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Fortsættes ...

DESCRIPTION

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[0001] This application is associated with and claims priority from Australian Provisional Patent Application No. 2008900463, filed on 1 February 2008.

FIELD

[0002] The present invention relates generally to the field of allergies. More particularly, the present invention provides a probiotic and a food allergen for use in treating an allergy in a subject by inducing tolerance to an allergen associated with the allergy. Medicinal kits useful in protocols to induce tolerance or reduce intolerance in a subject are also described herein.

BACKGROUND

[0003] Bibliographic details of the publications referred to by author in this specification are collected alphabetically at the end of the description.

[0004] Reference to any prior art in this specification is not, and should not be taken as, an acknowledgment or any form of suggestion that this prior art forms part of the common general knowledge in any country.

[0005] Rates of allergic disease have risen exponentially since 1980. While the prevalence of asthma, eczema and rhinitis may be stabilizing, food allergy and anaphylaxis continue to rise (Gupta et al, Thorax 62(1):91-96, 2007; Robertson et al, Med J Aust 180(6):273-276, 2004). In the UK, hospital admissions for food allergy and anaphylaxis have increased 500% and 700% respectively from 1991 to 2005 (Gupta *et al, supra* 2007). Prevalence of childhood peanut allergy has doubled between 1997 and 2002 (Sicherer et al, J Allergy Clin Immunol 112(6):1203-1207, 2003). Data from the Australian Institute of Health and Welfare show similar trends (Mullins, Med J Aust 186(12):618-621, 2007). Allergic disorders are now the most common chronic diseases affecting children in Western societies. It is estimated that 5%-8% of children have a food allergy (Bock, Pediatrics 79(5):683-688, 1987; Young et al, Lancet 343(8906):1127-1130, 1994), and 1.5% of children have peanut allergy (Grundy et al, J Allergy Clin Immunol 110(5):784-789, 2002).

[0006] Foods are the commonest triggers of severe allergic reactions (anaphylaxis) [Kemp et al, Arch Intern Med 155(16):1749-1754, 1995]. Peanut allergy is of particular concern as reactions to peanuts are usually severe, involving two or more organ systems in 41% of peanut allergic subjects, and involving the respiratory system (anaphylaxis) in 42% of peanut allergic subjects (Sicherer et al, Pediatrics 102(1):199-205, 1998). Reactions to peanuts caused 27% (Pumphrey, Curr Opin Allergy Cin Immunol 4(4):285-290, 2004) to 30% (Bock et al, J Allergy Clin Immunol 107(1):191-193, 2001) of deaths from food induced anaphylaxis. The threshold dose for reaction to peanut is often low - subjective and objective symptoms may be induced by as little as 100µg (<1/1000$_{th}$ of a peanut) and 2mg of peanut protein (< 1/100$_{th}$ of a peanut), respectively (Hourihane et al, J Allergy Clin Immunol 100(5):596-600, 1997). In double blind placebo controlled peanut challenges, 50% of peanut allergic subjects reacted to 3mg of peanut protein (1/100$_{th}$ of a peanut) [Wensing et al, J Allergy Clin Immunol 110(6):915-920, 2002]. Furthermore, subjects with severe reactions to peanut tend to react to lower doses of peanut than those with mild symptoms (Wensing *et al*, *supra* 2002). Therefore, most allergic reactions to peanut are severe, reactions may occur to low doses of allergen, and peanut induced reactions account for a large proportion of deaths from food allergy.

[0007] Most cases of peanut allergy first present in early childhood between the ages of 14 and 24 months (Sicherer *et al*, *supra* 1998). Unlike allergy to milk and egg which generally resolve by late childhood, peanut allergy usually persists. Only 18% (Hourihane et al, Bmj 316(7140):1271-1275, 1998) to 21% (Skolnick et al, J Allergy Clin Immunol 107(2):367-374, 2001) of children outgrow their peanut allergy (spontaneous development of tolerance), and there are no reliable predictors for resolution (Skolnick *et al, supra* 2001; Hourihane *et al, supra* 1998). Accidental ingestions of peanut in children with peanut allergy are common - 50% within 1 year and 75% within 5 years (Bock and Atkins, J Allergy Clin Immunol 83(5):900-904, 1989). Most reactions from accidental ingestion are life threatening (Vander Leek et al, J Pediatr 137(6):749-755, 2000). Only 25% of peanut allergic patients were able to achieve complete avoidance without reaction in a five year period (Bock and Atkins, *supra* 1989). Therefore, patients with peanut allergy remain at significant ongoing risk of severe reactions.

[0008] There has been no effective long term treatment for food allergy. Management involves avoidance of the food concerned, early recognition of symptoms of an allergic reaction and initiation of appropriate emergency treatment of allergic reactions, particularly anaphylaxis. Adrenaline is the first line therapy for anaphylaxis and is available as a self injectable device, the EpiPen (Registered) / EpiPen Jr (Registered) in Australia (and other devices in USA). The EpiPen (Registered) or EpiPen Jr (Registered) must be replaced regularly (12-18 months) and requires specific training in its use (Mehr et al, Paediatr Allergy Immunol 18(5):448-452, 2006). As the majority of reactions to peanut are severe, most children with peanut allergy are prescribed an EpiPen (Registered) which must be carried with them at all times. The EpiPen (Registered) should be administered if accidental exposure results in a severe reaction involving the respiratory or cardiovascular systems (anaphylaxis). However, most patients who have been prescribed an EpiPen (Registered) fail to use it at the time of a severe allergic reaction. Only 71% of patients prescribed an EpiPen had their EpiPen with them, 10% of these had expired, and only 32% were able to demonstrate its correct use (Sicherer et al, Pediatrics 105(2):359-362, 2000). The burden of living with peanut allergy and its management is significant - children with peanut allergy are reported by their parents to have a poorer quality of life than children with rheumatological conditions (Primeau et al, Clin Exp Allergy 30(8):1135-1143, 2000). Therefore, for peanut allergy, the high risk of repeated severe life-threatening reactions and the limited reliability of EpiPen (Registered) being used for the treatment of acute reactions in the community highlight the need for long term treatment options that can achieve immune modulation and tolerance.

