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(54) **LIVE ATTENUATED BORDETELLA STRAINS AS A SINGLE DOSE VACCINE AGAINST WHOOPING COUGH**

LEBENDE ABGESCHWÄCHTE BORDETELLA-STÄMME ALS EINZELDOSISIMPFSTOFF GEGEN KEUCHHUSTEN

SOUCHES VIVANTES ATTENUEES DE BORDETELLA SOUS FORME DE VACCIN UNIDOSE CONTRE LA COQUELUCHE

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Description

Field of the Invention

[0001] The application describes a mutated *Bordetella* strain comprising at least a mutated *ptx* gene, a deleted or mutated *dnt* gene and a heterologous ampG gene. The invention relates to a mutated *Bordetella* strain, more particularly to an attenuated *Bordetella* strain, comprising at least a mutated *ptx* gene, a deleted or mutated *dnt* gene and a heterologous ampG gene as defined in the claims. The attenuated mutated *Bordetella* strain can be used in an immunogenic composition or a vaccine for the treatment or prevention of a *Bordetella* infection. Use of the attenuated *Bordetella* strain for the manufacture of a vaccine or immunogenic compositions, as well as methods for protecting mammals against infection by *Bordetella* are described in the application. The invention relates to immunogenic compositions and vaccines as well as to uses of a strain of the invention in the manufacture of vaccines.

Background of the Invention and Related Prior Art

[0002] *Pertussis* is still among the principal causes of death world-wide, and its incidence is increasing even in countries with high vaccine coverage. Although all age groups are susceptible, it is most severe in infants too young to be protected by currently available vaccines.

[0003] Whooping cough or *pertussis* is a severe childhood disease responsible for high mortality rates before the introduction of effective vaccines in the second half of the 20th century. The success of these vaccines has led to the opinion that the disease is essentially under control, although world-wide 200,000 to 400,000 *pertussis*-linked deaths are still recorded annually, and the disease still ranks sixth among the causes of mortality due to infectious agents [1]. Although mostly prevalent in developing countries, the disease is also re-emerging in the developed world [2, 3], including the U.S.A., where the incidence has increased five-fold over the last twenty years [4]. Unexpectedly, the epidemiology of *pertussis* has changed in countries with high vaccine coverage, where cases of adolescent and adult *pertussis* are increasingly frequent [5]. This is probably due to progressive waning of vaccine-mediated immunity during adolescence. Often atypical and therefore difficult to diagnose, *pertussis* is generally not life-threatening in adults and in many cases remains unnoticed. However, infected adults constitute an important reservoir for transmission of the disease to very young children, too young to be fully vaccinated, and therefore at risk to develop severe disease associated with high mortality rates.

[0004] *Pertussis* vaccination usually begins at two months of age, and full protection requires at least three immunizations at one- to two-month intervals. Therefore, infants are not fully protected before the age of 6 months using the currently available vaccines. To reduce the incidence of *pertussis* in the very young and most vulnerable age groups, early immunization, possibly at birth, would thus be highly desirable. However, numerous studies in humans and in animal models have suggested that the neonatal immune system is too immature to effectively induce vaccine-mediated protective immunity [6, 7]. Especially the IFN-γ production, indicative of a Th1 response that is essential to the development of protective immunity to *pertussis* [8], appears to be significantly reduced in human newborns, compared to older children or adults [9]. This is also reflected by the fact that significant amounts of antigen-specific IFN-γ are only produced after several months ($\geq$ 6 months) in children vaccinated with *pertussis* vaccines, especially with acellular vaccines (aPV) [10].

[0005] Natural infection with *Bordetella pertussis* has long been considered to induce strong and long-lasting immunity, that wanes much later than vaccine-induced immunity [5, 11]. Furthermore, infection with *B. pertussis* induces measurable antigen-specific Th1 type immune responses even in very young children (as young as one month of age) [12]. These observations suggest that live vaccines applicable by the nasal route in order to mimic as closely as possible natural infection, may be attractive alternatives over the currently available vaccines.

[0006] There are many vaccinating compositions to treat *Bordetella* infections known in the art. However, these immunogenic compositions are not used to treat newborn children or in cases where an epidemic and rapid protective immunity is required.

[0007] Thus, French Patent FR 0206666 and PCT application WO 03/102170 discloses live *Bordetella* strains that have been rendered deficient in at least two toxins chosen from PTX, DNT, AC and TCT. This patent discloses the over expression of an endogenous ampG gene by the addition of a strong promoter, and the addition of 11 terminal amino acids of the ampG gene from *E. coli.*

[0008] Mielcarek et al, Vaccine (2006; 24S2: S2/54-S2-55) disclose a strain of *Bordetella pertussis* attenuated of PTX$^-$, DTN$^-$ and TCT$^-$ for use in the immunization of mice. This reference discloses that to reduce the production of tracheal cytotoxin, the ampG gene should be overexpressed. However, upon further evaluation, the authors realized that by overexpressing the *amp*G gene, there is an increase in tracheal cytotoxin and not a decrease as was originally thought.

[0009] Mielcarek et al in Advance Drug Delivery Review 51 (2001) pgs. 55-69 disclose that live vaccines can induce systemic and mucosal responses when administered by the oral or nasal route.

[0010] Roduit et al in Infection and Immunity (2002 Jul; 70(7): 3521-8) describe vaccinating neonatals and infants with mutated *Bordetella* strains with a DTP composition.

[0011] Mattoo et al, in Frontiers of Bioscience 6, e168-e186 (2001), suggest replacing the endogenous ampG gene in *Bordetella* with the *E. coli ampG* gene, which resulted in a decrease in the amount of TCT produced.

[0012] Mattoo and Cherry 2005 (Clinical Microbiology Reviews 18(2): 326-382) relates to the molecular pathogenesis, epidemiology and clinical manifestations of respiratory infections due to *Bordetella pertussis* and other *Bordetella* subspecies.

[0013] Thus, the prior art although disclosing various types of vaccinating compositions fails to address the problem of providing a vaccine or immunogenic composition that can provide protection to a newborn prior to six months. Furthermore, the prior art fails to disclose an immunogenic or a vaccine that provides rapid protective immunity against a *Bordetella* infection. The prior art also fails to disclose an immunogenic composition or vaccine that provides a rapid protective immunity against a *Bordetella* infection, said protective immunity increasing over at least the next two months following vaccination.

[0014] Therefore, it is an object of the invention to overcome the deficiencies in the prior art.

[0015] It is another object of the invention to produce a live attenuated vaccine candidate or immunogenic composition through genetic attenuation of a *Bordetella* strain such as *B. pertussis* or *B. parapertussis* to diminish pathogenicity, while maintaining the ability to colonize and induce protective immunity.

[0016] It is another object of the invention to produce a vaccine or immunogenic composition that induces protection in newborns after a single intranasal administration that is superior to the protection provided by the current aPV.

[0017] It is yet another object of the invention to provide protection against infection with *Bordetella parapertussis,* as well as *Bordetella pertussis* which was not seen after vaccination with aPV.

[0018] Another object of the invention is to induce strong protective immunity in newboms against *Bordetella* infection.

[0019] Yet another object of the invention is to provide a vaccine or immunogenic composition that induces mucosal and systemic immunity.

[0020] It is another object of the invention to produce a live attenuated *Bordetella pertussis* strain to be given as a single-dose nasal vaccine in early life, called BPZE1.

[0021] It is yet another object of the invention to provide a vaccine that can not only be used to vaccinate newborns, but can be used in all mammals of any age in the case of an epidemic of whooping cough.

[0022] Another object of the invention is to provide a vaccine against *Bordetella* infection that induces a rapid protective immunity and/or a protective immunity that increases over at least the next two months after the vaccination.

[0023] Yet another object of the invention is to provide prevention or treatment against *Bordetella* infection that is relatively low in production costs.

[0024] These and other objects are achieved by the invention as evidenced by the summary of the invention, description of the preferred embodiments and the claims.

Summary of the Invention

[0025] The claims define the matter for which protection is sought, any statement going beyond of that which is defined in the claims is provided for information only.

[0026] The invention provides a mutated *Bordetella* strain comprising at least a mutated *pertussis* toxin (*ptx*) gene, a deleted or mutated dermonecrotic toxin (*dnt*) gene, and a heterologous *ampG* gene as defined in the claims.

[0027] In another aspect the invention relates to an immunogenic composition comprising a mutated *Bordetella* strain comprising at least a mutated *pertussis* toxin (*ptx*) gene, a deleted or mutated *pertussis* dermonecrotic toxin (*dnt*) gene, and a heterologous *ampG* gene as defined in the claims.

[0028] In yet another aspect the invention provides a vaccine comprising the attenuated *Bordetella* strain comprising at least a mutated *pertussis* toxin (*ptx*) gene, a deleted or mutated *pertussis* dermonecrotic toxin (*dnt*) gene, and a heterologous *ampG* gene as defined in the claims.

[0029] It still another aspect, the invention provides the use of an attenuated *Bordetella* strain comprising at least a mutated *ptx* gene, a deleted or mutated *dnt* gene, and a heterologous *ampG* gene as defined in the claims for the manufacture of a vaccine for the prevention of a *Bordetella* infection.

[0030] In yet another aspect, the invention provides the use of an attenuated *Bordetella* strain comprising at least a mutated *ptx* gene, a deleted or mutated *dnt* gene, and a heterologous *ampG* gene as defined in the claims for the manufacture of a vaccine for the induction of an immune response directed preferentially toward the Th1 pathway against said attenuated *Bordetella.*

[0031] Also provided by the application is a method of protecting a mammal against disease caused by infection by *Bordetella pertussis* and *Bordetella parapertussis* comprising administering to said mammal in need of such treatment a mutated *Bordetella* strain comprising at least a mutated *ptx* gene, a deleted or mutated *dnt* gene, and a heterologous *ampG* gene.

[0032] A method of providing a rapid protective immunity against a *Bordetella* infection comprising administering to said mammal in need of such treatment a mutated *Bordetella* strain comprising at least a mutated *ptx* gene, a deleted or mutated *dnt* gene, and a heterologous *ampG* gene is also part of the application.

[0033] A method of providing a rapid protective immunity against a *Bordetella* infection comprising administering to a mammal in need of such treatment a mutated *Bordetella* strain comprising at least a mutated *ptx* gene, a deleted or mutated *dnt* gene, and a heterologous *ampG* gene or a vaccine comprising said mutated *Bordetella* strain, wherein said method provides further an increase in said protective immunity over at least two months after vaccination is still another aspect of the application.

[0034] Use of the mutated *Bordetella* strain comprising at least a mutated *ptx* gene, a deleted or mutated *dnt* gene and a heterologous *ampG* gene as defined in the claims for the preparation of a multivalent vaccine (*i.e.*, a vaccine for preventing or treating infections caused by different pathogens) to treat respiratory diseases is yet another aspect of the invention.

[0035] Use of an attenuated *Bordetella* strain of the invention, by administration to mammals in need of a rapid protective immunity against a *Bordetella* infection, wherein said protective immunity increases over at least two months after administration, is also part of the application.

[0036] A method to provide a mucosal response and a systemic response to treat or protect against *Bordetella* infections in mammals is still another aspect of the application.

[0037] The invention relates to a mutated *Bordetella* strain as defined in the claims, as well as to an immunogenic composition comprising a mutated *Bordetella* strain of the invention, to a vaccine comprising an attenuated *Bordetella* strain of the invention, to a mutated *Bordetella* strain of the invention for use as a vector for expressing at least one heterologous antigen, to the use of an attenuated *Bordetella* strain of the invention for the manufacture of a vaccine for the prevention of a *Bordetella* infection, to the use of an attenuated *Bordetella* strain of the invention for the manufacture of a vaccine for the simultaneous prevention against a *B. pertussis* and *B. parapertussis* infection, and to the use of a mutated *Bordetella* strain of the invention for the preparation of a multivalent vaccine to treat respiratory diseases.

Brief Description of the Drawings

[0038]

Fig. 1 is a bar graph illustrating the TCT present in culture supernatants of BPSM and BPZE1 expressed as means of nM/OD_{540nm} (± standard error) of 3 separate cultures for each strain.

Fig. 2 is an immunoblot analysis of PTX production in the culture supernatants of BPSM (lane 1) and BPZE1 (lane 2). The sizes of the Mr markers are expressed in kDa and given in the left margin.

Fig. 3 is a Southern-blot analysis of the *dnt* locus in BPSM (lane 1) and BPZE1 (lane 2). The lengths of the size markers are indicated in base pairs (bp) are shown in the left margin.

Fig. 4 is a graph illustrating the growth rates of BPSM (black line) and BPZE1 (dotted line) in liquid culture.

Fig. 5 are electron micrographs representative of BPSM (left) and BPZE1 (right) grown in liquid medium for 24 h.

Fig. 6 is a graph illustrating the i*n vitro* adherence of BPSM (black columns) and BPZE1 (white columns) to human pulmonary epithelial A549 cells (left) and murine macrophage-like J774 cells (right). The results are expressed as means of percentages of binding bacteria relative to the bacteria present in the inoculum from three different experiments.

Fig. 7 is a graph illustrating lung colonization by BPSM (black lines) and BPZE1 (dotted lines) of adult mice infected intranasally with 10^6 CFU of BPZE1 or BPSM. The results are expressed as mean (± standard error) CFUs from three to four mice per group and are representative of two separate experiments.*, P = 0.004.

Fig. 8 are photographs of a histological analysis of lungs from BPZE1 (upper panel) or BPSM-infected (middle panel) adult mice compared to controls given PBS (lower panel). One week after infection, the lungs were aseptically removed and fixed in formaldehyde. Sections were stained with hematoxylin and eosin and examined by light microscopy.

Fig. 9 are graphs illustrating the protection against *B. pertussis* in (a) adult and (b) infant mice or *B. parapertussis* in infant mice (d). Mice immunized with BPZE1, aPV or PBS (naive) were challenged with BPSM (a and b) or B. *parapertussis* (d), and lung CFU counts were determined 3 h (white bars) or 7 days (black bars) later. Results are expressed as mean (± standard error) CFUs from 3-4 mice per group and are representative of two separate experiments. (b,*, *P* = 0.009; d,*, P = 0.007) (c) CFU counts 3 h after BPSM challenge in adult mice vaccinated with BPZE1 or aPV, compared to controls. Results obtained from 3 separate experiments are expressed as percentages of CFUs of each mouse relative of the average of CFUs in non-immunized group from the same experiment.

Fig. 10 are bar graphs illustrating the immune responses induced by BPZE1 or aPV immunization. (a) Anti-FHA IgG(H+L) titers and (b) IgG1/IgG2a ratios before (white bars) or 1 week after BPSM challenge (black bars) in BPZE1 or aPV immunized mice, compared to controls. (c) IFN-γ to IL-5 ratios produced by FHA-, PTX- or ConA-stimulated splenocytes from mice vaccinated 2 months before with BPZE1 (black bars) or aPV (white bars), compared to controls (gray bars). Antibodies and cytokines were measured in individual mice, and the results are expressed as mean values (± standard error) for 4 mice per group tested in triplicate.

