mock) (lane 10); and, BrAccRM₁₉₇ (lane 11). A peptide specific monoclonal antibody from mice (248-6H9-806 A_β 17-28) was used as the primary antibody (antisera) (1:3000 dilution was found to be the best). Goat-Anti mouse IgG (H+L)-HPR was the secondary antibody (1:1000 dilution). It was observed that all the conjugates were recognized by the primary antibody, except for the mock conjugate and the activated CRM₁₉₇. (See FIG. 10.)

Protein Concentration

[0223] Protein concentrations of the conjugate samples were determined by the Pierce BCA assay. (See Table 15.)

Amino Acid Analysis

[0224] Amino acid analysis was carried out to determine the degree of conjugation. T degree of conjugation was calculated based on the CMCA (carboxymethylcycteamine) residues found in the conjugates. CMCA was used to cap the unreacted activated sites after conjugation with the peptides. (See Table 15.)

TABLE 15

Degree of Conjugation of Peptides with BrAcCRM ₁₉₇			
Conjugate Code	Peptide Sequence (SEQ ID NO:)	Final Concentration (mg/mL)	Degree of Conjugation (Based on CMCA)
L-28375 24/01	LVFFAEDV-C (SEQ ID NO: 44)	1.67	8/10
L-28375 24/02	KLVFFAED-C (SEQ ID NO: 45)	0.82	5/10
L-28375 24/03	VFFAEDVG-C (SEQ ID NO: 43)	1.43	8/10
L-28375 24/04	C-LVFFAEDV (SEQ ID NO: 47)	1.04	9/10
L-28375 24/05	C-KLVFFAED (SEQ ID NO: 48)	0.78	1/10
L-28375 24/06	C-VFFAEDVG (SEQ ID NO: 46)	0.97	9/10
L-28375 24/07	VFFAEDV-C (SEQ ID NO: 49)	1.00	7/10
L-28375 24/08	LVFFAED-C (SEQ ID NO: 50)	0.99	8/10
L-28375 24/09	(Mock)	1.89	10/11

[0225] All colorimetric assays were performed using microplate spectrophotometer and SOFTmax Pro.

EXAMPLE 11

Inmunogenic Studies of A β Peptide Conjugates in Swiss Webster Mice

[0226] Outbred Swiss Webster mice were immunized with VFFAEDVG-C (SEQ ID NO:43), LVFFAEDV-C (SEQ ID NO:44), KLVFFAED-C (SEQ ID NO:45), C-VFFAEDVG (SEQ ID NO:46), C-LVFFAEDV (SEQ ID NO:47), C-KLVFFAED (SEQ ID NO:48), VFFAEDV-C (SEQ ID NO:49), LVFFAED-C (SEQ ID NO:50) each conjugated to CRM₁₉₇, or with A β 1-7CRM₁₉₇, all formulated with the adjuvant RC 529 SE. Nine groups of 10 animals per group were immunized subcutaneously with one of the A β peptide conjugates at the beginning of the study (week 0) and subsequently at week 4. Serum was collected prior to, but on the same days as immunization.

Immunogenic Studies of A β Peptide Conjugates in Inbred Balb/c Mice

[0227] Inbred Balb/c mice were immunized as in the preceding paragraph, but were also boosted with conjugate and adjuvant at week 12.

Results

[0228] Sera from both studies are being collected for analysis of A β ₁₃₋₂₈ peptide-specific IgG antibody titer. Sera

from Balb/c mice are also collected for analysis one day prior to the week 12 boost, and one week thereafter. Spleen cells from animals used in Example 11 are evaluated for their potential to respond in-vitro to stimulation with an overlapping pool of peptides spanning A β ₁₋₄₂, full length A β ₁₋₄₂, CRM₁₉₇, or polyclonal activators. Analysis is comprised of Elispot readout for interleukins 4 and 5, and interferon-gamma. Upon completion, the A β peptide conjugates are evaluated as described above and as described in Example 6.

EXAMPLE 12

Inmunogenic Studies of A β Peptide Conjugates in PSAPP Mice

[0229] PSAPP mice are immunized with VFFAEDVG-C (SEQ ID NO:43), LVFFAEDV-C (SEQ ID NO:44), KLVFFAED-C (SEQ ID NO:45), C-VFFAEDVG (SEQ ID NO:46), C-LVFFAEDV (SEQ ID NO:47), C-KLVFFAED (SEQ ID NO:48), VFFAEDV-C (SEQ ID NO:49), LVFFAED-C (SEQ ID NO:50). The PSAPP mouse, a doubly transgenic mouse (PSAPP) overexpressing mutant APP and P51 transgenes, is described in Holcomb, et al. (1998) *Nature Medicine* 4:97-11.

Immunogenic Studies of A β Peptide Conjugates in PDAPP Mice

[0230] PDAPP mice are immunized with VFFAEDVG-C (SEQ ID NO:43), LVFFAEDV-C (SEQ ID NO:44), KLVFFAED-C (SEQ ID NO:45), C-VFFAEDVG (SEQ ID NO:46), C-LVFFAEDV (SEQ ID NO:47), C-KLVFFAED (SEQ ID NO:48), VFFAEDV-C (SEQ ID NO:49), LVFFAED-C (SEQ ID NO:50). The PDAPP mouse expresses a mutant form of human APP (APP^{V71F}) and develops Alzheimer's disease at a young age (Bard, et al. (2000) *Nature Medicine* 6:916-919; Masliah E, et al. (1996) *J Neurosci*. 15;16(18):5795-811).

Results

[0231] Sera from both studies are collected for analysis of A β ₁₃₋₂₈ peptide-specific IgG antibody titer. Upon completion, the A β peptide conjugates will be evaluated as described above and as described in Examples 6 and 11, as well as in the contextual fear conditioning (CFC) assay.

[0232] Contextual fear conditioning is a common form of learning that is exceptionally reliable and rapidly acquired in most animals, for example, mammals. Test animals learn to fear a previously neutral stimulus and/or environment because of its association with an aversive experience. (see, e.g., Fanselow, *Anim. Learn. Behav.* 18:264-270 (1990); Wehner et al., *Nature Genet.* 17:331-334. (1997); Caldarone et al., *Nature Genet.* 17:335-337 (1997)).

[0233] Contextual fear conditioning is especially useful for determining cognitive function or dysfunction, e.g., as a result of disease or a disorder, such as a neurodegenerative disease or disorder, an A β -related disease or disorder, an amyloidogenic disease or disorder, the presence of an unfavorable genetic alteration effecting cognitive function (e.g., genetic mutation, gene disruption, or undesired genotype), and/or the efficacy of an agent, e.g., an A β conjugate agent, on cognitive ability. Accordingly, the CFC assay provides a method for independently testing and/or validating the therapeutic effect of agents for preventing or treating a cognitive

disease or disorder, and in particular, a disease or disorder affecting one or more regions of the brains, e.g., the hippocampus, subiculum, cingulated cortex, prefrontal cortex, perirhinal cortex, sensory cortex, and medial temporal lobe.

[0234] Typically, the CFC assay is performed using standard animal chambers and the employment of conditioning training comprising a mild shock (e.g., 0.35 mA foot shock) paired with an auditory (e.g., a period of 85 db white noise), olfactory (e.g., almond or lemon extract), touch (e.g., floor cage texture), and/or visual cue (light flash). The response to the aversive experience (shock) is typically one of freezing (absence of movement except for respiration) but may also include eye blink, or change in the nictitating membrane reflex, depending on the test animal selected. The aversive response is usually characterized on the first day of training to determine a baseline for unconditioned fear, with aversive response results on subsequent test days, e.g., freezing in presence of the context and/or cue but in the absence of the aversive experience, being characterized as context and cue conditioned fear, respectively. For improved reliability, test animals are typically tested separately by independent technicians and scored over time. Additional experimental design details can be found in the art, for example, in Crawley, J N, *What's Wrong with my Mouse; Behavioral Phenotyping of Transgenic and Knockout Mice*, Wiley-Liss, NY (2000).

[0235] Exemplary test animals (e.g., model animals) include mammals (e.g. rodents or non-human primates) that exhibit prominent symptoms or pathology that is characteristic of an amyloidogenic disorder such as Alzheimer's. Model animals may be created by selective inbreeding for a desired or they may genetically engineered using transgenic techniques that are well-known in the art, such that a targeted genetic alteration (e.g. a genetic mutation, gene disruption) in a gene that is associated with the dementia disorder, leading to aberrant expression or function of the targeted gene. For example, several transgenic mouse strains are available that overexpress APP and develop amyloid plaque pathology and/or develop cognitive deficits that are characteristic of Alzheimer's disease (see for example, Games et al., supra, Johnson-Wood et al., *Proc. Natl. Acad. Sci. USA* 94:1550 (1997); Masliah E and Rockenstein E. (2000) *J Neural Transm Suppl.*;59:175-83).

[0236] Alternatively, the model animal can be created using chemical compounds (e.g. neurotoxins, anesthetics) or surgical techniques (e.g. stereotactic ablation, axotomy, transection, aspiration) that ablate or otherwise interfere with the normal function of an anatomical brain region (e.g. hippocampus, amygdala, perirhinal cortex, medial septal nucleus, locus coeruleus, mammillary bodies) or specific neurons (e.g. serotonergic, cholinergic, or dopaminergic neurons) that are associated with characteristic symptoms or pathology of the amyloidogenic disorder. In certain preferred embodiments, the animal model exhibits a prominent cognitive deficit associated with learning or memory in addition to the neurodegenerative pathology that is associated with a amyloidogenic disorder. More preferably, the cognitive deficit progressively worsens with increasing age, such that the disease progression in the model animal parallels the disease progression in a subject suffering from the amyloidogenic disorder.

[0237] Contextual fear conditioning and other in vivo assays to test the functionality of the conjugates described

herein may be performed using wild-type mice or mice having a certain genetic alteration leading to impaired memory or mouse models of neurodegenerative disease, e.g., Alzheimer's disease, including mouse models which display elevated levels of soluble A β in the cerebrospinal fluid (CSF) or plasma. For example, animal models for Alzheimer's disease include transgenic mice that overexpress the "Swedish" mutation of human amyloid precursor protein (hAPPswe; Tg2576) which show age-dependent memory deficits and plaques (Hsiao et al. (1996) *Science* 274:99-102). The in vivo functionality of the conjugates described herein can also be tested using the PS-1 mutant mouse, described in Duff, et al. (1996) *Nature* 383, 710-713. Other genetically altered transgenic models of Alzheimer's disease are described in Masliah E and Rockenstein E. (2000) *J Neural Transm Suppl.* 59:175-83.

[0238] In various aspects, the methods of the invention comprise the administration of an A β conjugate that is capable of improving cognition in a subject wherein the A β conjugate has been identified in using an assay which is suitably predictive of immunotherapeutic efficacy in the subject. In exemplary embodiments, the assay is a model animal assay that is based, at least in part, on comparing cognition, as determined from a contextual fear conditioning study, of an animal after administration of a test immunological reagent to the animal, as compared to a suitable control. The CFC assay evaluates changes in cognition of an animal (typically a mouse or rat) upon treatment with a potential therapeutic compound. In certain embodiments, the change in cognition evaluated is an improvement in memory impairment status or a reversal of memory deficit. Accordingly, the CFC assay provides a direct method for determining the therapeutic effect of agents for preventing or treating cognitive disease, and in particular, a disease or disorder affecting one or more regions of the brains, e.g., the hippocampus, subiculum, cingulated cortex, prefrontal cortex, perirhinal cortex, sensory cortex, and medial temporal lobe. Such CFC assays are discussed in copending U.S. Patent Application Ser. No. 60/XXX,XXX entitled "Contextual Fear Conditioning for Predicting Immunotherapeutic Efficacy" (bearing Attorney Docket No. ELN-058-1), filed on Dec. 15, 2004, and U.S. Patent Application Ser. No. 60/XXX,XXX entitled "Contextual Fear Conditioning for Predicting Immunotherapeutic Efficacy" (bearing Attorney Docket No. ELN-058-2) the entire contents of which are hereby incorporated by reference.

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- [0258] U.S. Pat. No. 5,360,897 November, 1994 Anderson et al.
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His	Ile	Ser	Val	Asn	Gly	Arg	Lys	Ile	Arg	Met	Arg	Cys	Arg	Ala	Ile
					450			455			460				
Asp	Gly	Asp	Val	Thr	Phe	Cys	Arg	Pro	Lys	Ser	Pro	Val	Tyr	Val	Gly

-continued

465	470	475	480
Asn Gly Val His Ala Asn Leu His Val Ala Phe His Arg Ser Ser Ser			
485	490	495	
Glu Lys Ile His Ser Asn Glu Ile Ser Ser Asp Ser Ile Gly Val Leu			
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Gly Tyr Gln Lys Thr Val Asp His Thr Lys Val Asn Ser Lys Leu Ser			
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Leu Phe Phe Glu Ile Lys Ser			
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Arg

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Cys Leu Val Phe Phe Ala Glu Asp
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Cys Lys Leu Val Phe Phe Ala Glu
1 5

1-369. (canceled)

370. A method for conjugating a peptide immunogen via a reactive group of an amino acid residue of the peptide immunogen to a protein/polypeptide carrier having one or more functional groups, the method comprising the steps of:

(a) derivatizing one or more of the functional groups of the protein/polypeptide carrier or optionally to a polypeptide linker attached to the protein/polypeptide carrier to generate a derivatized carrier with reactive sites;

(b) reacting the derivatized protein/polypeptide carrier of step (a) with a reactive group of an amino acid of the peptide immunogen under reaction conditions such that the peptide immunogen is conjugated to the derivatized protein/polypeptide carrier via the fictional groups; and

(c) further reacting the conjugate with a capping reagent to inactivate free, reactive unreacted functional groups on the derivatized protein/polypeptide carrier, thereby preserving the functionality of the carrier, such that it retains its ability to elicit the desired immune responses

against the peptide immunogen that would otherwise not occur without a carrier.

371. The method of claim 370, wherein the protein/polypeptide carrier is selected from the group consisting of human serum albumin, keyhole limpet hemocyanin (KLH), immunoglobulin molecules, thyroglobulin, ovalbumin, influenza hemagglutinin, PADRE polypeptide, malaria circumsporozoite (CS) protein, hepatitis B surface antigen (HBSAg₁₉₋₂₈), Heat Shock Protein (HSP) 65, *Mycobacterium tuberculosis*, cholera toxin, cholera toxin mutants with reduced toxicity, diphtheria toxin, CRM197 protein that is cross-reactive with diphtheria toxin, recombinant Streptococcal C5a peptidase, *Streptococcus pyogenes* ORF1224, *Streptococcus pyogenes* ORF1664, *Streptococcus pyogenes* ORF2452, *Chlamydia pneumoniae* ORF T367, *Chlamydia pneumoniae* ORF T858, Tetanus toxoid, HIV gp120 T1, components recognizing microbial surface adhesive matrix molecules (MSCRAMMS), growth factors, hormones, cytokines and chernokines.

372. The method of claim 371, wherein the protein/polypeptide carrier is CRM₁₉₇.

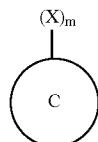
373. The method of claim 370, wherein the peptide immunogen is selected from the group consisting of a bacterial protein, a viral protein, and a eukaryotic protein.

374. The method of claim 370, wherein the functional group of one or more amino acid molecules of the protein/polypeptide carrier or of the optionally attached polypeptide linker is derivatized using a cross-linking reagent.

375. The method of claim 374, wherein the protein/polypeptide carrier is reacted with a haloacetyating agent.

376. The method of claim 370, wherein the capping reagent that is used to inactivate free reactive, functional groups on the activated protein/polypeptide carrier is selected from the reagent group consisting of cysteamine, N-acetylcysteamine, ethanolamine, sodium hydroxide, sodium carbonate, ammonium bicarbonate and ammonia.

377. A method for conjugating a peptide immunogen to a protein/polypeptide carrier having the structure:



wherein,

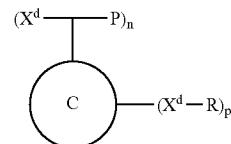
C is a protein/polypeptide carrier and X is a derivatizable functional group of an amino acid residue of the protein/polypeptide carrier or optionally of an amino acid residue of a peptide linker covalently attached to the protein/polypeptide carrier, and wherein m is an integer greater than 0, but less than or equal to 85, the method comprising the steps of:

(a) derivatizing one or more of the functional groups of the protein/polypeptide carrier or of the optionally attached linker molecule to generate a derivatized molecule with reactive sites;

(b) reacting the derivatized protein/polypeptide carrier of step (a) with a reactive group of an amino acid residue

of the peptide immunogen to form a covalently coupled peptide immunogen-protein/polypeptide carrier conjugate; and

(c) further reacting the said conjugate with a capping reagent to inactivate the free reactive functional groups on the activated protein/polypeptide carrier, such that the capped groups are not free to react with other molecules, thereby preserving the functionality of the carrier, such that it retains its ability to elicit the desired immune responses against the peptide immunogen that would otherwise not occur without a carrier, so as to generate a capped peptide immunogen-protein/polypeptide carrier conjugate having the formula:



wherein,

C is the protein/polypeptide carrier and X^d is a derivatized functional group of an amino acid residue of the protein/polypeptide carrier or optionally of an amino acid residue of a peptide linker covalently attached to the protein/polypeptide carrier, and, wherein,

P is a peptide immunogen molecule covalently attached to the derivatized functional group of the amino acid residue of the protein carrier or optionally of an amino acid residue of a peptide linker covalently attached to a protein/polypeptide carrier,

R is a capping molecule covalently attached to the derivatized functional group of an amino acid residue of the protein/polypeptide carrier or optionally of an amino acid residue of a peptide linker covalently attached to a protein/polypeptide carrier,

n is an integer greater than 0, but less than or equal to 85, and

p is an integer greater than 0, but less than 85.

378. The method of claim 377, wherein the protein/polypeptide carrier is selected from the group consisting of serum albumin, keyhole limpet hemocyanin (KLH), immunoglobulin molecules, thyroglobulin, ovalbumin, influenza hemagglutinin, PADRE polypeptide, malaria circumsporozoite (CS) protein, hepatitis B surface antigen (HBSAg₁₉₋₂₈), Heat Shock Protein (HSP) 65, *Mycobacterium tuberculosis*, cholera toxin, cholera toxin mutants with reduced toxicity, diphtheria toxin, CRM₁₉₇ protein that is cross-reactive with diphtheria toxin, recombinant Streptococcal C5a peptidase, *Streptococcus pyogenes* ORF1224, *Streptococcus pyogenes* ORF1664, *Streptococcus pyogenes* ORF2452, *Chlamydia pneumoniae* ORF T367, *Chlamydia pneumoniae* ORF T858, Tetanus toxoid, HIV gp120 T1, components recognizing microbial surface adhesive matrix molecules (MSCRAMMS), growth factors, hormones, cytokines and chemokines.

379. The method of claim 378, wherein the protein/polypeptide carrier is CR₁₉₇.

380. The method of claim 377, wherein the peptide immunogen is selected from a bacterial protein, a viral protein, and a eukaryotic protein.

381. The method of claim 377, wherein the functional group of one or more amino acid molecules of the protein/polypeptide carrier or of the optionally attached polypeptide linker is derivatized using a cross-linking reagent.

382. The method of claim 381, wherein the protein/polypeptide carrier is reacted with a haloacetyating agent.

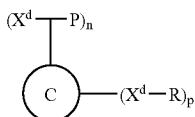
383. The method of claim 377, wherein the capping reagent that is used to inactivate free reactive, functional groups of the activated protein/polypeptide carrier is selected from the reagent group consisting of cysteamine, N-acetylcysteamine, ethanolamine, sodium hydroxide, sodium carbonate, ammonium bicarbonate and ammonia.

384. A peptide immunogen-protein/polypeptide carrier conjugate wherein the protein/polypeptide carrier has the formula:



wherein,

C is a protein/polypeptide carrier and X is a derivatizable functional group of an amino acid residue on the protein/polypeptide carrier or optionally of an amino acid residue of a peptide linker covalently attached to the protein/polypeptide carrier, and, wherein m is an integer greater than 0, but less than or equal to 85, and wherein the peptide immunogen-protein/polypeptide carrier conjugate has the formula:



wherein,

C is the protein/polypeptide carrier and X^d is a derivatized functional group of an amino acid residue of the protein/polypeptide carrier or optionally of an amino acid residue of a peptide linker covalently attached to the protein/polypeptide carrier, and, wherein, P is a peptide immunogen molecule covalently attached to the derivatized functional group of the amino acid residue of the protein carrier or optionally of an amino acid residue of a peptide linker covalently attached to a protein/polypeptide carrier, R is a capping molecule covalently attached to the derivatized functional group of an amino acid residue of the protein/polypeptide carrier or optionally of an amino acid residue of a peptide linker covalently attached to a protein/polypeptide carrier, thereby preserving the functionality of the carrier such that it retains its ability to elicit the desired immune responses against the peptide immunogen that would otherwise not occur without a carrier, n is an

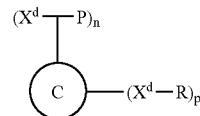
integer greater than 0, but less than or equal to 85, and p is an integer greater than 0, but less than 85.

385. The conjugate of claim 384, wherein the protein/polypeptide carrier is selected from the group consisting of serum albumin, keyhole limpet hemocyanin (KLH), immunoglobulin molecules, thyroglobulin, ovalbumin, influenza hemagglutinin, PADRE polypeptide, malaria circumsporozoite (CS) protein, hepatitis B surface antigen (HBsAg₁₉₋₂₈), Heat Shock Protein (HSP) 65, *Mycobacterium tuberculosis*, cholera toxin, cholera toxin mutants with reduced toxicity, diphtheria toxin, CRM₁₉₇ protein that is cross-reactive with diphtheria toxin, recombinant Streptococcal C5a peptidase, *Streptococcus pyogenes* ORF 1224, *Streptococcus pyogenes* ORF 1664, *Streptococcus pyogenes* ORF2452, *Chlamydia pneumoniae* ORF T367, *Chlamydia pneumoniae* ORF T858, Tetanus toxoid, HIV gp120 T1, components recognizing microbial surface adhesive matrix molecules (MSCRAMMS), growth factors, hormones, cytokines and chemokines.

386. The conjugate of claim 385, wherein the protein/polypeptide carrier is CRM₁₉₇.

387. The conjugate of claim 384, wherein the peptide immunogen is selected from the group consisting of a bacterial protein, a viral protein, and a eukaryotic protein.

388. A peptide immunogen-protein/polypeptide carrier conjugate generated according to the method of claim 377 and having the formula:



wherein,

C is the protein/polypeptide carrier and X^d is a derivatized functional group of an amino acid residue of the protein/polypeptide carrier or optionally of an amino acid residue of a peptide linker covalently attached to the protein/polypeptide carrier, and, wherein, P is a peptide immunogen molecule covalently attached to the derivatized functional group of the amino acid residue of the protein carrier or optionally of an amino acid residue of a peptide linker covalently attached to a protein/polypeptide carrier, R is a capping molecule covalently attached to the derivatized functional group of an amino acid residue of the protein/polypeptide carrier or optionally of an amino acid residue of a peptide linker covalently attached to a protein/polypeptide carrier, which preserves the functionality of the carrier, such that it retains its ability to elicit the desired immune responses against the peptide immunogen that would otherwise not occur without a carrier, n is an integer greater than 0, but less than or equal to 85, and p is an integer greater than 0, but less than 85.

389. The conjugate of claim 388, wherein the protein/polypeptide carrier is selected from the group consisting of human serum albumin, keyhole limpet hemocyanin (KLH), immunoglobulin molecules, thyroglobulin, ovalbumin, influenza hemagglutinin, PADRE polypeptide, malaria circumsporozoite (CS) protein, hepatitis B surface antigen (HBsAg₁₉₋₂₈), Heat Shock Protein (HSP) 65, *Mycobacter-*

rium tuberculosis, cholera toxin, cholera toxin mutants with reduced toxicity, diphtheria toxin, CRM₁₉₇ protein that is cross-reactive with diphtheria toxin, recombinant Streptococcal C5a peptidase, *Streptococcus pyogenes* ORF1224, *Streptococcus pyogenes* ORF1664, *Streptococcus pyogenes* ORF2452, *Chlamydia pneumoniae* ORF T367, *Chlamydia pneumoniae* ORF T858, Tetanus toxoid, HIV gp120 T1, components recognizing microbial surface adhesive matrix molecules (MSCRAMMS), growth factors, hormones, cytokines and chemokines.

390. The conjugate of claim 389, wherein the protein/polypeptide carrier is CRM₁₉₇.

391. The conjugate of claim 388, wherein the peptide immunogen is selected from the group consisting of a bacterial protein, a viral protein, a fungal protein, a parasite protein and a eukaryotic protein.

392. An immunogenic composition, comprising a conjugate of a peptide immunogen with a protein/polypeptide carrier generated by the method of claim 377, together with one or more pharmaceutically acceptable excipients, diluents, and/or adjuvants.

393. The immunogenic composition of claim 392, wherein the protein/polypeptide carrier is selected from the group consisting of human serum albumin, keyhole limpet hemocyanin (KLH), immunoglobulin molecules, thyroglobulin, ovalbumin, influenza hemagglutinin, PADRE polypeptide, malaria circumsporozoite (CS) protein, hepatitis B surface antigen (HBsAg₁₉₋₂₈), Heat Shock Protein (HSP) 65, *Mycobacterium tuberculosis*, cholera toxin, cholera toxin mutants with reduced toxicity, diphtheria toxin, CRM₁₉₇ protein that is cross-reactive with diphtheria toxin, recombinant Streptococcal C5a peptidase, *Streptococcus pyogenes* ORF1224, *Streptococcus pyogenes* ORF1664, *Streptococcus pyogenes* ORF2452, *Chlamydia pneumoniae* ORF T367, *Chlamydia pneumoniae* ORF T858, Tetanus toxoid, HIV gp120 T1, components recognizing microbial surface adhesive matrix molecules (MSCRAMMS), growth factors, hormones, cytokines and chemokines.

394. The immunogenic composition of claim 393, wherein the protein/polypeptide carrier is CRM₁₉₇.

395. The immunogenic composition of claim 392, wherein the peptide immunogen is selected from the group consisting of a bacterial protein, a viral protein, a fungal protein, a parasite protein, and a eukaryotic protein.

396. The immunogenic composition of claim 392, wherein one or more adjuvants are selected from the group consisting of GM-CSF, 529 SE, IL-12, aluminum phosphate, aluminum hydroxide, *Mycobacterium tuberculosis*, *Bordetella pertussis*, bacterial lipopolysaccharides, aminoalkyl glucosamine phosphate compounds, MPL™ (3-O-deacylated monophosphoryl lipid A), a polypeptide, Quil A, STIMULON™ QS-21, a pertussis toxin (PT), an *E. coli* heat-labile toxin (LT), IL-1 α, IL-1 β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-13, IL-14, IL-15, IL-16, IL-17, IL-18, interferon-α, interferon-β, interferon-γ, G-CSF, TNF-α and TNF-β.

tella pertussis, bacterial lipopolysaccharides, aminoalkyl glucosane phosphate compounds, MPL™ (3-O-deacylated monophosphoryl lipid A), a polypeptide, Quil A, STIMULON™ QS-21, a pertussis toxin (PT), an *E. coli* heat-labile toxin (LT), IL-1 α, IL-1 βIL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-13, IL-14, IL-15, IL-16, IL-17, IL-18, interferon-α, interferon-β, interferon-γ, G-CSF, TNF-α and TNF-β.