[0009] The mechanisms leading to the development of food allergy remain poorly understood. It is considered that food allergy is caused by a failure of oral tolerance. Oral tolerance can be induced by either a single high dose exposure to antigen or by repeated low dose exposures to antigen. High dose tolerance involves Fas-mediated apoptosis or anergy, while low dose tolerance is mediated by regulatory T cells (Treg). Recent studies suggest that anergy and induction of Treg may not be distinct mechanisms for tolerance, and most studies now focus on the role of Treg (reviewed in [Strobel and Mowat, Curr Opin Allergy Clin Immunol 6(3):207-213, 2006]). Several Treg subsets have been identified including Th3 cells, Tr1 cells, and CD4+CD25+ Treg. Th3 cells produce TGFβ and variable amounts of IL-4 and IL-10 (Chen et al, Science 265(5176):1237-1240, 1994). Tr1 cells secrete IL-10 (Groux et al, Nature 389(6652):737-742, 1997). CD4+CD25+ Treg express the transcription factor forkhead box P3 (FOXP3) and mediate their suppressive effects in part by cell surface bound TGFβ and to a lesser extent IL-10 (Chung et al, J Leukoc Biol 77(6):906-913, 2005). CD4+CD25+ Treg arise predominantly in the thymus, but may also develop in mesenteric lymph nodes, Peyer's patches and peripheral lymph nodes where they play a role in mucosal tolerance (Chung *et al, supra* 2005). Treg and the regulatory cytokines TGFβ and IL-10 have been shown to play important roles in oral tolerance induction and in food allergy. In a mouse model of food allergy, mice tolerized to β-lactoglobulin had higher numbers of antigen specific IgA secreting cells in Peyer's patches and higher levels of fecal IgA, as well as increased TGFβ and IL-10 production by Peyer's patch T cells as compared to sensitized mice (Frossard et al, J Allergy Clin Immunol 114(2):377-382, 2004).

[0010] Evidence of a role for Treg in tolerance induction and food allergy is also observed in human studies. Children with food allergy have fewer TGF_+ lymphocytes in the duodenal epithelium and lamina propria (Perez-Machado et al, Eur J Immunol 33(8):2307-2315, 2003), and show reduced TGF expression by milk specific duodenal lymphocytes (Beyer et al, J Allergy Clin Immunol 109(4):7070-713, 2002). Similar findings have been reported for patients with non-IgE mediated food allergies (food protein induced enterocolitis) [Chung et al, J Allergy Clin Immunol 109(1):150-154, 2002]. In subjects with cow's milk allergy, resolution of allergy was associated with increased numbers of CD4+CD25+ T cells and reduced β-lactoglobulin induced proliferation compared to those with ongoing allergy (Karlsson et al, J Exp Med 199(12):1679-1688, 2004). *In vitro* depletion of these CD4+CD25+ cells led to increased β-lactoglobulin induced proliferation suggesting that induction of oral tolerance was related to increased CD4+CD25+ cells Treg (Karlsson *et al, supra* 2004). Oral tolerance is also associated with increased IFNγ (Tureanu et al, J Clin Invest 111(7):1065-1072, 2003). Comparison of peanut specific immune responses in normal children, children with peanut allergy, and peanut allergic children who had outgrown their allergy showed Th2 skewed responses in peanut allergy and Th1 skewed responses in oral tolerance (normal children and children who outgrew their peanut allergy) [Tureanu *et al*, *supra* 2003). These findings suggest that food allergy is associated with loss of tolerance, reduced Treg and TGFβ, as well as reduced Th1 and increased Th2 responses.

[0011] Immunotherapy is used for the long term treatment of asthma, allergic rhinitis and insect venom anaphylaxis. Subcutaneous immunotherapy (SCIT) has been shown to reduce clinical symptoms and induce prolonged tolerance to allergens by modulation of immune responses (Norman, J Allergy Clin Immunol 113(6):1013-1023, 2004 ; Schmidt-Weber and Blaser, Spinger Semin Immunopathol 25(3-4):377-390, 2004). Mechanistic studies have shown that SCIT induces Treg and restores the disturbed balance of Th1/Th2 effector cells in allergic patients. SCIT leads to reduced allergen specific IgE, elevated allergen specific IgG4, reduced Th2 cytokine expression (IL-4, IL-5), and in most studies increased Th1 cytokine expression (IFNγ) [Norman, *supra* 2004; Schmidt-Weber and Blaser, *supra* 2004]. These effects have been shown to be mediated by increased numbers of CD4+CD25+ Treg, and induction of antigen specific CD4+CD25+ Treg with suppressive activity that is mediated by production of IL-10 and/or TGFβ (Norman, *supra* 2004; Schmidt-Weber and Blaser, *supra* 2004). Other immunological effects of

SCIT include increased apoptosis of allergen specific Th2 cells, reduced tissue mast cell numbers and reduced serum levels of TNFα and IL-1β (Norman, *supra* 2004). Sublingual immunotherapy (SLIT) has also been shown to be effective in reducing clinical symptoms in respiratory allergy (asthma, rhinitis), however, immunological effects are less well characterized. Increased specific IgG4 and reduced specific IgE have been reported in some but not all studies (Norman, *supra* 2004). Oral immunotherapy (OIT) has not been consistently effective when used for the treatment of respiratory allergy and was largely abandoned for treatment of these conditions.