Fig. 11 is the amino acid sequence of *pertussis* toxin (SEQ ID NO:1) (islet-activating protein S1). The first 34 amino acids are the signal sequence, while amino acids 35 to 269 are the mature chain.

Fig. 12 is the amino acid sequence of dermonecrotic toxin (SEQ ID NO:2).

Fig. 13 is the amino acid sequence of AmpG from *Bordetella pertussis* (SEQ ID NO:3).

Fig. 14 is the amino acid sequence of AmpG from *Escherichia coli* (SEQ ID NO:4).

Description of the Preferred Embodiments of the Invention

[0039] As used herein, the abbreviation "PTX" refers to *pertussis* toxin, which synthesizes and secretes an ADP-ribosylating toxin. PTX is composed of six polypeptides S1 to S5, the enzymatically active moiety is called S1. PTX has a 34 amino acid signal sequence, while the mature chain consists of amino acids 35 to 269. PTX is the major virulence factor expressed by *B. pertussis.* The A moiety of these toxins exhibit ADP-ribosyltransferase activity and the B portion mediates binding of the toxin to host cell receptors and the translocation of A to its site of action (57).

[0040] As used herein the abbreviation "DNT" refers to *pertussis* dermonecrotic toxin, which is a heat labile toxin that induces localized lesions in mice and other laboratory animals when it is injected intradermally. It is lethal to mice when it is injected in low doses intravenously (58 to 61). DNT is considered to be a virulence factor for the production of turbinate atrophy in porcine atrophic rhinitis (62, 63).

[0041] As used herein the abbreviation "TCT" refers to tracheal cytotoxin, which is a virulence factor synthesized by *Bordetellae.* TCT is a peptidoglycan fragment and has the ability to induce interleukin-1 production and nitric oxide synthase. It has the ability to cause stasis of cilia and has lethal effects on respiratory epithelial cells.

[0042] The term "mammal" encompasses any of various warm-blooded vertebrate animals of the class Mammalia, including humans, characterized by a covering of hair on the skin and, in the female, milk-producing mammary glands for nourishing the young.

[0043] The term "attenuated" means a weakened, less virulent *Bordetella* strain that is capable of stimulating an immune response and creating protective immunity, but does not cause any illness.

[0044] The terminology "rapid protective immunity" means that immunity against Bordetella is conferred in a short time after administration of the mutated *Bordetella* strain of the invention. By "short time" means vaccinated and challenged one week later. More specifically, there is a quick expansion of existing pathogen-specific peripheral lymphocytes, $CD8^+$ cytotoxic effectors (CTLs) and $CD4^+$ helper cells. The $CD4^+$ helper cells induce B cell maturation and antibody production. Thus, lymphocytes with the memory pool are poised to rapidly proliferate at the time of subsequent infection.

[0045] The term *"Bordetella* strain" encompasses strains from *Bordetella pertussis, Bordetella parapertussis* and *Bordetella bronchiseptica.*

[0046] The expression *"Bordetella* infection" means an infection caused by at least one of the three following strains: *Bordetella pertussis, Bordetella parapertussis* and *Bordetella bronchiseptica.*

[0047] By "child" is meant a person or a mammal between 6 months and 12 years of age.

[0048] By the term "newborn" is meant, a person or a mammal that is between 1 day old and 24 weeks of age.

[0049] The term "treatment" as used herein is not restricted to curing a disease and removing its causes but particularly covered means to cure, alleviate, remove or lessen the symptoms associated with the disease of interest, or prevent or reduce the possibility of contracting any disorder or malfunction of the host body.

[0050] The terms "protection" and "prevention" are used herein interchangeably and mean that an infection by *Bordetella* is impeded.

[0051] "Prophylaxis vaccine" means that this vaccine prevents *Bordetella* infection upon future exposure.

[0052] By "preferentially towards the Th1 pathway" is meant that the Th1 pathway is favored over the Th2 pathway.

[0053] The term "immunogenic composition" means that the composition can induce an immune response and is therefore antigenic. By "immune response" means any reaction by the immune system. These reactions include the alteration in the activity of an organism immune system in response to an antigen and may involve, for example, antibody production, induction of cell-mediated immunity, complement activation or development of immunological tolerance.

[0054] More specifically, the invention provides at least a triple mutated *Bordetella* strain that can be used as an immunogenic composition or a vaccine. It will be appreciated that the at least triple mutated *Bordetella* strain contains a mutated *ptx* gene, a deleted or mutated *dnt* gene and a heterologous ampG gene. The heterologous ampG gene product reduces in large quantities the amount of tracheal cytotoxin that is produced.

[0055] The invention is not limited to only the triple mutants described above. Other additional mutations can be undertaken such as adenylate cyclase (AC) deficient mutants (64), lipopolysaccharide (LPS) deficient mutants (65), filamentous hemagglutinin (FHA) (66) and any of the bvg-regulated components (67).

[0056] The starting strain which is mutated can be any *Bordetella* strain including *Bordetella pertussis, Bordetella parapertussis* and *Bordetella bronchiseptica.* In one aspect the starting strain used to obtain the mutated *Bordetella* strain is B. *pertussis.*

[0057] The construction of the mutated *Bordetella* strain starts with replacing the *Bordetella* ampG gene in the strain with a heterologous *ampG* gene. Any heterologous *ampG* gene can be used in the invention. These include all those gram-negative bacteria that release very small amounts of peptidoglycan fragments into the medium per generation. Examples of gram-negative bacteria include, but are not limited to *Escherichia coli, Salmonella, Enterobacteriaceae, Pseudomonas, Moraxella, Helicobacter, Stenotrophomonas, Legionella* and the like.

[0058] By replacing the *Bordetella ampG* gene with a heterologous *ampG* gene, the amount of tracheal cytoxin (TCT) produced in the resulting strain expresses less than 1% residual TCT activity. In another embodiment, the amount of TCT toxin expressed by the resulting strain is between 0.6% to 1% residual TCT activity or 0.4% to 3 % residual TCT activity or 0.3 % to 5% residual TCT activity.

[0059] PTX is a major virulence factor responsible for the systemic effects of *B. pertussis* infections, as well as one of the major protective antigens. Due to its properties, the natural *ptx* gene is replaced by a mutated version so that the enzymatically active moiety S1 codes for an enzymatically inactive toxin, but the immunogenic properties of the pertussis toxin are not affected. This can be accomplished by replacing the arginine (Arg) at position 9 of the sequence with a lysine (Lys). Furthermore, a glutamic acid (Glu) at position 129 is replaced with a glycine (Gly).

[0060] Other mutations can also be made such as those described in U.S. Patent 6,713,072 as well as any known or other mutations able to reduce the toxin activity to undetectable levels. Allelic exchange is first used to delete the *ptx* operon and then to insert the mutated version.

[0061] Finally, the *dnt* gene is then removed from the *Bordetella* strain by using allelic exchange. Besides the total removal, the enzymatic activity can also be inhibited by a point mutation. Since DNT is constituted by a receptor-binding domain in the N-terminal region and a catalytic domain in the C-terminal part, a point mutation in the *dnt* gene to replace Cys-1305 to Ala-1305 inhibits the enzyme activity of DNT (68). DNT has been identified as an important toxin in *Bordetella bronchiseptica* and displays lethal activity upon injection of minute quantities (26).

[0062] Besides allelic exchange to insert the mutated *ptx* gene and the inhibited or deleted *dnt* gene, the open reading frame of a gene can be interrupted by insertion of a genetic sequence or plasmid. This method is also contemplated in the invention.

[0063] The triple mutated strain of the invention is called a BPZE1 strain and has been deposited with the Collection Nationale de Cultures de Microorganismes (CNCM) in Paris, France on March 9, 2006 under the number CNCM I-3585. The mutations introduced into BPZE1 result in drastic attenuation, but allow the bacteria to colonize and persist. Thus, in another embodiment the invention provides BPZE1, which can induce mucosal immunity and systemic immunity when administered. In another aspect the BPZE1 is administered intranasally.

[0064] The mutated *Bordetella* strains of the invention can be used in immunogenic compositions. Such immunogenic compositions are useful to raise an immune response, either an antibody response and or preferably a T cell response in mammals. Advantageously, the T cell response is such that it protects a mammal against *Bordetella* infection or against its consequences.

[0065] The mutated *Bordetella* strains of the invention can be used as live strains or chemically or heat-killed strains in the vaccines or immunogenic compositions. In one aspect, the live strains are used for nasal administration, while the chemically-or heat killed strains can be used for systemic or mucosal administration.

[0066] The immunogenic composition may further comprise a pharmaceutically suitable excipient or carrier and/or vehicle, when used for systemic or local administration. The pharmaceutically acceptable vehicles include, but are not limited to, phosphate buffered saline solutions, distilled water, emulsions such as an oil/water emulsions, various types of wetting agents sterile solutions and the like.

[0067] The immunogenic composition of the invention can also comprise adjuvants, *i.e.,* any substance or compound capable of promoting or increasing a T-cell mediated response, and particularly a $CD4^+$-mediated or $CD8^+$-mediated immune response against the active principle of the invention. Adjuvants such as muramyl peptides such as MDP, IL-12, aluminium phosphate, aluminium hydroxide, Alum and/or Montanide® can be used in the immunogenic compositions of the invention.

[0068] It would be appreciated by the one skilled in the art that adjuvants and emulsions in the immunogenic compositions are used when chemically or heat treated mutated *Bordetella* strains are used in the vaccines or immunogenic compositions.

[0069] The immunogenic compositions of the invention further comprise at least one molecule having a prophylactic effect against a *Bordetella* infection or the detrimental effects of *Bordetella* infection, such as a nucleic acid, a protein, a polypeptide, a vector or a drug.

[0070] The immunogenic composition of the invention is used to elicit a T-cell immune response in a host in which the composition is administered. All immunogenic compositions described above can be injected in a host via different routes: subcutaneous (s.c.), intradermal (i.d.), intramuscular (i.m.) or intravenous (i.v.) injection, oral administration and intranasal administration or inhalation.

[0071] When formulated for subcutaneous injection, the immunogenic composition or vaccine of the invention preferably comprises between 10 and 100 μg of the *Bordetella* strain per injection dose, more preferably from 20 to 60 μg/dose, especially around 50 μg/dose, in a sole injection.

[0072] When formulated for intranasal administration, the *Bordetella* strain is administered at a dose of approximately 1 x 10^3 to 1 x 10^6 bacteria, depending on the weight and age of the mammal receiving it. In another aspect a dose of 1 x 10^4 to 5 x 10^6 can be used.

[0073] The mutated *Bordetella* strains of the invention can be used as an attenuated vaccine to protect against future *Bordetella* infection. In this regard, an advantage of the invention is that a single dose can be administered to mammals and the protection can last at least for a duration of longer than two months, particularly longer than six months. The vaccine of the invention can be administered to newborns and protects against infection of whooping cough. This is especially crucial since the fatality rate from *Bordetella pertussis* infections is about 1.3% for infants younger than 1 month.

[0074] Moreover, the vaccines of the invention can be used in adult mammals when there is a epidemic or in older adults over the age of 60, since their risk of complications maybe higher than that of older children or healthy adults.

[0075] The vaccines can be formulated with the physiological excipients set forth above in the same manner as in the immunogenic compositions. For instance, the pharmaceutically acceptable vehicles include, but are not limited to, phosphate buffered saline solutions, distilled water, emulsions such as an oil/water emulsions, various types of wetting agents sterile solutions and the like. Adjuvants such as muramyl peptides such as MDP, IL-12, aluminium phosphate, aluminium hydroxide, Alum and/or Montanide® can be used in the vaccines.

[0076] The vaccines of the invention are able to induce high titers of serum IgG against FHA. The analysis of the antigen-specific cytokine patterns revealed that administration with the mutated attenuated *Bordetella* strains of the invention favored a strong TH1 response.

[0077] The vaccines of the invention provide high level of protection against a *Bordetella* infection i.e., a level of protection higher than 90%, particularly higher than 95%, more particularly higher than 99% (calculated 7 days after infection as detailed on example 9). The level of protection of the vaccine comprising the BPZE1 strain reaches more than 99.999% compared to non-vaccinated (naive) mice, at least two months after vaccination.

[0078] The vaccines can be administered subcutaneous (s.c.), intradermal (i.d.), intramuscular (i.m.) or intravenous (i.v.) injection, oral administration and intranasal administration or inhalation. The administration of the vaccine is usually in a single dose. Alternatively, the administration of the vaccine of the invention is made a first time (initial vaccination), followed by at least one recall (subsequent administration), with the same strain, composition or vaccine, or with acellular vaccines, or a combination of both.

[0079] In one aspect, intranasal administration or inhalation of the vaccines is accomplished, which type of administration is low in costs and enables the colonization by the attenuated strains of the invention of the respiratory tract: the upper respiratory tract (nose and nasal passages, paranasal sinuses, and throat or pharynx) and/or the respiratory airways (voice box or larynx, trachea, bronchi, and bronchioles) and/or the lungs (respiratory bronchioles, alveolar ducts, alveolar sacs, and alveoli)

[0080] Intranasal administration is accomplished with an immunogenic composition or a vaccine under the form of liquid solution, suspension, emulsion, liposome, a cream, a gel or similar such multiphasic composition. Solutions and suspensions are administered as drops. Solutions can also be administered as a fine mist from a nasal spray bottle or from a nasal inhaler. Gels are dispensed in small syringes containing the required dosage for one application.

[0081] Inhalation is accomplished with an immunogenic composition or a vaccine under the form of solutions, suspensions, and powders; these formulations are administered via an aerosol or a dry powder inhaler. Compounded powders are administered with insufflators or puffers.

[0082] Use of the mutated *Bordetella* strains comprising at least a mutated *ptx* gene, a deleted or mutated *dnt* gene and a heterologous ampG gene for the preparation of a multivalent vaccine to treat respiratory diseases is yet another aspect of the invention. In this regard, the attenuated mutated *Bordetella* strain described above, can be used as an heterologous expression platform to carry heterologous antigens to the respiratory mucosa. Thus, such respiratory pathogens such as Neisseria, Pneumophila, yersinia, pseudomonas, mycobacteria, influenza and the like can prevent infection using the BPZE1 as a carrier.

[0083] Use of the live attenuated mutated *Bordetella* strains described herein for the manufacture of a vaccine for the treatment or prevention of *Bordetella* infection is also encompassed by the invention. In this regard, the vaccine can be used for the simultaneous treatment or prevention of an infection by *B. pertussis* and *B. parapertussis.*

[0084] Use of the vaccine to provide rapid protective immunity in case of a *pertussis* epidemic is also encompassed by the invention.

[0085] Use of the vaccine to provide a rapid protective immunity, increasing over the at least next two months following vaccination is also encompassed by the invention.

[0086] The vaccine or immunogenic composition is also provided in a kit. The kit comprises the vaccine or immunogenic composition and an information leaflet providing instructions for immunization.

[0087] The application a method for inducing T-cell mediated immune response and particularly a $CD4^+$-mediated immune response or a $CD8^+$-mediated immune response, comprising administering the live attenuated *Bordetella* strains of the application in a non-human mammal or a human mammal.