397. A method for inducing an immune response in a mammalian subject, which comprises administering an effective amount of the immunogenic composition of claim 392 to the subject.

398. The method of claim 397, wherein the protein/polypeptide carrier is selected from the group consisting of human serum albumin, keyhole limpet hemocyanin (KLH), immunoglobulin molecules, thyroglobulin, ovalbumin, influenza hemagglutinin, PADRE polypeptide, malaria circumsporozoite (CS) protein, hepatitis B surface antigen (HBsAg₁₉₋₂₈), Heat Shock Protein (HSP) 65, *Mycobacterium tuberculosis*, cholera toxin, cholera toxin mutants with reduced toxicity, diphtheria toxin, CRM₁₉₇ protein that is cross-reactive with diphtheria toxin, recombinant Streptococcal C5a peptidase, *Streptococcus pyogenes* ORF1224, *Streptococcus pyogenes* ORF1664, *Streptococcus pyogenes* ORF2452, *Chlamydia pneumoniae* ORF T367, *Chlamydia pneumoniae* ORF T858, Tetanus toxoid, HIV gp120 T1, components recognizing microbial surface adhesive matrix molecules (MSCRAMMS), growth factors, hormones, cytokines and chemokines.

399. The method of claim 398, wherein the protein/polypeptide carrier is CRM₁₉₇.

400. The method of claim 397, wherein the peptide immunogen is selected from the group consisting of a bacterial protein, a viral protein, and a eukaryotic protein.

401. The method of claim 397, further comprising administering one or more adjuvants selected from the group consisting of GM-CSF, 529 SE, IL-12, aluminum phosphate, aluminum hydroxide, *Mycobacterium tuberculosis*, *Bordetella pertussis*, bacterial lipopolysaccharides, aminoalkyl glucosamine phosphate compounds, MPL™ (3-O-deacylated monophosphoryl lipid A), a polypeptide, Quil A, STIMULON™ QS-21, a pertussis toxin (PT), an *E. coli* heat-labile toxin (LT), IL-1 α, IL-1 β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-13, IL-14, IL-15, IL-16, IL-17, IL-18, interferon-α, interferon-β, interferon-γ, G-CSF, TNF-α and TNF-β.

* * * * *



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(19) United States

(12) Patent Application Publication

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(54) CHIMERIC MOLECULE USEFUL IN
IMMUNOTHERAPY FOR LEISHMANIASIS,
WHICH INCLUDES A FRAGMENT OF THE
PFR1 PROTEIN OF LEISHMANIA INFANTUM
WITH SPECIFIC IMMUNODOMINANT
EPITOPES

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Científicas (CSIC), Madrid (ES)

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C12Q 1/68 (2006.01)

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CPC *C07K 14/44* (2013.01); *C12Q 1/6893*
(2013.01); *C12Q 1/04* (2013.01)
USPC ... 424/185.1; 536/23.5; 514/44 R; 435/320.1;
435/6.15; 435/34

(57) ABSTRACT

The present invention claims an isolated nucleotide sequence characterized by encoding the PFR1 protein of *Leishmania infantum* or a fragment thereof. This PFR1 protein or a fragment thereof comprises at least a selected immunodominant epitope between the following group: SEQ ID No: 1, SEQ ID No: 2, SEQ ID No: 3, SEQ ID No: 4, SEQ ID No: 5, SEQ ID No: 6, SEQ ID No: 7 and SEQ ID No: 8, where the immunodominant epitope is able to induce an antigen-specific T cell cytotoxic immune response in an animal, against the kinetoplastids causing the leishmaniasis disease. The immunodominant epitopes are cytotoxic T-lymphocyte activators and they present a high binding affinity for A2 type MHC Class I molecule.

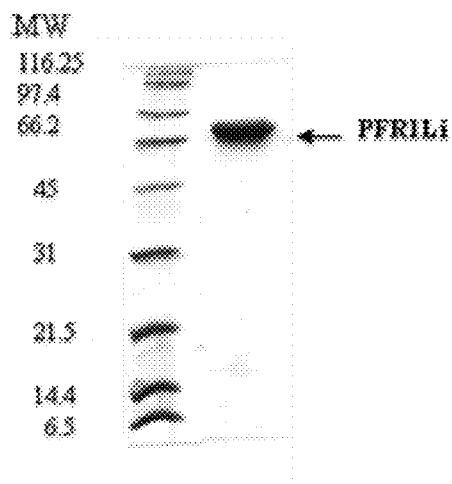


Fig. 1

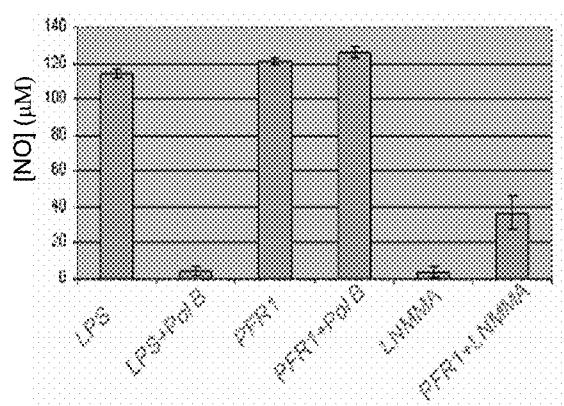
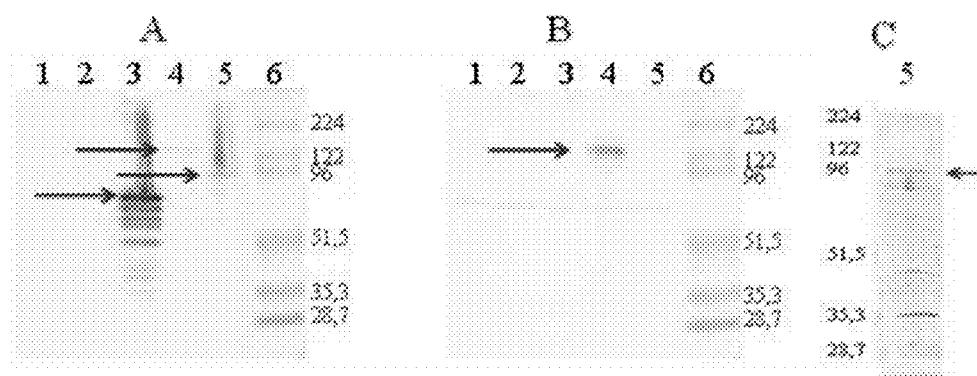


Fig. 2



Figs. 3A-3C

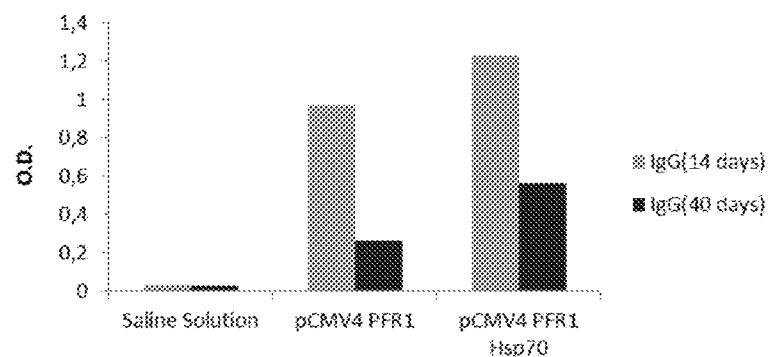


Fig. 4A

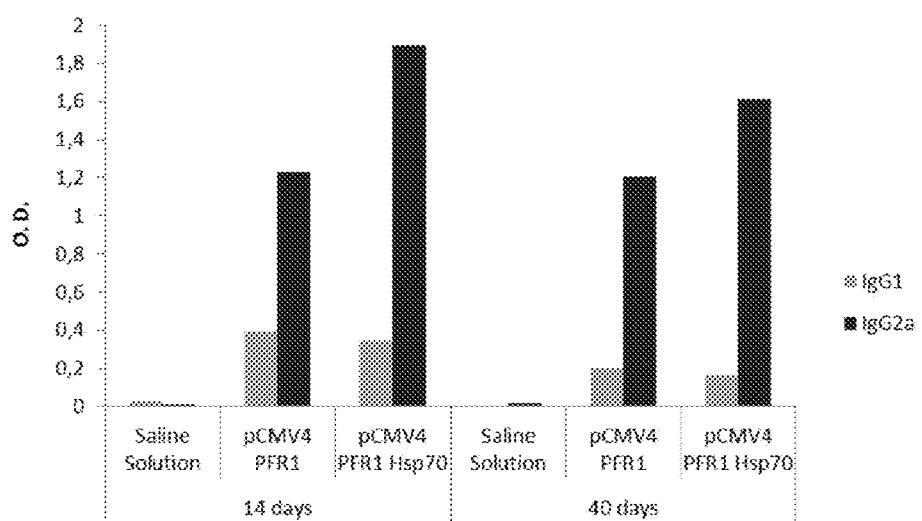


Fig. 4B

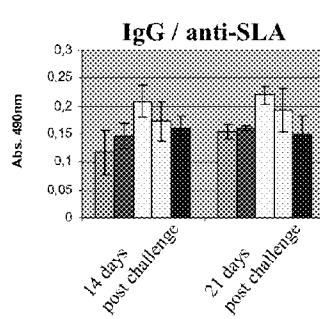


Fig. 5A

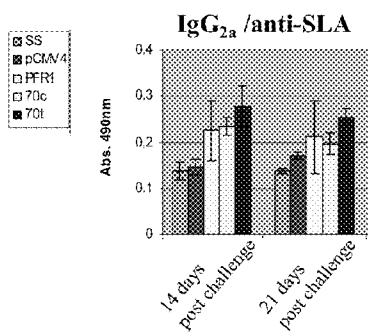


Fig. 5B

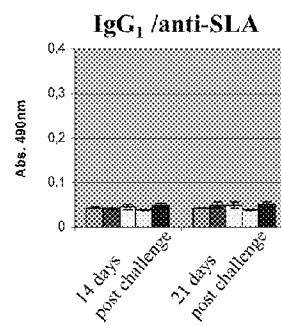


Fig. 5C

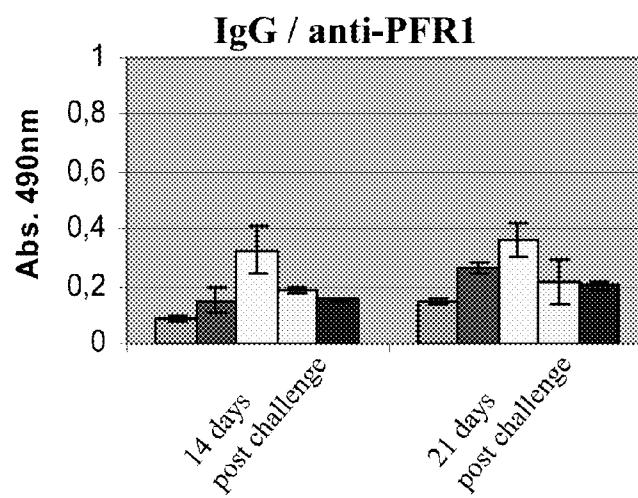


Fig. 5D

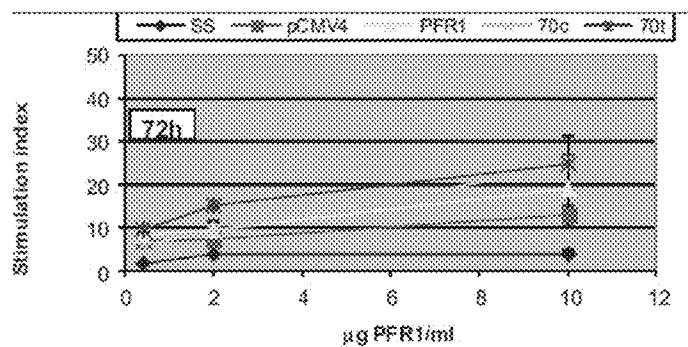


Fig. 6

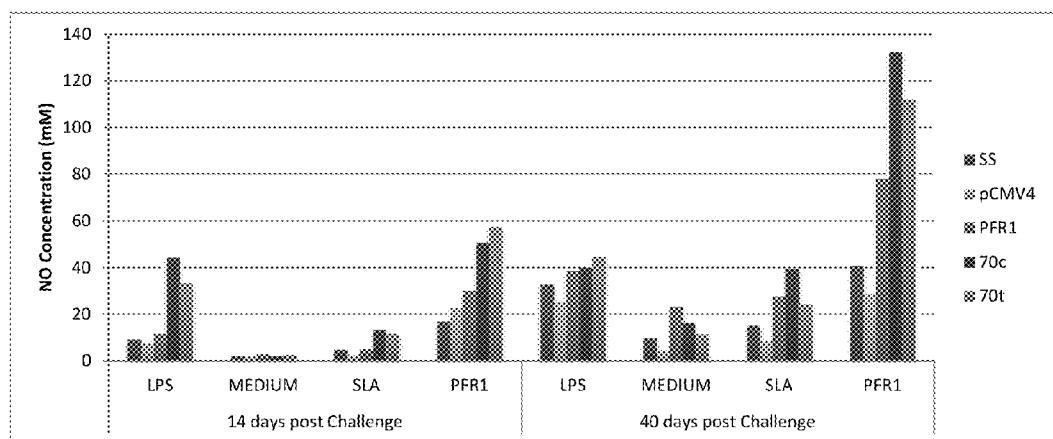


Fig. 7

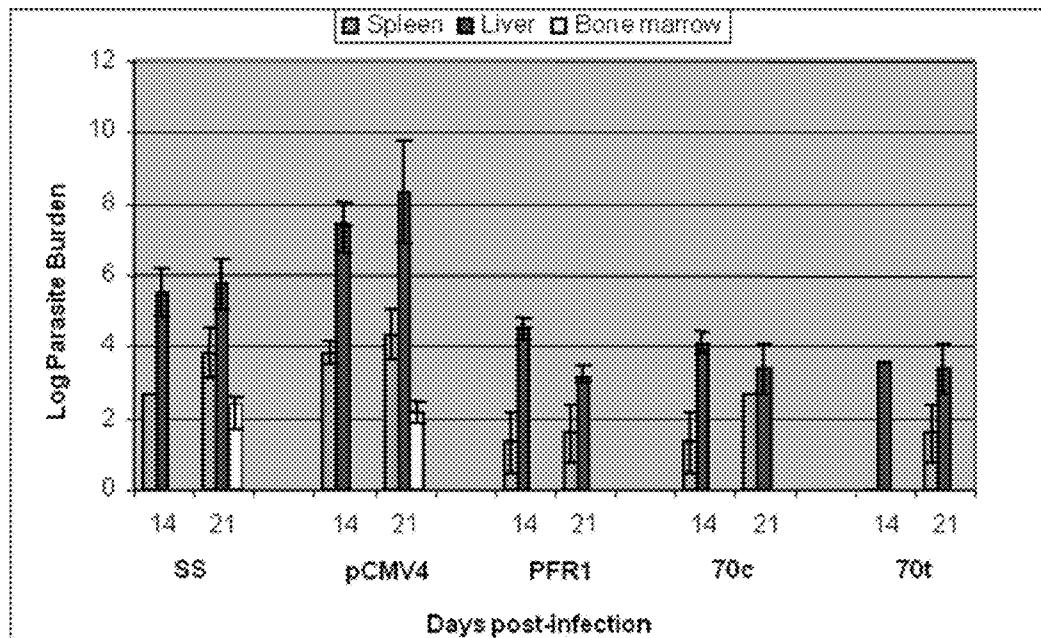


Fig. 8

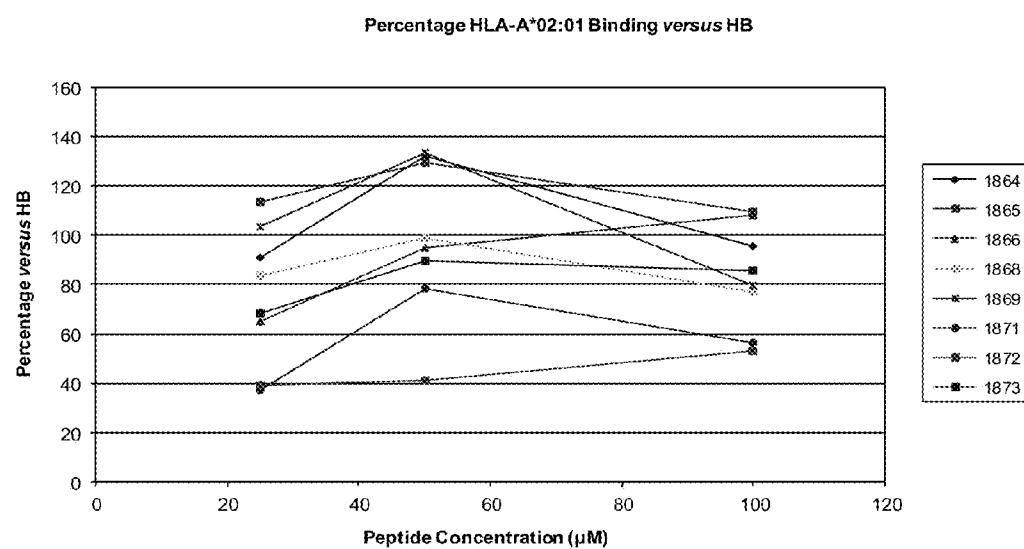


Fig. 9

Peptide	Saline Solution	pCMV4 PFR1	pCMV4 PFR1Hsp70
1864	-	-	-
1865	-	-	-
1866	-	-	430
1868	-	-	386
1869	-	-	452
1871	-	164	-
1872	-	-	230
1873	-	-	442

Fig. 10

Peptide	Non infected	Infected
1864	-	266
1865	-	-
1866	-	-
1868	-	-
1869	-	-
1871	-	376
1872	-	390
1873	-	466

Fig. 11

Peptide	Non infected	Infected
1864	-	-
1865	-	-
1866	-	-
1868	-	-
1869	-	-
1871	-	104
1872	-	87
1873	83	98

Fig. 12

Nonamers:

Position	SEQUENCE	SYFPEITHI	RANKPEP	BIMAS (min)
165 (1868)	K M M E D I M N A	20	54%	438
186	Q M Q T Q L A Q L	24	64%	35
204 (1869)	A M H D G E T Q V	23	59%	206
222 (1871)	Q L Q E R L I E L	28	68%	201
229	E L L K D K F G L	20	24%	34
395	L V S E G C A G V	21	29%	42
431 (1872)	M L Y L T L G S L	27	64%	35
532	V L T R R S K M V	19	62%	118
538 (1873)	K M V E Y K S H L	22	57%	222

Decamers:

Position	SEQUENCE	SYFPEITHI	RANKPEP	BIMAS (min)
1	M M T P E D A T G L	20	23%	26
103 (1864)	F M D I I G V K K V	24	25%	213
130	Q L I D N S I A K M	25	N.D.	47
150 (1865)	Q L D A T Q L A Q V	26	31%	64
165	K M M E D I M N A T	18	22%	657
222	Q L Q E R L I E L L	26	29%	99
373 (1866)	K L L E L T V Y N C	20	N.D.	606

Fig. 13

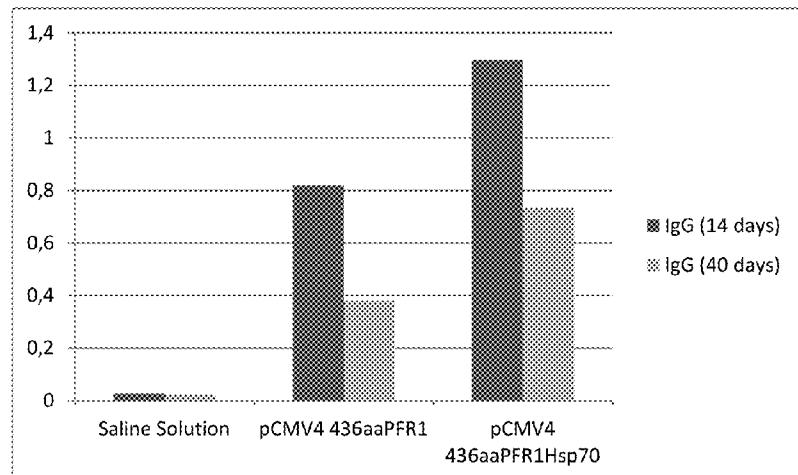


Fig. 14A

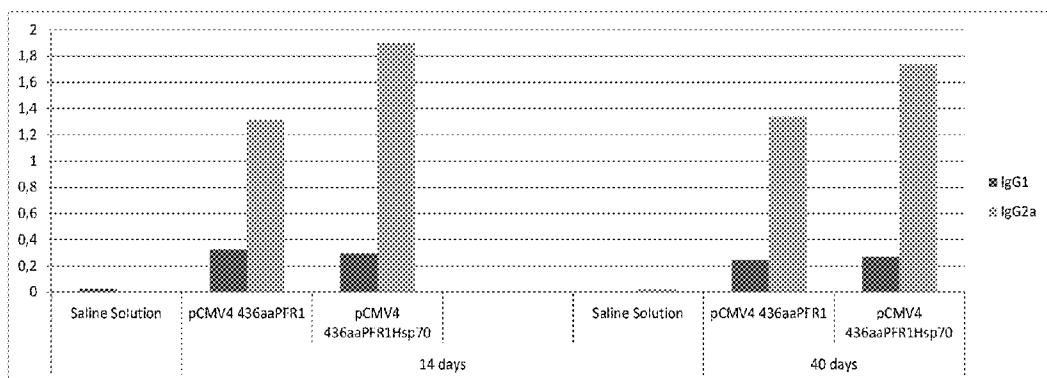


Fig. 14B

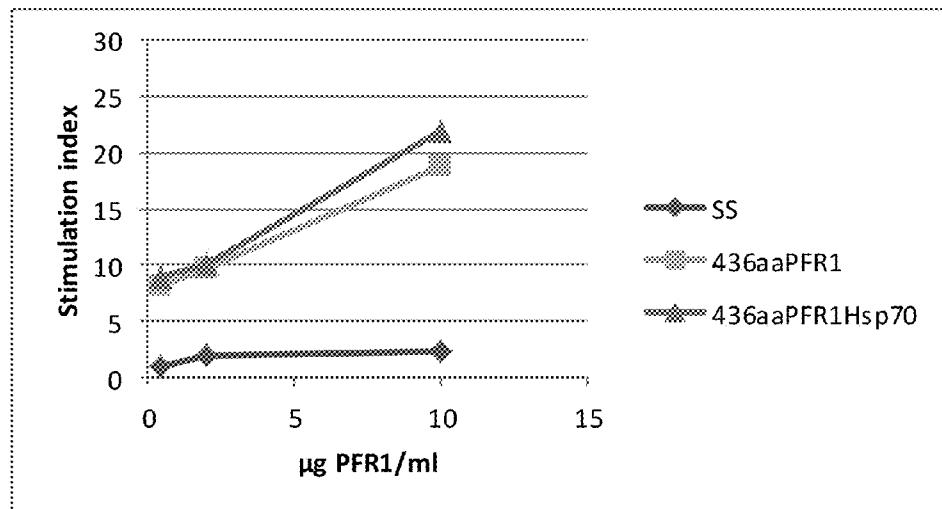


Fig. 15

Peptide	Saline Solution	pCMV4 436aaPFR1	pCMV4 436aaPFR1Hsp70
1864	-	-	-
1865	-	-	-
1866	-	-	419
1868	-	-	401
1869	-	-	466
1871	-	171	-
1872	-	-	233
1873	-	-	418

Fig. 16

**CHIMERIC MOLECULE USEFUL IN
IMMUNOTHERAPY FOR LEISHMANIASIS,
WHICH INCLUDES A FRAGMENT OF THE
PFR1 PROTEIN OF LEISHMANIA INFANTUM
WITH SPECIFIC IMMUNODOMINANT
EPITOPES**

[0001] The present invention is found within the chemical and pharmaceutical sector, and in the areas of medicine, molecular biology, immunology, parasitology, and veterinary, and refers to an immunological tool for combating leishmaniasis, a disease caused by different species belonging to the genus *Leishmania*.

STATE OF THE ART

[0002] The species of the genus *Leishmania*, Protozoan intracellular parasites, belonging to the order Kinetoplastida Trypanosomatidae family, are distributed by tropical and subtropical regions of the world, causing a broad spectrum of clinical symptoms, called leishmaniasis. Sorting by the causative species, leishmaniasis can be classified as cutaneous, mucocutaneous or visceral (in reference to their tissue tropism and the clinical causing symptoms). The incidence of leishmaniasis has increased enormously in recent years, so that it is now endemic in 88 countries across all continents and it is claimed that there are 350 million people at risk of contracting the disease (WHO report, <http://who.int/ctd/html/leidis.html>). It is estimated that worldwide there are about 12 million people affected by leishmaniasis. Accounting 2 million new cases annually, 500,000 are visceral leishmaniasis (VL). This disease is caused by *L. infantum* and *L. donovani* and symptoms of the disease include intermittent fever, anemia, splenomegaly, hepatomegaly, and lymphadenopathy. The outcome of the VL is usually death, caused by a concomitant infection, given the weakness of the immune system of the affected patient. *Leishmania/HIV* co-infection has revealed in recent years as a major health concern for the Mediterranean area countries. In these countries, the main host is the dog, acting as a reservoir of the disease for humans (Alvar et al. 2004 *Adv Parasitol.* 57: 1-88).

[0003] Vaccination is the most efficient medical treatment to prevent mortality and morbidity due to infectious agents. Despite this, currently there are very few high-effective vaccines against pathogens against which there is an effective vaccine. Front of the different *Leishmania* species, in recent years, it has been tested the use of antigen vaccines or thereof with disparate results (Kedzierski et al. 2010. *J Glob Infect Dis.* 2 (2): 177-85; Kedzierski et al. 2006. *Parasitology,* 133: 87-112; Requena et al. 2004. *Expert Opin Biol Ther.* 4 (9): 1505-17). However, none of the attempts to obtain a vaccine in humans has been fully satisfactory so far.

[0004] Another interesting option in the fight against the disease is therapeutic or, in its absence, the immunochemotherapy. Pasteurized adjuvanted complete parasites have been tested (Convit et al. 2003 *Med Hyg.* 97: 469-72), as well as a mixture of the parasite antigens (Badaro et al. 2006 *J Infect Dis.* 194: 1151-9), but, as in the case of vaccination, any ultimate success has failed (El-On J. 2009. *Isr Med Assoc J.* 11 (10): 623-8).

[0005] As it was outlined before, the dog is the main reservoir of the disease, so that several attempts have also been made from the immunological point of view in the fight against this human but also veterinary health problem. In fact, leishmaniasis is a serious parasitic disease in the dog. The

most common clinical symptom is loss of hair, especially around the eyes, ears, and nose. In a more advanced stage of the disease the dog lose weight but not appetite and they are common the presence of wounds in the skin, especially on the head and legs, as well as symptoms related to kidney failure. The disease is endemic in large parts of Spain, as well as in most of the countries of the Mediterranean region.

[0006] Likewise in humans, there have been made numerous attempts to control the disease from different angles, both prophylactic and immunotherapy, without a completely satisfactory success so far (Alvar et al. 2004. *Adv Parasitol.* 57: 1-88; Reis et al. 2010 *Trends Parasitol.* 26 (7): 341-9).