[0012] Various immunotherapy approaches have been attempted for the treatment of food allergy. Treatment with a humanized anti-IgE antibody was shown to increase the threshold dose required to induce a reaction, however, this approach is expensive and only provides a short term benefit without modifying the natural history of disease (Leung et al, N Engl J Med 348(11):986-993, 2003). SCIT for peanut anaphylaxis was effective in inducing desensitization and increasing the threshold dose required to induce a reaction (from 178mg to 2805mg, or from half a peanut to nine peanuts) in subjects who were able to continue on maintenance therapy (Nelson et al, J Allergy Clin Immunol 99(6 Ptl):744-751, 1997). However, serious systemic reactions were frequent (39% during maintenance) and this approach has been abandoned. Peptide and mutated protein SCIT are being investigated to avoid systemic reactions, however, translation to the clinic setting has been slow. SLIT has been used for the treatment of food allergy. A double blind placebo controlled study of SLIT with hazelnut extract for four months in 41 adults with hazelnut allergy resulted in an increased threshold for reaction in the active treatment group (from 2.29g to 11.56g) but not the placebo group (3.49g to 4.14g). 50% of the treatment group as compared to 9% of the placebo group were able to tolerate 20g of hazelnut during oral challenge performed 8-12 weeks after immunotherapy had been discontinued, indicating long-lasting tolerance. As further evidence of immune tolerance, the active treatment group demonstrated increased serum levels of IL-10 and hazelnut specific IgG. SLIT with fresh kiwi pulp also resulted in prolonged clinical tolerance to kiwi in a 29 year old female who demonstrated protective effects from SLIT even after it had been discontinued for a period of four months (Kerzl et al, J Allergy Clin Immunol 119(2):507-508, 2007). These findings confirm the potential for SLIT as a treatment for food allergy in adults with evidence of immunomodulatory effects and prolonged clinical protection.

[0013] However, a major disadvantage of SLIT limiting its applicability in children is the need to hold the extract under the tongue for a period of time (1-3 minutes) before swallowing or discharging (Enrique et al, J Allergy Clin Immunol 116(5): 1073-1079, 2005; Kerzl *et al*, *supra* 2007). OIT offers the advantage of improved acceptability and compliance in children (Buchanan et al, J Allergy Clin Immunol 119(1):199-205, 2007).

[0014] OIT has been used successfully for the treatment of food allergy. Case reports describe desensitization with OIT in milk allergy (Nucera et al, Dig Dis Sci 45(3):637-641, 2000; Bauer et al, Allergy 54(8):894-895, 1999). A 12 year old girl was desensitized to cow's milk and remained on OIT indefinitely (Bauer *et al*, *supra* 1999). A six year old girl with cow's milk allergy was desensitized to milk following four months of milk OIT, and experienced dramatic immunological changes including complete loss of SPT reaction to cow's milk, reduced serum levels of milk specific IgE, increased serum levels of milk specific IgG4 and IgA, as well as increased IFNγ and decreased IL-4 production in β-lactoglobulin stimulated PBMC cultures (Nucera *et al*, *supra* 2000). This suggests that OIT may induce tolerance in some circumstances. A large case control study of OIT in 51 patients aged 3 - 55 years with various food allergies showed successful desensitization in 83% (45/54) of subjects who remained on daily OIT (Patriarca et al, Aliment Pharmacol Ther 17(3):459-465, 2003). A reduction in peanut specific IgE and increase in peanut specific IgG4 was demonstrated suggesting the possibility of tolerance induction but this was not examined specifically (Patriarca *et al*, *supra* 2003). A double blind RCT of milk OIT (200ml maintenance dose) for six months in 21 children with milk allergy reported successful desensitization to milk in 71% (15/21) [tolerated 200 ml of milk on a daily basis], and partial desensitization in 3/21 (14%) [tolerated 40-80 ml of milk] (Meglio et al, Allergy 59(9):980-987, 2004). None of the children demonstrated a reduction in milk specific IgE suggesting that tolerance was not achieved. In all of these previous studies, it is not certain whether OIT was effective in inducing tolerance since DBPC food challenges were not performed after immunotherapy was discontinued. Rolinck-Werninghaus reported two patients in whom discontinuation of milk or egg OIT following 37 wk and 41 wk of OIT respectively resulted in loss of desensitization, indicating that tolerance had not been achieved (Rolinck-Werninghaus et al, Allergy 60(10):1320-1322, 2005).

[0015] Studies and investigations aimed at developing protocols to manage allergic disorders have been focused on prevention rather than treatment and have been based on animal models which poorly replicate the human allergic disease condition (e.g: Schabussora and Widermann, Curr Opin Allergy Clin Immunol 8(6):557-564, 2008; Daniel et al, Allergy 52(11):1237-1242, 2007; and Shida et al, Clin Exp Allergy 32:563-570, 2002). Neither a prevention nor treatment protocol based on probiotics or prebiotics alone has achieved large scale success. Initial studies of probiotics or prebiotics for the prevention of eczema had provided promising results (Osborne and Sinn, *Probiotics is infants for prevention of allergic disease and food hypersensitivity (Review),* Cochrane Database *Syst Rev* Art No. CD006475, 2007; Osborne and Sinn, *Probiotics in infants for prevention of allergic disease and food hypersensitivity (Review),* Cochrane Database *Syst Rev* Art No. CD006474, 2007). However, probiotics for the treatment of eczema has not proven successful as have the use of probiotics or prebiotics alone for the prevention or treatment of food

allergy (Boyle et al, Syst Rev, October 8, 2008 Issue 4, CD006135). Pan et al. J Allerg Clin Immunol 117: S327, 2006 compares the ability of two compositions to induce immunological tolerance to peanuts: one containing a probiotic food supplement (ImmuSoy) and the second one being LGG (*Lactobacillus rhamnosus* GG). The ability of *Lactobacillus* to manage allergy to cow's milk is disclosed in Majamaa et al. J Allerg Clin Immunol, 99(2): 179-185, 1997. In the experiments reported LGG was used in combination with allergen elimination. US 7060687 B2 uses transformed *Lactobacillus* or *Streptococcus* bacteria to induce immunological tolerance to airborne allegens.