[0088] A method of protecting a mammal against disease caused by infection by *Bordetella* comprising administering to said mammal in need of such treatment a mutated *Bordetella* strain comprising at least a mutated *ptx* gene, a deleted or mutated *dnt* gene, and a heterologous *ampG* gene is another embodiment of the application. This method encompasses treating or preventing infections against *Bordetella pertussis* and/or *Bordetella parapertussis*. In one aspect the BPZE1 strain is used in this method.

[0089] Also a method of providing a rapid protective immunity against a *Bordetella* infection comprising administering to said mammal in need of such treatment a mutated *Bordetella* strain comprising at least a mutated *ptx* gene, a deleted or mutated *dnt* gene, and a heterologous *ampG* gene is encompassed by the application. In one aspect the BPZE1 strain is used in this method.

[0090] Moreover, the mutated live attenuated *Bordetella* strains of the application induce mucosal immunity, as well as systemic immunity. Thus, in another aspect the application also relates to a method of inducing mucosal and systemic immunity by administering to a mammal in need of such treatment the mutated live attenuated *Bordetella* strains of the application. In one aspect the BPZE1 strain is used in this method.

[0091] Besides its role in the prevention and/or treatment of *Bordetella* infection, the mutated strain of the invention may be used as vector, to bear at least one further heterologous nucleic acid sequence encoding a RNA (such as antisense RNA) or a protein of interest. This means that the mutated strain bears at least one further heterologous nucleic acid sequence in addition to the heterologous ampG gene. In one aspect, the protein encoded by this at least one further heterologous nucleic acid sequence is a protein for which the expression is desired in the respiratory tract. In another aspect, the protein of interest is an antigen, such as a viral, a bacterial or a tumoral antigen, against which an immune response is desired. Therefore, the mutated *Bordetella* strain bearing at least one further heterologous nucleic acid sequence may also be used as a vaccine. The definitions given above for administration of the vaccine or immunogenic composition also apply to a vaccine comprising mutated *Bordetella* strain bearing at least one further heterologous nucleic acid sequence. Examples of heterologous proteins are antigens of pathogens causing infections of or diseases associated with the respiratory track: poliomyelitis, influenza *(influenza virus* from *Orthomyxoviridae* family) or antigens from pneumococcus (such as *Streptococcus pneumoniae).*

[0092] The application describes the following aspects:

1. A mutated *Bordetella* strain comprising at least a mutated *pertussis* toxin (*ptx*) gene, a deleted or mutated dermonecrotic (*dnt*) gene, and a heterologous *ampG* gene.
2. The mutated *Bordetella* strain according to aspect 1, that is a *Bordetella pertussis* strain.
3. The mutated *Bordetella* strain according to aspect 1 or 2, wherein the *Bordetella ampG* gene is replaced by an *E. coli ampG* gene.
4. The mutated *Bordetella* strain according to any one of aspects 1 to 3, wherein the resulting strain expresses less than 5% residual TCT activity.
5. The mutated *Bordetella* strain according to any one of aspects 1 to 3, wherein the resulting strain expresses less than 1% residual TCT activity.
6. The mutated *Bordetella* strain according to any one of aspects 1 to 5, wherein the mutation of the ptx gene consists in the substitution of one amino acid involved in the substrate binding and/or one amino acid involved in the catalysis.
7. The mutated *Bordetella* strain according to aspect 6, that is a *Bordetella pertussis* strain wherein the substitution of the amino acid involved in the substrate binding is R9K and the substitution of the amino acid involved in the catalysis is E129G.
8. The mutated *Bordetella* strain according to any one of aspects 1 to 7 that is a triple mutant strain.
9. The triple mutated *Bordetella* strain according to aspect 8, that it is a BPZE1 strain deposited with the Collection Nationale de Culture Microorganismes (C.N.C.M.) on March 9, 2006, under number I-3585.
10. The mutated *Bordetella* strain according to any one of aspects 1 to 9 that is attenuated.
11. An immunogenic composition comprising a mutated *Bordetella* strain according to any one of aspects 1 to 10.
12. The immunogenic composition according to aspect 11, further comprising a pharmaceutically suitable excipient, vehicle and/or carrier.
13. The immunogenic composition according to aspect 11 or 12, further comprising an adjuvant.
14. The immunogenic composition according to any one of aspects 11 to 13, further comprising a molecule having a prophylactic effect against a *Bordetella* infection or the detrimental effects of *Bordetella* infection.
15. The immunogenic composition according to any one of aspects 11 to 14, wherein said mutated *Bordetella* strain is BPZE1.
16. A vaccine comprising the attenuated *Bordetella* strain of aspect 10.
17. A vaccine according to aspect 16, formulated for intranasal administration.
18. A kit comprising a vaccine according to aspect 16 or 17, and an information leaflet.
19. The attenuated *Bordetella* strain according to aspect 10 for use as a vaccine against infections caused by

Bordetella species.

20. The attenuated *Bordetella* strain according to aspect 19 for use as a prophylaxis vaccine against infections caused by *Bordetella* species.

21. The attenuated *Bordetella* strain according to aspect 19 or 20, wherein the *Bordetella* species is *B. pertussis* or *B. parapertussis.*

22. The attenuated *Bordetella* strain of aspect 10 or of any one of aspects 19 to 21, wherein said mutated *Bordetella* strain is BPZE1.

23. Use of an attenuated *Bordetella* strain according to aspect 10 for the manufacture of a vaccine for the prevention of a *Bordetella* infection.

24. Use of an attenuated *Bordetella* strain according to aspect 10 for the manufacture of a vaccine for the simultaneous prevention against a *B. pertussis* and *B. parapertussis* infection.

25. Use of an attenuated *Bordetella* strain according to aspect 10 for the manufacture of a vaccine for the induction of an immune response directed preferentially toward the Th1 pathway against said attenuated *Bordetella.*

26. Use of an attenuated *Bordetella* according to any one of aspects 23 to 25, wherein the vaccine is administered by subcutaneous (s.c.), intradermal (i.d.), intramuscular (i.m.), intravenous (i.v.), oral or intranasal administration, by injection or by inhalation.

27. Use of an attenuated *Bordetella* according to any one of aspects 23 to 25, wherein the vaccine is administered intranasally.

28. Use of an attenuated *Bordetella* according to any one of aspects 23 to 25, wherein the vaccine is administrated to mammals in need of a rapid protective immunity against a *Bordetella* infection.

29. Use of an attenuated *Bordetella* according to aspect 28, wherein the vaccine is administrated to newborns.

30. Use of an attenuated *Bordetella* according to aspect 28, wherein the vaccine is administrated to children.

31. Use of an attenuated *Bordetella* according to any one of aspects 28 to 30, wherein the vaccine is administered intranasally.

32. Use of an attenuated *Bordetella* according to any one of aspects 28 to 31, wherein the vaccine is administered once in a single dose.

33. Use of an attenuated *Bordetella* according to any one of aspects 23 to 27, comprising a) administrating a strain according to aspect 10 or any one of aspects 19 to 22; and b) carrying out at least one recall with either the same strain or an acellular vaccine or a combination of both.

34. Use of an attenuated *Bordetella* according to aspect 23, wherein the vaccine is administrated to mammals in need of a rapid protective immunity against a *Bordetella* infection, wherein said protective immunity increases over at least two months after vaccination.

35. Use of a mutated *Bordetella* strain comprising at least a mutated *ptx* gene, a deleted or mutated *dnt* gene and a heterologous ampG gene for the preparation of a multivalent vaccine to treat respiratory diseases.

36. Use according to any one of aspects 23 to 35, wherein said mutated *Bordetella* strain is BPZE1.

37. Use according to any one of aspects 23 to 36, wherein a level of protection against a *Bordetella* infection is more than 95%, particularly more than 99%.

38. Use according to aspect 37, wherein said level of protection against a *Bordetella* infection reaches more than 99.999%.

39. A method of protecting a mammal against disease caused by infection by *Bordetella* comprising administering to said mammal in need of such treatment a mutated *Bordetella* strain comprising at least a mutated *ptx* gene, a deleted or mutated *dnt* gene, and a heterologous ampG gene.

40. A method of providing a rapid protective immunity against a *Bordetella* infection comprising administering to said mammal in need of such treatment a mutated *Bordetella* strain comprising at least a mutated *ptx* gene, a deleted or mutated *dnt* gene, and a heterologous ampG gene.

41. The method according to aspect 39 or 40, wherein said Bordetella infection is from *Bordetella pertussis* and *Bordetella parapertussis.*

42. The method according to any one of aspects 39 to 41, wherein said mammal is a newborn.

43. The method according to any one of aspects 39 to 41, wherein said mammal is a child.

44. The method according to any one of aspects 39 to 43, wherein said mutated *Bordetella* strain is administered subcutaneously (s.c.), intradermally (i.d.), intramuscularly (i.m.), intravenously (i.v.), orally or intranasally, by injection or by inhalation.

45. The method according to any one of aspects 39 to 43, wherein said mutated *Bordetella* strain is administered intranasally.

46. A method to provide a mucosal response and a systemic response to treat *Bordetella* infections in mammals said method comprising administering to mammals in need of such treatment the attenuated *Bordetella* strain according to aspect 10.

47. A method of providing a rapid protective immunity against a *Bordetella* infection comprising administering to

said mammal in need of such treatment a mutated *Bordetella* strain comprising at least a mutated *ptx* gene, a deleted or mutated *dnt* gene, and a heterologous ampG gene, wherein said method provides further an increase in said protective immunity over at least two months after vaccination.

48. The method of aspect 39,40 or 47, wherein said mutated *Bordetella* strain is BPZE1.

49. The method of any one of aspects 39 to 41 or of 47, wherein a level of protection against a *Bordetella* infection is more than 95%, particularly more than 99%.

50. The method according to aspect 49, wherein said level of protection against a *Bordetella* infection reaches more than 99.999%.

51. The mutated *Bordetella* strain according to any one of aspects 1 to 10, further comprising at least one heterologous nucleic acid sequence encoding an RNA or a protein.

52. The mutated *Bordetella* strain according to aspect 51, wherein said at least one heterologous nucleic acid sequence encodes an antigen.

53. The mutated *Bordetella* strain according to aspect 52, wherein said at least one heterologous nucleic acid sequence encodes a viral or bacterial antigen.

54. Use of a mutated *Bordetella* strain according to any one of aspects 1 to 10 as a vector for expressing at least one heterologous antigen.

55. A mutated *Bordetella* strain according to any one of aspects 51 to 53, for use as a vaccine.

EXAMPLES

MATERIALS AND METHODS

Example 1 - *Bordetella* strains and growth conditions

[0093] The *B. Pertussis* strains used in this study were all derived from *B. pertussis* BPSM [13], and *B. parapertussis* is a streptomycin-resistant derivative of strain 12822 (kindly provided by Dr. N. Guiso, Institut Pasteur Paris, France). All *Bordetella* strains were grown on Bordet-Gengou (BG) agar (Difco, Detroit, Mich.) supplemented with 1% glycerol, 20% defibrinated sheep blood, and 100 μg/ml streptomycin. For cell adherence assays, exponentially growing *B. pertussis* was inoculated at an optical density of 0.15 at 600 nm in 2.5 ml modified Stainer-Scholte medium [14] containing 1 g/l heptakis(2,6-di-o-methyl) ß-cyclodextrin (Sigma) and supplemented with 65 μCi/ml L-[^{35}S]methionine plus L-[^{35}S]cysteine (NEN, Boston, Mass.) and grown for 24 h at 37°C.The bacteria were then harvested by centrifugation, washed three times in phosphate-buffered saline (PBS) and resuspended in RPMI 1640 (Gibco, Grand Island, N. Y.) at the desired density.

Example 2 - Construction of *B. pertussis* BPZE1.

[0094] To construct *B. pertussis* BPZE1, the *B. pertussis ampG* gene was replaced by *Escherichia coli* ampG using allelic exchange. A PCR fragment named *met* and located at position 49,149 to 49,990 of the *B. pertussis* genome (http://www.sanger.ac.uk/Projects/B_pertussis/), upstream of the *B. pertussis ampG* gene, was amplified using oligonucleotides A : 5'-TATAAATCGATATTCCTGCTGGTTTCGTTCTC-3' (SEQ ID No:5) and B : 5'-TATAGCTAGCAAGTTGGGAAACGACACCAC-3' (SEQ ID No:6), and *B. pertussis* BPSM [13] genomic DNA as a template. This 634-bp fragment was inserted into Topo PCRII (InVitrogen Life Technology, Groningen, The Netherlands) and then excised as a *Cla*I-*Nhe*I fragment and inserted into *Cla*I- and *Nhe*I-digested pBP23 [50], a suicide vector containing the *E. coli ampG* gene with flanking *B. pertussis* DNA of 618 bp (from position 50,474 to 51,092 of the *B. pertussis* genome) and 379 bp (from position 52,581 to 52,960 of the *B. pertussis* genome) at the 5' and 3' end of *E. coli ampG,* respectively. The resulting plasmid was transferred into *E. coli* SM10 [51], which was then conjugated with BPSM, and two successive homologous recombination events were selected as described [52]. Ten individual colonies were screened by PCR as follows. The colonies were suspended in 100 μl H_2O, heated for 20 min. at 95°C, and centrifuged for 5 min at 15,000 x g. One μl of supernatants was then used as template for PCR using oligonucleotides A and C : 5'-TAAGAAGCAAAATAAGCCAGGCATT-3' (SEQ ID No:7) to verify the presence of *E. coli* ampG and using oligonucleotides D : 5'-TATACCATGGCGCCGCTGCTGGTGCTGGGC-3'(SEQ ID No:8) and E : 5'-TATATCTAGACGCTGGCCGTAACCTTAGCA-3'(SEQ ID No:9) to verify the absence of *B. pertussis ampG.* One of the strains containing *E. coli ampG* and lacking *B. pertussis ampG* was then selected, and the entire ampG locus was sequenced. This strain was then used for further engineering.

[0095] The *ptx* genes were deleted from the chromosome of this strain as described [21] and then replaced by mutated *ptx* coding inactive PTX. The *Eco*RI fragment containing the mutated *ptx* locus from pPT-RE [16] was inserted into the *Eco*RI site of pJQ200mp18rpsl [53]. The resulting plasmid was integrated into the *B. pertussis* chromosome at the *ptx* locus by homologous recombination after conjugation via *E. coli* SM10. The *ptx* locus in the chromosome of the resulting *B. pertussis* strain was sequenced to confirm the presence of the desired mutations. Toxin production was analyzed by immunoblotting using a mix of monoclonal antibodies IB7 [54] specific for subunit S1, and 11 E6 [55] specific for subunits S2 and S3 of PTX.