[0007] In any case, either human or canine leishmaniasis, one of the most promising ways of control of leishmaniasis points to the use of DNA vaccines that carry multiple genes coding for specific *leishmania* antigens or chimeric recombinant proteins containing different antigens of the parasite (De Oliveira et al. 2009 *Parasitol Int.* 58 (4): 319-24; Palatnik-de-Sousa, 2008. *Vaccine* 26: 1709-1724).

[0008] PFR proteins (Paraflagellar Rod proteins) represent a family of relevant specific antigens of tripanosomatids that are located in the paraflagellar region of these parasites (Fouts et al., 1998 *J Biol Chem.* 273 (34): 21846-21855; Clark et al., 2005. *Parasitol Res.* 96 (5): 312-320). Some members of this family of antigens, PFR1-3 proteins, stand out for their high immunogenicity (Michailowsky et al., *Infect Immun* 71 (6): 3165-3171). Immunization of mice with PFR1 and PFR2 proteins purified from *T. cruzi* induces a Th1 immune response capable of reducing, in experimental infection tests, *T. cruzi* load in the heart tissue of mice immunized and infected with the *T. cruzi* parasite (Morell et al. 2006 *Vaccine*, 24: 7046-7055). These results show that PFR proteins may be suitable candidates for use as vaccines. According to these results, it has been found that lymphocytes CD4+ isolated from mice immunized with PFR are capable of activating parasite-infected macrophages causing the death of the parasite by NO releasing (Wrightzman et al. 2000 *Vaccine* 18 (14): 1419-27, Miller et al. 1997 *J Immunol* 158 (11): 5330-7), and also being able to reduce the level of parasites in blood, in the absence of B cells, but not in the absence of functional CD4+ and CD8+T lymphocytes as well. From the point of view of gene immunization, different paraflagellar proteins of different kinetoplastids have been studied. The immunogenic and protective capacity of *L. mexicana* PFR2 protein after their inoculation as both DNA and recombinant protein has been demonstrated (Saravia et al. 2005 *Vaccine* 23: 984-995). Immunization of mice with DNA vectors containing the gene that encodes the PFR2 protein of *T. cruzi* alone or fused to the Hsp70 protein of the same pathogen induces high levels of IgG2a type anti-PFR antibodies. However, only the immunization with the chimeric vaccine stimulates the production of IL-12 and IFN- γ , and causes the decrease of IL-4 producing cells, triggering a protective response against *T. cruzi* (Morell et al. 2006 *Vaccine* 24: 7046-7055).

[0009] Hsp70 protein belongs to the heat shock proteins (HSP) family, which are highly conserved among the different species (eukaryotes and prokaryotes). They are fundamental in maintaining cellular homeostasis by their role as chaperone (Smith, Whitesell et al. 1998 *Pharmacological Reviews* 50 (4): 493-513). They are very interesting for their ability to activate the immune system, highlighting the Hsp70 family by its immunological versatility. Hsp70 proteins obtained from tumor cells or virus-infected cells are capable of activating CD8+CTL response both in vivo and in vitro

against various expressed antigens in the cells of which the immunogenic protein has been purified from (Srivastava. 2002 *Nat Rev Immunol* 2: 185-194; Wu et al., 2005. *Cancer Res.* 65 (11): 4947-4954). Thus, the extracellular Hsp70 can form complexes with antigenic peptides and activate APCs at the same time. This interaction triggers a cascade of events, gathering processes of peptides cross-presentation to CD8+ restricted MHC I and T CD4+ cells restricted MHC II, secretion of proinflammatory cytokines, and functional and phenotypic maturation of dendritic cells (DCs) (Asea et al. 2000 *Nat Med* 6: 435-442; Basu et al. 2001 *Immunity* 14: 303-313; Harmala et al. 2002 *J Immunol* 169: 5622-5629; Tobian et al. 2004 *J Immunol* 173: 5130-5137).

[0010] On in vitro tests carried out in our laboratory, the *Trypanosoma cruzi* Hsp70 protein has shown to have a unique stimulator effect on spleen and ganglion cells of naive mice (Marañón et al., 2000. *Int. Immunol.* 12 (12): 1685-1693), resulting in a quick and intense stimulation of T cells. In addition, this protein is able to induce in vivo and in vitro, against the associated hapten, a mixed immune response (IgG1 and IgG2a) which, interestingly, turns out to be independent of TLR2 and TLR4 receptors (Qazi et al. 2007 *Vaccine* 25 (6): 1096-1103). This *T. cruzi* HSP70 protein has also proven to be capable of triggering a specific response against KMP11 protein in mice when they are immunized with a vector bearing the sequences that encode for both proteins, by activating the production of IgG2a type specific antibodies against KMP11 (Thomas, Olivares et al. 2000 *DNA and Cell Biology* 19 (1): 47-57; Thomas, Olivares et al. 2000 *Acta Tropica* 75 (2): 203-210). Moreover, mice immunized with the KMP11-HSP70 fusion protein, but not those immunized with the isolated KMP11 protein, induce a cytotoxic lymphocyte response against Jurkat-A2/Kb cells expressing the KMP11 protein as well as against those cells loaded with KMP11-derived peptides of proven affinity to A2 molecule. Similar achievements have been obtained using the *Leishmania* genus itself, administering Hsp70 together to the gp63 metalloproteinase of *Leishmania donovani* (Kaur et al. 2011 *Parasite Immunol.* 33 (2): 95-103).

[0011] To fight against different species of *Leishmania*, in recent years, vaccines comprising the inactivated whole parasite and/or modified antigens or fragments thereof, as purified as recombinant, have been tested with different results. Thus, according to our own data, none of the previous attempts to obtain a fully successful vaccine for humans or dogs has been achieved so far. Even though chemotherapy currently in use has shown some effectiveness in many cases, it is unable to generate an enough parasite clearance, necessary for a full control and eradication of the disease.

[0012] Five years ago, it was carried out the identification of A*0201 molecule-restricted T cell binding epitopes contained in the PFR1 protein, as well as the generation of chimeric molecules (DNA vaccines) formed by the sequences coding for the *L. infantum* PFR1 protein or fragments derived from this protein, for their use both isolated and associated to the HSP70 protein and/or fragments thereof, as a carrier molecule. In addition, it was studied in immunized mice the PFR1-specific immune response induced by the referred chimeric molecules.

[0013] There are previous studies in the literature not recommending the use of peptides isolated from the PFR proteins of *T. cruzi* for induction of CTL response. In particular, Wrightsman R. A. et al., published an article in 2002 where various peptides isolated from *T. cruzi* PFR proteins were

tested and evaluated whether these peptides were efficiently processed and presented, within the context of a MHC-Class I, in the course of an experimental infection with this parasite and whether they were recognized by cytotoxic T cells (CTLs) in immunized animals (Wrightsman et al. 2002 *Parasite Immunol.* 24 (8): 401-12). In this study, PFR1 epitopes capable of binding to murine HLA class I molecules (H-2 Kb and H-2Db) are identified, but the authors conclude that mice immunization with PFR1 or PFR3 proteins does not induce CTLs against these proteins nor against the identified peptides.

[0014] Therefore, in view of the state of the art information, currently there is a need to demonstrate the efficiency of chimeric molecules (DNA and/or recombinant vaccines), formed by the coding sequence of antigenic proteins of *L. infantum* PFR1. The PFR1 protein comprises specific epitopes capable of inducing a CTL response, both isolated and associated with the HSP70 protein and/or fragments thereof, as carrier molecule. At the same time, it is necessary to test its efficacy and safety as prophylactic and therapeutic vaccine against *Leishmania* infection, both in humans and dogs.

BRIEF DESCRIPTION OF THE INVENTION

[0015] The present invention represents a useful tool in the fight against animal and human leishmaniasis. It is based on the use of the PFR1 protein-coding nucleotide sequences of *Leishmania infantum* or fragments thereof containing a series of peptides, alone or in conjunction with the Hsp70 protein of *Trypanosoma cruzi*, as well as plasmids containing their genetic encoding sequences. These peptides contained within the PFR1 protein of *L. infantum* correspond to immunodominant epitopes recognized specifically by CD8+T lymphocytes showing cytotoxic capacity, induced by the aforementioned protein used as immunogenicity agent. Where a preferential, the identified epitopes are presented by the human molecule HLA-A*0201. By means of these genetic and protein molecules the present invention provides a cellular and humoral immune response in the host against the protozoan parasite, with capacity for control, significantly, *Leishmania* infection or prevent it. The molecules object of the present invention are relevant for their role in immunotherapy against leishmaniasis used both as a preventive vaccine as therapeutic (isolated or in combination with various chemotherapy). Thus, the use of these molecules represents an industrial advantage over the lack of really effective preventive vaccines against this parasitic disease already in the market along with the difficulties and inconveniences that presents more traditional pharmacological treatments. Although they show some effectiveness in the majority of cases, in many other (especially dogs) do not lead to a complete clearance of the parasite, the whole treatments are quite expensive, they often have many side effects, and there is an increasing occurrence of cases of resistance, among their main setbacks.

DETAILED DESCRIPTION OF THE INVENTION

[0016] The present invention claims an isolated nucleotide sequence characterized by encoding the PFR1 protein of *Leishmania infantum* or a fragment thereof. This PFR1 protein or a fragment thereof comprises at least a selected immunodominant epitope between the following group: SEQ ID No: 1, SEQ ID No: 2, SEQ ID No: 3, SEQ ID No: 4, SEQ ID

No: 5, SEQ ID No: 6, SEQ ID No: 7 and SEQ ID No: 8, where the immunodominant epitope is able to induce an antigen-specific T cell cytotoxic immune response in an animal, against the kinetoplastids causing the leishmaniasis disease. The immunodominant epitopes are cytotoxic T-lymphocyte activators and they present a high binding affinity for A2 type MHC Class I molecule.

[0017] Within the context of the present invention "immunodominant epitope" is considered a fragment peptide capable of being recognized by lymphocyte receptors and of generating an epitope-specific cellular immune response.

[0018] In a preferred embodiment of the present invention the expression "animal" refers to a human or a pet, and more preferably a dog.

[0019] In a preferred embodiment of the present invention "nucleotide sequence" refers to any nucleotide sequence containing DNA, preferably genomic DNA, synthetic DNA, RNA, in vitro transcribed RNA, messenger RNA, being sense or antisense sequences. Therefore, the present invention refers to these sequences regardless of their obtaining conditions, thus including sequences obtained from a biological sample by cloning or by chemical synthesis or by enzymatic processes.

[0020] In a preferred embodiment of the present invention "nucleotide sequence encoding for a protein or a fragment thereof" refers to a nucleotide sequence that is capable, under adequate control of expression by their corresponding regulation elements (promoters, enhancers, transcription sites, etc.), of transcribing and translating one amino acid sequence of the protein of interest or a fragment thereof. The protein of interest as well as the fragments thereof referred to in the present invention is characterized by their amino acids sequence, but they can also be characterized by the cell where they are expressed, their maturation process and the existing environmental conditions during expression.

[0021] In a preferred embodiment of the present invention the term "amino acid sequence" refers to a sequence of an oligopeptide, peptide, protein, or their fragments, which are naturally or synthetically produced. As a sequence encoded by a nucleotide sequence is referred within the present invention, the term is not limited to the complete amino acid sequences, or native amino acid sequences associated with the aforementioned protein molecule, it is also referring to the variations that may suffer such native amino acid sequence.

[0022] In a particular embodiment, the present invention claims the nucleotide sequence defined above, encoding for the 1-595 amino acids of *Leishmania infantum* PFR1 protein, determined by the SEQ ID No: 12. This fragment encodes the complete protein.

[0023] In another particular embodiment, the present invention claims the nucleotide sequence defined above, encoding for the 160-595 amino acids of *Leishmania infantum* PFR1 protein, determined by the SEQ ID No: 9.

[0024] In another particular embodiment, the present invention claims the nucleotide sequence defined above, encoding for the 160-548 amino acids of *Leishmania infantum* PFR1 protein, determined by the SEQ ID No: 10.

[0025] In another particular embodiment, the present invention claims the nucleotide sequence defined above, encoding for the 160-385 amino acids of *Leishmania infantum* PFR1 protein, determined by the SEQ ID No: 11.

[0026] The present invention also protects a chimeric molecule comprising:

(a) a nucleotide sequence defined above, that is preferably fused to
(b) a nucleotide sequence encoding for the Hsp70 protein of *Trypanosoma cruzi*, or fragments thereof;

wherein the chimeric molecule is able to induce an antigen-specific cytotoxic T cell immune response in an animal, against the kinetoplastids causing the leishmaniasis disease.

[0027] In a preferred embodiment of the present invention "chimeric molecule" refers to a DNA molecule containing nucleotide sequences from two different species, preferably those species are *Leishmania infantum* and *Trypanosoma cruzi*.

[0028] In a particular embodiment, the present invention claims the chimeric molecule defined above, carrying the nucleotide sequence encoding for the Hsp70 protein of *Trypanosoma cruzi*, determined by SEQ ID No: 13, or fragments of the HSP70 with carrier activity.

[0029] In another particular embodiment, the present invention claims the chimeric molecule defined above, incorporating at the 3' end of the gene encoding fragment for the PFR1 protein fragment corresponding from 160 to 595 amino acids, characterized by the SEQ ID No: 9, and a nucleotide sequence encoding for the Hsp70 protein of *Trypanosoma cruzi*, determined by the SEQ ID no.: 13 or HSP70 fragments with carrier activity.

[0030] In another particular embodiment, the present invention claims the chimeric molecule defined above, incorporating at the 3' end of the gene encoding fragment for the PFR1 protein fragment corresponding from 160 to 548 amino acids, characterized by the SEQ ID No: 10, and a nucleotide sequence encoding for the Hsp70 protein of *Trypanosoma cruzi*, determined by the SEQ ID no.: 13 or HSP70 fragments with carrier activity.

[0031] In another particular embodiment, the present invention claims the chimeric molecule defined above, incorporating at the 3' end of the gene encoding fragment for the PFR1 protein fragment corresponding from 160 to 365 amino acids, characterized by the SEQ ID No: 11, and a nucleotide sequence encoding for the Hsp70 protein of *Trypanosoma cruzi*, determined by the SEQ ID no.: 13 or HSP70 fragments with carrier activity.

[0032] In another particular embodiment, the present invention claims the chimeric molecule defined above, incorporating at the 3' end of the gene encoding fragment a nucleotide sequence encoding for the Hsp70 protein of *Trypanosoma cruzi*, determined by the SEQ ID no.: 13 or HSP70 fragments with carrier activity.

[0033] The present invention also claims a recombinant or expression vector comprising:

(a) a nucleotide sequence defined above, or
(b) a chimeric molecule defined above.

[0034] The present invention also claims a recombinant plasmid comprising the vector defined above.

[0035] Another embodiment of the present invention claims a composition, preferably pharmaceutical or immunogenic, comprising:

(a) a nucleotide sequence defined above, or
(b) a chimeric molecule defined above.

[0036] Another embodiment claimed by the present invention concerns the use of the pharmaceutical or immunogenic

composition defined above, for the treatment and/or prevention of kinetoplastids infection that causes leishmaniasis disease in an animal.

[0037] Another embodiment claimed by the present invention concerns a method for the manufacture of a preventive or therapeutic vaccines for the treatment and/or prevention of kinetoplastids infection causing the leishmaniasis disease, through the use of the pharmaceutical or immunogenic composition defined above. Such use or method is characterized by comprising the following stages:

- a) identifying the encoding sequences of interest, preferably identify at least one nucleotide sequence defined above,
- b) amplifying at least one nucleotide sequence identified in a) by PCR using genomic DNA from the kinetoplastid that causes the leishmaniasis disease, and oligonucleotides containing restriction enzymes sites that allow the amplicon direct cloning into prokaryotic and eukaryotic expression vectors after its digestion with these restriction enzymes,
- c) cloning at least one nucleotide sequence amplified in b) for the production of the protein encoded by the said nucleotide sequence,
- d) purifying at least one of the proteins obtained in c), also known as recombinant proteins (chimeras or not), by affinity chromatography, and
- e) producing at least one endotoxin-free DNA vector for a safe inoculation of a preventive or therapeutic vaccine for the treatment and/or prevention of kinetoplastids infection causing the leishmaniasis disease.

[0038] Another embodiment claimed by the present invention concerns the use of a nucleotide sequence, a chimeric molecule, a recombinant vector, a recombinant plasmid, or a pharmaceutical or immunogenic composition, as defined above, as markers in methods for controlling the degree of infection of kinetoplastids that cause leishmaniasis disease in an animal.

[0039] Preferably, the present invention claims the use of a nucleotide sequence, a chimeric molecule, a recombinant vector, a recombinant plasmid, or a pharmaceutical or immunogenic composition, defined above, to generate a protective immunological memory against the infection of the kinetoplastids causing the leishmaniasis disease in an uninfected animal.

[0040] More preferably, the present invention claims the use of a nucleotide sequence, a chimeric molecule, a recombinant vector, a recombinant plasmid, or a pharmaceutical or immunogenic composition, defined above, to clarify or generate partial or total clearance of the kinetoplastids causing the leishmaniasis disease of the tissues in an infected animal.

[0041] The present invention has mainly two clear options of immuno-therapeutic applications, a preventive vaccination and its use in immune therapies against infection by *Leishmania*. Likewise, the molecules/products object of the present invention can be used in the form of recombinant proteins, as DNA plasmids (genetic vaccine), or in a combined form.

[0042] Thus, a first implementation of the claimed products would be the use of these molecules to generate protective immunological memory against infection by the parasite protozoan mentioned, either in humans or dogs, getting control of the disease in endemic areas (vaccination). Another application would be the use of these molecules as immunotherapy in individuals already infected, in order to enhance the immune response of the host against the parasite and control it. This treatment can be isolated or in combination with other

existing chemotherapeutic, chasing the total clearance of the parasite. Thus, activating and modulating the immune response of the host against the parasite and especially by inducing a T cytotoxic antigen-specific response the immune system could eradicate the parasites stationed in tissues, such as bone marrow or spleen that the majority of chemotherapeutic treatments fail to clarify.

[0043] Throughout the description and claims the word "comprise" and its variants do not exclude other technical features, additives, components or steps. For experts in the field, other objects, advantages and features of the invention come off as part of the description and as part of the practice of the invention. The following figures and examples are provided for illustration and they are not intended to limit the scope of the present invention.

FIGURE LEGENDS

[0044] FIG. 1. Purified PFR-1 recombinant protein was electrophoresed in a 10% SDS-PAGE gel and visualized by coomassie blue staining. MW. Protein molecular weight marker.

[0045] FIG. 2. Measurement of Nitric Oxide (NO) concentration in the supernatant of macrophages culture stimulated with lipopolysaccharide (LPS); lipopolysaccharide+polymyxin B (LPS+PolB); purified PFR-1 protein (PFR-1); purified PFR-1 protein+polymyxin B (PFR1+PolB); L-NG-monomethyl arginine citrate (LNMMMA); purified PFR-1 protein+L-NG-monomethyl arginine citrate (PFR1+LNMMMA). Concentration is expressed in $\mu\text{mol/litre}$.

[0046] FIG. 3. Western blot analysis of PFR-1 and PFR1-Hsp70 protein expression in COS-7 cells transfected with the pCMV4 empty vector (lane 2) and pCMV4 PFR1 (lane 3); pCMV4 PFR1-Hsp70 (lane 4) and pCMV4 PFR1-Th70 (lane 5) constructs. No transfected cells are used as control (lane 1). Protein molecular weight marker (Lane 6). Poly clonal antibody against LiPFR1 (Panel A), TcHsp70 (Panel B and C).

[0047] FIG. 4. Panel A. IgG antibody level against PFR-1 recombinant protein in sera from mice inoculated with saline solution and immunized with pCMV4 PFR-1 and pCMV4 PFR1Hsp70 vectors, 14 (grey Bars) and 40 days (black bars) after the fourth immunization. Panel B. Level of IgG1 (grey bars) and IgG2a (black bars) antibodies specific of the PFR-1 recombinant protein detected in the immunized mice referred in Panel A at 14 and 40 days post fourth immunization. Bars represent the mean value of optical density of each group.

[0048] FIG. 5. Antibody level against *Leishmania* soluble antigens (SLA, panels A, B and C) and PFR1 recombinant protein (Panel D) in sera from C57BL/6 mice inoculated with saline solution (SS) and, pCMV4 plasmid (pCMV4) or immunized with pCMV4 PFR-1 (PFR1), pCMV4 PFR-1-Hsp70 (70c) and pCMV4 PFR-1 truncated Hsp70 (70t) recombinant vectors, 14 and 21 days after challenge with *Leishmania infantum* infective promastigotes. Bars represent the mean of optical density of each group.

[0049] FIG. 6. Lymphoproliferative response to PFR-1 protein in mice inoculated with saline solution (SS) and pCMV4 empty vector (pCMV4) and immunized with pCMV4-PFR1 (PFR1), pCMV4 PFR1-HSP70 (70c) and pCMV4 PFR1-Truncated Hsp70 (70t). Stimulation index was calculated as [(arithmetic mean of cpm (stimulated culture)-arithmetic mean of cpm (control culture))/arithmetic mean of cpm]. The results represent the mean and standard deviation of three independent immunization experiments.

[0050] FIG. 7. Nitric Oxide production (NO) by peritoneal macrophages from immunized C57BL/6 mice inoculated with saline solution (SS) and pCMV4 empty vector (pCMV4) or immunized with pCMV4 PFR1 (PFR1), pCMV4 PFR1-Hsp70 (70c) and pCMV4 PFR1-truncated Hsp70 (70t) constructs after *Leishmania infantum* challenge in response to lipopolysaccharide (LPS), culture media (Medium), *Leishmania* soluble antigens (SLA) and PFR1 recombinant protein.

[0051] FIG. 8. Parasite burden in spleen, liver and bone marrow from mice inoculated with saline solution (SS) and empty vector pCMV4 (pCMV4) or immunized with pCMV4 PFR1 (PFR1), pCMV4 PFR1-Hsp70 (70c) and pCMV4 PFR1-truncated Hsp70 (70t) recombinant vectors at 14 and 21 days post challenged with *Leishmania infantum* infective promastigotes.

[0052] FIG. 9. Binding assay of HLA*02:01-restricted PFR1-derived peptides to TAP-deficient T2 cells. Percentage of maximal complex stabilization was calculated with the HB-ENV₃₃₄₋₃₄₂ peptide fluorescence index as a reference. The binding of each peptide was determined at different concentrations of each peptide.

[0053] FIG. 10. Cytotoxic activity of the CD8⁺ T lymphocytes specific for the eight selected PFR1 peptides evaluated by the secretion of GzB through ELISPOT in splenocytes from B6-A2/K^b mice inoculated with saline solution (SS) or immunized with pCMV4 PFR1 (PFR1) or pCMV4 PFR1-Hsp70 (PFR1-HSP70) recombinant vectors. Spots were visualized using a KS ELISPOT device (Zeiss). Only large spots with fuzzy borders were scored as spot-forming cells (SFC). Responses were considered significant if (i) a minimum of 150 SFC/106 splenocytes were detected after subtraction of the negative control (splenocytes without peptide), and additionally, (ii) the response was at least over two fold the negative control.

[0054] FIG. 11. Cytotoxic activity of the CD8⁺ T lymphocytes specific for the eight selected PFR1 peptides evaluated by the secretion of GzB through ELISPOT in splenocytes from B6-A2/K^b mice infected with *Leishmania infantum* infective promastigotes. Non-inoculated animals were used as control. Spots were visualized using a KS ELISPOT device (Zeiss). Only large spots with fuzzy borders were scored as spot-forming cells (SFC). Responses were considered significant if (i) a minimum of 250 SFC/106 splenocytes were detected after subtraction of the negative control (splenocytes without peptide), and additionally, (ii) the response was at least over two fold the negative control.

[0055] FIG. 12. Cytotoxic activity of the CD8⁺ T lymphocytes specific for the eight selected PFR1 peptides evaluated by the secretion of GzB through ELISPOT in hepatocytes from B6-A2/K^b mice infected with *Leishmania infantum* infective promastigotes. Non-inoculated animals were used as control. Spots were visualized using a KS ELISPOT device (Zeiss). Only large spots with fuzzy borders were scored as spot-forming cells (SFC). Responses were considered significant if (i) a minimum of 75 SFC/106 hepatocytes detected after subtraction of the negative control (hepatocytes without peptide), and additionally, (ii) the response was at least over two fold the negative control.

[0056] FIG. 13. The prediction of potential HLA-A*02:01 ligands contained in *L. Infantum* PFR1 protein was carried out through the screening of the deduced amino acid sequence of PFR1 gene using three HLA-A2-binding affinity algorithms: SYPFEITHI (<http://www.syfpeithi.de>), RANK-

PEP (<http://immunax.dfci.harvard.edu/Tools/rankpep.html>) and BIMAS (theoretical half-time dissociation, www.bimas.cit.nih.gov/molbio/hla_bind/).

[0057] FIG. 14. Panel A. IgG antibody level against PFR1 recombinant protein in sera from mice inoculated with saline solution and immunized with pCMV4 436aaPFR1 and pCMV4 436aaPFR1 Hsp70 vectors, 14 (black bars) and 40 days (grey bars) after the fourth immunization. Panel B. Level of IgG1 (black bars) and IgG2a (grey bars) antibodies specific of the PFR-1 recombinant protein detected in the immunized mice referred in Panel A at 14 and 40 days post fourth immunization. Bars represent the mean value of optical density of each group.

[0058] FIG. 15. Lymphoproliferative response to PFR-1 protein in mice inoculated with saline solution (SS) or immunized with pCMV4 436aaPFR1 (436aaPFR1) and pCMV4 436aa PFR1-HSP70 (436aa PFR1 HSP70). Stimulation index was calculated as [(arithmetic mean of cpm (stimulated culture)-arithmetic mean of cpm (control culture))/arithmetic mean of cpm]. The results represent the mean and standard deviation of three independent immunization experiments.

[0059] FIG. 16. Cytotoxic activity of the CD8⁺ T lymphocytes specific for the eight selected PFR1 peptides evaluated by the secretion of GzB through ELISPOT in splenocytes from B6-A2/K^b mice inoculated with saline solution or immunized with pCMV4 436aaPFR1 or pCMV4 436aa PFR1-Hsp70 recombinant vectors. Spots were visualized using a KS ELISPOT device (Zeiss). Only large spots with fuzzy borders were scored as spot-forming cells (SFC). Responses were considered significant if (i) a minimum of 150 SFC/106 splenocytes were detected after subtraction of the negative control (splenocytes without peptide), and additionally, (ii) the response was at least over two fold the negative control.

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EXAMPLES

[0093] The following specific examples that are provided in this patent document are intended to illustrate the nature of the present invention. These examples are only for illustrative purposes and should not be interpreted as limitations to the invention that is claimed here. Therefore, the examples described below illustrate the invention without limiting the field of application of the same.