[0016] New strategies to treat allergies which enhance tolerance induction are required.

SUMMARY

[0017] The present invention contemplates the use of allergen immunotherapy (in particular a food allergen) and probiotic agents to induce tolerance in subjects, such as humans and non-human animals, to the allergen. "Allergen immunotherapy" includes the administration of the allergen or an antigen component or modified form thereof by any means such as by oral, subcutaneous, sublingual, inhalation, intravenous, rectal or intraperitoneal means.

[0018] The probiotic agent to be used in the invention is a probiotic agent selected from the list consisting of a species of *Lactobacillus, Bifidobacterium, Escherichia, Bacillus, Saccharomyces* and/or *Streptococcus.* In one embodiment, the probiotic agent is *Lactobacillus rhamnosus.*

[0019] The allergen immunotherapy and probiotic agent(s) may be sequentially administered or given simultaneously. Reference to "administration" includes sequential or simultaneous administration of the allergen and probiotic.

[0020] The allergen includes *inter alia* any food allergens. Food allergies such as to milk, eggs, legumes (e.g. peanuts), tree nuts, fish, shellfish, soy and wheat and bread are particularly contemplated herein.

[0021] Hence, use protocols to induce allergen tolerance or reduce intolerance in a subject in need thereof forms part of the present invention. The term "induce tolerance" includes reducing sensitivity to an allergen and reducing sensitivity to an allergy. In particular, the present invention is directed to reducing intolerance to a food allergen by the sequential or simultaneously administration of a food allergen and a probiotic to a subject in need of treatment.

[0022] Accordingly, the present invention provides a probiotic and a food allergen for use in a) treating intolerance to said allergen in a subject wherein said probiotic and food allergen are to administered sequentially or simultaneously to said subject; b) treating an allergy induced by the allergen in a subject by inducing tolerance to said allergen, wherein said probiotic is to be used in combination with said food allergen; or c) inducing tolerance to said allergen in a subject wherein said probiotic and food allergen are to be administered sequentially or simultaneously to said subject; wherein the probiotic in (a), (b) and (c) is selected from the list consisting of a species of *Lactobacillus, Bifidobacterium, Escherichia, Saccharomyces, Streptococcus* and *Bacillus.*

[0023] The present invention also provides the use of a probiotic and a food allergen in the manufacture of a medicament to a) treat intolerance to said allergen in a subject wherein said probiotic and food allergen are to administered sequentially or simultaneously to said subject; b) treat an allergy induced by the allergen in a subject by inducing tolerance to said allergen, wherein said probiotic is to be used in combination with said food allergen; or c) induce tolerance to said allergen in a subject wherein said probiotic and food allergen are to be administered sequentially or simultaneously to said subject; wherein the probiotic in (a), (b) and (c) is selected from the list consisting of a species of *Lactobacillus, Bifidobacterium, Escherichia, Saccharomyces, Streptococcus* and *Bacillus.*

[0024] Whilst not intending to limit the present invention to any one theory or mode of action, the combined effect of the probiotic agent with allergen immunotherapy is proposed to increase Th1 responses compared to Th2 responses.

[0025] By 'subject" is meant a human or non-human animal such as a companion animal, livestock animal or captured wild animal. The subject is generally in need of treatment.

DETAILED DESCRIPTION

[0026] Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as

"comprises" or "comprising", is understood to imply the inclusion of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers.

[0027] As used in the subject specification, the singular forms "a", "an" and "the" include plural aspects unless the context clearly dictates otherwise. Thus, for example, reference to "a biotic agent" includes a single biotic agent, as well as two or more biotic agents (which includes two or more probiotics or prebiotics or a probiotic and a prebiotic); reference to "an allergen" includes a single allergen, as well as two or more allergens; reference to "the invention" includes single and multiple aspects of an invention; and so forth.

[0028] The present application discloses a medicinal protocol for treating a subject with an allergy by generating tolerance in the subject to an allergen. The protocol comprises providing the subject with a biotic agent and the allergen or a modified form thereof to which tolerance is desired. In particular the present application provides a probiotic and a food allergen for use in treating an allergy induced by the allergen in a subject by inducing tolerance to said allergen, wherein said probiotic is to be used in combination with said food allergen, wherein the probiotic is selected from the list consisting of a species of *Lactobacillus, Bifidobacterium, Escherichia, Saccharomyces, Streptococcus* and *Bacillus.*

[0029] "Inducing tolerance" includes reducing sensitivity to an allergen or an allergen associated with an allergy. Hence, it encompasses reducing sensitivity to an allergy as well as reducing intolerance to an allergen - induced allergy.

[0030] Hence, the present application discloses a method for treating allergen intolerance in a subject, the method comprising administering sequentially or simultaneously to the subject, a biotic and the allergen or an antigenic component or fragment or analog thereof in an amount effective to induce tolerance to the allergen. In particular the present invention provides a probiotic and a food allergen for use in treating intolerance to said allergen in a subject wherein said probiotic and food allergen are to be administered sequentially or simultaneously to said subject, wherein the probiotic is selected from the list consisting of a species of *Lactobacillus*, *Bifidobacterium*, *Escherichia*, *Saccharomyces*, *Streptococcus* and *Bacillus.*

[0031] The allergen is provided to initiate and/or boost and/or maintain an immune response. Reference to an "allergen" includes any substance which is capable of stimulating a typical hypersensitivity reaction in atopic subjects. Allergens contemplated herein include any substance in food, drugs, perfume, plants, the environment or biological systems (e.g. prokaryotic or eukaryotic cells or viruses), as well as chemical allergens. Types of allergens include animal products (e.g. cats, fur and dander, cockroach calyx, dust mite excretion); drugs (e.g. penicillin, sulfonamides, salicylates (also found naturally in numerous fruits), local anaesthetics); foods (e.g. celery, celeriac, corn or maize, eggs (typically albumen, the white), fruit, pumpkin, legumes (e.g. beans, peas, peanuts, soybeans), milk, seafood, sesame, soy, tree nuts (e.g. pecans almonds), wheat, insect stings (e.g. bee sting venom, wasp sting venom, mosquito stings); mold spores, latex, metal; and plant pollens (e.g. grass - ryegrass, timothy-grass, weeds - ragweed, plantago, nettle, Artemisia, vulgaris, chenopodium album, sorrel, trees - birch alder, hazel, hornbeam, aesculus, willow, poplar, platanus, tilia, olea).