[0096] Finally, the *dnt* gene was deleted from the resulting *B. pertussis* strain as the *dnt* flanking regions were amplified by PCR using BPSM genomic DNA as a template and oligonucleotides F: 5'-TATAGAATTCGCTCGGTTCGCTGGT-CAAG G-3' (SEQ ID No:10) and G: 5'-TATATCTAGAGCAATGCCGATTCATCTTTA-3' (SEQ ID No:11) for the *dnt* upstream region, and H: 5'-TATATCTAGAGCGGCCTT TATTGCTTTTCC-3' (SEQ ID No:12) and I: 5'-TATAAAGCT-TCTCATGCACGCCG GCTTCTC-3' (SEQ ID No:13) for the *dnt* downstream region, as primers. The resulting 799-bp and 712-bp DNA fragments were digested with EcoRI/XbaI and *Xba*I/*Hin*dIII, respectively, and linked together using the Fast Link kit (Epicentre Biotechnologies, Madison, WI). The ligated fragment was amplified by PCR using oligonucleotides F and I, and the 1505-bp PCR fragment was then inserted into pCR2.1-Topo (Invitrogen), re-isolated from the resulting plasmid as an *Eco*RI fragment and inserted into then unique *Eco*RI site of pJQmp200rpsL18. The resulting plasmid was introduced into *B. pertussis* by conjugation via *E. coli* SM10. Successful deletion of the *dnt* gene by allelic exchange was verified by Southern blot analysis on PvuII-digested *B. pertussis* genomic DNA using the PCR fragment corresponding to the *dnt* upstream region as a probe. The probe was labeled with digoxigenin (DIG) using the DIG Easy Hyb labeling kit (Roche, Meylan, France). The sizes of the hybridizing bands were determined from the migration distance of the Dig-labeled DNA molecular marker III (Roche). The *dnt* locus of this final strain, named BPZE1 was sequenced.

Example 3 - Analysis of TCT production.

[0097] For sensitive quantitation of TCT production, culture supernatants of *B. pertussis* grown to logarithmic phase were collected, subjected to solid phase extraction [15] and derivatized with phenylisothiocyanate (PITC, Pierce). The resulting phenylthiocarbamyl (PTC) derivatives were separated by reversed-phase HPLC using a C8 column (Perkin Elmer) and detected at 254 nm. The amount of *B. pertussis* PTC-TCT in each sample was determined by comparing the peak area and elution time with an identically processed TCT standard.

Example 4 - Cell-adherence assay.

[0098] To analyze adherence properties of the *B. pertussis* strains, their attachment rates to the human pulmonary epithelial cell line A549 (ATCC n°CCL-185) and the murine macrophage cell line J774 (ATCC n°TIB-67) were measured as previously described [16].

Example 5 - Transmission electron microscopy.

[0099] The single droplet-negative staining procedure was used as described previously [17] with the following modifications. 20 μl of a suspension at approximately 10^9 bacteria/ml were absorbed for 2 min. onto form formvard carbon-coated nickel grids (400 mesh; Electron Microscopy Sciences EMS, Washington, PA). After 30 seconds air-drying the grids were stained for 2 minutes with 20 μl of 2% phosphotungstic acid (pH7; EMS) and examined after air-drying under a transmission electron microscope (Hitachi 7500, Japan) at 60 kvolts and high resolution.

Example 6 - Intranasal infection and vaccination.

[0100] 3-week and 8-week old female Balb/C were kept under specific pathogen-free conditions, and all experiments were carried out under the guidelines of the Institut Pasteur de Lille animal study board. Mice were intranasally infected with approximately $4x10^6$ bacteria in 20 μl PBS, and kinetics of CFU in the lungs were measured as previously described [18]. For vaccination with aPV (Tetravac; Aventis-Pasteur, France), mice were immunized intraperitoneally (i.p.) with 20% of the human dose and boosted one month later using the same dose.

Example 7 - Antibody determination.

[0101] Sera were collected, and antibody titers were estimated by enzyme-linked immunosorbent assays (ELISA) as previously described [18].

Example 8 - Cytokine assays.

[0102] Spleen cells from individual mice were tested at different time points after immunization for *in vitro* cytokine production in response to heat-killed *B. pertussis* BPSM (10^6 cells/ml), 5.0 μg/ml PTX (purified from *B. pertussis* BPGR4 [19] as previously described [20] and heat-inactivated at 80°C for 20 min), 5.0 μg filamentous hemagglutinin (FHA, purified from *B. pertussis* BPRA [21] as previously described [22]), 5 μg/ml concanavalin A (Sigma Chemical Co., St.

Louis, Mo.) or medium alone as control. Supernatants were removed from triplicate cultures after 72 h incubation at 37°C and 5% CO_2, and IFN-γ and IL-5 concentrations were determined by immunoassays (BD OptEIA set, Pharmingen).

Example 9 - Intranasal infection and vaccination: challenge at 1, 2, 3 and 4 weeks.

[0103] An infant (3 weeks-old) mouse model [29] was used to compare the efficiency of vaccination with BPZE1 with the one of vaccination with acellular *pertussis* vaccine (aPv). Female Balb/C mice were intranasally infected with approximately $1x10^6$ BPZE1 strain in 20 μl PBS. For vaccination with aPv (Tetravac; Aventis-Pasteur, France), mice were immunized intraperitoneally with 20% of the human dose. One, two, three or four weeks after vaccination with BPZE1 or aPv, mice were intranasally challenged with virulent *B. pertussis* BPSM/bctA-lacZ strain [53]. This strain is a BPSM-derivative gentamycin-resistant which allows the discrimination with BPZE1 (gentamycin-sensitive) on Bordet-Gengou agar plates containing 10μg/ml of gentamycin and 100μg/ml of streptomycin (BGgs). Control group corresponds to naive mice challenged with BPSM/bctA-lacZ. One week after challenge infection, lungs were aseptically removed, homogenized and plates on BGgs for CFU determination as previously described [18].

[0104] Mice were vaccinated with BPZE1 or aPv and challenged with virulent *B. pertussis* one, two, three or four weeks after vaccination. Lung CFUs counts were determined 3 hours or 7 days later. Results are expressed as mean (± standard error) CFUs from three to five mice per group. Levels of protection are calculated for each challenge infection as mean percentages of CFUs of each group relative of the average of CFUs in non-immunized group, 7 days after challenge infection (Tables 2 to 5).

Example 10 - Statistical analysis.

[0105] The results were analyzed using the unpaired Student's *t* test and the Kruskal-Wallis test followed by the Dunn's post-test (GraphPad Prism program) when appropriate. Differences were considered significant at P ≤0.05.

RESULTS

Construction of *B. pertussis* BPZE1

[0106] Three virulence factors were genetically targeted: tracheal cytotoxin (TCT), pertussis toxin (PTX) and dermonecrotic toxin (DNT).

[0107] TCT is responsible for the destruction of ciliated cells in the trachea of infected hosts [24, 25] and may thus be involved in the cough syndrome. TCT is a breakdown product of peptidoglycan in the cell wall of Gram-negative bacteria, which generally internalize it into the cytosol by then AmpG transporter protein to be re-utilized during cell wall biosynthesis. *B. pertussis* AmpG is inefficient in the internalization of peptidoglycan breakdown products. We therefore replaced the B. *pertussis* ampG gene by *E. coli ampG.* The resulting strain expressed less than 1 % residual TCT activity (Fig. 1).

[0108] PTX is a major virulence factor responsible for the systemic effects of B. *pertussis* infections and is composed of an enzymatically active moiety, called S1, and a moiety responsible for binding to target cell receptors (for review, see 26). However, it is also one of the major protective antigens, which has prompted us to replace the natural *ptx* genes by a mutated version coding for an enzymatically inactive toxin. This was achieved by replacing Arg-9 by Lys and Glu-129 by Gly in S1, two key residues involved in substrate binding and catalysis, respectively. Allelic exchange was used to first delete the *ptx* operon, and then to insert the mutated version. The presence of the relevant toxin analogues in the *B. pertussis* culture supernatants was evaluated by immunoblot analysis **(Fig. 2).**

[0109] Finally, allelic exchange was used to remove the *dnt* gene **(Fig. 3).** Although the role of DNT in the virulence of *B. pertussis* is not certain, it has been identified as an important toxin in the closely related species *Bordetella bronchiseptica* and displays lethal activity upon injection of minute quantities (for review, see 26).

***In vitro* characterization of** *B. pertussis* **BPZE1**

[0110] Since some of the genetic alterations in BPZE1 may potentially affect the bacterial cell wall synthesis, the size and shape, as well as the *in vitro* growth rate of BPZE1 was compared with those of the parental strain BPSM. The growth rate of BPZE1 did not differ from that of BPSM **(Fig. 4),** and no difference in bacterial shape or size was detected between BPZE1 and BPSM, as evidenced by electron microscopy analysis **(Fig. 5).** However, the cell wall of BPZE1 appeared to be consistently somewhat thinner than that of BPSM.

[0111] To determine whether the absence or alterations of any of the targeted toxins in BPZE1 affects adherence properties of *B. pertussis,* the attachment rates of BPZE1 was compared with those of BPSM, using the human pulmonary epithelial cell line A549 and the murine macrophage cell line J774, as two cellular models often used to study the adherence of *B. pertussis.* No significant difference in the adherence capacities to either cell line was observed between the two strains **(Fig. 6).**

Attenuation of *B. pertussis* BPZE1

[0112] To determine whether the mutations introduced into *B. pertussis* BPZE1 have resulted in attenuation, yet allow the organism to colonize the respiratory tract, Balb/C mice were intranasally infected with BPZE1 or BPSM, and colonization was followed over time. BPZE1 was able to colonize and persist in the lungs of mice as long as BPSM **(Fig. 7).** However, the peak of multiplication seen 7 days after infection with BPSM was consistently lacking in mice infected with BPZE1. Studies done with strains mutated in individual toxin genes indicated that this is due to the mutations in the *ptx* locus (data not shown). When the lungs were examined for histopathological changes and inflammatory infiltration, infection with BPSM was found to induce strong peri-bronchiovascular infiltrates and inflammatory cell recruitment 7 days after infection, associated with a strong hypertrophy of the bronchiolar epithelial cells **(Fig. 8).** In contrast, no such changes were seen in BPZE1-infected animals, and the histology of the BPZE1-infected mice was similar to that of the control mice that had received PBS instead of the bacteria. The BPSM-infection induced inflammation lasted for at least two months (data not shown). These results indicate that the mutations introduced into BPZE1 have resulted in drastic attenuation, but allow the bacteria to colonize and persist in the lungs.

Protection against *B. pertussis* challenge after intranasal vaccination of adult mice with BPZE1

[0113] To evaluate the protection offered by BPZE1, the effect of a single intranasal administration of this strain to 8-weeks old Balb/C mice on the subsequent colonization by the wild type challenge strain BPSM was compared with that of two i. p. immunizations with 1/5 of a human dose of aPV. This aPV immunization protocol has been described as the best to correlate with pertussis vaccine efficacy in human clinical trials [27, 28]. As shown by the total clearance of bacterial colony counts in the lungs seven days after challenge infection, a single intranasal administration of BPZE1 and two i.p. immunizations with aPV provided similar levels of protection **(Fig. 9a).** High bacterial loads were found in the control mice that had received two injections of PBS instead of the vaccine.

Protection against *B. pertussis* challenge after intranasal vaccination of infant mice with BPZE1

[0114] Since the principal targets of novel *pertussis* vaccines are young infants, that are not protected with the currently available vaccines, an infant (3 weeks-old) mouse model [29] was developed and used to compare the efficiency of vaccination with BPZE1 with that of vaccination with aPV. A single nasal administration of BPZE1 fully protected infant mice against challenge infection (**Fig**. **9b),** as complete bacterial clearance was observed in the lungs one week after challenge. In contrast, substantial numbers of bacteria remained in the aPV-vaccinated animals one week after challenge infection. The difference in bacterial load between the BPZE1-vaccinated and the aPV-vaccinated mice was statistically significant, indicating that in the infant mouse model a single intranasal administration with BPZE1 provides better protection than two systemic administrations of aPV.

[0115] In addition, a strong reduction in the bacterial load of the challenge strain 3 hours after administration when the mice had been immunized with BPZE1 was consistently observed compared to the aPV-immunized animals **(Fig. 9c),** indicating that vaccination with BPZE1 reduces the susceptibility to infection by the challenge strain. This effect was seen in both 8-weeks old and in infant mice. In contrast, aPV had no effect on the bacterial counts 3 hours after infection, when compared to the control mice.

Protection against *B. parapertussis* challenge after intranasal vaccination with BPZE1

[0116] There is increasing concern about *B. parapertussis* infection in children, especially in immunized populations [30, 31]. *B. parapertussis* causes a milder *pertussis*-like syndrome, the frequency of which is probably largely underestimated. Furthermore, the incidence of *B. parapertussis* infections has been increasing over the last decades, possibly due to the fact that *pertussis* vaccines are known to have very low or no protective efficacy against ***B.*** *parapertussis* [32, 33]. In contrast, infection by *B. pertussis* has recently been reported to protect against *B. parapertussis* infection [34]. BPZE1 was also assessed for protection against *B. parapertussis* using the infant mouse model. Whereas two administrations of aPV did not provide any protection against *B. parapertussis,* as previously reported, a single intranasal administration of BPZE1 provided strong protection, as measured by the low numbers of *B. parapertussis* counts in the lungs of the vaccinated mice 1 week after challenge **(Fig. 9d).**

Immune responses induced by BPZE1 vaccination

[0117] Although the mechanisms of protective immunity against *B. pertussis* infection are not yet completely understood, clear evidence of a role for both B cells and IFN-γ has been demonstrated in mice [28]. Vaccination with either one nasal dose of BPZE1 or two i. p. administrations of aPV induced high titers of serum IgG against FHA, a major surface antigen of *B. pertussis* [35], also present in aPV (**Fig. 10a**). Following *B. pertussis* challenge, positive anamnestic responses were measured in BPZE1- and in aPV-vaccinated animals, as indicated by an increase in anti-FHA IgG titers, compared to primary responses before *B. pertussis* infection. Examination of the anti-FHA IgG1/IgG2a ratios showed that theses ratios were higher after aPV administration, characteristic of a Th2 type response, than after BPZE1 vaccination (**Fig. 10b**). Although the anti-FHA-IgG1/IgG2a decreased after challenge in the aPV vaccinated mice, it remained still substantially higher than in the BPZE1-vaccinated animals after *B. pertussis* challenge.

[0118] Analysis of *B. Pertussis* antigen-specific cytokine patterns induced by BPZE1 or aPV vaccination confirmed that BPZE1 administration favors a stronger Th1 type response than aPV vaccination. This was revealed by the fact that the ratios of IFN-γ over IL-5 produced by splenocytes stimulated with FHA or PT, or with the polyclonal activator ConA were significantly higher in BPZE1 vaccinated mice than in aPV vaccinated mice (**Fig. 10c**).

Protective immunity of BPZE1 over time (from 1 week to 4 weeks)

[0119] As shown in Tables 1 to 5 below, whereas administration of aPv provided limited protection (reduction of 75% of bacterial load compared to non-vaccinated mice at 1 week) against *B. pertussis,* a single intranasal administration of BPZE1 already provided high level of protection (reduction of 97.64 % of bacterial load) against a *B. pertussis* challenge infection performed one week after vaccination. If challenge infection occurred two weeks after vaccination, the level of protection induced by BPZE1 reached more than 99.999 % compared to non-vaccinated mice and is significantly superior to the protection induced by aPv vaccine (approximately 92 % compared to non-vaccinated mice). Therefore, vaccine efficacy of BPZE1 against *B. pertussis* challenge is already significant one week after vaccination and is increasing over the at least next two months.