Example 1

1.1. The PFR1 Protein Induces the Expression of Nitric Oxide (NO)

[0094] PFR1 protein of *Leishmania infantum* is encompassed within the family of characteristic proteins of paraflagellar rod proteins from kinetoplastids. In FIG. 1, we show the PFR1 recombinant protein of *L. infantum* expressed in a prokaryotic expression system and purified by affinity chromatography. For this protein we have demonstrated that it has the ability to activate the production of nitric oxide (NO), one of the main mechanisms that the macrophages have to eliminate pathogens that have phagocytosed into alveolar macrophages of nave rat, i.e. without any treatment or previous infection. This activation of nitric oxide production confers to the aforementioned protein a relevant immunological feature because NO favors the clearance of the amastigotes of *Leishmania* that multiply within the host macrophages. In FIG. 2, the values of NO production by these alveolar macrophages are collected by stimulating them with LPS (control) and the PFR1 protein. Interestingly, unlike the one detected for PFR1 protein, the activation observed for LPS is inhibited by the presence of polymyxin B (LPS activity-induced inhibitor), which discards any contamination with LPS from the PFR1 recombinant protein. On the other hand, the addition of L-N⁶-monomethyl Arginine (LNMMMA), an inhibitor of the enzyme nitric oxide synthase, inhibits the NO activation induced by the PFR1 protein, indicating the

mechanism of action of this protein. Similar results are obtained by the fusion PFR1-HSP70 protein.

1.2. Expression of PFR1 Protein in Eukaryotic Cells

[0095] The determination of the correct expression of the proteins to study in eukaryotic cells is determined by the analysis of COS-7 transfected cells with genes encoding for PFR1 protein and chimeric fusion proteins HSP70-PFR1 and PFR1-H70T cloned into pCMV4 plasmid. The visualization of different proteins is made by Western blots analysis of cells extracts transfected with the respective abovementioned plasmids and induced PFR1-protein polyclonal antibodies. Thus, in FIG. 3 is observed, respectively, the presence of recognition bands with sizes of 70 kDa (corresponding to the PFR1 protein in the cells lane with the pCMV4 PFR1), 140 kDa in the lane of COS-7 cells transfected with the pCMV4 PFR1-Hsp70 plasmid and 96 kDa in the cells lane with the pCMV4 PFR1-Hsp70T plasmid. Cells containing the empty pCMV4 plasmid are not detected.

1.3 Antigen-Specific Humoral Response Induced by the Tested Molecules

[0096] As example we show the results obtained after intramuscularly immunization in groups of 12 mice of the C57BL/6 strain, with 100 µg of different plasmids under study, as well as negative controls (empty plasmid and saline solution). Each mouse was immunized 4 times every two weeks. Six weeks after the fourth immunization, six mice in each group were challenged intravenously with 105 infective promastigotes of the JPCM5 (MCAN/s/98/LLM-724) strain of *L. infantum*.

[0097] To analyze the humoral response generated in different groups of mice, blood samples were collected two weeks after each immunization and specific antibody measured. The obtained results indicate the presence of anti-PFR1 IgG antibodies 15 days post-second immunization in the group of mice immunized by the isolated PFR1 gene and two weeks after the third immunization in those immunized by the fused HSP70-PFR1 or PFR1-HSP70T genes. FIG. 4 shows the results for each molecule at 14 and -40 days post-fourth immunization. IgG levels registered in sera from mice immunized by the PFR1 gene fused to the HSP70 gene were higher than those detected in mice immunized with the isolated PFR1 gene, peaking within two weeks post-fourth immunization. In all cases the level of anti-PFR1 antibodies slightly descends after six weeks post-fourth immunization. The isotype analysis reflects that generated antibodies show a clear polarization of the response towards the IgG2a isotype (Th1-type immune response). Similar results were obtained for the PPFR1-H70T molecule.

[0098] The results obtained in PFR1 protein humoral response assays and total antigens of *Leishmania* (SLA) occurring in mice immunized by the molecules under study and challenged with *L. infantum* are shown in FIG. 5. Analysis of these results indicates that infection with *Leishmania* does not induce significant variation in IgG levels against PFR1 and the same polarization towards the IgG2a isotype is observed prior to the infection.

1.4 Antigen-Specific Cell Response Induced by the Molecules Under Study

[0099] Lymphoproliferation tests were carried out three weeks after the fourth immunization. The spleens were

extracted in sterile conditions and the obtained splenocytes were cultivated in vitro, with an increasing concentration of the recombinant PFR1 protein (0.4, 2 and 10 µg/ml). In addition, in this assay a mitogen (ConA) and unstimulated splenocytes were included as a positive and negative control, respectively. From the results obtained, shown in FIG. 6, a significant cellular proliferation rate is observed as the recombinant PFR1 is present and, in addition, this stimulation is dose-dependent in the splenocytes from mice immunized by the recombinant testing molecules. Interestingly, this index was significantly higher in the groups receiving the PFR1 gene fused to the HSP70 and HSP70T. The proliferation rate (IE) for the splenocytes from mice immunized with these fusion molecules are approximately 25, while the measured index for splenocytes from mice immunized with the plasmid containing the isolated PFR1 gene is about 20. In both cases, above the controls; mice inoculated with the empty plasmid, IE=13 and saline solution, IE=4. These proliferation rates of splenic cells of mice immunized with the testing molecules remain with similar values after eight weeks post-fourth immunization. In addition, stimulation capacity is maintained after the challenge, noting a maximum of proliferation rate (cellular response) in mice immunized by the isolated PFR1 gene and after stimulating with 2 µg/ml of the PFR1 recombinant protein.

1.5 Expression of Nitric Oxide (NO) in Mice Immunized and Infected by *L. infantum*

[0100] As shown in FIG. 7, it can be seen a significant greater ability to produce nitric oxide (NO) in peritoneal macrophages of mice immunized with the testing molecules, especially with the fused ones (PFR1-HSP70 or PFR1-HSP70T) comparing to the control mice, both stimulated and unstimulated. In addition, this NO releasing ability of the mentioned immunized groups of mice increases significantly after stimulation with the recombinant PFR1 protein. On the other hand, such production was significantly higher in the infected mice versus the unchallenged ones.

1.6 Determination of the Capability of Inducing Protection Against *L. infantum* Infection

[0101] The parasite load was analyzed by limiting dilution (Buffet et al., 1995, *Antimicrob. Agents Chemother.* 39 (9): 2167-2168), in the liver, spleen and bone marrow (target tissues of the parasite), after 14 and 28 days post-infection, showing a summary of the results in FIG. 8. Thus, after 14 days post-infection all groups of mice had parasites in the liver, however, in control groups (ss and pCMV4) the parasite load was significantly higher than the detected in the groups of animals immunized with different testing molecules, showing higher values in at least one order of magnitude. In addition, unlike the control groups, vaccinated mice with the testing molecules do not show an increase in hepatic parasitic load at 28 days post-infection. In fact, mice immunized with PFR1 gene fused to the full HSP70 gene (pPFR1-HSP70), showed an important parasite clearance in the liver. The analysis of the parasite load in spleen shows a similar pattern. Thus, after 14 days post-infection it is observed a significant higher parasite load (at least one order of magnitude) in splenic tissue of control mice (ss and pCMV4) versus vaccinated mice. In fact, mice vaccinated with PFR1 gene fused to the HSP70T gene (pPFR1-H70T) show no parasites in spleen after two weeks post-infection. Regarding bone marrow, only

control groups (ss and pCMV4) account parasites, detected just after 28 days post-infection. Interestingly, none of the vaccinated mice presents parasites in this tissue. In summary, all vaccinated groups showed in all analyzed tissues during the infection a significantly lower parasitic load than the control groups (decrease between two to four orders of magnitude), pointing that these molecules confer a high level of protection against *L. infantum* infection, as intravenously administered.

[0102] The analysis of the expression pattern of cytokines of splenocytes of vaccinated mice versus control groups (cytometry measures in the supernatant of the cell culture using the Mouse Th1/Th2 Cytokine CBA—BD Biosciences kit) shows the existence of higher levels of TNF- α and IFN- γ and with statistical significance in vaccinated mice with PFR1-HSP70 and PFR1-H70T chimeric constructs, stimulated or unstimulated with rPFR1 and/or SLA, versus the control groups ($P<0.01$). However, there is not a statistically significant variation among groups regarding IL-2 or IL-4 levels. Likewise, the analysis, after the challenge with *Leishmania*, of the level of the splenic macrophage activation of all the mice groups, problem and control, is measured by the expression pattern of CD80, CD86 and CD40 surface molecules. The results show that after 21 days post-infection the expression of CD86 and CD40 was significantly higher in the vaccinated group with chimeric constructions PFR1-HSP70 and PFR1-H70T versus the control ($P<0.01$).

1.7 Identification of T CD8+Epitopes T in the PFR1 Protein of *L. infantum*

[0103] The identification of epitopes in the sequence of the PFR1 protein of *L. infantum* able to be recognized by CD8+ T cells and, as a result of activating a cytotoxic antigen-specific response in the host, was carried out trying to identify epitopes capable of binding to MHC-class I HLA. To do this, it was selected, by *in silico* analyses, different peptides capable of binding to HLA-A*0201 (expressed in the half of the human population), using three programs: SYFPEITHI, RANKPEP, and BIMAS. The deduced sequence of the PFR1 protein of *Leishmania infantum* (gene number: AY702344) was introduced in each of the programs and it was selected the algorithm of binding to HLA-A*0201. The first two programs deliver a score to each peptide based on its theoretical affinity with the HLA molecule. On the other hand BIMAS scores the stability of binding with the HLA molecule, focusing on the theoretical binding time. Combining the results, eight theoretical epitopes of high binding affinity to class I HLA molecule were selected and their corresponding peptides were synthesized: SEQ ID No: 1-1864 (FMDIIGVKKV), SEQ ID No: 2-1865 (QLDATQLAQV), SEQ ID No: 3-1866 (KLLELTVYNC), SEQ ID No: 4-1868 (KMMDIMNA), SEQ ID No: 5-1869 (AMHDGETQV), SEQ ID No: 6-1871 (QLQERLIEL), SEQ ID No: 7-1872 (MLYLTLGSL) and SEQ ID No: 8-1873 (KMVEYKSHL). The FIG. 13, tables 1 and 2, includes scores for the different peptides in the mentioned software.

[0104] To determine the binding capacity to the HLA-A*0201 molecule, it was performed binding tests to T2 cells, having a low expression capacity of transport-antigen molecules. The results, shown in FIG. 9, indicate that all the testing peptides performed a good or very good binding affinity to the HLA-A*0201 molecule, being in some cases superior to the HB-ENV₃₃₄₋₂₄₂ peptide affinity used as control, for which a percentage of 100% binding is expected.

[0105] The analysis of the effective presentation capacity of these epitopes in the context of an experimental immunization with the testing molecules, took place in C57BL/6-A2/K^b transgenic mice (they were modified to express the product of the chimeric gene HLA-A2.1/K^b, where alpha1 and alpha2 domains are the same as the HLA-A*0201 human molecule and the alpha3 domains, both transmembrane and cytoplasmic corresponding to the H-2K^b murine molecule) immunized intramuscularly with pPFR1 and pPFR1-HSP70 molecules. Mice injected with saline solution (Sigma) were used as negative control. Six weeks after the fourth immunization the splenocytes of the different mice groups were stimulated with the testing peptides in the context of an ELISPOT assay using an anti-granzyme B antibody as probe. The results obtained (FIG. 10) show that five of the peptides gave a positive response to granzyme B (activation of antigen-specific CTLs) in vaccinated mice with the chimeric molecule pPFR1-HSP70 and one in those immunized with the molecule containing the isolated PFR1 gene. These results demonstrate that the above testing molecules are efficiently processed and presented in the context of the MHC-Class I. Furthermore it points that in particular those epitopes for which positive values are obtained are recognized by cytotoxic T lymphocytes (CTLs) of the immunized mice.

[0106] The analysis of the capacity to generate cytotoxic response in the course of an experimental infection with *L. infantum* was evaluated in C57BL/6-A2/K^b transgenic mice infected via i.v. with 10⁶ infective promastigotes of *L. infantum* (strain JPCM5). 170 Days after infection mice were sacrificed and their splenocytes or their not-parenchymal liver cells were exposed to the testing peptides in the context of an ELISPOT assay using Granzima B as detector antibody. The results show (FIGS. 11 and 12) that four of the tested peptides gave a positive response to Granzyme B in splenocytes and three of them also in not-parenchymal liver cells. Therefore, these peptides are presented to the CTLs in the course of experimental infection by the parasite. Interestingly, only one out of these four peptides also gave positive response in immunized mice, showing that the antigen presentation differs between immunization with plasmids and the course of the experimental infection.

CONCLUDING REMARKS

[0107] Example 1 conclusion: the results show that immunized mice with the testing molecules and subsequently challenged, do not present parasites in bone marrow, while those groups inoculated with the pCMV4empty plasmid or saline control (control) present a high parasite load in the same tissue after 28 days post-infection. In addition, the parasite load in spleen and liver of immunized mice are between two to four orders of magnitude lower than the detected in the control groups (empty pCMV4 plasmid or saline solution). Immunized mice with the pPFR1-H70T molecule and *Leishmania*-infected, do not show parasites in spleen after 14 days post-infection.

Example 2

2.1 Antigen-Specific Humoral Response

[0108] As an example, it is shown the results obtained after intramuscularly immunization of 12-mice-groups of the C57BL/6 strain, with 100 µg of the different testing plasmids, as well as the negative controls (mice inoculated with saline

solution). In this case, the tested plasmids contain the sequence corresponding to the fragment of the PFR1 protein of *Leishmania infantum* (436 amino acids length) comprised between amino acids 160 and 595 of the PFR1 protein. Each mouse was immunized 4 times every other week.

[0109] To analyze the generated humoral response in different groups of mice, blood collection was made two weeks after each immunization and antibody level detection in animal sera was measured. The results indicate that the presence of anti-PFR1 IgG antibodies appears 15 days post-fourth immunization in the immunized group with the isolated 436aaPFR1 gen and two weeks after the third immunization in those which carry fused 436aaPFR1-HSP70 genes. FIG. 14 (A) shows the results for each testing molecule at 14 and 40 days post-fourth immunization. IgG levels in sera from immunized mice with the 436aaPFR1 gen fused to the HSP70 gene were higher than those detected in the group immunized with the isolated 436aaPFR1 gene, with a maximum of antibodies within two weeks post-fourth immunization. In all cases, the level of anti-PFR1 antibodies slightly descends at six weeks post-fourth immunization. Analysis of isotypes (B) shows a clear polarization of the response towards the IgG2a isotype (Th1 type immune response) in released antibodies.

2.2 Antigen-Specific Cell Response

[0110] Lymphoproliferation tests were carried out three weeks after the fourth immunization. The spleens were extracted in sterile conditions and the obtained splenocytes were in vitro cultivated in the presence of an increasing concentration of the rPFR1 protein (0.4, 2 and 10 µg/ml). In addition, a mitogen (ConA) and unstimulated splenocytes were included in this assay as a positive and negative control, respectively. From the results obtained, shown in FIG. 15, a significant cellular proliferation rate is observed as the recombinant PFR1 (rPFR1) is present and, in addition, this stimulation is dose-dependent in the splenocytes of mice immunized by the recombinant testing molecules. Interestingly, this index was significantly higher in the groups receiving the PFR1 gene fused to the HSP70 and HSP70T. The proliferation rate (IE) for the splenocytes of mice inoculated with these fusion molecules are approximately 22, while the measured index for splenocytes from mice immunized with the plasmid containing the isolated PFR1 gene is about 18. In both cases, above the controls; saline solution, IE=4. These proliferation rates of splenic cells of mice immunized with the testing molecules remain with similar values after eight weeks post-fourth immunization.

[0111] The analysis of the effective presentation capacity of these epitopes in the context of an experimental immunization with the testing molecules, took place in C57BL/6-A2/K^b transgenic mice (they were modified to express the product of the chimeric gene HLA-A2.1/K^b, where alpha1 and alpha2 domains are the same as the HLA-A*0201 human molecule and the alpha3 domains, both transmembrane and cytoplasmic corresponding to the H-2K^b murine molecule) immunized intramuscularly with 436aaPFR1 and 436aaPFR1-HSP70 molecules. Mice injected with saline solution (Sigma) were used as negative control. Six weeks after the fourth immunization the splenocytes of the different mice groups were stimulated with the testing peptides in the context of an ELISPOT assay using an anti-granzyme B antibody as probe. The results obtained (FIG. 16) show that five of the peptides gave a positive response to granzyme B (activation of antigen-specific CTLs) in vaccinated mice with the

chimeric molecule 436aaPFR1-HSP70 and one in those immunized with the molecule containing the isolated 436aaPFR1 gene. These results demonstrate that the above testing molecules are efficiently processed and presented in the context of the MHC-Class I. Furthermore it points that in particular those epitopes for which positive values are obtained are recognized by cytotoxic T lymphocytes (CTLs) of the immunized mice.

[0112] Conclusion Example 2: the results show that the 436 amino acids fragment of the PFR1 protein performs very similarly to the complete protein, getting a similar pattern of immune response (humoral, cellular and cytotoxic). Therefore, since these parameters are those involved in protection against the parasite, we can conclude that this fragment behaves in all terms to study as the complete protein.

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<223> OTHER INFORMATION: "High affinity MCH class I, type A2 molecule
immunodominant epitope"

<400> SEQUENCE: 6
Gln Leu Gln Glu Arg Leu Ile Glu Leu
1 5

<210> SEQ ID NO 7
<211> LENGTH: 9
<212> TYPE: PRT
<213> ORGANISM: Leishmania infantum
<220> FEATURE:
<221> NAME/KEY: SOURCE
<222> LOCATION: 1..9
<223> OTHER INFORMATION: /mol_type="protein"
/organism="Leishmania infantum"
<220> FEATURE:
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<222> LOCATION: 1..9
<223> OTHER INFORMATION: "Sequence 1872 corresponds to Li PFR1 protein
(amino acids 431-440)"
<220> FEATURE:
<221> NAME/KEY: PEPTIDE
<222> LOCATION: 1..9
<223> OTHER INFORMATION: "Cytotoxic T lymphocytes activator
immunodominant epitope"
<220> FEATURE:
<221> NAME/KEY: PEPTIDE
<222> LOCATION: 1..9
<223> OTHER INFORMATION: "High affinity MCH class I, type A2 molecule
immunodominant epitope"

<400> SEQUENCE: 7
Met Leu Tyr Leu Thr Leu Gly Ser Leu
1 5

<210> SEQ ID NO 8
<211> LENGTH: 9
<212> TYPE: PRT
<213> ORGANISM: Leishmania infantum
<220> FEATURE:
<221> NAME/KEY: SOURCE
<222> LOCATION: 1..9

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<223> OTHER INFORMATION: /mol_type="protein"
      /organism="Leishmania infantum"
<220> FEATURE:
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<222> LOCATION: 1..9
<223> OTHER INFORMATION: "Sequence 1873 corresponds to Li PFRI protein
      (amino acids 538-546)"
<220> FEATURE:
<221> NAME/KEY: PEPTIDE
<222> LOCATION: 1..9
<223> OTHER INFORMATION: "High affinity MCH class I, type A2 molecule
      immunodominant epitope"
<220> FEATURE:
<221> NAME/KEY: PEPTIDE
<222> LOCATION: 1..9
<223> OTHER INFORMATION: "Cytotoxic T lymphocytes activator
      immunodominant epitope"

<400> SEQUENCE: 8

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Lys Met Val Glu Tyr Lys Ser His Leu
1           5

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```

<210> SEQ ID NO 9
<211> LENGTH: 436
<212> TYPE: PRT
<213> ORGANISM: Leishmania infantum
<220> FEATURE:
<221> NAME/KEY: SOURCE
<222> LOCATION: 1..436
<223> OTHER INFORMATION: /mol_type="protein"
      /organism="Leishmania infantum"
<220> FEATURE:
<221> NAME/KEY: REGION
<222> LOCATION: 1..436
<223> OTHER INFORMATION: "PFR1 fragment aminoacids 160-595"
<220> FEATURE:
<221> NAME/KEY: PEPTIDE
<222> LOCATION: 6..14
<223> OTHER INFORMATION: "SEQ ID No: 4"
<220> FEATURE:
<221> NAME/KEY: PEPTIDE
<222> LOCATION: 45..53
<223> OTHER INFORMATION: "SEQ ID No: 5"
<220> FEATURE:
<221> NAME/KEY: PEPTIDE
<222> LOCATION: 63..71
<223> OTHER INFORMATION: "SEQ ID No: 6"
<220> FEATURE:
<221> NAME/KEY: PEPTIDE
<222> LOCATION: 214..223
<223> OTHER INFORMATION: "SEQ ID No: 3"
<220> FEATURE:
<221> NAME/KEY: PEPTIDE
<222> LOCATION: 272..280
<223> OTHER INFORMATION: "SEQ ID No: 7"
<220> FEATURE:
<221> NAME/KEY: PEPTIDE
<222> LOCATION: 379..387
<223> OTHER INFORMATION: "SEQ ID no: 8"

<400> SEQUENCE: 9

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

```

```

Ile Gln Asn Ala Leu Ala Ser Thr Asp Asp Gln Met Gln Thr Gln Leu
20          25          30

```

```

Ala Gln Leu Glu Lys Thr Asn Glu Ile Gln Asn Val Ala Met His Asp
35          40          45

```

```

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

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Gln	Glu	Arg	Leu	Ile	Glu	Leu	Leu	Lys	Asp	Lys	Phe	Gly	Leu	Ile	Gly
65					70			75					80		
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Lys	Cys	Glu	Glu	Asn	Ala	Gln	Phe	Lys	Glu	Ile	Tyr	Glu	Val	Gln	
								85	90				95		
<hr/>															
Lys	Gln	Ala	Asn	Gln	Glu	Thr	Ser	Gln	Met	Lys	Asp	Ala	Lys	Arg	Arg
								100	105			110			
<hr/>															
Leu	Arg	Gln	Arg	Cys	Glu	Thr	Asp	Leu	Lys	His	Ile	Gln	Asp	Ala	Ile
								115	120			125			
<hr/>															
Gln	Lys	Ala	Asp	Leu	Glu	Asp	Ala	Glu	Ala	Val	Lys	Arg	Tyr	Pro	Arg
	130				135				140						
<hr/>															
Asn	Lys	Glu	Arg	Ser	Glu	Arg	Ala	Ile	Lys	Glu	Asn	Glu	Glu	Met	Gln
145					150				155				160		
<hr/>															
Glu	Glu	Ala	Trp	Asn	Lys	Ile	Gln	Asp	Leu	Glu	Arg	Gln	Leu	Gln	Asn
	165							170				175			
<hr/>															
Leu	Gly	Thr	Asp	Arg	Phe	Asp	Glu	Val	Lys	Arg	Arg	Ile	Glu	Glu	Val
	180							185				190			
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Asp	Arg	Glu	Glu	Lys	Arg	Arg	Val	Glu	Asn	Ala	Gln	Phe	Leu	Glu	Ile
	195						200				205				
<hr/>															
Ala	Ala	Gln	His	Lys	Lys	Leu	Leu	Glu	Leu	Thr	Val	Tyr	Asn	Cys	Asp
	210				215				220						
<hr/>															
Leu	Ala	Met	Arg	Cys	Thr	Gly	Leu	Val	Glu	Glu	Leu	Val	Ser	Glu	Gly
225					230				235			240			
<hr/>															
Cys	Ala	Gly	Val	Lys	Ala	Arg	Tyr	Asp	Lys	Thr	Asn	Gln	Asp	Leu	Ala
	245						250				255				
<hr/>															
Ala	Leu	Arg	Leu	Glu	Val	His	Lys	Glu	His	Leu	Glu	Tyr	Phe	Arg	Met
	260						265				270				
<hr/>															
Leu	Tyr	Leu	Thr	Leu	Gly	Ser	Leu	Ile	Tyr	Lys	Lys	Glu	Lys	Arg	Leu
	275						280			285					
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Glu	Glu	Ile	Asp	Arg	Asn	Ile	Arg	Leu	Ala	His	Ile	Gln	Leu	Glu	Phe
	290						295			300					
<hr/>															
Cys	Val	Glu	Thr	Phe	Asp	Pro	Asn	Ala	Lys	Lys	His	Ala	Asp	Met	Lys
305					310				315			320			
<hr/>															
Lys	Glu	Leu	Tyr	Arg	Leu	Arg	Gln	Gly	Val	Glu	Glu	Glu	Leu	Ala	Met
	325						330				335				
<hr/>															
Leu	Lys	Glu	Gln	Ala	Ala	Ala	Leu	Asp	Asp	Phe	Lys	Glu	Ser	Glu	
	340						345				350				
<hr/>															
Glu	Ala	Leu	Asp	Ala	Ala	Gly	Ile	Glu	Phe	Ser	His	Pro	Val	Asp	Glu
	355						360			365					
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Asn	Asn	Glu	Glu	Val	Leu	Thr	Arg	Arg	Ser	Lys	Met	Val	Glu	Tyr	Lys
	370						375			380					
<hr/>															
Ser	His	Leu	Thr	Lys	Glu	Glu	Val	Arg	Ile	Ala	Ala	Glu	Arg	Glu	
385					390			395				400			
<hr/>															
Glu	Ile	Lys	Arg	Ala	Arg	Leu	Leu	Arg	Ser	Gly	Gly	Glu	Ser	Ala	Ala
	405						410			415					
<hr/>															
Ala	Gln	Ile	Thr	Ser	Gly	Ser	Met	Asn	Ala	Asp	Tyr	Ala	Ala	Ser	Ala
	420						425			430					
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Gln	Leu	Glu	Leu												
	435														

<210> SEQ ID NO 10
<211> LENGTH: 389
<212> TYPE: PRT

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<213> ORGANISM: Leishmania infantum
<220> FEATURE:
<221> NAME/KEY: SOURCE
<222> LOCATION: 1..389
<223> OTHER INFORMATION: /mol_type="protein"
    /organism="Leishmania infantum"
<220> FEATURE:
<221> NAME/KEY: REGION
<222> LOCATION: 1..389
<223> OTHER INFORMATION: "PFR1 fragment aminoacids 160-548"
<220> FEATURE:
<221> NAME/KEY: PEPTIDE
<222> LOCATION: 6..14
<223> OTHER INFORMATION: "SEQ ID No: 4"
<220> FEATURE:
<221> NAME/KEY: PEPTIDE
<222> LOCATION: 45..53
<223> OTHER INFORMATION: "SEQ ID No: 5"
<220> FEATURE:
<221> NAME/KEY: PEPTIDE
<222> LOCATION: 63..71
<223> OTHER INFORMATION: "SEQ ID No: 6"
<220> FEATURE:
<221> NAME/KEY: PEPTIDE
<222> LOCATION: 214..223
<223> OTHER INFORMATION: "SEQ ID No: 3"
<220> FEATURE:
<221> NAME/KEY: PEPTIDE
<222> LOCATION: 272..280
<223> OTHER INFORMATION: "SEQ ID No: 7"
<220> FEATURE:
<221> NAME/KEY: PEPTIDE
<222> LOCATION: 379..387
<223> OTHER INFORMATION: "SEQ ID no: 8"

<400> SEQUENCE: 10

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

Ile Gln Asn Ala Leu Ala Ser Thr Asp Asp Gln Met Gln Thr Gln Leu
20          25          30

Ala Gln Leu Glu Lys Thr Asn Glu Ile Gln Asn Val Ala Met His Asp
35          40          45

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

Gln Glu Arg Leu Ile Glu Leu Leu Lys Asp Lys Phe Gly Leu Ile Gly
65          70          75          80

Lys Cys Glu Glu Asn Ala Gln Phe Lys Glu Ile Tyr Glu Val Gln
85          90          95

Lys Gln Ala Asn Gln Glu Thr Ser Gln Met Lys Asp Ala Lys Arg Arg
100         105         110

Leu Arg Gln Arg Cys Glu Thr Asp Leu Lys His Ile Gln Asp Ala Ile
115         120         125