[0032] The present invention is directed to food allergens such as found in milk, eggs, peanuts, tree nuts, fish, shellfish, soy and wheat.

[0033] In one embodiment, the present invention is directed to inducing tolerance to legume allergens and in particular peanut allergens.

[0034] Reference to the "allergen" includes the allergen in a purified or substantially purified or isolated form or when incorporated as part of a substance such as food, a biological system, or chemical composition. Furthermore, the allergen to be administered may also be a modified form including an antigenic derivative or component or homologue or chemical analog. An "allergen" encompasses a mixture of allergens as well as genetically modified or chemically modified allergens.

[0035] The present invention is directed to inducing tolerance or reducing sensitivity to a food allergen or an allergy associated with the food allergen as well as reducing intolerance to a food allergen - induced allergy.

[0036] The term "biotic" encompasses both a probiotic and a prebiotic. A probiotic is generally a live eukaryotic or a prokaryotic organism which has a beneficial property when given to a subject. In one aspect, the probiotic complements the existing microflora in the subject. Hence, the probiotic agent is a live microorganism which can confer a health benefit to a host subject. The probiotic agent may be a culture of microorganisms or provided in a dietary supplement or may be freeze dried and reconstituted prior to use. A prebiotic is an agent which facilitates or confers growth, maintenance and/or beneficial properties of or on the subject's microflora. A prebiotic includes an oligosaccharide and soluble or insoluble fibre material. A probiotic and a prebiotic may also be sequentially or simultaneously administered. In the present invention probiotics are used.

[0037] Examples of probiotic agents include species of *Lactobacillus, Escherichia, Bacillus*, *Bifidobacterium*, *Saccharomyces* and *Streptococcus.*

[0038] Particularly useful probiotic agents are from the genus *Lactobacillus* such as *Lactobacillus acidophilus* NCFM, *Lactobacillus casei*, *Lactobacillus casei* Shirota, *Lactobacillus casei* immunitass, *Lactobacillus johnsonii*, *Lactococcus lactis*, *Lactobacillus plantarum, Lactobacillus reuteri, Lactobacillus rhamnosus, Lactobacillus salivarius* and *Lactobacillus helvetirus.*

[0039] *Lactobacillus rhamnosus* GG (LGG) is considered herein to be a particularly useful probiotic agent.

[0040] The microorganisms may be naturally occurring, attenuated or genetically modified to introduce new or to alter existing traits. In one embodiment, the probiotic has been genetically modified to introduce an allergen gene or part or fragment or portion thereof which is expressed to produce recombinant microorganisms which release or expose the subject's immune system to the allergen or an antigenic fragment thereof Hence, the probiotic and allergen may be given to the subject as a single entity. As indicated above, a probiotic and a prebiotic may also be administered, together with the allergen or the allergen may be produced by the probiotic.

[0041] The present application discloses a method for inducing tolerance in a subject to an allergen, the method comprising administering to the subject an amount of allergen or antigenic fragment, component or analog thereof and a biotic agent effective to induce tolerance in the subject to the allergen.

[0042] In particular, the present invention provides a probiotic and a food allergen for use in inducing tolerance in a subject to said allergen, wherein said probiotic and food allergen are to be administered sequentially or simultaneously to said subject, wherein the probiotoic is selected from the list consisting of a species of *Lactobacillus, Bifidobacterium, Escherichia, Saccharomyces*, *Streptococcus* and *Bacillus.*

[0043] In one particular embodiment, the food allergen is a legume such as a peanut.

[0044] One or more allergens may be administered generally at an amount which does not cause distress to the subject such as in the form of anaphylaxis. As indicated above, the legume allergen may also be produced by a probiotic form of the biotic.

[0045] In a particular embodiment, probiotic is a species of *Lactobacillus,* preferably *L. rhamnosus.*

[0046] Another aspect of the present invention, therefore, is a probiotic and a food allergen for use in inducing tolerance in a subject to a legume allergen, wherein said probiotic and legume allergen are to be administered sequentially or simultaneously to said subject. The probiotic agent is selected from the list consisting of *L. acidophilus* NCFM, *L. casei*, *L. casei* Shirota, *L. casei* immunitass, *L. johnsonii*, *L. lactis*, *L. plantarum, L. reuteri, L. rhamnosus, L. salivarius* and *L. helvetirus.*

[0047] The present application further discloses, a method for inducing tolerance in a subject to a legume allergen, the method comprising administering to the subject an amount of the legume allergen or an antigenic fragment, component or analog thereof and a biotic agent is a prebiotic agent selected from the list consisting of an oligosaccharide and a fibre effective to induce tolerance in the subject to the allergen.

[0048] The prebiotic may be an oligosaccharide or a soluble or insoluble fibre.

[0049] The present invention further contemplates, a probiotic and a food allergen for use in inducing tolerance in a subject to a peanut allergen wherein said probiotic and peanut allergen are to be administered sequentially or simultaneously to said subject wherein the probiotic agent selected from the list consisting of *L. acidophilus* NCFM, *L. casei*, *L. casei* Shirota, *L. casei* immunitass, *L. johnsonii, L. lactis*, *L. plantarum, L. reuteri*, *L. rhamnosus, L. salivarius* and *L. helvetirus.*

[0050] The present application also discloses a method for inducing tolerance in a subject to a peanut allergen, the method comprising administering to the subject an amount of the peanut allergen or an antigenic fragment, component or analog thereof and a prebiotic agent selected from the list consisting of an oligosaccharide and a fibre effective to induce tolerance in the subject to the allergen.