Table 1 : Kinetics of vaccines efficacy against *B. pertussis* challenge in infant mice.

Time between vaccination and challenge	Time between lungs recovery and challenge	Log10 cfu / lungs of mice		
		Naive	**aPv-vaccinated**	**BPZE1-vaccinated**
1 week	**3 hours**	5.71 ± 0.03	5.8 ± 0.07	5.74 ± 0.01
	7 days	6.71 ± 0.06	5.97 ± 0.20	4.86 ± 0.35
2 **weeks**	**3 hours**	5.77 ± 0.10	5.60 ± 0.02	5.49 ± 0.05
	7 days	6.49 ± 0.08	5.31 ± 0.16	3.22 ± 0.33
3 weeks	**3 hours**	6.03 ± 0.11	5.88 ± 0.04	5.33 ± 0.08
	7 days	6.58 ± 0.09	5.62 ± 0.11	3.14 ± 0.38
4 weeks	**3 hours**	6.31 ± 0.01	6.15 ± 0.02	5.83 ± 0.05
	7 days	6.36 ± 0.04	5.21 ± 0.11	1.83 ± 0.46

Table 2: level of protection of aPv-vaccinated and BPZE1-vaccinated mice as compared to non-vaccinated mice at week 1.

Nonvaccinated mice	**Number of bacteria in lungs**	**Mean number of bacteria**	
Non-vaccinated 1	4.7×10^6		

(continued)

Nonvaccinated mice	Number of bacteria in lungs	Mean number of bacteria		
Non-vaccinated 2	3.8×10^6	$5.36.10^6$		
Non-vaccinated 3	8.2×10^6			
Non-vaccinated 4	4.1×10^6			
Non-vaccinated 5	6×10^6			
	Number of bacteria in lungs	**Percentage of remaining bacteria (1)**	**Mean percentage of remaining bacteria**	**Level of protection**
aPv-vaccinated mice				
aPv1	1.95×10^6	36.38	25%	75%
aPv2	2.9×10^6	54.1		
aPv3	2.9×10^5	5.41		
aPv4	3.6×10^5	6.72		
aPv5	1.2×10^6	22.39		
BPZE1-vaccinated mice				
BPZE1-1	3.2×10^5	5.97	2.36%	97.64%
BPZE1-2	2×10^4	0.004		
BPZE1-3	6×10^4	1.12		
(1) Percentage of remaining bacteria = number of bacteria for each particular mouse / mean number of bacteria of all non-vaccinated mice				

Table 3: Level of protection of aPv-vaccinated and BPZE1-vaccinated mice as compared to non-vaccinated mice at week 2.

Nonvaccinated mice	Number of bacteria in lungs	Mean number of bacteria	
Non-vaccinated 1	5×10^6	3.34×10^6	
Non-vaccinated 2	3.6×10^6		
Non-vaccinated 3	1.7×10^6		
Non-vaccinated 4	2.4×10^6		
Non-vaccinated 5	4×10^6		

(continued)

	Number of bacteria in lungs	Percentage of remaining bacteria (1)	Mean percentage of remaining bacteria	Level of protection
aPv-vaccinated mice				
aPv1	9.5×10^4	2.84	8.11%	91.89%
aPv2	2.9×10^5	8.68		
aPv3	1×10^5	2.99		
aPv4	6.8×10^5	20.36		
aPv5	1.9×10^5	5.69		
BPZE1-vaccinated mice				
BPZE1-1	9.5×10^3	2.8×10^{-3}	1.03×10^{-3} %	99.999 %
BPZE1-2	450	1.35×10^{-4}		
BPZEI-3	3500	1.05×10^{-3}		
BPZE1-4	500	1.5×10^{-4}		
(1) Percentage of remaining bacteria = number of bacteria for each particular mouse / mean number of bacteria of all non-vaccinated mice				

Table 4: Level of protection of aPv-vaccinated and BPZE1-vaccinated mice as compared to non-vaccinated mice at week 3.

Nonvaccinated mice	Number of bacteria in lungs	Mean number of bacteria		
Non-vaccinated 1	1.8×10^6	$4.04\text{x}10^6$		
Non-vaccinated 2.	5.75×10^6			
Non-vaccinated 3	4.7×10^6			
Non-vaccinated 4	3.2×10^6			
Non-vaccinated 5	4.75×10^6			
	Number of bacteria in lungs	**Percentage of remaining bacteria (1)**	**Mean percentage of remaining bacteria**	**Level of protection**
aPv-vaccinated mice				
aPv1	1.99×10^5	4.94	11.26 %	88.74 %
aPv2	6×10^5	14.85		
aPv3	6×10^5	14.85		
aPv4	4.2×10^5	10.40		

(continued)

BPZE1-vaccinated mice				
BPZE1-1	3640	9.01×10^{-4}	8.65×10^{-4} %	99.999 %
HPZE1-2	9720	2.4×10^{-3}		
BPZE1-3	300	7.43×10^{-5}		
BPZE1-4	340	8.42×10^{-5}		
(1) Percentage of remaining bacteria = number of bacteria for each particular mouse / mean number of bacteria of all non-vaccinated mice				

Table 5: Level of protection of aPv-vaccinated and BPZE1-vaccinated mice as compared to non-vaccinated mice at week 4.

Nonvaccinated mice	**number of bacteria in lungs**	**Mean number of bacteria**		
Non-vaccinated 1	2.1×10^{6}	$2.36x10^{6}$		
Non-vaccinated 2	2.2×10^{6}			
Non-vaccinated 3	3.1×10^{6}			
Non-vaccinated 4	2.6×10^{6}			
Non-vaccinated 5	1.8×10^{6}			
	Number of bacteria in lungs	**Percentage of remaining bacteria (1)**	**Mean percentage of remaining bacteria**	**Level of protection**
aPv-vaccinated mice				
aPv1	2.52×10^{5}	10.68	7.76%	92.24%
aPv2	3.28×10^{5}	13.90		
aPv3	1.04×10^{5}	4.41		
aPv4	8.4×10^{5}	3.56		
aPv5	1.48×10^{5}	6.27		
BPZE1-vaccinated mice				
BPZE1-1	190	8.05×10^{-5}	7.13×10^{-5} %	99.999 %
BPZE1-2	0	0		
BPZE1-3	110	4.66×10^{-5}		
BPZE1-4	320	1.36×10^{-4}		
BPZE1-5	220	9.32×10^{-5}		
(1) **Percentage** of remaining bacteria = number of bacteria for each particular mouse / mean number of bacteria of all non-vaccinated mice				

DISCUSSION

[0120] *Pertussis* is the first infectious disease whose incidence is increasing in countries with high vaccine coverage. This paradoxical situation is most likely linked to the epidemiological changes observed since the massive introduction of highly efficacious vaccines. In contrast to the pre-vaccination era, cases of adolescent and adult *pertussis* are now increasingly more frequent. Although generally not life-threatening in that age group, *B. pertussis-infected* adults are an important reservoir for infection of the very young children, too young to be protected by vaccination. Early vaccination, possibly at birth, would therefore be highly desirable, but is hampered by the immaturity of the immune system of neonates and infants. However, the fact that natural *B. pertussis* infection, even very early in life, is able to induce a strong Th1 response in infants [12] prompted us to develop a live attenuated *B. pertussis* vaccine strain to be given by the nasal route as an alternative over the currently available vaccines.

[0121] Based on experimental infections of primates, Huang *et al.* had already in 1962 come to the conclusion that ultimate protection against whooping cough probably best follows a live *B. pertussis* inoculation [36]. In veterinary medicine, attenuated *Bordetella* strains have been used to vaccinate against bordetellosis in dogs and piglets. A live attenuated *Bordetella bronchiseptica* strain has been shown to provide strong protection against kennel cough in dogs [37] after nasal administration. This protection was seen as early as 48 h after vaccination. Intranasal vaccination with live attenuated *B. bronchiseptica* has also been shown to protect against atrophic rhinitis in two-days old piglets [38], indicating that in a live attenuated form *Bordetella* vaccines can be highly active in new-bom animals.

[0122] Previous attempts to genetically attenuate *B. pertussis* as a live vaccine candidate have met with rather limited success. Based on a strategy used for the successful attenuation of *Salmonella* vaccine strains [39], Roberts *et al.* have deleted the *aroA* gene of *B. pertussis* [40]. The *aroA* mutant was indeed highly attenuated, but had also lost its capacity to colonize the respiratory tract of the intranasally vaccinated animals and induced protective immunity only after repeated administrations of high doses. We took advantage of the knowledge on the molecular mechanisms of *B. pertussis* virulence and developed the highly attenuated strain BPZE1. This strain contains genetic alterations leading to the absence or inactivation of three major toxins, PTX, TCT and DNT. In contrast to the *aroA* mutant, this strain was able to colonize the mouse respiratory tract and to provide full protection after a single intranasal administration. The protection in adult mice was indistinguishable from that induced by two administrations of 1/5 of a human dose of aPV. An important difference, however, was seen in infant mice, where a single administration of BPZE1 fully protected, whereas aPV only offered partial protection. In the context of the difficulties to induce protection in infants with the currently available vaccines early in life, these results provide hope for the development of novel vaccination strategies that may be used in the very young children, possibly at birth. In addition, BPZE1 protected against *B. parapertussis,* whereas aPV did not. Therefore the use of BPZE1 should also have an impact on the incidence of whooping cough caused by *B. parapertussis* in infants.

[0123] Although the recent replacement of first generation whole-cell. vaccines by new aPV in many countries has significantly reduced the systemic adverse reactions observed with whole-cell vaccines, it has not abolished the need for repeated vaccination to achieve protection. This makes it unlikely to obtain protection in very young children (<6 months) that present the highest risk to develop severe disease. In addition, the wide-spread use of aPV has revealed new, unforeseen problems. Repeated administration of aPV may cause extensive swelling at the site of injection [41], which was not observed with whole-cell vaccines. In approximately 5 % of the cases this swelling involves almost the entire limb and lasts for more than a week. Although the mechanism of this swelling has not been characterized yet, it has been proposed to be due to an Arthus hypersensitivity reaction caused by high antibody levels induced by the primary immunization [42]. However, it could also be related to the Th2 skewing of the immune response, as, compared to whole-cell vaccines, aPV administration induces more Th2-type cytokines in vaccinated children [10] and causes a delay in the Th1 development (Mascart *et al.,* in preparation). Delayed maturation of Th1 function has been associated with a risk for atopy in genetically pre-disposed individuals [33]. The two mechanisms are not mutually exclusive. Compared to aPV, the immune response to BPZE1 administration is less biased towards the Th2 arm, and since BPZE1 is administered mucosally, no swelling reaction can occur.

[0124] The use of live attenuated bacteria as vaccines raises the issue of their biosafety. As such, they fall under the directives and guidelines for genetically modified organisms susceptible to be released into the environment. These guidelines and directives describe several objectives that have to be met, including hazard identification and environmental risk assessment [44]. Potential pathogenicity needs to be carefully considered, especially in immuno-compromized individuals, such as those infected with HIV. The natural biology of *B. pertussis* is particularly interesting in that regard. Although pertussis in HIV-infected subjects has been described occasionally, it is rather rare in AIDS patients [45]. In its genetically attenuated form, *B. pertussis* would therefore not be expected to cause major problems in HIV-infected children, especially if severe AIDS is an exclusion criterion, as it is for many vaccines. *B. pertussis* colonization is strictly limited to the respiratory epithelium, without extrapulmonary dissemination of the bacteria, which naturally excludes systemic bacteremia of the BPZE1 vaccine strain. If nevertheless unforeseeable safety problems occurred, the vaccine strain can easily be eliminated by the use of macrolide antibiotics, such as erythromycin, to which essentially all *B. pertussis* isolates are highly sensitive.

[0125] A further concern, like for any live vaccine, is the potential release of the vaccine strain in the environment and the consequences of such a release. *B. pertussis* is a strictly human pathogen, and there is no animal vector or reservoir. Moreover, unlike *B. bronchiseptica,* survival of wild-type *B. pertussis* in the environment is extremely limited [46]. *Pertussis* is only spread by coughing individuals, and there appears to be no asymptomatic carriage [47]. Coughing cannot be assessed in the mouse models used in this study. However, due to the nature of the genetic alterations in BPZE1, in particular the strong reduction of TCT and the genetic inactivation of PTX, this strain would not be expected to induce coughing. Active PTX has been shown to be required for cough induction in a coughing rat model, although the mechanism is not known [48]. If the vaccine strain were nevertheless to be transmitted to non-vaccinated individuals, this would at worst result in increased vaccine coverage. The consequences of each of these potential hazards can thus be graded as negligible and can easily and rapidly be controlled by antibiotic treatment if necessary.

[0126] Advantages of the use of BPZE1 include the relatively low production costs, making it especially attractive for developing countries, its needle-free easy and safe mode of administration and its potential to induce mucosal immunity in addition to systemic immunity. Although the role of mucosal immunity against *pertussis* has surprisingly not been much addressed, the fact that *B. pertussis* is a strictly mucosal pathogen, makes it likely that mucosal immune responses may contribute significantly to protection. None of the currently available vaccines induces any significant mucosal response.

[0127] Other advantages of the use of BPZE1 in vaccination are:

- the rapid protective immune response obtained after a single intranasal dose of BPZE1, since induction of the immunity can be detected 1 week after vaccination,
- an increase of the protective immunity over the at least next two months after vaccination, and
- the complete protective immunity, since a level of protection of more than 99.999% is obtained 2 weeks after vaccination.

[0128] The use of live attenuated *B. pertussis* for mucosal vaccination offers yet another advantage. *B. pertussis* can be used for the presentation of heterologous antigens to the respiratory mucosa (for review see 49). The use of BPZE1 as a heterologous expression platform may thus be helpful for the generation of multivalent vaccines against a variety of respiratory pathogens. However, since intranasal delivery of BPZE1 also induces strong systemic immune responses, as shown here by both the high levels of anti-FHA antibodies and of antigen-specific IFN-γ production, it may also be used for the production of antigens to which systemic immune responses are desired.

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Claims

1. A mutated *Bordetella* strain comprising at least:

   (a) a mutated *pertussis* toxin (*ptx*) gene, wherein the S1 moiety of said mutated *ptx* gene codes for a toxin, which is enzymatically inactive but immunologically not affected,
   (b) a deleted dermonecrotic (*dnt*) gene or a mutated *dnt* gene, wherein said mutated *dnt* gene is mutated by point mutation or by insertion of a genetic sequence or plasmid interrupting the open reading frame of the *dnt* gene, and wherein said mutated *dnt* gene codes for an enzymatically-inhibited DNT protein, and
   (c) an *E. coli ampG* gene which replaces the *Bordetella ampG* gene,

   wherein said strain expresses less than 5% residual TCT activity.

2. The mutated *Bordetella* strain according to claim 1, wherein said point mutation of the *dnt* gene results in the replacement of Cys-1305 by Ala-1305.

3. The mutated *Bordetella* strain according to claim 1 or 2, which, is a *Bordetella pertussis* strain.

4. The mutated *Bordetella* strain according to claim 3, wherein said enzymatically inactive toxin which keeps its immunogenic properties is obtained by replacing the arginine at position 9 by a lysine and the glutamic acid at position 129 by a glycine.