Gln Lys Ala Asp Leu Glu Asp Ala Glu Ala Val Lys Arg Tyr Pro Arg
130         135         140

Asn Lys Glu Arg Ser Glu Arg Ala Ile Lys Glu Asn Glu Glu Met Gln
145         150         155         160

Glu Glu Ala Trp Asn Lys Ile Gln Asp Leu Glu Arg Gln Leu Gln Asn
165         170         175

Leu Gly Thr Asp Arg Phe Asp Glu Val Lys Arg Arg Ile Glu Glu Val
180         185         190

Asp Arg Glu Glu Lys Arg Arg Val Glu Asn Ala Gln Phe Leu Glu Ile
195         200         205

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Ala Ala Gln His Lys Lys Leu Leu Glu Leu Thr Val Tyr Asn Cys Asp
 210 215 220

Leu Ala Met Arg Cys Thr Gly Leu Val Glu Glu Leu Val Ser Glu Gly
 225 230 235 240

Cys Ala Gly Val Lys Ala Arg Tyr Asp Lys Thr Asn Gln Asp Leu Ala
 245 250 255

Ala Leu Arg Leu Glu Val His Lys Glu His Leu Glu Tyr Phe Arg Met
 260 265 270

Leu Tyr Leu Thr Leu Gly Ser Leu Ile Tyr Lys Lys Glu Lys Arg Leu
 275 280 285

Glu Glu Ile Asp Arg Asn Ile Arg Leu Ala His Ile Gln Leu Glu Phe
 290 295 300

Cys Val Glu Thr Phe Asp Pro Asn Ala Lys Lys His Ala Asp Met Lys
 305 310 315 320

Lys Glu Leu Tyr Arg Leu Arg Gln Gly Val Glu Glu Glu Leu Ala Met
 325 330 335

Leu Lys Glu Lys Gln Ala Ala Ala Leu Asp Asp Phe Lys Glu Ser Glu
 340 345 350

Glu Ala Leu Asp Ala Ala Gly Ile Glu Phe Ser His Pro Val Asp Glu
 355 360 365

Asn Asn Glu Glu Val Leu Thr Arg Arg Ser Lys Met Val Glu Tyr Lys
 370 375 380

Ser His Leu Thr Lys
 385

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<210> SEQ_ID NO 11
<211> LENGTH: 226
<212> TYPE: PRT
<213> ORGANISM: Leishmania infantum
<220> FEATURE:
<221> NAME/KEY: SOURCE
<222> LOCATION: 1..226
<223> OTHER INFORMATION: /mol_type="protein"
/organism="Leishmania infantum"
<220> FEATURE:
<221> NAME/KEY: REGION
<222> LOCATION: 1..226
<223> OTHER INFORMATION: "PFR1 fragment aminoacids 160-385"
<220> FEATURE:
<221> NAME/KEY: PEPTIDE
<222> LOCATION: 6..14
<223> OTHER INFORMATION: "SEQ ID No: 4"
<220> FEATURE:
<221> NAME/KEY: PEPTIDE
<222> LOCATION: 45..53
<223> OTHER INFORMATION: "SEQ ID No: 5"
<220> FEATURE:
<221> NAME/KEY: PEPTIDE
<222> LOCATION: 63..71
<223> OTHER INFORMATION: "SEQ ID No: 6"
<220> FEATURE:
<221> NAME/KEY: PEPTIDE
<222> LOCATION: 214..223
<223> OTHER INFORMATION: "SEQ ID No: 3"

<400> SEQUENCE: 11

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

Ile Gln Asn Ala Leu Ala Ser Thr Asp Asp Gln Met Gln Thr Gln Leu
 20 25 30

Ala Gln Leu Glu Lys Thr Asn Glu Ile Gln Asn Val Ala Met His Asp

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35	40	45
Gly Glu Thr Gln Val Ala Glu Glu Gln Met Trp Thr Lys Val Gln Leu		
50	55	60
Gln Glu Arg Leu Ile Glu Leu Leu Lys Asp Lys Phe Gly Leu Ile Gly		
65	70	75
Lys Cys Glu Glu Asn Ala Gln Phe Lys Glu Ile Tyr Glu Val Gln		
85	90	95
Lys Gln Ala Asn Gln Glu Thr Ser Gln Met Lys Asp Ala Lys Arg Arg		
100	105	110
Leu Arg Gln Arg Cys Glu Thr Asp Leu Lys His Ile Gln Asp Ala Ile		
115	120	125
Gln Lys Ala Asp Leu Glu Asp Ala Glu Ala Val Lys Arg Tyr Pro Arg		
130	135	140
Asn Lys Glu Arg Ser Glu Arg Ala Ile Lys Glu Asn Glu Glu Met Gln		
145	150	155
Glu Glu Ala Trp Asn Lys Ile Gln Asp Leu Glu Arg Gln Leu Gln Asn		
165	170	175
Leu Gly Thr Asp Arg Phe Asp Glu Val Lys Arg Arg Ile Glu Glu Val		
180	185	190
Asp Arg Glu Glu Lys Arg Arg Val Glu Asn Ala Gln Phe Leu Glu Ile		
195	200	205
Ala Ala Gln His Lys Lys Leu Leu Glu Leu Thr Val Tyr Asn Cys Asp		
210	215	220
Leu Ala		
225		

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<210> SEQ ID NO 12
<211> LENGTH: 595
<212> TYPE: PRT
<213> ORGANISM: Leishmania infantum
<220> FEATURE:
<221> NAME/KEY: SOURCE
<222> LOCATION: 1..595
<223> OTHER INFORMATION: /mol_type="protein"
    /organism="Leishmania infantum"
<220> FEATURE:
<221> NAME/KEY: REGION
<222> LOCATION: 1..595
<223> OTHER INFORMATION: "PFR1 protein aminoacids"
<220> FEATURE:
<221> NAME/KEY: REGION
<222> LOCATION: 160..595
<223> OTHER INFORMATION: "SEQ ID No: 9"
<220> FEATURE:
<221> NAME/KEY: REGION
<222> LOCATION: 160..548
<223> OTHER INFORMATION: "SEQ ID No: 10"
<220> FEATURE:
<221> NAME/KEY: REGION
<222> LOCATION: 160..385
<223> OTHER INFORMATION: "SEQ ID No: 11"
<220> FEATURE:
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<222> LOCATION: 103..113
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<223> OTHER INFORMATION: "SEQ ID No: 2"
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<221> NAME/KEY: PEPTIDE
<222> LOCATION: 165..173
<223> OTHER INFORMATION: "SEQ ID No: 4"

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<220> FEATURE:
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<222> LOCATION: 204..212
<223> OTHER INFORMATION: "SEQ ID No: 5"
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<222> LOCATION: 222..230
<223> OTHER INFORMATION: "SEQ ID No: 6"
<220> FEATURE:
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<222> LOCATION: 373..382
<223> OTHER INFORMATION: "SEQ ID No: 3"
<220> FEATURE:
<221> NAME/KEY: PEPTIDE
<222> LOCATION: 431..440
<223> OTHER INFORMATION: "SEQ ID No: 7"
<220> FEATURE:
<221> NAME/KEY: PEPTIDE
<222> LOCATION: 538..546
<223> OTHER INFORMATION: "SEQ ID No: 8"