[0051] As indicated above, the fibre may be soluble or insoluble.

[0052] A method is also disclosed for reducing a subject's sensitivity to an allergy, the method comprising administering to the subject a biotic agent in conjunction with an allergen associated with the allergy or an antigenic fragment or component or analog of the allergen for a time and under conditions sufficient for a level of tolerance to be induced in the subject.

[0053] The "administering" includes sequential and simultaneous administration of a probiotic and/or prebiotic and allergen.

[0054] Whilst not intending to limit the present invention to any one theory or mode of action, the combined effect of the biotic agent with allergen immunotherapy is proposed to increase Th1 responses compared to Th2 responses.

[0055] The present application also discloses a medicinal kit comprising in compartmental form a first compartment or series of compartments comprising biotic agents and a second compartment or series of compartments comprising an allergen or source of allergens or antigenic fragments, components or analogs thereof with instructions for use.

[0056] The instructions for use include a medicinal protocol to use the biotic agents in conjunction with an allergen or source of allergen to induce tolerance or reduced sensitivity to an allergen.

[0057] An "effective amount" or "therapeutically effective amount" means an amount necessary to at least partially attain the desired immunological effect of tolerance or to delay the onset or inhibit progression or halt altogether, the onset of progression of an allergic response to an allergen in the subject in need of treatment. It is expected that the amount will fall in a relatively broad range that can be determined through routine trials. The "effective amount" relates to the allergen and biotic, individually or combined. Conveniently, administration of at least the allergen includes a "rush" amount followed by a "maintenance" amount. Examples of effective amounts range from 0.05mg to 2000mg per day, week or month. For peanut allergens, 0.1mg to 300mg per day is effective.

[0058] The uses of the present invention may have utility in the treatment of a subject in need thereof rather than prophylaxis. That said, a prophylactic component is contemplated.

[0059] As used herein, the terms "treating" or "treatment" encompass the administration of an agent which induces tolerance to an allergen.

[0060] The present application further discloses a method for inducing a level of tolerance to an allergy in a subject, the method comprising providing to the subject effective amounts of a biotic and an allergen associated with the allergy.

[0061] As indicated above, a single allergen may be provided or multiple allergens are provided.

[0062] The "level" of tolerance includes complete tolerance or an increased threshold in the amount of allergen to which a subject may be exposed prior to inducing an adverse allergic reaction.

[0063] The term "subject" as used herein refers to an animal, particularly a mammal and more particularly a primate including a lower primate and even more particularly, a human who can benefit from the uses of the present invention and methods described herein. Generally, the subject is in need of treatment as the present disclosure is particularly directed to treatment of an allergen-induced allergy. Genetic testing of subjects or embryos *in utero* may also identify subjects at risk of developing an allergy. A subject regardless of whether a human or non-human animal or embryo, may be referred to as an individual, subject, animal, patient, host or recipient. The present invention, therefore, has both human and veterinary applications. For convenience, an "animal" specifically includes livestock animals such as cattle, horses, sheep, pigs, camelids, goats and donkeys. With respect to horses, these include horses used in the racing industry as well as those used recreationally or in the livestock industry. The non-human animal may also include a companion animal such as a dog or cat or captured wild animal.

[0064] The present invention extends to any subject having an allergy or predisposed to an allergic reaction. Hence, the subject may have a family history, genetic trait or predisposition to the development of an allergy and accordingly may be administered doses of the probiotic and allergen to induce some level of tolerance to the allergen.

[0065] The biotic and allergen are given in conjunction with each other. Insofar as the biotic is a probiotic, the allergen or a genetically modified form or fragment thereof, may be produced by the microorganism.

[0066] By "in conjunction" is meant simultaneous administration in the same formulation or in two different formulations *via* the same or different routes or sequential administration by the same or different routes. The term "in conjunction" also includes the use of two or more allergens in the same therapeutic protocol. By "sequential" administration is meant a time difference of from

seconds, minutes, hours or days between the administration of the two types of molecules. The biotic and allergen may be administered in any order. The probiotic form of the biotic may also produce the allergen. The biotic (i.e. probiotic and/or prebiotic) may be sequentially or simultaneously administered with the allergen.

[0067] As used herein "administering" or "administration" or "providing" an agent to a subject includes delivery *via* any route such as oral, subcutaneous, sub lingual, nasal, intravenous, anal or intra-peritoneal routes. The biotic may be given over a period of time prior to the allergen *vice versa*. Alternatively, both agents may be given at approximately the same time.

[0068] Standard formulations may be employed for each or either of the biotic and allergen. As indicated above, the biotic may be in freeze dried form which is then reconstituted prior to use or the biotic may be given as a dietary supplement. The freeze dried formulation may also comprise the allergen in a similar form. The biotic may also be given with a source of allergen such as milk, eggs, bread, soy and the like.

[0069] The present application further discloses diagnostic assays to monitor immune mechanisms underlying tolerance. Examples of immune mechanisms include monitoring IgE, IgG4 and IgA levels as well as regulatory T-cell levels (Tregs).

[0070] The present invention is further described by the following non-limiting Examples.

EXAMPLE 1

Effects of probiotic and allergen,

Subject recruitment, treatment and sample collection

[0071] Eleven healthy non-atopic adults were treated with $1.8x10^{10}$ CFU LGG (Dicoflor, Dicofarm SpA, Rome) daily for 7 days. Ten ml venous blood was harvested prior to treatment and at day 7. Blood samples were collected in polypropylene tubes containing heparinized RPMI tissue-culture medium (Invitrogen, Carlsbad, CA).