5. The mutated *Bordetella* strain according to any one of claims 1 to 4, which is a triple mutant strain.

6. The triple mutated *Bordetella* strain according to claim 5, which is a BPZE1 strain deposited with the Collection Nationale de Culture Microorganismes (C.N.C.M.) on March 9, 2006, under number I-3585.

7. The mutated *Bordetella* strain according to any one of claims 1 to 6, which is attenuated.

8. The mutated *Bordetella* strain according to any one of claims 1 to 7, further comprising at least one heterologous nucleic acid sequence encoding an RNA or a protein.

9. The mutated *Bordetella* strain according to claim 8, wherein said at least one heterologous nucleic acid sequence encodes an antigen.

10. The attenuated *Bordetella* strain according to claim 7 for use as a vaccine against infections caused by *Bordetella* species, wherein said *Bordetella* species is/are at least one of *B. pertussis, B. parapertussis,* and *B. bronchiseptica.*

11. The attenuated *Bordetella* strain according to claim 10 for use as a prophylaxis vaccine against infections caused by *Bordetella* species, wherein said *Bordetella* species is/are at least one of *B. pertussis, B. parapertussis,* and *B. bronchiseptica.*

12. An immunogenic composition comprising a mutated *Bordetella* strain according to any one of claims 1 to 7.

13. The immunogenic composition according to claim 12, further comprising a pharmaceutically suitable excipient, vehicle and/or carrier.

14. The immunogenic composition according to claim 12 or 13, further comprising an adjuvant.

15. A vaccine comprising the attenuated *Bordetella* strain of claim 7.

16. A vaccine according to claim 15, formulated for intranasal administration.

17. A kit comprising a vaccine according to claim 15 or 16 and an information leaflet.

18. Use of an attenuated *Bordetella* strain according to claim 7 for the manufacture of a vaccine for the prevention of a *Bordetella* infection, wherein said *Bordetella* species is/are at least one of *B. pertussis, B. parapertussis,* and *B. bronchiseptica.*

19. Use of an attenuated *Bordetella* strain according to claim 7 for the manufacture of a vaccine for the simultaneous prevention against a *B. pertussis* and *B. parapertussis* infection.

20. Use of an attenuated *Bordetella* strain according to claim 18 or 19, wherein the vaccine is administered by subcutaneous (s.c.), intradermal (i.d.), intramuscular (i.m.), intravenous (i.v.), oral or intranasal administration, by injection or by inhalation.

21. Use of an attenuated *Bordetella* strain according to claim 18 or 19, wherein the vaccine is administered intranasally.

22. Use of an attenuated *Bordetella* strain according to claim 18 or 19, wherein the vaccine is administrated to mammals in need of a rapid protective immunity against a *Bordetella* infection.

23. Use of an attenuated *Bordetella* strain according to claim 22, wherein the vaccine is administrated to newborns.

24. Use of an attenuated *Bordetella* strain according to claim 22, wherein the vaccine is administrated to children.

25. Use of an attenuated *Bordetella* strain according to any one of claims 22 to 24, wherein the vaccine is administered intranasally.

26. Use of an attenuated *Bordetella* strain according to any one of claims 22 to 25, wherein the vaccine is administered once in a single dose.

27. Use of an attenuated *Bordetella* strain according to claim 18 or 19, comprising a) administrating a strain according to claim 7; and b) carrying out at least one recall with either the same strain or an acellular vaccine or a combination of both.

28. Use of a mutated *Bordetella* strain comprising at least (a) a mutated *ptx* gene, wherein the S1 moiety of said mutated ptx gene codes for a toxin, which is enzymatically inactive but immunologically not affected, (b) a deleted *dnt* gene or a mutated *dnt* gene, wherein said mutated *dnt* gene is mutated by point mutation or by insertion of a genetic sequence or plasmid interrupting the open reading frame of the *dnt* gene, and wherein said mutated *dnt* gene codes for an enzymatically-inhibited DNT protein, and (c) an *E. coli ampG* gene replacing the *Bordetella ampG* gene, said strain expressing less than 5% residual TCT activity, for the preparation of a multivalent vaccine to treat respiratory diseases.

29. The use of claim 28, wherein said point mutation of the *dnt* gene results in the replacement of Cys-1305 by Ala-1305.

30. Use according to any one of claims 18 to 29, wherein a level of protection against a *Bordetella* infection is more than 95%, preferably more than 99%.

31. Use of an attenuated *Bordetella* strain according to claim 7, for the manufacture of a vaccine to provide a mucosal response and a systemic response to treat *Bordetella* infections in mammals.

32. The mutated *Bordetella* strain according to any one of claims 1 to 9, for use as a vector for expressing at least one heterologous antigen.

Patentansprüche

1. Mutierter *Bordetella-Stamm* umfassend mindestens:

(a) ein mutiertes *Pertussis*-Toxin (*ptx*) Gen, wobei der S1-Anteil des mutierten *ptx* Gens ein Toxin kodiert, das enzymatisch inaktiv aber immunologisch nicht beeinträchtigt ist,
(b) ein deletiertes dermonekrotisches (*dnt*)-Gen oder ein mutiertes *dnt*-Gen, wobei das mutierte *dnt*-Gen durch Punktmutation oder durch Insertion einer genetischen Sequenz oder durch ein Plasmid, welches das offene Leseraster des *dnt*-Gens unterbricht, mutiert ist, und wobei das mutierte *dnt*-Gen ein enzymatisch-inhibiertes DNT-Protein kodiert, und
(c) ein *E. coli ampG*-Gen, welches das *Bordetella ampG*-Gen ersetzt,

wobei der Stamm weniger als 5 % restliche TCT-Aktivität aufweist.

2. Mutierter *Bordetella-Stamm* nach Anspruch 1, wobei die Punktmutation des *dnt* Gens in dem Austausch von Cys-1305 durch Ala-1305 resultiert.

3. Mutierter *Bordetella-Stamm* nach Anspruch 1 oder 2, der ein *Bordetella Pertussis*-Stamm ist.

4. Mutierter *Bordetella-Stamm* nach Anspruch 3, wobei das enzymatisch inaktive Toxin, welches seine immunogenen Eigenschaften behält, durch Austausch des Arginins an Position 9 durch ein Lysin und der Glutaminsäure an Position 129 durch ein Glycin, erhalten wird.

5. Mutierter *Bordetella-Stamm* nach einem beliebigen der Ansprüche 1 bis 4, der ein dreifach mutierter Stamm ist.

6. Dreifach mutierter *Bordetella-Stamm* nach Anspruch 5, der ein BPZE1-Stamm ist, der mit der Collection Nationale de Culture Microorganismes (C.N.C.M.) am 9. März 2006, unter Nummer I-3585 hinterlegt wurde.

7. Mutierter *Bordetella-Stamm* nach einem beliebigen der Ansprüche 1 bis 6, der attenuiert ist.

8. Mutierter *Bordetella-Stamm* nach einem beliebigen der Ansprüche 1 bis 7, des Weiteren umfassend mindestens eine heterologe Nukleinsäure-Sequenz, die eine RNA oder ein Protein kodiert.

9. Mutierter *Bordetella-Stamm* nach Anspruch 8, wobei die mindestens eine heterologe Nukleinsäure-Sequenz ein Antigen kodiert.

10. Attenuierter *Bordetella-Stamm* nach Anspruch 7 zur Verwendung als ein Impfstoff gegen Infektionen, die durch *Bordetella*-Spezies verursacht werden, wobei die *Bordetella*-Spezies mindestens eine von *B. Pertussis, B. Parapertussis,* und *B. Bronchiseptica* ist/sind.

11. Attenuierter *Bordetella-Stamm* nach Anspruch 10 zur Verwendung als ein Prophylaxis-Impfstoff gegen Infektionen, die durch *Bordetella*-Spezies verursacht werden, wobei die *Bordetella*-Spezies mindestens eine von *B. Pertussis, B. Parapertussis,* und *B. Bronchiseptica* ist/sind.

12. Immunogene Zusammensetzung umfassend einen mutierten *Bordetella-Stamm* gemäß einem beliebigen der Ansprüche 1 bis 7.

13. Immunogene Zusammensetzung nach Anspruch 12, des Weiteren umfassend einen pharmazeutisch-verträglichen Hilfsstoff, ein Vehikel und/oder einen Träger.

14. Immunogene Zusammensetzung nach Anspruch 12 oder 13, des Weiteren umfassend ein Adjuvans.

15. Impfstoff umfassend den attenuierten *Bordetella-Stamm* gemäß Anspruch 7.

16. Impfstoff gemäß Anspruch 15, für intranasale Verabreichung formuliert.

17. Kit umfassend einen Impfstoff gemäß Anspruch 15 oder 16 und einen Beipackzettel.

18. Verwendung eines attenuierten *Bordetella-Stamms* gemäß Anspruch 7 für die Herstellung eines Impfstoffs für die Prävention einer *Bordetella*-Infektion, wobei die *Bordetella*-Spezies mindestens eine von *B. Pertussis, B. Parapertussis,* und *B. Bronchiseptica* ist/sind.

19. Verwendung eines attenuierten *Bordetella-Stamms* gemäß Anspruch 7 für die Herstellung eines Impfstoffs für die gleichzeitige Prävention gegen *B. Pertussis* und *B. Parapertussis*-Infektion.

20. Verwendung eines attenuierten *Bordetella-Stamms* nach Anspruch 18 oder 19, wobei der Impfstoff durch subkutane (s.c.), intradermale (i.d.), intramuskuläre (i.m.), intravenöse (i.v.), orale oder intranasale Verabreichung, durch Injektion oder durch Inhalation verabreicht wird.

21. Verwendung eines attenuierten *Bordetella-Stamms* nach Anspruch 18 oder 19, wobei der Impfstoff intranasal ver-

abreicht wird.

22. Verwendung eines attenuierten *Bordetella-Stamms* nach Anspruch 18 oder 19, wobei der Impfstoff an Säuger verabreicht wird, die eine schnelle Schutzimmunität gegenüber einer *Bordetella*-Infektion benötigen.

23. Verwendung eines attenuierten *Bordetella-Stamms* nach Anspruch 22, wobei der Impfstoff an Neugeborene verabreicht wird.

24. Verwendung eines attenuierten *Bordetella-Stamms* nach Anspruch 22, wobei der Impfstoff an Kinder verabreicht wird.

25. Verwendung eines attenuierten *Bordetella-Stamms* nach einem der beliebigen der Ansprüche 22 bis 24, wobei der Impfstoff intranasal verabreicht wird.

26. Verwendung eines attenuierten *Bordetella-Stamms* nach einem der beliebigen der Ansprüche 22 bis 25, wobei der Impfstoff einmal in einer Einzeldosis verabreicht wird.

27. Verwendung eines attenuierten *Bordetella-Stamms* gemäß Anspruch 18 oder 19, umfassend a) Verabreichen eines Stamms gemäß Anspruch 7; und b) Ausführen von mindestens einer Folgeverabreichung mit entweder dem gleichen Stamm oder einem azellulären Impfstoff oder einer Kombination der beiden.

28. Verwendung eines muttierten *Bordetella-Stamms* umfassend mindestens (a) ein mutiertes *ptx*-Gen, wobei der S1-Anteil des mutierten ptx-Gens ein Toxin kodiert, das enzymatisch inaktiv aber immunologisch nicht beeinflusst ist, (b) ein deletiertes *dnt*-Gen oder ein mutiertes *dnt*-Gen, wobei das mutierte *dnt*-Gen durch Punktmutation oder durch Insertion einer genetischen Sequenz oder durch ein Plasmid, welches das offene Leseraster des *dnt*-Gens unterbricht, mutiert ist, und wobei das mutierte *dnt*-Gen ein enzymatisch-inhibiertes DNT-Protein kodiert, und (c) ein *E. coli ampG*-Gen, welches das *Bordetella ampG*-Gen ersetzt, wobei der Stamm weniger als 5 % restliche TCT-Aktivität aufweist, für die Herstellung eines multivalenten Impfstoffs für die Behandlung von respiratorischen Krankheiten.

29. Verwendung nach Anspruch 28, wobei die Punktmutation des *dnt*-Gens in dem Austausch von Cys-1305 durch Ala-1305 resultiert.

30. Verwendung nach einem beliebigen der Ansprüche 18 bis 29, wobei ein Schutzgrad gegen eine *Bordetella*-Infektion höher als 95 %, vorzugsweise höher als 99 % ist.

31. Verwendung eines attenuierten *Bordetella-Stamms* nach Anspruch 7, für die Herstellung eines Impfstoffs, um eine mucosale Antwort und eine systemische Antwort bereitzustellen, um Bordetella-Infektionen in Säugern zu behandeln.

32. Mutierter *Bordetella-Stamm* gemäß einem beliebigen der Ansprüche 1 bis 9, zur Verwendung als ein Vektor für das Exprimieren von mindestens einem heterologen Antigen.

Revendications

1. Souche de *Bordetella* mutée comprenant au moins :

(a) un gène de toxine *pertussis (ptx)* muté, dans lequel la partie S1 dudit gène *ptx* muté code pour une toxine, qui est enzymatiquement inactive mais non immunologiquement affectée,
(b) un gène dermonécrotique (dnt) délété, ou un gène *dnt* muté, dans lequel ledit gène *dnt* muté est muté par mutation ponctuelle ou par insertion d'une séquence génétique ou d'un plasmide interrompant le cadre de lecture ouvert du gène *dnt,* et dans lequel ledit gène *dnt* muté code pour une protéine DNT enzymatiquement inhibée, et
(c) un gène *ampG* de *E. coli* qui remplace le gène *ampG* de *Bordetella,*

dans laquelle ladite souche exprime moins de 5 % d'activité TCT résiduelle.

2. Souche de *Bordetella* mutée selon la revendication 1, dans laquelle ladite mutation ponctuelle du gène *dnt* entraine le remplacement de Cys-1305 par Ala-1305.

3. Souche de *Bordetella* mutée selon la revendication 1 ou 2, qui est une souche de *Bordetella pertussis.*

4. Souche de *Bordetella* mutée selon la revendication 3, dans laquelle ladite toxine enzymatiquement inactive qui conserve ses propriétés immunogènes est obtenue par remplacement de l'arginine en position 9 par une lysine et de l'acide glutamique en position 129 par une glycine.

5. Souche de *Bordetella* mutée selon l'une quelconque des revendications 1 à 4, qui est une souche triplement mutante.

6. Souche de *Bordetella* triplement mutée selon la revendication 5, qui est une souche BPZE1 déposée à la Collection Nationale de Culture de Microorganismes (CNCM) le 9 mars 2006, sous le numéro 1-3585.

7. Souche de *Bordetella* mutée selon l'une quelconque des revendications 1 à 6, qui est atténuée.

8. Souche de *Bordetella* mutée selon l'une quelconque des revendications 1 à 7, comprenant en outre au moins une séquence d'acide nucléique hétérologue codant pour un ARN ou une protéine.

9. Souche de *Bordetella* mutée selon la revendication 8, dans laquelle ladite au moins une séquence d'acide nucléique hétérologue code pour un antigène.