<400> SEQUENCE: 12

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

Lys Ile His Asn Leu Lys Leu Lys Thr Ala Cys Leu Glu Asn Glu Glu
20 25 30

Leu Val Gln Glu Leu His Ile Ser Asp Trp Ser Glu Thr Gln Arg Gln
35 40 45

Lys Leu Arg Gly Ala His Glu Lys Gly Glu Glu Leu Leu Ala Ser Val
50 55 60

Glu Val Gly Thr Lys Trp Asn Leu Met Glu Ala Tyr Asp Leu Ala Lys
65 70 75 80

Leu Met Arg Val Cys Gly Leu Glu Met Ser Gln Arg Glu Leu Tyr Arg
85 90 95

Pro Glu Asp Lys Pro Gln Phe Met Asp Ile Ile Gly Val Lys Lys Val
100 105 110

Leu Gln Asp Leu Arg Gln Asn Arg Asn Lys Thr Arg Val Val Ser Phe
115 120 125

Thr Gln Leu Ile Asp Asn Ser Ile Ala Lys Met Glu Lys Val Glu Glu
130 135 140

Glu Leu Arg Arg Ser Gln Leu Asp Ala Thr Gln Leu Ala Gln Val Pro
145 150 155 160

Thr Arg Thr Val Lys Met Met Glu Asp Ile Met Asn Ala Thr Gln Ile
165 170 175

Gln Asn Ala Leu Ala Ser Thr Asp Asp Gln Met Gln Thr Gln Leu Ala
180 185 190

Gln Leu Glu Lys Thr Asn Glu Ile Gln Asn Val Ala Met His Asp Gly
195 200 205

Glu Thr Gln Val Ala Glu Glu Gln Met Trp Thr Lys Val Gln Leu Gln
210 215 220

Glu Arg Leu Ile Glu Leu Leu Lys Asp Lys Phe Gly Leu Ile Gly Lys
225 230 235 240

Cys Glu Glu Glu Asn Ala Gln Phe Lys Glu Ile Tyr Glu Val Gln Lys
245 250 255

Gln Ala Asn Gln Glu Thr Ser Gln Met Lys Asp Ala Lys Arg Arg Leu
260 265 270

Arg Gln Arg Cys Glu Thr Asp Leu Lys His Ile Gln Asp Ala Ile Gln
275 280 285

```

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Lys Ala Asp Leu Glu Asp Ala Glu Ala Val Lys Arg Tyr Pro Arg Asn
290 295 300

Lys Glu Arg Ser Glu Arg Ala Ile Lys Glu Asn Glu Glu Met Gln Glu
305 310 315 320

Glu Ala Trp Asn Lys Ile Gln Asp Leu Glu Arg Gln Leu Gln Asn Leu
325 330 335

Gly Thr Asp Arg Phe Asp Glu Val Lys Arg Arg Ile Glu Glu Val Asp
340 345 350

Arg Glu Glu Lys Arg Arg Val Glu Asn Ala Gln Phe Leu Glu Ile Ala
355 360 365

Ala Gln His Lys Lys Leu Leu Glu Leu Thr Val Tyr Asn Cys Asp Leu
370 375 380

Ala Met Arg Cys Thr Gly Leu Val Glu Glu Leu Val Ser Glu Gly Cys
385 390 395 400

Ala Gly Val Lys Ala Arg Tyr Asp Lys Thr Asn Gln Asp Leu Ala Ala
405 410 415

Leu Arg Leu Glu Val His Lys Glu His Leu Glu Tyr Phe Arg Met Leu
420 425 430

Tyr Leu Thr Leu Gly Ser Leu Ile Tyr Lys Lys Glu Lys Arg Leu Glu
435 440 445

Glu Ile Asp Arg Asn Ile Arg Leu Ala His Ile Gln Leu Glu Phe Cys
450 455 460

Val Glu Thr Phe Asp Pro Asn Ala Lys Lys His Ala Asp Met Lys Lys
465 470 475 480

Glu Leu Tyr Arg Leu Arg Gln Gly Val Glu Glu Glu Leu Ala Met Leu
485 490 495

Lys Glu Lys Gln Ala Ala Ala Leu Asp Asp Phe Lys Glu Ser Glu Glu
500 505 510

Ala Leu Asp Ala Ala Gly Ile Glu Phe Ser His Pro Val Asp Glu Asn
515 520 525

Asn Glu Glu Val Leu Thr Arg Arg Ser Lys Met Val Glu Tyr Lys Ser
530 535 540

His Leu Thr Lys Glu Glu Glu Val Arg Ile Ala Ala Glu Arg Glu Glu
545 550 555 560

Ile Lys Arg Ala Arg Leu Leu Arg Ser Gly Gly Glu Ser Ala Ala Ala
565 570 575

Gln Ile Thr Ser Gly Ser Met Asn Ala Asp Tyr Ala Ala Ser Ala Gln
580 585 590

Leu Glu Leu
595

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<210> SEQ ID NO 13
<211> LENGTH: 680
<212> TYPE: PRT
<213> ORGANISM: Trypanosoma cruzi
<220> FEATURE:
<221> NAME/KEY: SOURCE
<222> LOCATION: 1..680
<223> OTHER INFORMATION: /mol_type="protein"
/organism="Trypanosoma cruzi"
<220> FEATURE:
<221> NAME/KEY: REGION
<222> LOCATION: 1..680
<223> OTHER INFORMATION: "HSP70 protein aminoacid sequence"
<220> FEATURE:

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<221> NAME/KEY: PEPTIDE
<222> LOCATION: 64..145
<223> OTHER INFORMATION: "Carrier activity fragment"
<220> FEATURE:
<221> NAME/KEY: PEPTIDE
<222> LOCATION: 100..145
<223> OTHER INFORMATION: "Carrier activity fragment"

<400> SEQUENCE: 13

Met Thr Tyr Glu Gly Ala Ile Gly Ile Asp Leu Gly Thr Thr Tyr Ser
1           5          10          15

Cys Val Gly Val Trp Gln Asn Glu Arg Val Glu Ile Ile Ala Asn Asp
20          25          30

Gln Gly Asn Arg Thr Thr Pro Ser Tyr Val Ala Phe Thr Asp Ser Glu
35          40          45

Arg Leu Ile Gly Asp Ala Ala Lys Asn Gln Val Ala Met Asn Pro Arg
50          55          60

Asn Thr Val Phe Asp Ala Lys Arg Leu Ile Gly Arg Lys Phe Ser Asp
65          70          75          80

Pro Val Val Gln Ser Asp Met Lys His Trp Pro Phe Lys Val Ile Thr
85          90          95

Lys Gly Asp Asp Lys Pro Val Ile Gln Val Gln Phe Arg Gly Glu Thr
100         105         110

Lys Thr Phe Asn Pro Glu Glu Val Ser Ser Met Val Leu Ser Lys Met
115         120         125

Lys Glu Ile Ala Glu Ser Tyr Leu Gly Lys Gln Val Lys Lys Ala Val
130         135         140

Val Thr Val Pro Ala Tyr Phe Asn Asp Ser Gln Arg Gln Ala Thr Lys
145         150         155         160

Asp Ala Gly Thr Ile Ala Gly Leu Glu Val Leu Arg Ile Ile Asn Glu
165         170         175

Pro Thr Ala Ala Ala Ile Ala Tyr Gly Leu Asp Lys Val Glu Asp Gly
180         185         190

Lys Glu Arg Asn Val Leu Ile Phe Asp Leu Gly Gly Thr Phe Asp
195         200         205

Val Thr Leu Leu Thr Ile Asp Gly Gly Ile Phe Glu Val Lys Ala Thr
210         215         220

Asn Gly Asp Thr His Leu Gly Gly Glu Asp Phe Asp Asn Arg Leu Val
225         230         235         240

Ala His Phe Thr Asp Glu Phe Lys Arg Lys Asn Lys Gly Lys Asp Leu
245         250         255

Ser Thr Asn Leu Arg Ala Leu Arg Arg Leu Arg Thr Ala Cys Glu Arg
260         265         270

Ala Lys Arg Thr Leu Ser Ser Ala Ala Gln Ala Thr Ile Glu Ile Asp
275         280         285

Ala Leu Phe Asp Asn Val Asp Phe Gln Ala Thr Ile Thr Arg Ala Arg
290         295         300

Phe Glu Glu Leu Cys Gly Glu Leu Phe Arg Gly Thr Leu Gln Pro Val
305         310         315         320

Glu Arg Val Leu Gln Asp Ala Lys Met Asp Lys Arg Ala Val His Asp
325         330         335

Val Val Leu Val Gly Gly Ser Thr Arg Ile Pro Lys Val Met Gln Leu
340         345         350

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Val Ser Asp Phe Phe Arg Gly Lys Glu Leu Lys Lys Ser Ile Gln Pro			
355	360	365	
Asp Glu Ala Val Ala Tyr Gly Ala Ala Val Gln Ala Phe Ile Leu Thr			
370	375	380	
Gly Gly Lys Ser Lys Gln Thr Glu Gly Leu Leu Leu Leu Asp Val Thr			
385	390	395	400
Pro Leu Thr Leu Gly Ile Glu Thr Ala Gly Gly Val Met Thr Ser Leu			
405	410	415	
Ile Lys Arg Asn Thr Thr Ile Pro Thr Lys Ser Gln Ile Phe Ser			
420	425	430	
Thr Tyr Ala Asp Asn Gln Pro Gly Val His Ile Gln Val Phe Glu Gly			
435	440	445	
Glu Arg Ala Met Thr Lys Asp Cys His Leu Leu Gly Thr Phe Glu Leu			
450	455	460	
Ser Gly Ile Pro Pro Pro Pro Arg Gly Val Pro Gln Ile Glu Val Thr			
465	470	475	480
Phe Asp Leu Asp Ala Asn Gly Ile Leu Asn Val Ser Ala Glu Glu Lys			
485	490	495	
Gly Thr Gly Lys Arg Asn Gln Ile Val Leu Thr Asn Asp Lys Gly Arg			
500	505	510	
Leu Ser Arg Ala Glu Ile Glu Arg Met Val Arg Glu Ala Ala Lys Tyr			
515	520	525	
Glu Ala Glu Asp Lys Asp Gln Val Arg Gln Ile Asp Ala Lys Asn Gly			
530	535	540	
Leu Glu Asn Tyr Ala Phe Ser Met Lys Asn Ala Val Asn Asp Pro Asn			
545	550	555	560
Val Ala Gly Lys Ile Glu Glu Ala Asp Lys Lys Thr Ile Thr Ser Ala			
565	570	575	
Val Glu Glu Ala Leu Glu Trp Leu Asn Asn Asn Gln Glu Ala Ser Lys			
580	585	590	
Glu Glu Tyr Glu His Arg Gln Lys Glu Leu Glu Asn Leu Cys Thr Pro			
595	600	605	
Ile Met Thr Asn Met Tyr Gln Gly Met Ala Gly Ala Gly Met Pro Gly			
610	615	620	
Gly Met Pro Gly Gly Met Pro Gly Gly Met Pro Gly Gly Met Pro Gly			
625	630	635	640
Gly Met Pro Gly Gly Met Pro Gly Gly Met Pro Gly Gly Met Pro Gly			
645	650	655	
Gly Met Pro Gly Gly Met Pro Gly Gly Ala Asn Pro Ser Ser Ser Ser			
660	665	670	
Gly Pro Glu Val Glu Val Asp			
675	680		

1. A nucleotide sequence coding for the PFR1 protein of *Leishmania infantum* of a fragment thereof comprising at least one immunodominant epitope selected from the epitope group consisting of: SEQ ID No: 1, SEQ ID No: 2, SEQ ID No: 3, SEQ ID No: 4, SEQ ID No: 5, SEQ ID No: 6, SEQ ID No: 7 and SEQ ID No: 8, wherein the immunodominant epitope is able to induce an antigen-specific T cell cytotoxic immune response in an animal against the kinetoplastids causing the leishmaniasis disease.
2. The nucleotide sequence according to claim 1, coding for the 1-595 amino acids of *Leishmania infantum* PFR1 protein, determined by the SEQ ID No: 12.
3. The nucleotide sequence according to claim 1, coding for the 160-595 amino acids of *Leishmania infantum* PFR1 protein, determined by the SEQ ID No: 9.
4. The nucleotide sequence according to claim 1, coding for the 160-548 amino acids of *Leishmania infantum* PFR1 protein, determined by the SEQ ID No: 10.
5. The nucleotide sequence according to claim 1, coding for the 160-385 amino acids of *Leishmania infantum* PFR1 protein, determined by the SEQ ID No: 11.
6. A chimeric molecule comprising:
 - (a) at least one nucleotide sequence selected from the group consisting of:
 - a nucleotide sequence coding for the PFR1 protein of *Leishmania infantum* of a fragment thereof comprising at least one immunodominant epitope selected from the epitope group consisting of: SEQ ID No: 1, SEQ ID No: 2, SEQ ID No: 3, SEQ ID No: 4, SEQ ID No: 5, SEQ ID No: 6, SEQ ID No: 7 and SEQ ID No: 8, wherein the immunodominant epitope is able to induce an antigen-specific T cell cytotoxic immune response against kinetoplastids causing leishmaniasis disease in an animal;
 - a nucleotide sequence coding for the 1-595 amino acids of *Leishmania infantum* PFR1 protein, determined by the SEQ ID No: 12, and comprising at least one immunodominant epitope able to induce an antigen-specific T cell cytotoxic immune response against kinetoplastids causing leishmaniasis disease in an animal;
 - a nucleotide sequence coding for the 160-595 amino acids of *Leishmania infantum* PFR1 protein, determined by the SEQ ID No: 9, and comprising at least one immunodominant epitope able to induce an antigen-specific T cell cytotoxic immune response against kinetoplastids causing leishmaniasis disease in an animal;
 - a nucleotide sequence coding for the 160-548 amino acids of *Leishmania infantum* PFR1 protein, determined by the SEQ ID No: 10, and comprising at least one immunodominant epitope able to induce an antigen-specific T cell cytotoxic immune response against kinetoplastids causing leishmaniasis disease in an animal;
 - a nucleotide sequence coding for the 160-385 amino acids of *Leishmania infantum* PFR1 protein, determined by the SEQ ID No: 11, and comprising at least one immunodominant epitope able to induce an antigen-specific T cell cytotoxic immune response against kinetoplastids causing leishmaniasis disease in an animal, wherein the at least one nucleotide is fused to
 - (b) a nucleotide sequence coding for the Hsp70 protein of *Trypanosoma cruzi*, or for a fragment thereof, wherein the chimeric molecule is capable of inducing an antigen-specific T cell cytotoxic immune response in an animal against the kinetoplastids causing the leishmaniasis disease.
7. The chimeric molecule according to claim 6, wherein the nucleotide sequence coding for the Hsp70 protein of *Trypanosoma cruzi*, or for the fragment thereof, is determined by the SEQ ID No: 13 or a fragment thereof.
8. A recombinant vector comprising:
 - (a) at least one nucleotide sequence selected from the group consisting of:
 - a nucleotide sequence coding for the PFR1 protein of *Leishmania infantum* of a fragment thereof comprising at least one immunodominant epitope selected from the epitope group consisting of: SEQ ID No: 1, SEQ ID No: 2, SEQ ID No: 3, SEQ ID No: 4, SEQ ID No: 5, SEQ ID No: 6, SEQ ID No: 7 and SEQ ID No: 8, wherein the immunodominant epitope is able to induce an antigen-specific T cell cytotoxic immune response against kinetoplastids causing leishmaniasis disease in an animal;
 - a nucleotide sequence coding for the 1-595 amino acids of *Leishmania infantum* PFR1 protein, determined by the SEQ ID No: 12, and comprising at least one immunodominant epitope able to induce an antigen-specific T cell cytotoxic immune response against kinetoplastids causing leishmaniasis disease in an animal;
 - a nucleotide sequence coding for the 160-595 amino acids of *Leishmania infantum* PFR1 protein, determined by the SEQ ID No: 9, and comprising at least one immunodominant epitope able to induce an antigen-specific T cell cytotoxic immune response against kinetoplastids causing leishmaniasis disease in an animal;
 - a nucleotide sequence coding for the 160-548 amino acids of *Leishmania infantum* PFR1 protein, determined by the SEQ ID No: 10, and comprising at least one immunodominant epitope able to induce an antigen-specific T cell cytotoxic immune response against kinetoplastids causing leishmaniasis disease in an animal; and
 - a nucleotide sequence coding for the 160-385 amino acids of *Leishmania infantum* PFR1 protein, determined by the SEQ ID No: 11, and comprising at least one immunodominant epitope able to induce an antigen-specific T cell cytotoxic immune response against kinetoplastids causing leishmaniasis disease in an animal, or
 - (b) a chimeric molecule, comprising: at least one nucleotide sequence from (a) fused to a nucleotide sequence coding for the Hsp70 protein of *Trypanosoma cruzi*, or for a fragment thereof, wherein the chimeric molecule is capable of inducing an antigen-specific T cell cytotoxic immune response in an animal against the kinetoplastids causing the leishmaniasis disease; or comprising at least one nucleotide sequence from (a) fused to a nucleotide sequence coding for the Hsp70 protein of *Trypanosoma cruzi* determined by the SEQ ID No: 13 or a fragment thereof.
9. The recombinant vector of claim 8, as part of a recombinant plasmid comprising the vector.

10. A pharmaceutical composition comprising:

(a) at least one nucleotide sequence selected from the group consisting of:

a nucleotide sequence coding for the PFR1 protein of *Leishmania infantum* of a fragment thereof comprising at least one immunodominant epitope selected from the epitope group consisting of: SEQ ID No: 1, SEQ ID No: 2, SEQ ID No: 3, SEQ ID No: 4, SEQ ID No: 5, SEQ ID No: 6, SEQ ID No: 7 and SEQ ID No: 8, wherein the immunodominant epitope is able to induce an antigen-specific T cell cytotoxic immune response against kinetoplastids causing leishmaniasis disease in an animal;

a nucleotide sequence coding for the 1-595 amino acids of *Leishmania infantum* PFR1 protein, determined by the SEQ ID No: 12, and comprising at least one immunodominant epitope able to induce an antigen-specific T cell cytotoxic immune response against kinetoplastids causing leishmaniasis disease in an animal;

a nucleotide sequence coding for the 160-595 amino acids of *Leishmania infantum* PFR1 protein, determined by the SEQ ID No: 9, and comprising at least one immunodominant epitope able to induce an antigen-specific T cell cytotoxic immune response against kinetoplastids causing leishmaniasis disease in an animal;

a nucleotide sequence coding for the 160-548 amino acids of *Leishmania infantum* PFR1 protein, determined by the SEQ ID No: 10, and comprising at least one immunodominant epitope able to induce an antigen-specific T cell cytotoxic immune response against kinetoplastids causing leishmaniasis disease in an animal; and

a nucleotide sequence coding for the 160-385 amino acids of *Leishmania infantum* PFR1 protein, determined by the SEQ ID No: 11, and comprising at least one immunodominant epitope able to induce an antigen-specific T cell cytotoxic immune response against kinetoplastids causing leishmaniasis disease in an animal, or

(b) a chimeric molecule, comprising: at least one nucleotide sequence from (a) fused to a nucleotide sequence coding for the Hsp70 protein of *Trypanosoma cruzi*, or for a fragment thereof, wherein the chimeric molecule is capable of inducing an antigen-specific T cell cytotoxic immune response in an animal against the kinetoplastids causing the leishmaniasis disease; or comprising at least one nucleotide sequence from (a) fused to a nucleotide sequence coding for the Hsp70 protein of *Trypanosoma cruzi* determined by the SEQ ID No: 13 or a fragment thereof.

11. A method for treatment and/or prevention of kinetoplastid infections that cause leishmaniasis disease in an animal, comprising use of at least one nucleotide sequence selected from the group consisting of:

a nucleotide sequence coding for the PFR1 protein of *Leishmania infantum* of a fragment thereof comprising at least one immunodominant epitope selected from the epitope group consisting of: SEQ ID No: 1, SEQ ID No: 2, SEQ ID No: 3, SEQ ID No: 4, SEQ ID No: 5, SEQ ID No: 6, SEQ ID No: 7 and SEQ ID No: 8, wherein the immunodominant epitope is able to induce an antigen-

specific T cell cytotoxic immune response against the kinetoplastids causing leishmaniasis disease in an animal;

a nucleotide sequence coding for the 1-595 amino acids of *Leishmania infantum* PFR1 protein, determined by the SEQ ID No: 12, and comprising at least one immunodominant epitope able to induce an antigen-specific T cell cytotoxic immune response against kinetoplastids causing leishmaniasis disease in an animal;

a nucleotide sequence coding for the 160-595 amino acids of *Leishmania infantum* PFR1 protein, determined by the SEQ ID No: 9, and comprising at least one immunodominant epitope able to induce an antigen-specific T cell cytotoxic immune response against kinetoplastids causing leishmaniasis disease in an animal;

a nucleotide sequence coding for the 160-548 amino acids of *Leishmania infantum* PFR1 protein, determined by the SEQ ID No: 10, and comprising at least one immunodominant epitope able to induce an antigen-specific T cell cytotoxic immune response against kinetoplastids causing leishmaniasis disease in an animal; and

a nucleotide sequence coding for the 160-385 amino acids of *Leishmania infantum* PFR1 protein, determined by the SEQ ID No: 11, and comprising at least one immunodominant epitope able to induce an antigen-specific T cell cytotoxic immune response against kinetoplastids causing leishmaniasis disease in an animal.

12. The method of claim 11, wherein the animal is a human or a dog.

13. A method for the manufacture of a therapeutic or preventive vaccine, comprising use of at least one nucleotide sequence selected from the group consisting of:

a nucleotide sequence coding for the PFR1 protein of *Leishmania infantum* of a fragment thereof comprising at least one immunodominant epitope selected from the epitope group consisting of: SEQ ID No: 1, SEQ ID No: 2, SEQ ID No: 3, SEQ ID No: 4, SEQ ID No: 5, SEQ ID No: 6, SEQ ID No: 7 and SEQ ID No: 8, wherein the immunodominant epitope is able to induce an antigen-specific T cell cytotoxic immune response against kinetoplastids causing leishmaniasis disease in an animal;

a nucleotide sequence coding for the 1-595 amino acids of *Leishmania infantum* PFR1 protein, determined by the SEQ ID No: 12, and comprising at least one immunodominant epitope able to induce an antigen-specific T cell cytotoxic immune response against kinetoplastids causing leishmaniasis disease in an animal;

a nucleotide sequence coding for the 160-595 amino acids of *Leishmania infantum* PFR1 protein, determined by the SEQ ID No: 9, and comprising at least one immunodominant epitope able to induce an antigen-specific T cell cytotoxic immune response against kinetoplastids causing leishmaniasis disease in an animal;

a nucleotide sequence coding for the 160-548 amino acids of *Leishmania infantum* PFR1 protein, determined by the SEQ ID No: 10, and comprising at least one immunodominant epitope able to induce an antigen-specific T cell cytotoxic immune response against kinetoplastids causing leishmaniasis disease in an animal; and

a nucleotide sequence coding for the 160-385 amino acids of *Leishmania infantum* PFR1 protein, determined by the SEQ ID No: 11, and comprising at least one immunodominant epitope able to induce an antigen-specific T

cell cytotoxic immune response against kinetoplastids causing leishmaniasis disease in an animal.

- 14.** The method of claim **13**, comprising the steps of:
- identifying at least one nucleotide sequence selected from the group consisting of:
 - a nucleotide sequence coding for the PFR1 protein of *Leishmania infantum* of a fragment thereof comprising at least one immunodominant epitope selected from the epitope group consisting of: SEQ ID No: 1, SEQ ID No: 2, SEQ ID No: 3, SEQ ID No: 4, SEQ ID No: 5, SEQ ID No: 6, SEQ ID No: 7 and SEQ ID No: 8, wherein the immunodominant epitope is able to induce an antigen-specific T cell cytotoxic immune response in the animal against the kinetoplastids causing the leishmaniasis disease;
 - a nucleotide sequence coding for the 1-595 amino acids of *Leishmania infantum* PFR1 protein, determined by the SEQ ID No: 12, and comprising at least one immunodominant epitope able to induce an antigen-specific T cell cytotoxic immune response against the kinetoplastids causing leishmaniasis disease in the animal;
 - a nucleotide sequence coding for the 160-595 amino acids of *Leishmania infantum* PFR1 protein, determined by the SEQ ID No: 9, and comprising at least one immunodominant epitope able to induce an antigen-specific T cell cytotoxic immune response against the kinetoplastids causing leishmaniasis disease in the animal;
 - a nucleotide sequence coding for the 160-548 amino acids of *Leishmania infantum* PFR1 protein, determined by the SEQ ID No: 10, and comprising at least one immunodominant epitope able to induce an antigen-specific T cell cytotoxic immune response against the kinetoplastids causing leishmaniasis disease in the animal; and
 - a nucleotide sequence coding for the 160-385 amino acids of *Leishmania infantum* PFR1 protein, determined by the SEQ ID No: 11, and comprising at least one immunodominant epitope able to induce an antigen-specific T cell cytotoxic immune response against the kinetoplastids causing leishmaniasis disease in the animal;
 - amplifying the at least one nucleotide sequence identified in a) by PCR using genomic DNA from the kinetoplastid that causes the leishmaniasis disease, and oligonucleotides containing restriction enzymes sites that allow the amplicon direct cloning into prokaryotic and eukaryotic expression vectors after its digestion with these restriction enzymes,
 - cloning the at least one nucleotide sequence amplified in b) for the production of the protein encoded by the said at least one nucleotide sequence,
 - purifying the at least one protein obtained in c) by affinity chromatography, and
 - producing at least one endotoxin-free DNA vector for a safe inoculation.

15. A method for measuring the degree of infection of kinetoplastids that cause leishmaniasis disease in an animal, comprising use of a marker comprising a nucleic acid sequence selected from the group consisting of a nucleotide sequence coding for the PFR1 protein of *Leishmania infantum* of a fragment thereof comprising at least one immunodominant epitope selected from the epitope group consisting

of: SEQ ID No: 1, SEQ ID No: 2, SEQ ID No: 3, SEQ ID No: 4, SEQ ID No: 5, SEQ ID No: 6, SEQ ID No: 7 and SEQ ID No: 8, wherein the immunodominant epitope is able to induce an antigen-specific T cell cytotoxic immune response against kinetoplastids causing leishmaniasis disease in an animal;

a nucleotide sequence coding for the 1-595 amino acids of *Leishmania infantum* PFR1 protein, determined by the SEQ ID No: 12, and comprising at least one immunodominant epitope able to induce an antigen-specific T cell cytotoxic immune response against kinetoplastids causing leishmaniasis disease in an animal;

a nucleotide sequence coding for the 160-595 amino acids of *Leishmania infantum* PFR1 protein, determined by the SEQ ID No: 9, and comprising at least one immunodominant epitope able to induce an antigen-specific T cell cytotoxic immune response against kinetoplastids causing leishmaniasis disease in an animal;

a nucleotide sequence coding for the 160-548 amino acids of *Leishmania infantum* PFR1 protein, determined by the SEQ ID No: 10, and comprising at least one immunodominant epitope able to induce an antigen-specific T cell cytotoxic immune response against kinetoplastids causing leishmaniasis disease in an animal; and

a nucleotide sequence coding for the 160-385 amino acids of *Leishmania infantum* PFR1 protein, determined by the SEQ ID No: 11 and comprising at least one immunodominant epitope able to induce an antigen-specific T cell cytotoxic immune response against kinetoplastids causing leishmaniasis disease in an animal.

16. The method of claim **15**, wherein the animal is a human or a dog.

17. A method for generating protective immunological memory against the infection of the kinetoplastids causing the leishmaniasis disease in an uninfected animal, comprising use of at least one nucleotide sequence selected from the group consisting of:

a nucleotide sequence coding for the PFR1 protein of *Leishmania infantum* of a fragment thereof comprising at least one immunodominant epitope selected from the epitope group consisting of: SEQ ID No: 1, SEQ ID No: 2, SEQ ID No: 3, SEQ ID No: 4, SEQ ID No: 5, SEQ ID No: 6, SEQ ID No: 7 and SEQ ID No: 8, wherein the immunodominant epitope is able to induce an antigen-specific T cell cytotoxic immune response against kinetoplastids causing leishmaniasis disease in an animal;

a nucleotide sequence coding for the 1-595 amino acids of *Leishmania infantum* PFR1 protein, determined by the SEQ ID No: 12, and comprising at least one immunodominant epitope able to induce an antigen-specific T cell cytotoxic immune response against kinetoplastids causing leishmaniasis disease in an animal;

a nucleotide sequence coding for the 160-595 amino acids of *Leishmania infantum* PFR1 protein, determined by the SEQ ID No: 9, and comprising at least one immunodominant epitope able to induce an antigen-specific T cell cytotoxic immune response against kinetoplastids causing leishmaniasis disease in an animal;

a nucleotide sequence coding for the 160-548 amino acids of *Leishmania infantum* PFR1 protein, determined by the SEQ ID No: 10, and comprising at least one immunodominant epitope able to induce an antigen-specific T cell cytotoxic immune response against kinetoplastids causing leishmaniasis disease in an animal; and

a nucleotide sequence coding for the 160-385 amino acids of *Leishmania infantum* PFR1 protein, determined by the SEQ ID No: 11, and comprising at least one immunodominant epitope able to induce an antigen-specific T cell cytotoxic immune response against kinetoplastids causing leishmaniasis disease in an animal.

18. The method of claim 17, wherein the animal is a human or a dog.

19. A method for clarifying or generating partial or total clearance of the kinetoplastids causing the leishmaniasis disease of the tissues in an infected animal, comprising use of at least one nucleotide sequence selected from the group consisting of:

a nucleotide sequence coding for the PFR1 protein of *Leishmania infantum* of a fragment thereof comprising at least one immunodominant epitope selected from the epitope group consisting of: SEQ ID No: 1, SEQ ID No: 2, SEQ ID No: 3, SEQ ID No: 4, SEQ ID No: 5, SEQ ID No: 6, SEQ ID No: 7 and SEQ ID No: 8, wherein the immunodominant epitope is able to induce an antigen-specific T cell cytotoxic immune response against kinetoplastids causing leishmaniasis disease in an animal;

a nucleotide sequence coding for the PFR1 protein of *Leishmania infantum* of a fragment thereof comprising at least one immunodominant epitope selected from the epitope group consisting of: SEQ ID No: 1, SEQ ID No: 2, SEQ ID No: 3, SEQ ID No: 4, SEQ ID No: 5, SEQ ID No: 6, SEQ ID No: 7 and SEQ ID No: 8, wherein the immunodominant epitope is able to induce an antigen-

specific T cell cytotoxic immune response against kinetoplastids causing leishmaniasis disease in an animal;

a nucleotide sequence coding for the 1-595 amino acids of *Leishmania infantum* PFR1 protein, determined by the SEQ ID No: 12, and comprising at least one immunodominant epitope able to induce an antigen-specific T cell cytotoxic immune response against kinetoplastids causing leishmaniasis disease in an animal;

a nucleotide sequence coding for the 160-595 amino acids of *Leishmania infantum* PFR1 protein, determined by the SEQ ID No: 9, and comprising at least one immunodominant epitope able to induce an antigen-specific T cell cytotoxic immune response against kinetoplastids causing leishmaniasis disease in an animal;

a nucleotide sequence coding for the 160-548 amino acids of *Leishmania infantum* PFR1 protein, determined by the SEQ ID No: 10, and comprising at least one immunodominant epitope able to induce an antigen-specific T cell cytotoxic immune response against kinetoplastids causing leishmaniasis disease in an animal; and

a nucleotide sequence coding for the 160-385 amino acids of *Leishmania infantum* PFR1 protein, determined by the SEQ ID No: 11, and comprising at least one immunodominant epitope able to induce an antigen-specific T cell cytotoxic immune response against kinetoplastids causing leishmaniasis disease in an animal.

20. The method of claim 19, wherein the animal is a human or a dog.

* * * * *

RESEARCH ARTICLE

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Immunoreactivity of synthetic peptides derived from proteins of *Cryptococcus gattii*

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ABSTRACT: Aim: To determine the immunoreactivity of synthetic *Cryptococcus*-derived peptides. Materials & methods: A total of 63 B-cell epitopes from previously identified *Cryptococcus gattii* immunoreactive proteins were synthesized and evaluated as antigens in ELISAs. The peptides were first evaluated for their ability to react against sera from immunocompetent subjects carrying cryptococcal meningitis. Peptides that yielded high sensitivity and specificity in the first test were then retested with sera from individuals with other fungal pathologies for cross-reactivity determination. Results: Six of 63 synthetic peptides were recognized by antibodies in immunoassays, with a specificity of 100%, sensitivity of 78% and low cross-reactivity. Conclusion: We successfully determined the immunoreactivity of selected synthetic peptides of *C. gattii* derived proteins.