Reparation of blood samples and cell culture

[0072] Mononuclear cells were separated by density centrifugation and cryopreserved for future batched analyses (Dunstan et al, J Allergy Clin Immunol 112:1178-1184, 2003). Two million cells/ml were incubated with or without antigen in AIM-V serum free medium (Invitrogen) for 48 h, or in RPMI with 10% v/v autologous plasma for 6 days in proliferation assays. Heat killed LGG (HKL) was prepared by incubating LGG at 75°C for 45 minutes and was used at 5:1 HKL to mononuclear cell ratio. OVA (Sigma, St Louis, MO) was used at 100µg/ml. IFNγ-1b (Boehringer Ingelheim, Germany) and LPS (Sigma) were both used at 10ng/ml. All cell culture reagents were tested for endotoxin contamination (Cape Cod Associates, E. Falmouth, MA). OVA required endotoxin removal over polymyxin B columns prior to use (Pierce, Rockford, IL).

Flow cytometry

[0073] Cell pellets were stained with fluorochrome-conjugated monoclonal antibodies in 50 µl staining volumes. Lineage cocktail-FITC (anti-CD3, 14, 16, 19, 20, 56), HLA-DR-Peridinin chlorogphyll protein (PerCP), CD123-PE and CD11c- Allophycocyanin (APC) were used to identify DC phenotypes as $CD11c^{hi}CD123w^{lo}$ myeloid DC (mDC), $CD123^{hi}CD11c^{lo}$ plasmacytoid DC (pDC) and $CD11c^{lo}CD123w^{lo}$ immature DC (iDC), CD3-APC, CD4-PerCP, CD25-PE-Cy7 (BD Bioscience, San Jose, CA), CD25-FITC and FoxP3-PE (E-Bioscience, San Diego, CA) were used to identify $CD25^{hi}FoxP3^{hi}$ T-cell populations. CFSE was used as a cell tracking dye, and aminostiblbamidine methanesulfonate as a viability dye (Molecular Probes, Eugene, OR). CBMC or PBMC were incubated with fluorochrome-labeled antibodies or isotype controls for 30 minutes, and for intracellular staining cells were subsequently permeabilized, fixed and stained with FoxP3-PE antibody or isotype control (E-Bioscience). Data were acquired on a 4-color LSR III (BD Bioscience) and analyzed with FACSDiva v4.1 software using well defined gating strategies.

ELISA

[0074] Concentrations of IL-10, IFN-γ, IL-13, IL-12p40 and TNF-α were determined in CBMC culture supernatants harvested at 48 h by multiplex cytokine bead assay using a Luminex 100 analyzer (Luminex Corporation, Austin, TX). Anti-cytokine beads and matched anti-cytokine biotinylated reporters were used according to the manufacturer's instructions (Millipore, Billerica, MA). Data were analyzed with Luminex IS 2.3 Software using a five-parameter regression formula to calculate sample concentrations from standard curves. concentrations of TGF-β1 were evaluated using a commercial human TGF-β1 ELISA kit according to the manufacturer's protocol (BD Biosciences). Supernatants were analyzed undiluted in duplicate with recombinant cytokine as a positive and culture medium as a negative control. TGF-β1 concentrations were determined based on a standard curve generated using the KCjunior v1.40.3 program (Bio-Tek Instruments, Winooski, VT) with a four-parameter equation. ELISA data were analyzed both as dichotomous data-detected versus not detected; and as continuous data - mean level in each group.

Real time PCR

[0075] RNA was extracted using the RNAeasy Mini Kit (Qiagen, Hilden, Germany) and reverse transcribed to cDNA using the Superscript First Strand Synthesis System and oligo(dT) primers (Invitrogen). All reactions included a'RT minus' control with no reverse transcriptase to control for the possibility of contaminating DNA. FoxP3 and IL-4 mRNA were quantified by real time PCR using FAM-labeled Taqman Gene Expression Assays on an ABI Prism 7300HT system (Applied Biosystems, Foster City, CA). Eukaryotic translation elongation factor 1 alpha (EEF1A1) which is stably expressed in human mononuclear cell cultures was used as a reference gene (Hamalaninen et al, Anal Biochem 299:63-70, 2001). The mean level of gene expression in cDNA samples was expressed as a ratio to mean EEF1A1 expression.

Statistics

[0076] The clinical trial was designed with a sample size of 250 in order to have 90% power to detect a 40% difference in eczema risk between probiotic and placebo groups. Secondary outcomes included immune outcomes reported herein. All available CBMC samples were evaluated and primary analyses were by intention to treat. Data were assessed using histograms and skewed data were logo transformed. Parametric paired data were analyzed using the paired t-test, and non-parametric paired data using Wilcoxon signed rank test and Sign test. Parametric unpaired data were analyzed using the independent t-test, and non-parametric unpaired data using Mann Whitney U test. Continuous data are presented as arithmetic means ± 1 SEM, or medians with inter-quartile ranges. Categorical data were analyzed using X^2 test or Fisher's exact test. P value <0.05 was considered statistically significant, with due caution in interpreting the results of multiple comparisons. Where the significance of findings was unclear a sensitivity analysis was undertaken by excluding participants in whom: (1) treatment compliance data (returned capsule counts) were not available (n=9); (ii) capsule counts suggested compliance levels <50% (n=2); or (iii) no treatment capsules were taken due to premature delivery between randomization and 36 weeks gestation (n=1). Analyses were performed using SPSS v 16.0 for Windows (SPSS Inc., Chicago, IL).

Effects of LGG treatment on BPMC proliferation

[0077] The effects of orally administered LGG were evaluated in healthy adults. PBMC were harvested from 11 adults prior to and upon completion of 7 days LGG treatment. Treatment was associated with a 30% reduction (95% CI 11 to 50%; P=0.03) in mean $CD4^+$ T-cell proliferative response to heat killed LGG (HKL) compared with proliferative responses from the same subjects before LGG treatment. In contrast, there was no changed in $CD4^+$ T-cell proliferation to OVA (P=0.2) or medium alone (P=0.06) after LGG treatment.