10. Souche atténuée de *Bordetella* selon la revendication 7, destinée à une utilisation en tant que vaccin contre les infections causées par des espèces de *Bordetella,* dans laquelle ladite/lesdites espèce(s) de *Bordetella* est/sont au moins une espèce parmi *B. pertussis, B. parapertussis* et *B. bronchiseptica.*

11. Souche de *Bordetella* atténuée selon la revendication 10, destinée à une utilisation en tant que vaccin prophylactique contre les infections causées par des espèces de *Bordetella,* dans laquelle ladite/lesdites espèce(s) de *Bordetella* est/sont au moins une espèce parmi *B. pertussis, B. parapertussis* et *B. bronchiseptica.*

12. Composition immunogène comprenant une souche de *Bordetella* mutée selon l'une quelconque des revendications 1 à 7.

13. Composition immunogène selon la revendication 12, comprenant en outre un excipient, un véhicule et/ou un support pharmaceutiquement approprié.

14. Composition immunogène selon la revendication 12 ou 13, comprenant en outre un adjuvant.

15. Vaccin comprenant la souche atténuée de *Bordetella* selon la revendication 7.

16. Vaccin selon la revendication 15, formulé pour une administration intranasale.

17. Kit comprenant un vaccin selon la revendication 15 ou 16 et une notice d'information.

18. Utilisation d'une souche atténuée de *Bordetella* selon la revendication 7, pour la fabrication d'un vaccin destiné à la prévention d'une infection par *Bordetella,* dans laquelle ladite/lesdites espèce(s) de *Bordetella* est/sont au moins une espèce parmi *B. pertussis, B. parapertussis* et *B. bronchiseptica.*

19. Utilisation d'une souche atténuée de *Bordetella* selon la revendication 7, pour la fabrication d'un vaccin destiné à la prévention simultanée contre une infection par *B. pertussis* et B. *parapertussis.*

20. Utilisation d'une souche atténuée de *Bordetella* selon la revendication 18 ou 19, dans laquelle le vaccin est administré par voie sous-cutanée (s.c.), intradermique (i.d.), intramusculaire (i.m.), intraveineuse (i.v.), administration orale ou intranasale, par injection ou par inhalation.

21. Utilisation d'une souche atténuée de *Bordetella* selon la revendication 18 ou 19, dans laquelle le vaccin est administré par voie intranasale.

22. Utilisation d'une souche atténuée de *Bordetella* selon la revendication 18 ou 19, dans laquelle le vaccin est administré à des mammifères ayant besoin d'une immunité protectrice rapide contre une infection par *Bordetella.*

23. Utilisation d'une souche atténuée de *Bordetella* selon la revendication 22, dans laquelle le vaccin est administré à des nouveau-nés.

24. Utilisation d'une souche atténuée de *Bordetella* selon la revendication 22, dans laquelle le vaccin est administré à des enfants.

25. Utilisation d'une souche atténuée de *Bordetella* selon l'une quelconque des revendications 22 à 24, dans laquelle le vaccin est administré par voie intranasale.

26. Utilisation d'une souche atténuée de *Bordetella* selon l'une quelconque des revendications 22 à 25, dans laquelle le vaccin est administré une fois en une seule dose.

27. Utilisation d'une souche atténuée de *Bordetella* selon la revendication 18 ou 19, comprenant a) l'administration d'une souche selon la revendication 7 ; et b) la réalisation d'au moins un rappel avec soit la même souche soit un vaccin acellulaire soit une combinaison des deux.

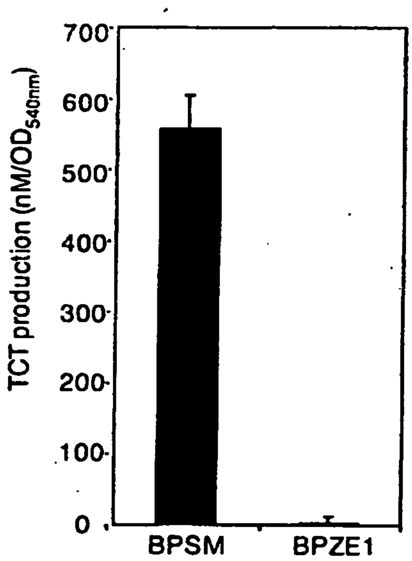
28. Utilisation d'une souche de *Bordetella* mutée comprenant au moins (a) un gène *ptx* muté, dans lequel la partie S1 dudit gène ptx muté code pour une toxine, qui est enzymatiquement inactive mais non immunologiquement affectée, (b) un gène *dnt* délété ou un gène *dnt* muté, dans lequel ledit gène *dnt* muté est muté par mutation ponctuelle ou par insertion d'une séquence génétique ou d'un plasmide interrompant le cadre de lecture ouvert du gène *dnt,* et dans lequel ledit gène *dnt* muté code pour une protéine DNT enzymatiquement inhibée, et (c) un gène *ampG* de *E. coli* remplaçant le gène *ampG* de *Bordetella,* ladite souche exprimant moins de 5 % d'activité TCT résiduelle, pour la préparation d'un vaccin multivalent destiné à traiter des maladies respiratoires.

29. Utilisation selon la revendication 28, dans laquelle ladite mutation ponctuelle du gène *dnt* entraine le remplacement de Cys-1305 par Ala-1305.

30. Utilisation selon l'une quelconque des revendications 18 à 29, dans laquelle un taux de protection contre une infection par *Bordetella* est supérieur à 95 %, de préférence supérieur à 99 %.

31. Utilisation d'une souche atténuée de *Bordetella* selon la revendication 7, pour la fabrication d'un vaccin destiné à procurer une réponse muqueuse et une réponse systémique pour traiter des infections par *Bordetella* chez des mammifères.

32. Souche de *Bordetella* mutée selon l'une quelconque des revendications 1 à 9, destinée à une utilisation en tant que vecteur pour l'expression d'au moins un antigène hétérologue.