Cryptococcosis is an important mycosis that affects humans and animals worldwide. *Cryptococcus neoformans* and *C. gattii* are the main pathogens that infect humans. *C. neoformans* mainly affects immunocompromised hosts, whereas *C. gattii* usually affects immunocompetent hosts [1,2]. Globally, almost 1 million cases of cryptococcal meningitis in HIV-infected subjects are estimated to occur every year, causing over 600,000 deaths [3]. *C. gattii* merits special attention because an outbreak has already occurred on Vancouver Island and has been shown to have spread rapidly to the Pacific coast of Canada and the USA [4–6].

Cryptococcosis is not a disease of compulsory notification in Brazil, thus surveillance for this mycosis does not exist. Its prevalence and annual incidence can only be roughly estimated through reports of cases by specialized services. Nevertheless, it is well known that the clinical cases caused by the

KEYWORDS

- antigens • cryptococcosis
- diagnosis • ELISA
- synthetic peptides

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Table 1. Synthetic peptides derived from immunoreactive proteins of *Cryptococcus gattii* and the amino acid sequences.

Peptide name	Protein	Amino acid sequence
S4	sks2	VIDKDGSPYVEDVY
S13	sks2	PVYEGERTQCKDNR
H18	Hsp70	VDVNGEIGVKVNYL
H21	Hsp70	RHFGRDFDYALVQ
H24	Hsp70	KEAGNPDEDTELVV
H26	Hsp70	EASYADPATLPKGI
H27	Hsp70	EEEVTVGEGEDAKT
H30	Hsp70	EEGEDASKSAYVQK
G36	GrpE	TALKHPQPIPAEN
E43	ENO1	SIEDPFHEDDFDAW
Hy49	CGNB 1302	PEAKSGGASANETA
Hy50	CGNB 1302	QKSANANKGEEKTE
X56	CGNB 1079	SQNQGYGQQPGGYG
X57	CGNB 1079	GGYGQQENYGGQNQ

two species of *Cryptococcus* are differently distributed throughout Brazil. *C. neoformans* predominates in all regions of the country and infects mainly immunocompromised hosts, especially AIDS patients; *C. gattii* (mainly genotype VGII) predominates in the northern and northeastern regions in immunocompetent adults, adolescents and children, with high morbidity and mortality rates, ranging from 37 to 49% [7–11].

The diagnosis of cryptococcosis is attained through microscopy, culture followed by biochemical tests and the detection of cryptococcal capsular antigen by enzyme immunoassay, latex agglutination or lateral flow assay [12–14]. The lateral flow assay is increasingly used in the cryptococcosis diagnosis, and it is widely used in sub-Saharan Africa and its performance has been widely shown in immunosuppressed patients and insufficiently reported in immunocompetent patients [15,16]. In this scenario, some gaps remain to be filled as detection of sub-clinical forms, differential diagnosis of species, among others.

Using immunoproteomics and bioinformatics approaches, Martins *et al.* identified B-cell epitopes with antigenic and/or immunogenic potential [17]. The application of synthetic peptides aiming to improve the diagnosis and development of vaccines for infectious diseases has been reported, but little has been achieved with regard to systemic mycoses [18]. This methodology has demonstrated a potential for discovering antigenic targets in leishmaniasis, Chagas disease, paracoccidioidomycosis and tuberculosis [18–23]. In this study, new antigenic targets for diagnostic tests for cryptococcosis were evaluated using synthetic peptides obtained from immunoreactive proteins of *C. gattii*.

Materials & methods

• Study population

Serum specimens from ten healthy individuals and 138 patients, comprising 70 with cryptococcosis, 17 with coccidioidomycosis, 22 with paracoccidioidomycosis, six with histoplasmosis, eight with aspergillosis and 15 with

Table 2. Diagnostic performance of synthetic peptides in serum samples from 70 cryptococcosis patients and ten healthy individuals with the respective immunoassay accuracy values.

Peptides	Sp	Se	AUC	Youden Index J	Cut-off	p-value
S4	90.00	61.43	0.780	0.5143	0.1193	0.0001
H18	90.00	60.00	0.757	0.5000	0.0583	<0.0001
H21	100.00	78.57	0.873	0.7857	0.0632	<0.0001
H26	90.00	60.00	0.750	0.5000	0.0666	0.0004
Hy49	90.00	70.00	0.786	0.6000	0.0751	<0.0001
Hy50	100.00	55.71	0.700	0.5571	0.0948	0.0007

The cut-off points were performed using Youden Index J.
AUC: Area under the curve; Se: Sensitivity; Sp: Specificity.

sporotrichosis, were included in this study, as shown in **Supplementary Table 1** (see online at www.futuremedicine.com/doi/suppl/10.2217/fmb.14.49).

• Synthetic peptides

Synthetic peptides were derived from the mapping of B-cell epitopes of immunoreactive proteins of *C. gattii* (genotype VGII) identified from an immunoproteomics approach. Only peptides that were simultaneously identified by the ABCpred and BCpreds programs were considered to be putative antigens for immunoassays for cryptococcosis [17].

A total of 63 overlapping peptides (20 mer amino acids, with an overlap of 14 amino acids) covering six immunogenic *C. gattii* proteins (Hsp70, sks2, GrpE, enolase, conserved hypothetical protein CGNB 1302 and conserved hypothetical protein CGNB 1079) were synthesized (PEPTIDE 2.0, Chantilly, VA, USA; **Supplementary Table 2**) [37]. Experiments were previously approved by the Human Ethics Committee at the State University of Piauí under protocol number 079/2008.

• ELISA

First, we optimized the ELISA reaction with optimal peptide concentrations (20 µg/ml), serum dilution (1:200) and conjugated immunoglobulins (1:10,000). Using these parameters, we had a better separation between the cryptococcosis patient's serum and uninfected individuals.

Briefly, Falcon flexible microtiter plates (Becton Dickinson, France) were coated with 100 µl/well of synthetic peptides diluted in bicarbonate buffer overnight at 4°C. The synthetic peptides were then blocked with 5% powdered skim milk in PBS-Tween20 (0.05%; PBST) for 1 h at 37°C. Serum samples diluted in PBST-milk (0.5%) were added to the wells for 1 h at 37°C. The plates were washed three-times with PBST, and diluted peroxidase-conjugated anti-human immunoglobulin G with PBST-milk (0.5%) was added. Subsequently, the micro-plates were incubated for 1 h at 37°C and washed three-times with PBST; the enzyme substrate Fast-OPD™ (Sigma-Aldrich, St Louis, MO, USA) was then added. The plates were incubated for 30 min in the dark at room temperature. The reactions were stopped with 2 M H₂SO₄, and the absorbance was measured at 492 nm using a Multiskan Plate Reader (MCC/340; Thermo Fisher Scientific, MA, USA).

Once the ELISA reaction was optimized, all peptides were screened for reactivity with separate pools of sera from ten cryptococcosis subjects with *C. gattii* infection and ten healthy individuals. The peptides that produced a high optical density (OD) difference between the cryptococcosis subjects and controls were considered potential candidate antigens for further assays using individual serum from infected (n = 70) and healthy (n = 10) subjects. As there was no OD difference ($p > 0.05$) between the serum from subjects infected by *C. gattii* and

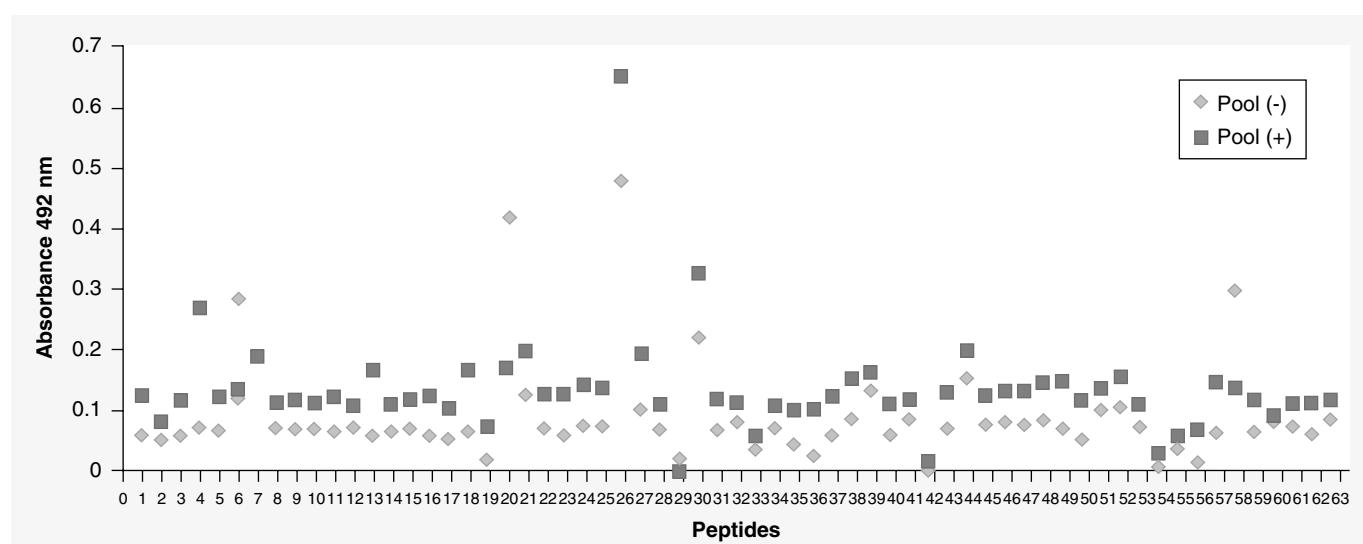


Figure 1. Immunoreactivity of 63 synthetic peptides derived from immunoreactive proteins of *Cryptococcus gattii* with a pool of sera from ten cryptococcosis patients (+) and a pool of sera from ten healthy individuals (-).

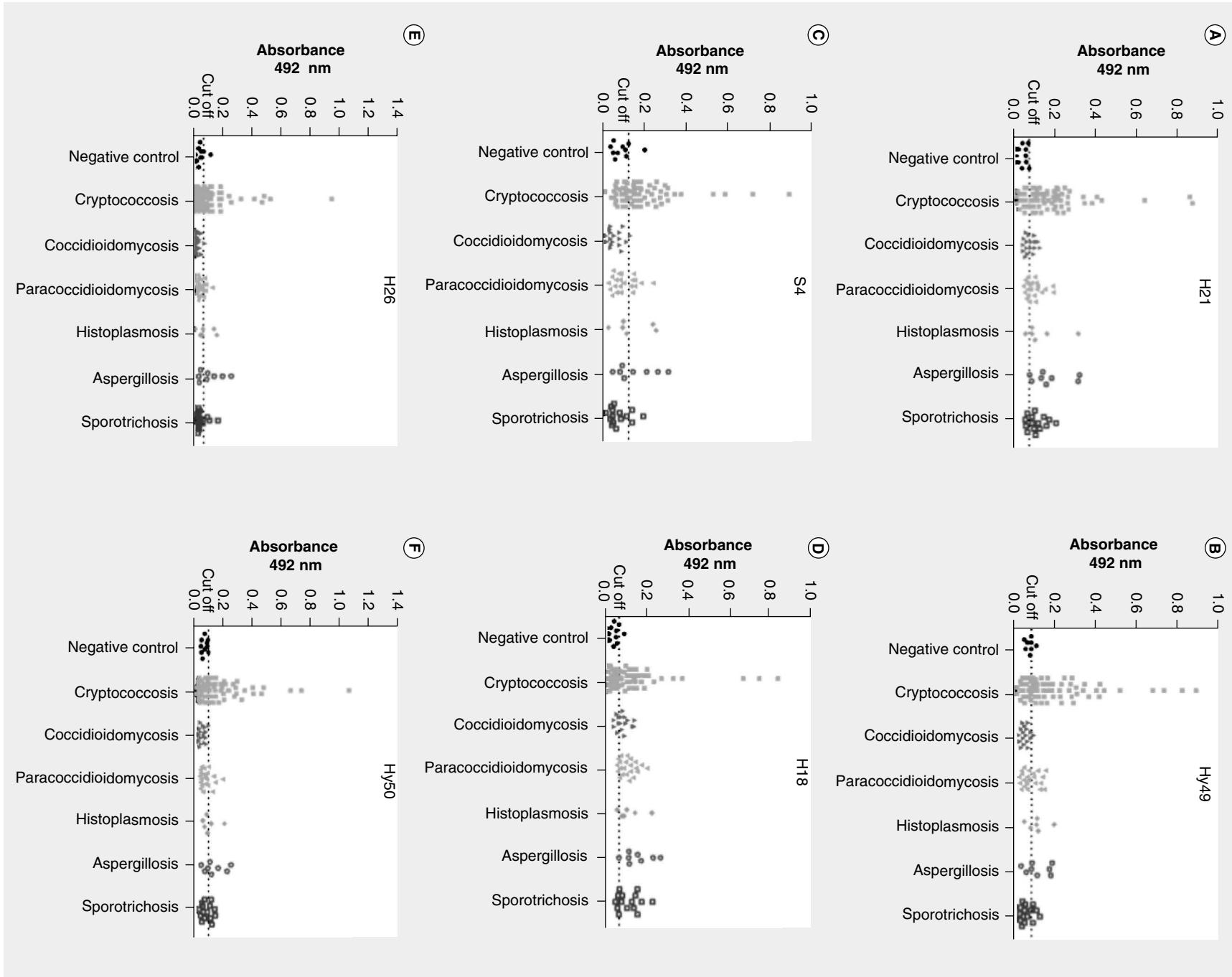


Figure 2. ELISA reactivity of the sera from subjects with cryptococcosis and other fungal infections against peptides (see facing page). (A) H21, (B) Hy49, (C) S4, (D) H18, (E) H26 and (F) Hy50. ELISA was performed with sera from ten healthy subjects and 138 patients with different fungal infections: 70 with cryptococcosis; 17 with coccidioidomycosis; 22 with paracoccidioidomycosis; six with histoplasmosis; eight with aspergillosis; and 15 with sporotrichosis. The samples were tested against six synthetic peptides derived from immunoreactive proteins of *Cryptococcus gattii*.

C. neoformans, analyses were performed by combining these sera in the cryptococcosis group. In this phase, those peptides with a sensitivity and specificity that were on average greater than or equal to 70% were evaluated for crossreactivity patterns against the subjects infected with other fungal diseases, as reported above. All samples ($n = 148$) were analyzed by the mean value of triplicate reactions.

• Statistical analysis

For each peptide, the sensitivity and specificity was determined by means of receiver operating characteristic curve analysis. The accuracy of each test was evaluated according to the area under the curve (AUC), as follows: no value ($AUC = 0.5$), low accuracy ($0.5 < AUC < 0.7$), moderate accuracy ($0.7 < AUC < 0.9$), high accuracy ($0.9 < AUC < 1.0$) and a perfect test ($AUC = 1$). Additionally, the Youden Index (J) [24] was used to choose the best cut-off point of each peptide for the healthy subjects and all fungal pathology tests. J has been defined as the accuracy of a test in clinical epidemiology and shows the point of the curve with the least chance of being random [25,26].

All statistical analyses were performed using GraphPad Prism™ (version 6.0) and MedCalc™ (version 11.5.0). A p-value of <0.05 was considered statistically significant.

Results

Figure 1 shows the OD observed between a pool of sera from cryptococcosis subjects and a pool of sera from negative controls against the 63 synthetic peptides tested in the screening. A total of 14 peptides (**Table 1**) showed an OD difference above 0.04 and were retested with individual serum. This cut-off was the smallest OD difference able to distinguish cryptococcosis patients from negative controls.

The receiver operating characteristic curve analysis showed that six of the 14 peptides (S4, H18, H21, H26, Hy49 and Hy50) had high sensitivity and specificity. All of them were considered to have moderate accuracy ($0.7 < AUC < 0.9$) (**Table 2**). The best recognized peptide was H21 (sensitivity 79% and specificity 100%),

followed by Hy49 (sensitivity 70% and specificity 90%). These results were confirmed by the Youden Index ($H21 = 0.7857$; $Hy49 = 0.60$). **Table 2** summarizes the AUC data and the Youden Index.

The analysis of cross-reactivity based on the cutoffs obtained by the Youden Index showed that the peptides tested are recognized chiefly by the antisera from cryptococcosis patients. The recognition by antibodies of sera from patients with other fungal diseases was found to be heterogeneous, with OD values much lower than those observed for cryptococcosis (p-values ranging from $p < 0.0001$ to $p = 0.0007$), with the exception of H18 (**Figure 2**).

Approaching the theoretical additive reactivities of the six peptides (S4, H18, H21, H26, HP49 and Hy50) showed that the association of five peptides (S4, H18, H21, H26 and HP49) improved the test accuracy to a high level ($AUC = 0.9143$ and $IJ = 0.8143$), as shown in **Table 3**. With this combination of peptides, the specificity is 100%, and the sensitivity increased to 81.4%.

Discussion

Efforts for a diagnostic test for cryptococcosis have advanced in the last years. The focus of these tests is the search for cryptococcal antigens. This work shows a new approach of diagnosis aiming at the detection of antibodies.

Here, we report the validation of the immunoproteomics approach as a strategy to identify new antigenic targets for the development of diagnostic tests for cryptococcosis. We studied 63 peptides derived from six proteins. Six peptides were identified as good antigenic candidates for developing new diagnostic tests for cryptococcosis. Of these, three are derived from Hsp70 (H18, H21 and H26). This chaperone possesses both regions conserved during evolution and variable regions that allow a distinction between species [27], and it may present immunogenic epitopes. In fact, recent works have characterized Hsps as key antigens inducing humoral responses to *C. neoformans* and *C. gattii* [17,28,29]. Furthermore, Rodrigues *et al.* [30] reported that Hsps are secreted by extracellular

Table 3. Theoretical additive reactivities with several combinations of peptides derived from immunoreactive proteins of *Cryptococcus gattii*.

Peptide combination	Area under the curve
H21 + Hy49 + S4 + H18 + H26 + Hy50	0.8186
H21 + Hy49 + S4 + H18 + H26	0.9143
H21 + Hy49 + S4 + H18	0.8486
H21 + Hy49 + S4	0.8543
H21 + Hy49	0.8557
H21	0.8730

vesicles and that these vesicles are involved in cryptococcal pathogenesis. Recently, Silveira *et al.* [31] showed the involvement of Hsp70 in the adhesion and phagocytosis processes of *C. neoformans*.

The remaining peptides are derived from two proteins for which information in the literature is scarce: peptides Hy49 and Hy50 (hypothetical protein CGNB 1302) and peptide S4 (protein Sks2) [17].

The cross-reactivity pattern between the sera of individuals with cryptococcosis and other fungal infections observed may be explained, at least in part, by the high homology between the sequences of the proteins studied [17]. However, the recognition of antibodies from patients carrying other mycoses by the peptides analyzed resulted in low ODs when compared with the ODs using samples from cryptococcosis patients (*p* value ranged from *p* < 0.0001 to *p* = 0.0007), except for H18. Houghton *et al.* [32] and Khan *et al.* [33] showed that further optimization by constructing chimerical multiepitope peptides or peptides attached to multiplexed microbeads may help in the accuracy of immunoassays. Our theoretical additive reactivities were similar, corroborating studies in other pathologies [19,21,22,34].

The peptides studied are promising, as they showed a satisfactory specificity, being a good starting point for further studies in clinical practice, as it can exclude other morbidities with similar clinical manifestations. In addition, the search for an alternative to diagnose cryptococcosis in advance, together with the lack of studies using synthetic peptides in developing serological tests in mycoses, makes the validation of synthetic peptides for developing a diagnostic test of cryptococcosis of utmost importance. This work makes us pioneers in this field and may be an alternative in the weaponry of diagnostic tests for analyzing serum reactivity for cryptococcosis, independent of the antigenic

charge of the fungus, currently used in tests like the lateral flow assay. Furthermore, after the identification of promising peptides for the use in immunoassay, opens up the prospect of its use in associations between the peptides, including peptides and proteins and even the development of new molecules as chimeric protein.

Studies with synthetic peptides to improve performance in the diagnosis of infectious diseases have shown promising results. Caldini *et al.* [18] showed a sensitivity of 100% and a specificity of 94.59% for the diagnosis of paracoccidioidomycosis using synthetic peptides. Other studies also showed good sensitivity (>88%) and specificity (>95%) in the diagnosis of infectious diseases [19–21,35].

In this work, we were able to successfully select some synthetic peptides that could contribute in an ELISA-based diagnostic test for cryptococcosis. In addition to the advantage of synthetic peptides being less expensive and fast to produce [19,36], our findings reveal peptides as promising candidate antigens for a diagnostic test for cryptococcosis.

Conclusion

In conclusion, using immunoproteomics and bioinformatics techniques, we were able to identify new antigenic promising targets for developing diagnostic tests for cryptococcosis. In this study, six synthetic peptides proved to be promising antigens in the immunodiagnosis of cryptococcosis. In addition, theoretical additive reactivities showed that the combination of peptides is an improved accuracy strategy for developing new antigenic targets.

Future perspective

Cryptococcosis is an important systemic mycosis that affects immunocompetent and immunosuppressed individuals. Cryptococcosis due to *C. gattii* has received much attention in

recent years due to outbreaks and the spread to countries not previously plagued by this pathogen. The early diagnosis of this infection is a challenge that science and health systems need to address because, in most cases, it is diagnosed late, resulting in significant mortality. Thus, efforts must be made in the search for a rapid, sensitive and specific diagnostic test. Our results have highlighted several peptides that can be used as antigens in immunoassays for the cryptococcosis diagnosis. The extension of this work in the field must be performed to confirm whether these peptides have an acceptable accuracy for the diagnosis of pulmonary cryptococcosis and/or the identification of subclinical infections in an endemic area for cryptococcosis.

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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

EXECUTIVE SUMMARY

Objectives of the study

- The objective was to identify new antigens for cryptococcosis immunodiagnostic tests.
- The study aimed to evaluate synthetic peptide performance against sera from subjects with cryptococcosis and other fungal infections.

Methods

- Synthesis of B-cell epitopes from *Cryptococcus gattii* immunoreactive proteins.
- Synthetic peptides were tested using ELISA to detect circulating antibodies in the serum of subjects with cryptococcosis.

Conclusion

- Synthetic peptides were found to be promising as new antigenic targets in cryptococcosis diagnosis.
- Six synthetic peptides had good performance with a high specificity and sensitivity in ELISA.

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Immunoproteomics and immunoinformatics analysis of *Cryptococcus gattii*: novel candidate antigens for diagnosis

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Aim: To identify immunoreactive proteins of *Cryptococcus gattii* genotype VGII and their B-cell epitopes. **Materials & methods:** We combined 2D gel electrophoresis, immunoblotting and mass spectrometry to identify immunoreactive proteins from four strains of *C. gattii* genotype VGII (CG01, CG02, CG03 and R265). Next, we screened the identified proteins to map B-cell epitopes. **Results:** Sixty-eight immunoreactive proteins were identified. The strains and the number of proteins we found were: CG01 (12), CG02 (12), CG03 (18) and R265 (26). In addition, we mapped 374 peptides potentially targeted by B cells. **Conclusion:** Both immunoreactive proteins and B-cell epitopes of *C. gattii* genotype VGII that were potentially targeted by a host humoral response were identified. Considering the evolutionary relevance of the identified proteins, we may speculate that they could be used as the initial targets for recombinant protein and peptide synthesis aimed at the development of immunodiagnostic tools for cryptococcosis.

Cryptococcosis has become a significant public global health problem. The CDC estimate the occurrence of over 1 million new cases per year of cryptococcosis worldwide in patients with AIDS and, despite the recent improvements in the diagnosis and treatment of cryptococcosis, cryptococcal meningitis is responsible for over 600,000 deaths per year worldwide. This potentially fatal fungal disease is caused by one of two species of the same genus: *Cryptococcus neoformans* (genotypes VNI–VNIV) or *Cryptococcus gattii* (genotypes VGI–VGIV). The first species is best known as the cause of severe meningoencephalitis or meningitis in immunocompromised patients and has been considered to be the most medically important species for many years [1–5]. It is important for clinical microbiology laboratories to accurately differentiate one species from the other. Rapid identification of *C. neoformans* and *C. gattii* is imperative for favoring the prompt treatment of cryptococcosis and for understanding the epidemiology of the disease [6–11]. *C. gattii* merits more attention so that its

environmental occurrence and role in cryptococcosis can be accurately determined, as this information will be helpful in devising strategies to manage potential outbreaks of cryptococcosis. Today, the differentiation between *C. neoformans* and *C. gattii* is a difficult task since: the available commercial differentiation methods (e.g., API® 20C AUX [bioMérieux, France], Vitek® [bioMérieux] and MicroScan® [Siemens, Germany]) do not differentiate between *C. neoformans* and *C. gattii*; and the methods capable of differentiating the two species (multiplex PCR, liquid array detection of pathogens, PCR restriction fragment length polymorphism [12,13] and matrix-assisted laser desorption/ionization mass spectrometry [MS] [14–16]) are not routinely used.

Recently, however, increased medical attention has been paid to *C. gattii*, which mainly affects immunocompetent individuals [1,2,17]. The interest in clinical studies of this species has arisen due to its rapid spread in Asia, Africa, Australia, Europe and South America, along with outbreaks in Vancouver Island, BC, Canada, and in parts

Keywords

- B cell ■ cryptococcosis
- *Cryptococcus gattii*
- diagnosis ■ epitopes
- immunoinformatics
- immunoproteomics

of continental North America. The potential for *C. gattii* to cause illness in immunocompetent patients and its rapid spread worldwide justify the implementation of a public health effort to increase the awareness of both the public and healthcare professionals [1,18–24]. The mechanism of *C. gattii* dispersion is not well understood. However, it is thought that the emergence of industrialization, as well as the subsequent development of high population densities, concurrent with processes such as the export of trees and wood products, transport of bacteria via air currents, water currents and biotic agents (e.g., birds, animals and insects) and global warming, may be the main catalysts for the spread of this pathogen [1,2]. As cryptococcosis does not require compulsory notification in Brazil, and there is currently no surveillance of this mycosis, its prevalence and annual incidence can only be approximately estimated based on publications of cases occurring in specialized services. Nevertheless, it is well known that the clinical cases attributed to these two species of the *Cryptococcus* genus are distributed differently throughout Brazil, with *C. neoformans* being predominant in the south and southeast and *C. gattii* (mainly genotype VGII) being predominant in the north and northeast regions. In these latter two regions, *C. gattii* affects immunocompetent adults, adolescents and children, and is associated with high morbidity and mortality rates (ranging from 37 to 49%). Information regarding the conditions that support the development of cryptococcosis in immunocompetent individuals remains elusive. However, it is possible that environmental factors play an important role in this process, considering that people living in endemic areas are at high risk for developing the disease [21,25–30]. Preventative measures that effectively combat infection by *C. gattii* are not available at present. Hence, the best approach for controlling cryptococcosis is through early diagnosis and treatment. Although recent advances directed at early diagnosis of cryptococcosis have been published [31–33], a diagnostic method with high specificity and sensitivity to guide the management and control of cryptococcosis remains to be developed.

Information from the recently published *C. gattii* genome, combined with data obtained via proteomic approaches, has created opportunities for the development of diagnostic tools and therapeutic targets in the context of cryptococcosis caused by *C. gattii* [18]. The development of such tools has been improved by the production of antigens, which increases the range of alternative tests for immunoassay-based

pathogen detection. In this study, we identify both immunoreactive proteins of *C. gattii* and predicted B-cell epitopes for their potential use as antigens in new serologic tests.

Materials & methods

Study patients & sera

The study included three immunocompetent subjects with cryptococcal meningitis recruited at the Natan Portella Institute of Tropical Diseases, a reference center for infectious diseases in Teresina, the capital of the northeastern state of Piauí, Brazil. As negative controls, we used sample sera from uninfected subjects ($n = 3$) who did not present clinical indicators or positive serological tests for cryptococcosis. The sera obtained from these patients were stored in a freezer at -20°C until use. The demographic, epidemiological, laboratory and baseline clinical characteristics of each patient were obtained from medical records and transferred to an epidemiological sheet. Detailed information regarding the patients involved in this study was published by Martins *et al.* [26]. Sera from three immunocompetent patients with cryptococcal meningitis caused by *C. gattii* genotype VGII were selected to produce a pool of serum containing each antigen at a titer of 1:1024 as determined by the latex agglutination test for diagnosing cryptococcosis. This pooled serum was used for all western blot experiments.

C. gattii strains

C. gattii proteins were screened in four different strains. The first strain, R265 (ATCC number: MYA 4093), was previously identified by Kidd *et al.* [20] and was kindly provided by MH Vainstein from the culture collection of the Laboratory of Fungi of Medical and Biotechnological Importance from the Biotechnology Center, Federal University of Rio Grande do Sul (UFRGS), Brazil. The other three *C. gattii* strains (CG01, CG02 and CG03) molecular type/genotype VGII involved in this study were isolated from immunocompetent patients who had cryptococcal meningitis (two children and one adult, from the state of Piauí, northeastern Brazil). These isolates were submitted to strain identification [26], which was performed via PCR restriction fragment length polymorphism analysis using *URA5* as a target gene, as described by Meyer *et al.* [34].

Culture conditions

Strains were recovered from 15% skimmed milk stocks, stored at -20°C prior to use. The strains

were maintained on yeast extract–peptone–dextrose (YPD) media (1% yeast extract, 2% peptone, 2% dextrose and 2% BactoTM Agar, Becton Dickinson, NJ, USA). Each isolate was inoculated and grown in 200 ml of YPD broth at 37°C for 24 h with shaking at 200 rpm. To obtain a better protein yield, the cultures were subsequently quantified in a Neubauer chamber, reseeded to an equivalent of 4×10^7 cells/ml in 200 ml YPD final broth and incubated under the same conditions as described above. After 24 h, the cells were collected via centrifugation at 7500 g for 10 min at 10°C and washed three times in cold, sterile Milli-Q[®] water (Millipore Corp., MA, USA). The cell pellet obtained was stored at -80°C.

Preparation of protein extracts

Prior to the extraction process, the samples were lyophilized (FreeZone[®] lyophilizer, Labconco, MI, USA) and macerated in liquid nitrogen until a fine powder was obtained. The samples were suspended in a lysis buffer with protease inhibitors and detergents (50 mM Tris-HCl pH 7.5, 1 mM EDTA, 1 mM phenylmethanesulfonyl fluoride, 50 μM N-p-tosyl-L-phenylalanine chloromethyl ketone, 5 mM iodoacetamide, 4% w/v 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate (CHAPS) and 0.25% v/v TritonTM X-100). The proteins were solubilized by vortexing the suspensions for 5 min at intervals of 1 min on ice, followed by centrifugation at 10,000 g for 20 min at 8°C. The supernatants were collected and preserved at -20°C. The remaining cell debris was suspended in the same buffer, followed by vortexing for 5 min with intervals on ice every 1 min. The supernatant was collected after centrifugation, pooled with the first supernatant and stored at -80°C. The protein content was determined by the Bradford method [35] using known concentrations of bovine serum albumin as the standard.

2D gel electrophoresis

Samples containing 150 μg of protein were precipitated using a 2D gel electrophoresis (2DE) clean-up kit (GE Healthcare, UK) following the manufacturer's instructions, then solubilized in 150 μl of isoelectric focusing (IEF) buffer containing 9 M urea, 4% (w/v) CHAPS, 1% (w/v) dithiothreitol (DTT) and 0.2% (v/v) ampholytes pH 4–7 (Bio-Rad, CA, USA) with 0.002% orange G. Next, 7-cm immobilized pH gradient (IPG) strips (linear gradient, pH 4–7, Bio-Rad) were passively rehydrated for 16–18 h. IEF was performed using the PROTEAN[®] IEF Cell

System (Bio-Rad) at 20°C with the following conditions: 250 V for 15 min, 250–4000 V for 2 h and 4000 V until reaching 10,000 V/h, with a maximum current of 50 μA/strip. Focused IPG strips were equilibrated for 15 min in equilibration buffer I, containing 30% (v/v) glycerol, 6 M urea, 1% DTT, 2% (w/v) sodium dodecyl sulfate (SDS), 0.375 M Tris pH 8.8 and 0.002% Bromophenol Blue, and then alkylated for 15 min in equilibration buffer II (equilibration buffer I in which the DTT was replaced with 4% iodoacetamide). SDS-PAGE was performed using the method developed by Laemmli [36]. The IPG strips were then placed on a 12% SDS-PAGE gel, and the second dimension of separation was performed in two steps at 10°C: 50 V/gel for 30 min and 100 V/gel until the tracking dye reached the bottom of the gels in a Mini-PROTEAN Tetra Cell Chamber (Bio-Rad), according to the manufacturer's instructions. The gels were stained with Coomassie Brilliant Blue and scanned with a GS-800TM scanner (Bio-Rad). Three technical replicates of classical 2DE western blots for each of the four independent biological samples were performed for each strain. A Coomassie Brilliant Blue G250 gel and the other duplicate gel were transferred onto a polyvinylidene difluoride membrane for subsequent western blot analysis [37].