Effects of LGG treatment on DC phenotype

[0078] DC phenotype was investigated in cultured PBMC harvested from adults before and after LGG treatment. Plasmacytoid DC (pDC) increased from 3.20% to 5.29% of total DDC (P=0.02) after LGG treatment, in PBMC cultured for 48 h with HKL. A trend towards increased ratio of pDC to myeloid (mDC) was also seen after LGG treatment in PBMC cultured with HKL (mean ratio

0.36 pre-treatment, 0.58 post-treatment; P=0.07). LGG treatment was not associated with any significant change in DC phenotype in PBMC cultured with OVA or medium alone.

[0079] The data show that oral administration of LGG to healthy adults leads to systemically detectable changes in T-cell proliferative responses and DC phenotype. Both the decrease in $CD4^+$ T-cell proliferation and the increase in pDC numbers in PBMC cultured with HKL are consistent with antigen-specific tolerance induction (Colonna et al, Nat Immunol 5:1219-1226, 2004).

[0080] Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications insofar as they fall within the scope of the appended claims. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features insofar as they fall within the scope of the appended claims.

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PATENRKRAV

1. Probiotikum og fødevareallergen til anvendelse i:

(a) behandling af intolerance over for allergenet hos et individ, hvor probiotikummet og fødevareallergenet er beregnet til at blive administreret sekventielt eller simultant til individet;

(b) behandling af en allergi, der er induceret af allergenet hos et individ, ved at inducere tolerance over for allergenet, hvor probiotikummet er beregnet til at blive anvendt i kombination med fødevareallergenet; eller

(c) induktion af tolerance over for allergenet hos et individ, hvor probiotikummet og fødevareallergenet er beregnet til at blive administreret sekventielt eller simultant til individet;

hvor probiotikummet i (a), (b) og (c) er udvalgt fra listen bestående af en art af *Lactobacillus, Bifidobacterium, Escherichia, Saccharomyces, Streptococcus* og *Bacillus.*

2. Probiotikum og fødevareallergen til anvendelse ifølge krav 1, hvor probiotikummet og fødevareallergenet er beregnet til at blive administreret i to forskellige formuleringer.

3. Probiotikum og fødevareallergen til anvendelse ifølge krav 1 eller 2, hvor probiotikummet er en art af *Lactobacillus.*

4. Probiotikum og fødevareallergen til anvendelse ifølge krav 3, hvor arten af *Lactobacillus* er udvalgt fra listen bestående af *Lactobacillus acidophilus* NCFM, *Lactobacillus casei, Lactobacillus casei* Shirota, *Lactobacillus casei* immunitass, *Lactobacillus johnsonii, Lactococcus lactis, Lactobacillus plantarum, Lactobacillus reuteri, Lactobacillus rhamnosus, Lactobacillus salivarius* og *Lactobacillus helvetirus.*

5. Probiotikum og fødevareallergen til anvendelse ifølge krav 4, hvor probiotikummet er *Lactobacillus rhamnosus.*

6. Probiotikum og fødevareallergen til anvendelse ifølge et hvilket som helst af kravene 1 til 5, hvor fødevareallergenet er et bælgplanteallergen.

7. Probiotikum og fødevareallergen til anvendelse ifølge krav 6, hvor bælgplanteallergenet er et jordnøddeallergen.

8. Probiotikum og fødevareallergen til anvendelse ifølge et hvilket som helst af kravene 1 til 7, hvor individet er et menneske.

9. Anvendelse af et probiotikum og et fødevareallergen i fremstillingen af et medikament til at:

a) behandle intolerance over for allergenet hos et individ, hvor probiotikummet og fødevareallergenet er beregnet til at blive administreret sekventielt eller simultant til individet;

(b) behandle en allergi, der er induceret af allergenet hos et individ, ved at inducere tolerance over for allergenet, hvor probiotikummet er beregnet til at blive anvendt i kombination med fødevareallergenet; eller

(c) inducere tolerance over for allergenet hos et individ, hvor probiotikummet og fødevareallergenet er beregnet til at blive administreret sekventielt eller simultant til individet;

hvor probiotikummet i (a), (b) og (c) er udvalgt fra listen bestående af en art af *Lactobacillus, Bifidobacterium, Escherichia, Saccharomyces, Streptococcus* og *Bacillus.*

10. Anvendelse af et probiotikum og et fødevareallergen ifølge krav 9, hvor probiotikummet er en art af *Lactobacillus.*

11. Anvendelse af et probiotikum og et fødevareallergen ifølge krav 10, hvor arten af *Lactobacillus* er udvalgt fra listen bestående af *Lactobacillus acidophilus* NCFM, *Lactobacillus casei, Lactobacillus casei* Shirota, *Lactobacillus casei* immunitass, *Lactobacillus johnsonii, Lactococcus lactis, Lactobacillus plantarum, Lactobacillus reuteri, Lactobacillus rhamnosus, Lactobacillus salivarius* og *Lactobacillus helvetirus.*

12. Anvendelse af et probiotikum og et fødevareallergen ifølge krav 11, hvor probiotikummet er *Lactobacillus rhamnosus.*

13. Anvendelse af et probiotikum og et fødevareallergen ifølge et hvilket som helst af kravene 9 til 12, hvor fødevareallergenet er et bælgplanteallergen.

14. Anvendelse af et probiotikum og et fødevareallergen ifølge krav 13, hvor bælgplanteallergenet er et jordnøddeallergen.

15. Anvendelse af et probiotikum og et fødevareallergen ifølge et hvilket som helst af kravene 9 til 14, hvor individet er et menneske.