TCT production (nM/OD540nm)
700
600
500
400
300
200
100
0
BPSM
BPZE1

Fig. 1

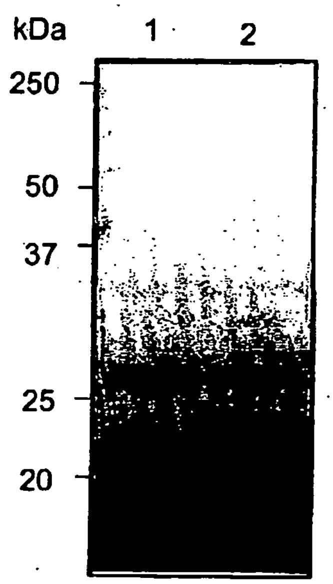


Fig. 2

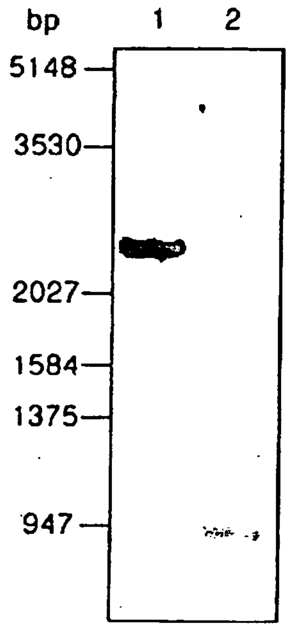


Fig. 3

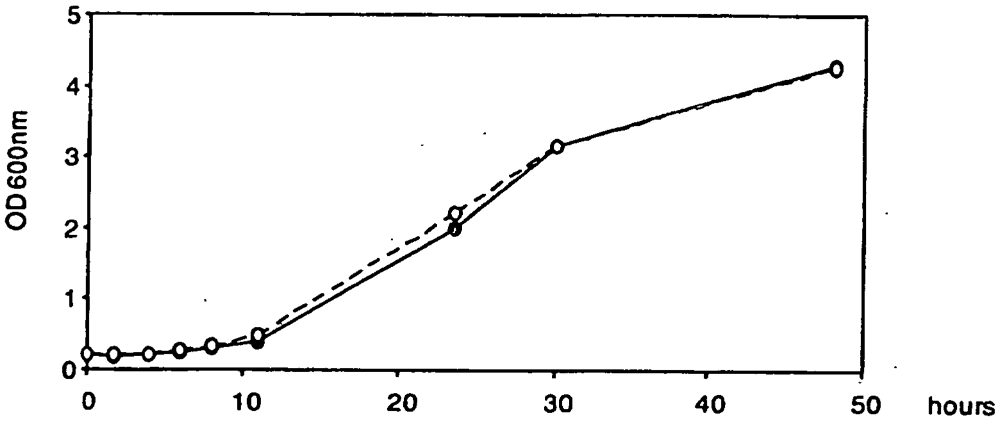


Fig. 4

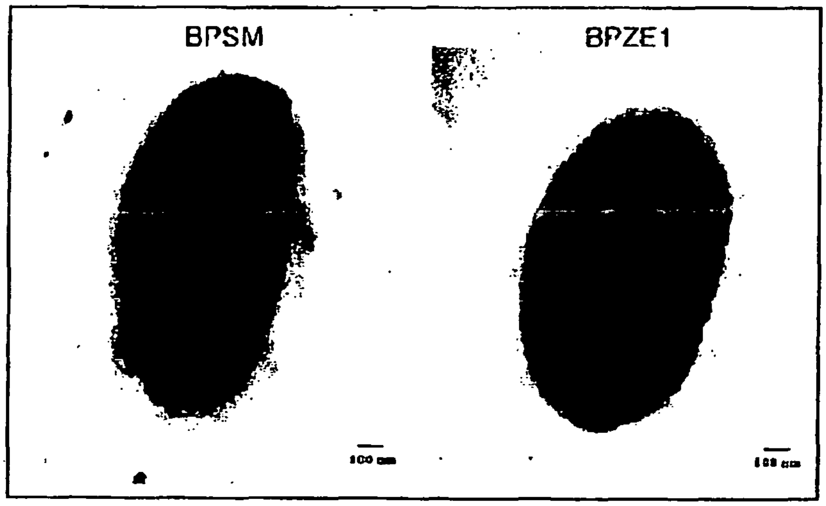


Fig. 5

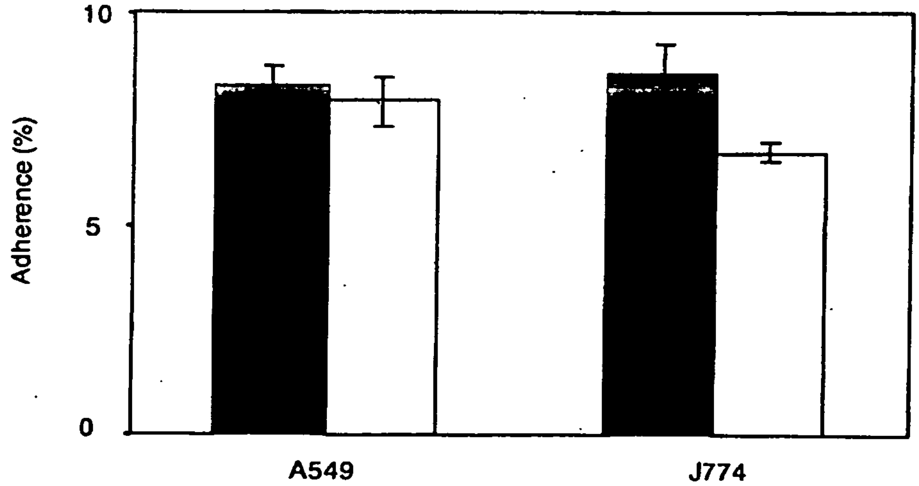


Fig. 6

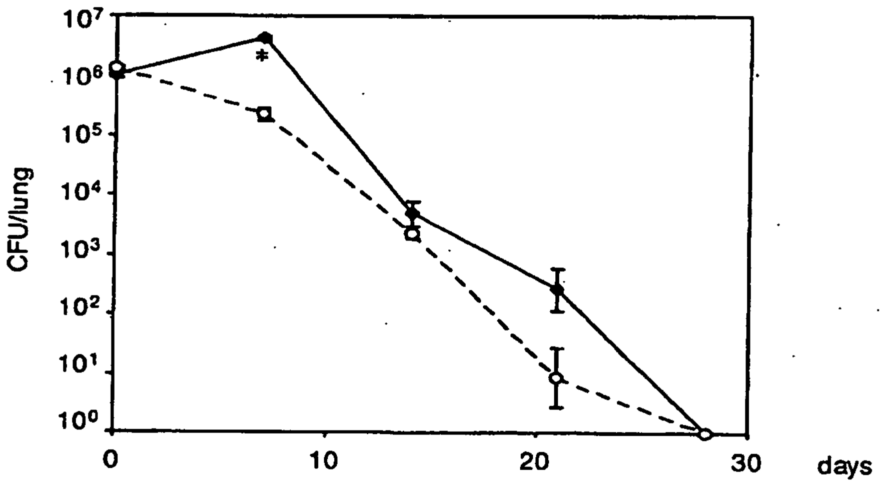


Fig. 7

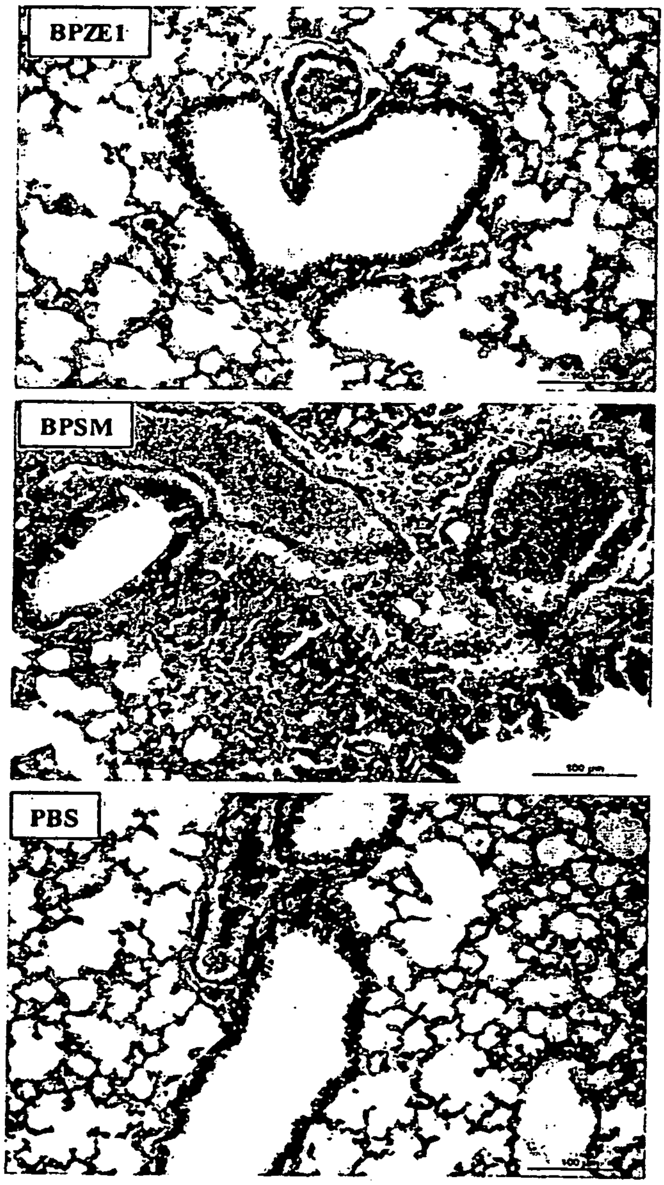


Fig. 8

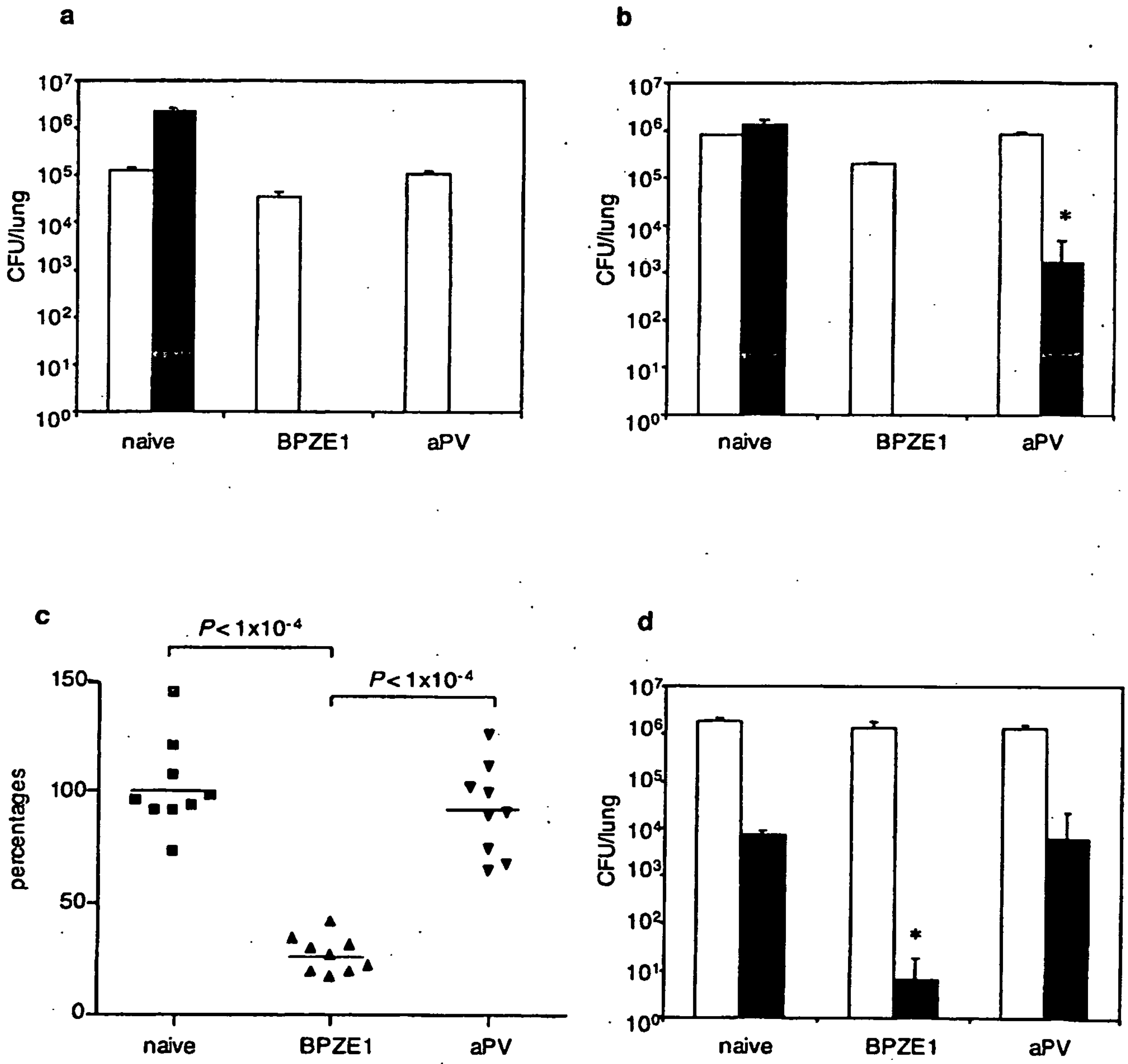

a
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
CFU/lung
naive
BPZE1
aPV
b
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
CFU/lung
*
naive
BPZE1
aPV
c
$P< 1x10^{-4}$
$P< 1x10^{-4}$
150
100
50
0
percentages
naive
BPZE1
aPV
d
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
CFU/lung
*
naive
BPZE1
aPV

Fig. 9

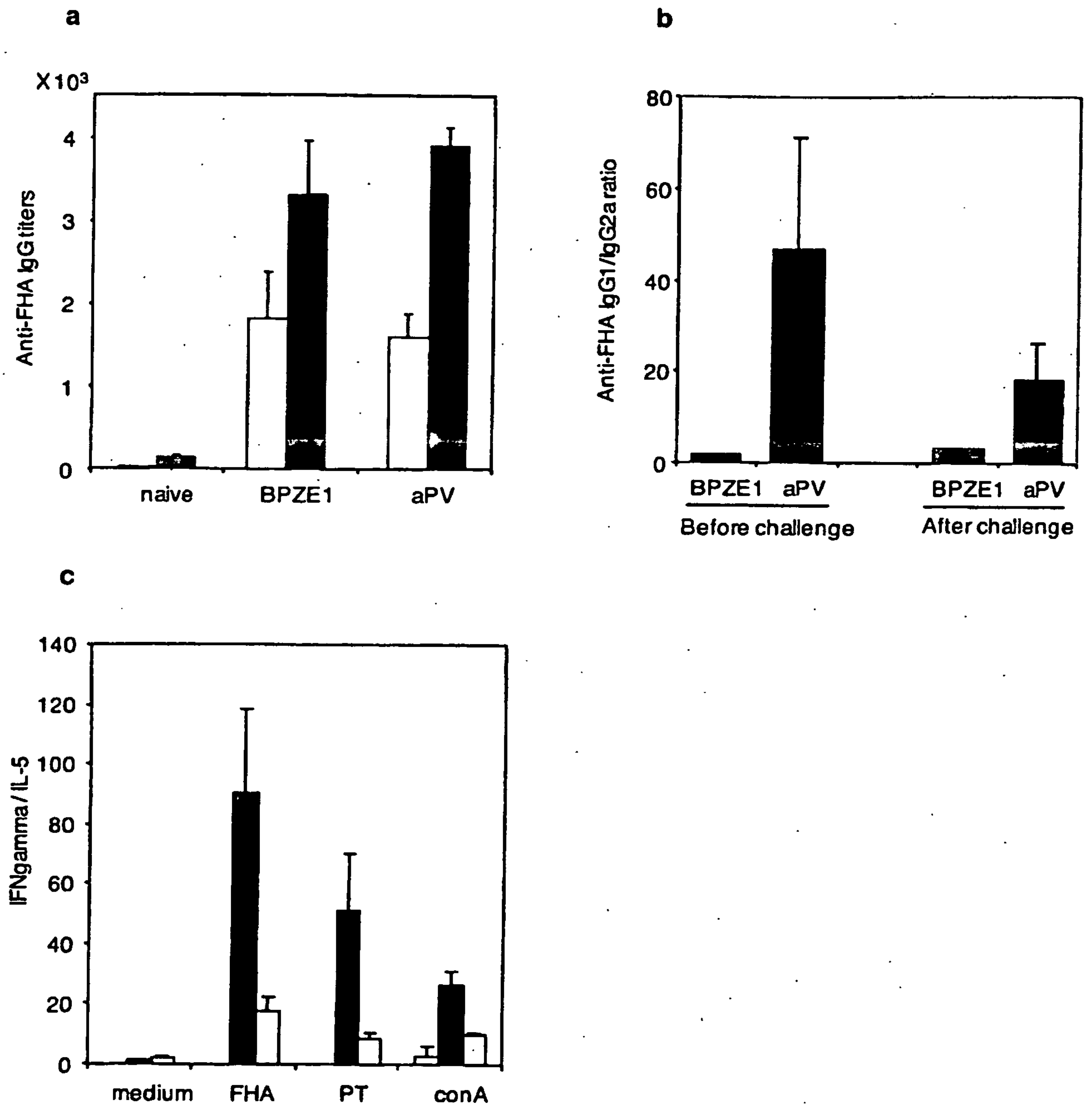

a
X10³
Anti-FHA IgG titers
0
1
2
3
4
naive
BPZE1
aPV
b
Anti-FHA IgG1/IgG2a ratio
0
20
40
60
80
BPZE1
aPV
Before challenge
BPZE1
aPV
After challenge
c
IFNgamma / IL-5
0
20
40
60
80
100
120
140
medium
FHA
PT
conA

Fig. 10

Islet-activating protein S1 (NP_882282)

MRCTRAIRQTARTGWLTWLAILAVTAPVTSPAWADDPPATVYRYDSRPPEDVF
QNGFTAWGNNDNVLDHLTGRSCQVGSSNSAFVSTSSSRRYTEVYLEHRMQEAV
EAERAGRGTGHFIGYIYEVRADNNFYGAASSYFEYVDTYGDNAGRILAGALAT
YQSEYLAHRRIPPENIRRVTRVYHNGITGETTTTEYSNARYVSQQTRANPNPY
TSRRSVASIVGTLVRMAPVIGACMARQAESSEAMAAWSERAGEAMVLVYYESI
AYSF

Fig. 11

Dermonecrotic toxin (NP_881965)

MDKDESALRQLVDMALVGYDGVVEELLALPSEESGDLAGGRAKREKAEFALFS
EAPNGDEPIGQDARTWFYFPKYRPVAVSNLKKMQVAIRARLEPESLILQWLIA
LDVYLGVLIAALSRTVISDLVFEYVKARYEIYYLLNRVPHPLATAYLKRRRQR
PVDRSGRLGSVFEHPLWFAYDELAGTVDLDADIYEQALAESIERRMDGEPDDG
SLDTAEHDVWRLCRDGINRGEQAIFQASGPYGVVADAGYMRTVADLAYADALA
DCLHAQLRIRAQGSVDSPGDEMPRKLDAWEIAKFHLAATQQARVDLLEAAFAL
DYAALRDVRVYGDYRNALALRFIKREALRLLGARRGNASTMPAVAAGEYDEIV
ASGAANDAAYVSMAAALIAGVLCDLESAQRTLPVVLARFRPLGVLARFRRLEQ
ETAGMLLGDQEPEPRGFISFTDFRDSDAFASYAEYAAQFNDYIDQYSILEAQR
LARILALGSRMTVDQWCLPLQKVRHYKVLTSQPGLIARGIENHNRGIEYCLGR
PPLTDLPGLFTMFQLHDSSWLLVSNINGELWSDVLANAEVMQNPTLAALAEPQ
GRFRTGRRTGGWFLGGPATEGPSLRDNYLLKLRQSNPGLDVKKCWYFGYRQEY
RLPAGALGVPLFAVSVALRHSLDDLAAHAKSALYKPSEWQKFAFWIVPFYREI
FFSTQDRSYRVDVGSIVFDSISLLASVFSIGGKLGSFTRTQYGNLRNFVVRQR
IAGLSGQRLWRSVLKELPALIGASGLRLSRSLLVDLYEIFEPVPIRRLVAGFV
SATTVGGRNQAFLRQAFSAASSSAGRTGGQLASEWRMAGVDATGLVESTSGGR
FEGIYTRGLGPLSECTEHFIVESGNAYRVIWDAYTHGWRVVNGRLPPRLTYTV
PVRLNGQGHWETHLDVPGRGGAPEIFGRIRTRNLVALAAEQAAPMRRLLNQAR
RVALRHIDTCRSRLALPRAESDMDAAIRIFFGEPDAGLRQRIGRRLQEVRAYI
GDLSPVNDVLYRAGYDLDDVATLFNAVDRNTSLGRQARMELYLDAIVDLHARL
GYENARFVDLMAFHLLSLGHAATASEVVEAVSPRLLGNVFDISNVAQLERGIG
NPASTGLFVMLGAYSESSPAIFQSFVNDIFPAWRQASGGGPLVWNFGPAAISP
TRLDYANTDIGLLNHGDISPLRARPPLGGRRDIDLPPGLDISFVRYDRPVRMS
APRALDASVFRPVDGPVHGYIQSWTGAEIEYAYGAPAAAREVMLTDNVRIISI
ENGDEGAIGVRVRLDTVPVATPLILTGGSLSGCTTMVGVKEGYLAFYHTGKST
ELGDWATAREGVQALYQAHLAMGYAPISIPAPMRNDDLVSIAATYDRAVIAYL
GKDVPGGGSTRITRHDEGAGSVVSFDYNAAVQASAVPRLGQVYVLISNDGQGA
RAVLLAEDLAWAGSGSALDVLNERLVTLFPAPV

Fig. 12

AmpG protein (NP_878961.1)

MAPLLVLGFASGLPLALSSGTLQAWATVENVSLQSIGFLTLAGTAYTLKFLWA
PLIDRYVPPFLGRRRGWMLLTQVLLAAAIMVMGMLSPGSALLPLALVAVLVAF
LSASQDIAFDAYSTDVLRQEERGAGAAMRVMGYRLAMIVSGGLALIVADRWLG
WGNTYVLMGGLMLACALGTLWAPEPERPANPPRDLGAAVVEPFREFFSRRGAI
DMLLLIVLYKLGDAFAGALSTTFLLRGAGFSATEVGTVNKVLGLAATIVGALA
GGSIMTRWGLYRSLMAFGLLQAVSNLGYWLIAVSPKNLYLMGLAVGVENLCGG
LGTASFVALLMAMCRQQFSATQFALLSALAAVGRTYLAGPLTPVLVEWLDWPG
FFIVTVLIALPGLWLLRLRRNVIDELDAQTAR

Fig. 13

AmpG protein (NP_752478.1)

MSSQYLRIFQQPRSAILLILGFASGLPLALTSGTLQAWMTVENIDLKTIGFFS
LVGQAYVFKFLWSPLMDRYTPPFFGRRRGWLLATQILLLVAIAAMGFLEPGTQ
LRWMAALAVVIAFCSASQDIVFDAWKTDVLPAEERGAGAAISVLGYRLGMLVS
GGLALWLADKWLGWQGMYWLMAALLIPCIIATLLAPEPTDTIPVPKTLEQAVV
APLRDFFGRNNAWLILLLIVLYKLGDAFAMSLTTTFLIRGVGFDAGEVGVVNK
TLGLLATIVGALYGGILMQRLSLFRALLIFGILQGASNAGYWLLSITDKHLYS
MGAAVFFENLCGGMGTSAFVALLMTLCNKSFSATQFALLSALSAVGRVYVGPV
AGWFVEAHGWSTFYLFSVAAAVPGLILLLVCRQTLEYTRVNDNFISRTEYPAG
YAFAMWTLAAGISLLAVWLLLLTMDALDLTHFSFLPALLEVGVLVALSGVVLG
GLLDYLALRKTHLM

Fig. 14

REFERENCES CITED IN THE DESCRIPTION

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