Western blot: 2DE analysis

The gels were transferred to polyvinylidene difluoride membranes (Hybond ECLTM, GE Healthcare) at 400 mA for 1 h in a transfer buffer (25 mM Tris, 192 mM glycine, 2% w/v SDS and 20% v/v methanol). The membranes were blocked at 4°C overnight with 5% (w/v) nonfat dry milk in phosphate-buffered saline pH 7.4 with Tween[®]-20 (PBS-T; 10 mM Na₂HPO₄, 1.7 mM KH₂PO₄, 137 mM NaCl, 2.7 mM KCl, and 0.1% v/v Tween-20, Sigma Aldrich, MO, USA) and washed three times for 10 min. The membranes were then incubated with pools of sera from three patients with cryptococcal meningitis, diluted 1:7500 in blocking buffer for 2 h at 24°C. This primary antibody dilution was previously determined based on performing western blotting using a 1D gel with serum dilutions ranging from 1:2000 to 1:10,000. After washing three times with PBS-T, the blots were incubated with antihuman IgG (GE Healthcare) diluted 1:2000 in blocking buffer for 1 h at 24°C. The membranes were washed three times with PBS-T buffer for 15 min and twice with phosphate-buffered saline for 10 min. Finally, the 2DE blots were processed with the ECL detection

reagent (GE Healthcare) according to the manufacturer's instructions. Images were prepared using the VersaDoc™ 4000 MP imaging system (Bio-Rad). The western blots were evaluated in technical triplicates. The immunoreactivity of each spot, representing a positive signal in the western blot analysis, was identified by matching with the position of the corresponding spot on the gel stained with Coomassie Brilliant Blue. To select spots, the images from membranes and gels containing protein extracts were analyzed using an ImageMaster 2D Platinum 6.0® (GE Healthcare). In order to identify any nonspecific reactions, we discarded all spots that were also reactive with the pool of sera from negative controls. Only the infected reactive spots sera were manually excised, destained, digested with trypsin and subjected to MS to identify the immunoreactive proteins.

Protein identification by liquid chromatography-electrospray ionization-quadrupole-time of flight tandem MS

Gel plugs were treated through three washing steps with 100 µl of 50% acetonitrile (ACN) and 50 mM ammonium bicarbonate (NH_4HCO_3) for 15 min, followed by one washing step with 100 µl of ACN. After washing, the gel plugs were dried via vacuum centrifugation (CentriVap® Benchtop Centrifugal Vacuum concentrator, Labconco) and subjected to trypsin digestion for 18–24 h at 37°C using 20 µl of 10 µg/ml trypsin (Trypsin Gold, MS grade, Promega, WI, USA) diluted in 25 mM NH_4HCO_3 . Peptide extraction was performed twice for 15 min with 100 µl of a 100% ACN and 5% formic acid solution. Trypsin digests were then concentrated in a SpeedVac® concentrator to approximately 10 µl and resuspended in 10 µl of 0.1% trifluoroacetic acid.

The resulting peptides were analyzed by liquid chromatography-electrospray ionization-quadrupole-time of flight tandem mass spectrometry (MS/MS) using a Waters nanoACQUITY UPLC™ system coupled to a Q-TOF Ultima™ API mass spectrometer (Waters MS Technologies, UK) at the Unit of Protein Chemistry and Mass Spectrometry (Uniprote-MS, Biotechnology Center, UFRGS, Brazil). The peptides were eluted from the reverse-phase column into the mass spectrometer at a flow rate of 600 nL/min with a 10–50% water/ACN 0.1% formic acid linear gradient over 30 min. The MS survey scan was set to 1 s (0.1 s interscan delay) and recorded at 200–2000 Da. MS/MS scans were

performed from 50 to 2000 m/z, and the scan and interscan rates were set as for MS. For each survey scan, the three most intense multiple-charged ions over a threshold of eight counts were selected for the MS/MS analysis. The collision energies for peptide fragmentation were set using the charge state recognition files for +2 and +3 peptide ions provided by MassLynx™ (Waters). The raw MS/MS data were processed using Mascot Distiller 2.2.1 software (Matrix Science, MA, USA) to form peak lists that were exported in the Mascot generic format.

Database searches

The peak lists for each protein spot were analyzed with the aid of the 'MS/MS Ion Search' engine of Mascot (version 2.1) software [101]. The Mascot search parameters were as follows: oxidation of methionine, modification of cysteine by carbamidomethylation, partial cleavage leaving one internal cleavage site, a peptide tolerance of 0.2 Da and a MS/MS tolerance of 0.1 Da. The significance threshold was set at $p < 0.05$, and identification required that each protein contained at least one peptide with an expected p-value < 0.05 . Thus, we compared the calculated molecular mass and isoelectric point values from the identified proteins with the observed values on the 2DE gel. Additionally, if a protein with a nonsignificant score was part of a horizontal series of spots with the adjacent identical proteins, we included this identification. All amino acid sequences were downloaded as FASTA-format files from the Broad Institute protein database [102]. The Blast2GO tool [103] was used to obtain functional categories of proteins. This tool assigns gene ontology (GO) terms based on the Basic Local Alignment Search Tool (BLAST) definitions. This assignment was accomplished by submitting the FASTA sequences of the identified proteins to the Blast2GO platform and comparing them against the National Center for Biotechnology Information (NCBI) databases [104]. Briefly, Blast2GO used BlastP with the default parameters to identify similar proteins with GO annotations.

Mapping B-cell epitopes

To map linear B-cell epitopes in the immunoreactive proteins selected by western blotting, we used two different programs: ABCPred [105], which is based on machine-learning methods that apply a recurrent neural network [38], and BCPred [106], which is also based on machine-learning methods, but involves methods that apply a support vector machine [39]. Only those

peptides that were simultaneously identified by the two programs were considered to be putative antigens for the development of immunoassays for cryptococcosis. This approach was based on the work of Faria *et al.*, which showed that the use of the default scores of prediction software programs associated with the overlap predictions of more than one software program can be preferable to the use of a single type of prediction [40].

Results

2DE proteome profiling of *C. gattii* strains

Proteins from *C. gattii* strain R265 and our isolates (CG01, CG02 and CG03) were separated using 2DE and analyzed for reactivity to the serum by immunoblotting. In fact, the capsule was a major obstacle to obtaining protein. The key points to improve protein recovery were background culture in liquid medium and lyophilization with maceration in liquid nitrogen. After these steps, proteins were obtained by solubilization in a lysis buffer containing 4% detergent (CHAPS). Protein samples obtained in this manner are mainly cytoplasmatic proteins and a trace quantity of membrane proteins associated to carbohydrates.

A representative image of the protein patterns is shown in FIGURE 1. Image analysis demonstrated approximately 350 spots for each strain of *C. gattii* (FIGURE 1), all of which optimally resolved at pH 4–7, and their molecular weights ranged from 12 to 225 kDa.

Only 68 of these spots showed reactivity based on immunoblotting against a pool of sera from cryptococcosis patients. One hypothesis to explain the low number of proteins identified by immunoblotting is that most of the proteins were cytoplasmic, together with the fact that many surface antigens of this fungus are located in capsules and should be represented mainly by carbohydrates.

The distribution of the spots for each strain is as follows: 12 spots from CG01; 12 from CG02; 18 from CG03; and 26 from R265. All of the immunoreactive spots selected for identification with MS were classified according to their biological activity (TABLE 1 & SUPPLEMENTARY TABLE 1) (see online at: www.futuremedicine.com/doi/suppl/10.2217/FMB.13.22). The data generated by the Blast2GO analysis are presented at GO multilevels to illustrate the general functional categories according to biological processes and molecular functions (SUPPLEMENTARY FIGURES 1 & 2) [103]. Of the 68 spots corresponding to 48 proteins, 34 (70.8%) were selected from only one

strain, and 14 (29.2%) were simultaneously derived from at least two strains; among the latter 14 proteins, six were simultaneously selected from three strains (FIGURE 2).

Prediction of B-cell linear epitopes

We sought to determine whether the 48 reactive proteins identified in this study shared epitopes that were potentially recognized by patient antibodies. Therefore, we screened these proteins for predictive B-cell epitopes using two different bioinformatics tools. The ABCPred program yielded 4747 peptides, whereas BCPreds yielded 475. By comparing the B-cell epitopes generated by these two different approaches, we were able to identify 374 concordant peptides (the peptide sizes ranged from nine to 14 amino acids) (SUPPLEMENTARY TABLE 2). We believe that the B-cell prediction was efficient because the majority of the proteins (41/48; 85.4%) presented in a high percentage (greater than 25%) of the epitopes (TABLE 2). This percentage of epitopes takes into account the total number of predicted peptides and the total number of amino acids in the mapped protein. Two hypothetical proteins (CNBG_1302 and CNBG_1079) contained the highest number of predicted peptides. Proteins and peptides with a greater potential of being B-cell epitopes deserve further investigation as candidate antigens for use in diagnostic testing.

Discussion

In this immunoproteomic study, we identified immunoreactive proteins in *C. gattii*, as well as the putative B-cell epitopes of these predicted proteins using immunoinformatics tools. The proteins recognized *in vitro* by antibodies in this work probably have the same antigenic determinants that induce antibodies *in vivo*. This approach has frequently been used to identify immunogenic proteins and, consequently, new targets to diagnose several diseases [41–54]. Although similar results have been achieved in cryptococcosis using murine [55] and koala models [56], this is the first report in human beings. In addition, the use of sample isolates obtained directly from patients would be very difficult, because they are hard to obtain directly from the brain or lung during infection, and the sample collected would probably not have a sufficient amount of protein for a proteomic approach.

We identified 68 immunoreactive proteins and highlight that only six of them were reactive in three isolates simultaneously without a reaction for negative sera. Due to their

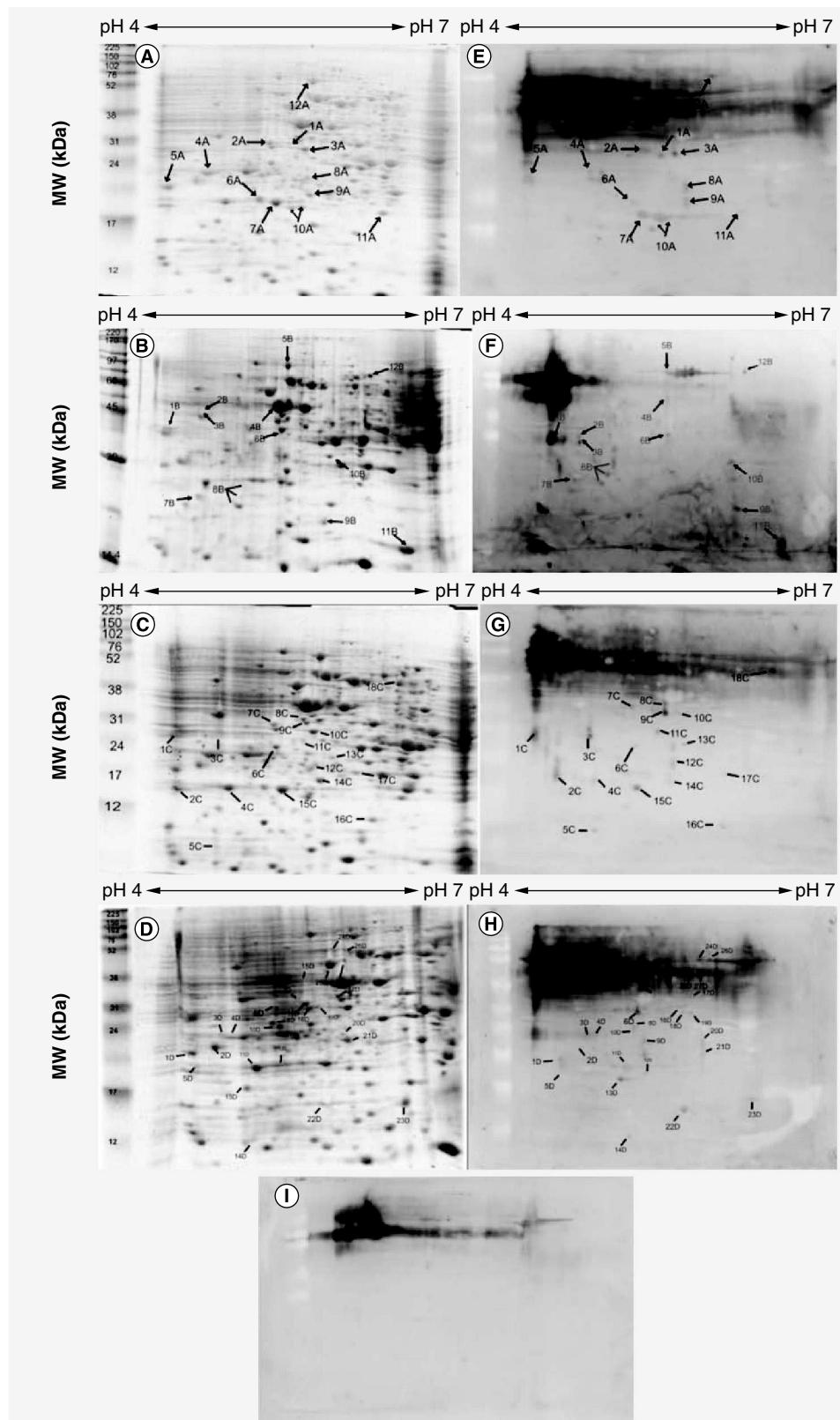


Figure 1. Western immunoblot analysis of representative serum samples. *Cryptococcus gatti* proteins in 2D gel electrophoresis: (A) CGO1, (B) CGO2, (C) CGO3 and (D) R265. Representative results of immunoblot from the pool of patient sera with cryptococcosis: (E) CGO1, (F) CGO2, (G) CGO3 and (H) R265 strains. (I) Result of immunoblot from the sera of uninfected person with cryptococcosis. The numbers refer to the spot identification used in TABLE 1.
MW: Molecular weight.

Table 1. Immunodominant proteins identified by serologic proteome analysis.

Spot/strain	Protein	Accession code	Mascot ID	Biological process	Molecular function
11A/CG01	Microtubule motor	E6R0Y5	CNBG_0115	Microtubule-based movement	ATP binding; microtubule motor activity
25D/R265	Heat shock protein sks2	E6R0K8	CNBG_0239	Response to stress	ATP binding
21D/R265	HHE domain-containing protein	E6R0I8	CNBG_0257	Unknown	Unknown
11C/CG03	Cytoplasm protein	E6R0G3	CNBG_0282	Unknown	Binding
5A/CG01	Conserved hypothetical protein	E6R0C0	CNBG_0290	Unknown	Unknown
7C/CG03	Conserved hypothetical protein	E6R068	CNBG_0372	Unknown	RNA binding
3D/R265	Ubiquitin carboxyl-terminal hydrolase	E6R058	CNBG_0379	Ubiquitin-dependent protein catabolic process	Ubiquitin thiolesterase activity
17D/R265	Disulfide-isomerase	E6QZV4	CNBG_0482	Cell redox homeostasis	Electron carrier activity; isomerase activity; protein disulfide oxidoreductase activity
9B/CG02	Conserved hypothetical protein	E6R3U6	CNBG_0624	Unknown	Unknown
12B/CG02	Aconitase	E6R432	CNBG_0705	Tricarboxylic acid cycle	Four-iron, four-sulfur cluster binding; aconitate hydratase activity
22D/R265	Cytoplasm protein	E6R4T7	CNBG_0959	Unknown	Unknown
8D/R265	Conserved hypothetical protein	E6R568	CNBG_1079	Unknown	Unknown
6B/CG02	Succinyl-CoA ligase β-chain	E6QYK0	CNBG_1185	Tricarboxylic acid cycle	ATP binding; succinate-CoA ligase (ADP-forming) activity
9C/CG03	Succinyl-CoA ligase β-chain	E6QYK0	CNBG_1185	Reductive tricarboxylic acid cycle	ATP binding
18D/R265	Succinyl-CoA ligase β-chain	E6QYK0	CNBG_1185	Tricarboxylic acid cycle	ATP binding
12C/CG03	Conserved hypothetical protein	E6QY79	CNBG_1302	Unknown	Unknown
3B/CG02	Endopeptidase	E6QY25	CNBG_1355	Proteolysis	Aspartic-type endopeptidase activity
8C/CG03	3-isopropylmalate dehydrogenase	E6QXQ4	CNBG_1460	Oxidation reduction	NAD or NADH binding
16D/R265	3-isopropylmalate dehydrogenase	E6QXQ4	CNBG_1460	Oxidation reduction	3-isopropylmalate dehydrogenase activity; NAD binding
4B/CG02	ATP synthase β-subunit	E6R8N5	CNBG_1632	ATP hydrolysis-coupled proton transport	ATP binding; hydrogen ion-transporting ATP synthase activity
15D/R265	ATP synthase β-subunit	E6R8N5	CNBG_1632	ATP catabolic process	Proton-transporting ATPase activity, rotational mechanism
6D/R265	Ketol-acid reductoisomerase	E6R847	CNBG_1816	Oxidation reduction	Coenzyme binding; isomerase activity; ketol-acid reductoisomerase activity
1C/CG03	Glyceraldehyde-3-phosphate dehydrogenase	E6R7Z5	CNBG_1866	Glycolysis	NAD binding; glyceraldehyde-3-phosphate dehydrogenase (NAD ⁺) phosphorylating activity
1B/CG02	Mannitol-1-phosphate dehydrogenase	E6RA07	CNBG_2079	Oxidation reduction	Oxidoreductase activity; zinc ion binding

The data are sorted by Mascot ID in crescent order.

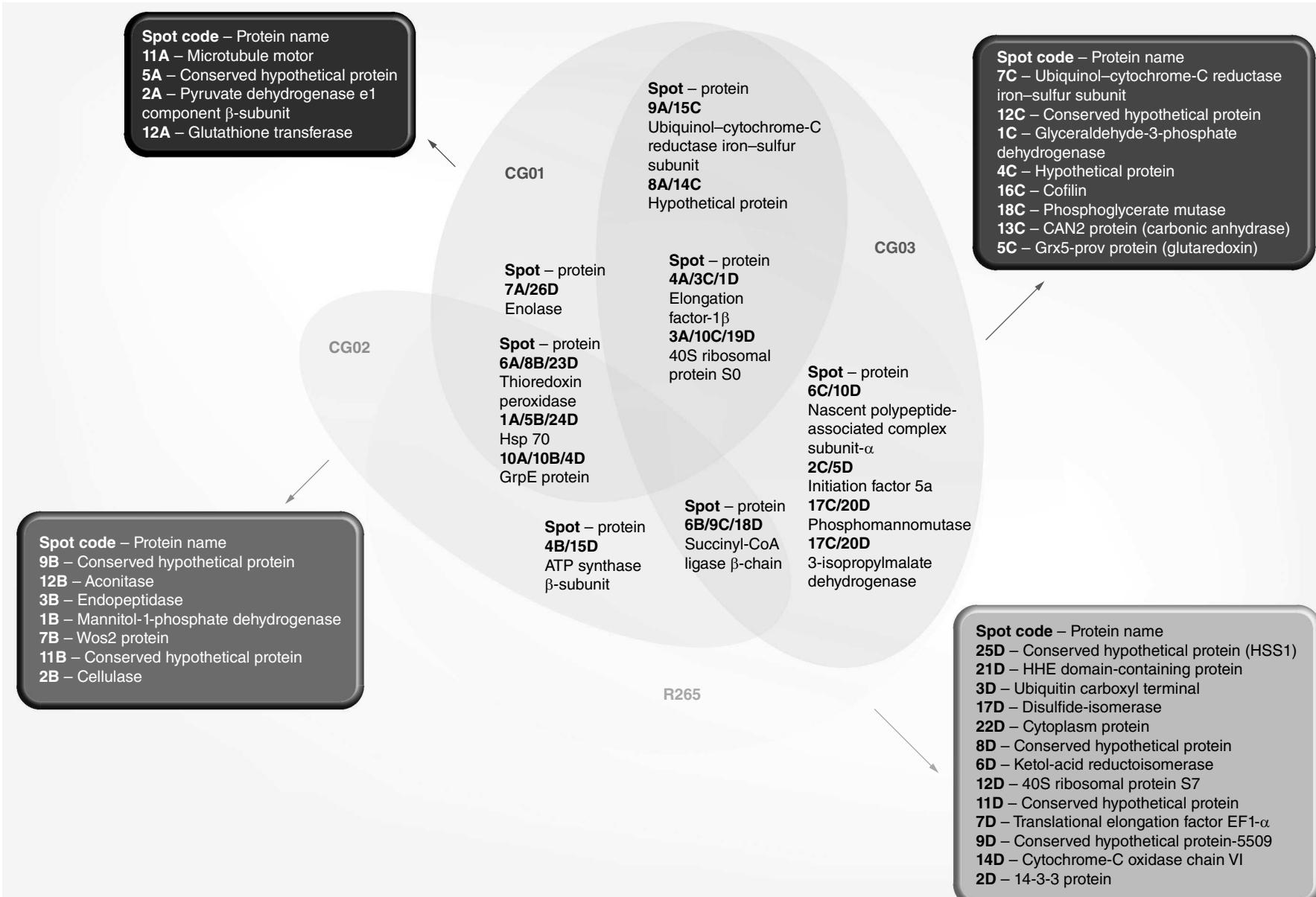


Figure 2. Venn diagram showing immunodominant proteins identified by serologic proteome from four *Cryptococcus gattii* strains. Strains, spots and protein names are according to TABLE 1.

Table 2. Proteins and B-cell epitopes mapped by ABCPred and BCPreds algorithms identified by immunoblot sera from patients with cryptococcosis.

Protein ID (aa [†])	Accession code	Name of protein	WB strain [‡]	Epitopes (n)	Epitopes (%) [§]
CNBG_1302 [§] (222)	E6QY79	Conserved hypothetical protein	CG03	9	47
CNBG_1079 [§] (302)	E6R568	Conserved hypothetical protein	R265	11	42
CNBG_3163 (225)	E6RDR5	Wos2 protein	CG02	8	41
CNBG_0115 (595)	E6R0Y5	Microtubule motor	CG01	21	40
CNBG_2923 (292)	E6RBG9	40S ribosomal protein S0	CG01, CG03, R265	9	35
CNBG_0705 (780)	E6R432	Aconitase	CG02	23	34
CNBG_0282 (334)	E6R0G3	Cytoplasm protein	CG03	10	34
CNBG_4548 (239)	E6R5R6	CAN2 protein	CG03	7	34
CNBG_4834 (459)	E6RF86	Translation elongation factor EF1- α	R265	13	33
CNBG_4027 (281)	E6R6Y7	Ubiquinol-cytochrome-C reductase iron-sulfur subunit	CG01, CG03	8	33
CNBG_3631 (138)	E6R286	Cofilin	CG03	4	33
CNBG_5365 (431)	E6R762	Cellulase	CG02	12	32
CNBG_5509 (213)	E6QZ83	Conserved hypothetical protein	R265	6	32
CNBG_3378 (216)	E6R3K5	Elongation factor 1 β	CG01, CG03, R265	5	32
CNBG_1866 (336)	E6R7Z5	Glyceraldehyde-3-phosphate dehydrogenase	CG03	9	31
CNBG_4560 (225)	E6R5Q4	Conserved hypothetical protein	R265	6	31
CNBG_6164 (187)	E6RED0	Conserved hypothetical protein	CG01, CG03	5	31
CNBG_3060 (228)	E6RDF4	GrpE protein	CG01, CG02, R265	6	30
CNBG_4692 (191)	E6R5B7	Nascent polypeptide-associated complex subunit- α	CG03, R265	5	30
CNBG_0959 (152)	E6R4T7	Cytoplasm protein	R265	4	30
CNBG_1632 (547)	E6R8N5	ATP synthase β -subunit	CG02, R265	14	29
CNBG_3753 (531)	E6RDZ5	Phosphoglycerate mutase	CG03	14	29

[†]Position of the first amino acid of the epitope identified.[‡]According to TABLE 1.[§]Highest number of predicted peptides.

WB: Western blot.

Table 2. Proteins and B-cell epitopes mapped by ABCPred and BCpreds algorithms identified by immunoblot sera from patients with cryptococcosis (cont.).

Protein ID (aa [†])	Accession code	Name of protein	WB strain [‡]	Epitopes (n)	Epitopes (%) [§]
CNBG_1355 (432)	E6QY25	Endopeptidase	CG02	11	29
CNBG_2617 (199)	E6RAJ9	40S ribosomal protein S7	R265	5	29
CNBG_5941 (158)	E6RBX1	Initiation factor 5a	CG03, R265	4	29
CNBG_0239 (614)	E6R0K8	Heat shock protein sks2	R265	15	28
CNBG_4851 (167)	E6RFA3	Cytoplasm protein	R265	4	28
CNBG_1185 (418)	E6QYK0	Succinyl-CoA ligase β-chain	CG02, CG03, R265	10	27
CNBG_2079 (420)	E6RA07	Mannitol-1-phosphate dehydrogenase	CG02	10	27
CNBG_0290 (251)	E6R0C0	Conserved hypothetical protein	CG01	6	27
CNBG_5765 (256)	E6RCQ9	14-3-3 protein	R265	6	27
CNBG_0379 (168)	E6R058	Ubiquitin carboxyl-terminal hydrolase	R265	4	27
CNBG_4912 (756)	E6RFH1	Heat shock protein (Hsp70)	CG01, CG02, R265	17	26
CNBG_2318 (175)	E6R9A9	Hypothetical protein	CG03	4	26
CNBG_4789 (135)	E6RF38	Conserved hypothetical protein	CG02	3	26
CNBG_0482 (408)	E6QZV4	Disulfide-isomerase	R265	9	25
CNBG_4625 (407)	E6R5I5	Pyruvate dehydrogenase e1 component β-subunit	CG01	9	25
CNBG_1460 (373)	E6QXQ4	3-isopropylmalate dehydrogenase	CG03, R265	8	25
CNBG_2499 (270)	E6RA79	Phosphomannomutase	CG03, R265	6	25
CNBG_6043 (233)	E6RCV4	Glutathione transferase	CG01	5	25
CNBG_0372 (291)	E6R068	Conserved hypothetical protein	CG03	6	24
CNBG_1816 (401)	E6R847	Ketol-acid reductoisomerase	R265	8	23
CNBG_5485 (152)	E6QZ59	Grx5-prov protein	CG03	3	23
CNBG_0257 (221)	E6R0I8	HHE domain-containing protein	R265	4	21
CNBG_0624 (125)	E6R3U6	Conserved hypothetical protein	CG02	2	18

[†]Position of the first amino acid of the epitope identified.[‡]According to TABLE 1.[§]Highest number of predicted peptides.

WB: Western blot.

Table 2. Proteins and B-cell epitopes mapped by ABCPred and BCPred algorithms identified by immunoblot sera from patients with cryptococcosis (cont.).

Protein ID (aa [†])	Accession code	Name of protein	WB strain [‡]	Epitopes (n)	Epitopes (%) [§]
CNBG_2132 (197)	E6R9V1	Thioredoxin peroxidase tpx1	CG01, CG02, R265	3	17
CNBG_3703 (433)	Q5KLA7	Phosphopyruvate hydratase (enolase)	CG01, R265	6	16

[†]Position of the first amino acid of the epitope identified.[‡]According to TABLE 1.[§]Highest number of predicted peptides.

WB: Western blot.

antigenicity in different strains, we believe that these proteins would be most promising for testing as antigens. However, after the analysis of the overlapping protein sequences in *C. gattii* (data not shown), we observed a high similarity (above 94%) between proteins from *C. gattii* genotypes VGII and VGII, except for the enolase protein (51%). Taken together, assessing the specificity of these antigens will be the next step to validate them for the diagnosis of cryptococcosis.

In addition, the fact that only six immunoreactive proteins were common to the three strains of *C. gattii* genotype VGII tested in our study is not surprising. For example, we recently demonstrated the significant genetic diversity of the *C. gattii* genotype VGII in Brazil using the multi-locus sequence typing technique [57]. Of the six immunoreactive proteins identified here (TABLE 1), three are constitutive and involved in the cell cycle, cell division or the tricarboxylic acid cycle and were therefore excluded from this discussion.

Two of the three antigenic targets identified here, Hsp70 and GrpE, are members of the 70-kDa heat shock protein family. Hsp70 was identified as an antigenic target in sera from cryptococcosis patients infected with the strains CG01, CG02 and R265. This finding corroborates previous studies that identified proteins of the Hsp family as potential biomarkers for cryptococcosis [58–60]. Hsp70 is a phylogenetically conserved chaperone protein whose expression increases in response to temperature variation and environmental stress [61]. The role of this protein as an antigenic target in the context of cryptococcosis has been reported by various studies in both humans (by Rodrigues *et al.* [62]) and animal models in the clinical and sub-clinical phases (by Jobbins *et al.* [56] and Young *et al.* [55]), thus supporting our findings.

To our knowledge, this is the first report of the involvement of the second type of protein in the chaperone family with a size of 70 kDa (GrpE) as an antigenic target in the context of cryptococcosis. These new data have the potential to be used

in the development of immunoassays because the same serum pool of neurocryptococcosis patients showed reactivity to GrpE in the three distinct strains of *C. gattii* genotype VGII.

The third protein identified in our study as an interesting antigenic target for the development of an immunoassay is the thiol peroxidase tpx1. We identified this protein in the aforementioned CG01, CG02 and R265 *C. gattii* genotype VGII strains. It is interesting to note that thiol peroxidase, a protein that acts to remove peroxides, also acts in response to oxidative stress, similar to Hsp70. Such proteins have previously been identified as being essential for intracellular survival, virulence and resistance to oxidative and nitrosative stress in *C. neoformans*, as reported by Missall *et al.* [63–65] and Wang *et al.* [66]. The finding presented here that thiol peroxidase is an antigenic target in *C. gattii* genotype VGII corroborates the findings of Jobbins *et al.* [56] and supports the hypothesis that this protein is involved in the pathogenesis of *C. gattii* genotype VGII infection in humans [62,67–69].

Other important proteins identified in the current study include the following: enolase and ATP synthase (found in strains CG01 and R265); phosphomannomutase (strains CG03 and R265); aconitase and Wos2 (strain CG02); and cofilin and CAN2 (strain CG03).

Enolase and ATP synthase appear to be important in the process of host invasion by the fungus. During the invasion of the brain and the CNS, penetration of the blood–brain barrier is a prerequisite for the establishment of meningoencephalitis by the opportunistic fungal pathogen *C. neoformans*. The fungal cells require a high level of energy to traverse this barrier, and enolase, which is an enzyme in the glycolysis pathway, could be essential for this purpose during the infection process [58]. In addition, the relative importance of plasminogen in infectious diseases is indicated by the surface-associated plasminogen-binding properties manifested by diverse species of human pathogens. Several

proteins, including enolase, have been found to play a major role in the microbial recruitment of plasminogen [68].

Phosphomannomutase can be considered to be an indirect virulence factor for *Cryptococcus* spp. This statement is justified by the fact that this enzyme is responsible for the synthesis of mannose, which is a carbohydrate comprising up to two-thirds of the main cryptococcal virulence factor, the polysaccharide capsule. The capsular polysaccharide of *C. neoformans* helps to protect yeast against the host immune system due to its function as either an antiphagocytic factor and/or an antigenic polysaccharide that is extruded into tissue and fluids and produces an immunosuppressive effect. The capsular polysaccharide, GXM, comprises three major sugars: mannose, xylose and glucuronic acid. The identification of this protein as an antigenic target is not surprising because it is involved in the production of mannoprotein–mannose complexes that elicit a cytokine-mediated inflammatory immune response. Important factors for proliferation in host tissue include enzymes involved in central carbon metabolism (gluconeogenesis and acetylCoA synthesis), regulators of capsule and melanin synthesis and proteins involved in resistance to phagocytosis [17,70–72].

The identification of the proteins aconitase (an enzyme involved in the metabolism of carbohydrates) and Wos2 (a cochaperone of Hsp90) corroborates the results of a study by Crestani *et al.* [73], which showed that aconitase and Wos2 expression levels increase in *C. gattii* (strain R265) in response to a lack of iron in the culture medium. It is important to note that the common factors between our study and that of Crestani *et al.* [73] are the stress conditions to which *C. gattii* was exposed and that Wos2 has been suggested to be an early marker of *C. gattii* infection in koalas [56]. Similar to enolase, the protein cofilin identified herein may play a relevant role in the process of the fungus spreading to the CNS. The rationale for this hypothesis is that cofilin interferes with the polymerization of F-actin and G-actin strands, thereby modulating the cytoskeleton of the target cell. This idea is in accordance with the results reported by Wang *et al.* [66], who demonstrated differential expression of cofilin in human umbilical vascular endothelial cells infected by *C. neoformans*.

The identification of CAN2 as an antigenic target in the context of cryptococcosis may be explained, at least in part, by this protein experiencing an increase in pressure in response to

the change in the environmental CO₂ tension (0.033%) relative to that found in the host (5%). This protein works as a sensor for this CO₂ tension change and alerts the cell to the need for capsule thickening. This thickening is, in turn, an important virulence mechanism in cryptococcosis [74,75].

Importantly, the applied B-cell epitope prediction was efficient because the majority of the proteins (85.4%) presented a high percentage of epitopes. The high percentage of epitopes in nearly all of the identified proteins can be explained by the selection based on western blot analysis. It is important to highlight the fact that two proteins that were previously defined as hypothetical proteins showed a higher percentage (>42%) of epitopes. These hypothetical proteins exhibit a conserved homology in other species of *Cryptococcus*. The identified epitopes can be produced as synthetic peptides and could be tested for use in the diagnosis of cryptococcosis.

Conclusion

In conclusion, the applied combination of immunoproteomics and immunoinformatics methods was demonstrated to be a specific and powerful tool for identifying novel antigens from mycological pathogens. The major finding of this work was the identification of *C. gattii* proteins recognized as molecular targets by antibodies produced by patients with cryptococcal meningitis. In addition, we identified potential antigens that may be used for the development of more accurate serological diagnoses in human cryptococcal meningitis.

Future perspective

Cryptococcosis caused by *C. gattii* genotype VGII has resulted in significant repercussions over the years owing to the occurrence of outbreaks in Canada, mainly affecting immunocompetent individuals, causing high morbidity and mortality. Therefore, significant efforts have been devoted to the search for diagnostic tests that are sensitive, specific and can detect infection as early as possible. The immunogenic proteins and B-cell epitopes identified herein need to be investigated as antigens in serological tests to diagnose cryptococcal infection. These proteins could present cross-reaction with cryptococcal infection caused by other species. Although these proteins can react with multiple fungal species, some of these fungi may not be present in the patient. However, this is the first important step to selecting new target antigens to facilitate the immunodiagnosis of cryptococcosis.

Executive summary

Objectives of the study

- The objective was to identify immunoreactive proteins using sera from patients with cryptococcosis.
- The study aimed to map B-cell epitopes from immunoreactive proteins from *Cryptococcus gattii* genotype VGII.

Methods

- 2D gel electrophoresis integrated with immunoblotting and mass spectrometry was used to identify immunoreactive proteins.
- ABCPred and BCpreds programs were utilized to predict epitopes for B cells.

Conclusion

- A total of 68 immunoreactive proteins were identified.
- The ABCPred and BCpreds programs generated 374 concordant peptides.
- The Hsp70, thioredoxin peroxidase and GrpE proteins were immunoreactive in at least three strains of *C. gattii* genotype VGII.

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Ethical conduct of research

The study protocol was approved by the State University of Piauí-Brazil Institutional Review Board (CEP 079/2008) and by the Brazilian National Ethics Committee (CONEP). All patients enrolled in the study signed forms providing free and informed consent.

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Immunoproteomics and immunoinformatics analysis of *Cryptococcus gattii*: novel candidate antigens for diagnosis

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Abstract

Aim: To identify immunoreactive proteins of *Cryptococcus gattii* genotype VGII and their B-cell epitopes.

Materials & methods: We combined 2D gel electrophoresis, immunoblotting and mass spectrometry to identify immunoreactive proteins from four strains of *C. gattii* genotype VGII (CG01, CG02, CG03 and R265). Next, we screened the identified proteins to map B-cell epitopes.

Results: Sixty-eight immunoreactive proteins were identified. The strains and the number of proteins we found were: CG01 (12), CG02 (12), CG03 (18) and R265 (26). In addition, we mapped 374 peptides potentially targeted by B cells.

Conclusion: Both immunoreactive proteins and B-cell epitopes of *C. gattii* genotype VGII that were potentially targeted by a host humoral response were identified. Considering the evolutionary relevance of the identified proteins, we may speculate that they could be used as the initial targets for recombinant protein and peptide synthesis aimed at the development of immunodiagnostic tools for cryptococcosis.

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