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(54) **IMMUNOMODULATING COMPOSITIONS AND METHODS OF USE THEREOF**

IMMUNMODULIERENDE ZUSAMMENSETZUNGEN UND VERFAHREN ZU IHRER VERWENDUNG

COMPOSITIONS IMMUNOMODULANTES ET LEURS PROCÉDÉS D'UTILISATION

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- **BONALDO ALESSIO ET AL: "The influence of dietary beta-glucans on the adaptive and innate immune responses of European sea bass (Dicentrarchus labrax) vaccinated against vibriosis", ITALIAN JOURNAL OF ANIMAL SCIENCE, vol. 6, no. 2, April 2007 (2007-04), pages 151-164 URL, XP55003746, ISSN: 1594-4077**
- **I. RUBIN-BEJERANO ET AL.: 'Phagocytosis by human neutrophils is stimulated by a unique fungal cell wall component.' CELL HOST MICROBE. vol. 2, no. 1, 12 July 2007, pages 55 - 67, XP008146190**
- **Y. NISHIKAWA ET AL.: 'Polysaccharides in lichens and fungi. III. Further investigation on the structrures and the antitumor activity of the polysacchrides from Gyrophora esculenta Miyoshi and Lasallia papulosa Llano.' CHEM. PHARM. BULL. vol. 17, no. 8, 1969, pages 1910 - 1916, XP008131359**

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Description

BACKGROUND OF THE INVENTION

[0001] The cell walls of fungi evoke a powerful immuno-stimulatory response, and have been proposed for use as potential anti-infective and anti-tumor drugs. Fungal cells can also activate dendritic cells and prime class II restricted antigen specific T cell responses. The majority of the cell wall (50-60%) of pathogenic (*Candida albicans*) and non-pathogenic fungi (*Saccharomyces cerevisiae)* is composed of an inner layer of β-glucan (β-1,3- and (β-1,6-glucan) covalently linked to a variety of cell surface mannoproteins [Klis, F. M. et al. Med Mycol 39 Suppl 1, 1-8, 2001; Klis, F. M. et al., FEMS Microbiol Rev 26, 239-56, 2002].

[0002] Recognition of β-glucans by macrophages is carried out mainly through Dectin-1 with cooperation of TLRs, including TLR2 [Brown, G. D. et al. Nature 413, 36-7, 2001]. Dectin-1 activity is inhibited by β-1,3-glucans and β-1,6-glucans, with the β-1,3-glucan laminarins having the highest effect. However, oligosaccharide microarray results show that Dectin-1 binds specifically to β-1,3-glucans. Neutrophils are professional killers, whose role in phagocytosis and killing of bacteria and fungi is well characterized. Neutropenic individuals are much more susceptible to bacterial and fungal infections, with return to normal counts playing an important role in resolution of infection. Neutrophils, unlike macrophages, require serum for optimal phagocytosis and killing. The main opsonic receptors are the complement receptor CR3 and the immunoglobulin- binding receptor FcγR. CR3 has a lectin domain [Brown, G. D. et al. Immunity 19, 311-5, 2003] that mediates increased neutrophil motility towards a mixture of β-1,3-glucan and β-1,6-glucan (PGG-glucan) [Wakshull, E. et al. Immunopharmacology 41, 89-107, 1999].

[0003] β-1,6-glucans have been found to provide potent anti-fungal activity, and *inter alia*, possess adjuvant activity and activate complement.

[0004] The complement (C) system of humans and other mammals involves more than 20 components that participate in an orderly sequence of reactions resulting in complement activation. Products derived from the activation of C components include non-self recognition molecules C3b, C4b and C5b, as well as the anaphylatoxins C3a, C4a and C5a that influence a variety of cellular immune responses (Hugli et al (1982) 15th International Leucocyte Culture Conference, Asilomar, CA (Abstract); Fujii et al. (1993) Protein Science 2:1301-1312; Morgan et al. (1982) J.Exp.Med. 155:1412-1426; Morgan (1993) Complement Today 1:56-75; Morgan et al. (1983) J.Immunol. 130:1257-1261). Complement activation occurs primarily via the "classical" pathway or the "alternative" pathway. The classical pathway is initiated by the binding of the first complement component (C1) to immune complexes through C1q, a subcomponent involved in binding to antibody. The cl complex is composed of C1q and two homologous serine proteases, C1r and C1s (1:2:2 molar ratio). After binding to the immune complexes C1q undergoes a conformational change resulting in the conversions of C1r and C1s to their activated forms. Activated C1s cleaves C4 and C2 to generate a complex of their fragments C4b2a, which in turn cleaves C3 into C3a and C3b. C3b binds to immune complexes.

[0005] The alternative pathway is activated without involvement of antibody. C3b molecules generated from C3 by interaction of C3 with two serine proteases, factors B and D, are deposited on the microbial surface where activation of C3 is amplified. C3b produced by activation of either pathway acts as a central molecule in the subsequent formation of membrane attack complexes that can lyse microbes and also as an opsonin.

[0006] It is unknown whether β-1,6-glucans produce a robust immune response in all subjects and by what mechanism such response is generated.

[0007] US5480642 discloses immune response regulation to an antigen with polyanionic polysaccharide derivatives: (1) having a molecular weight of between 1,000 and 600,000; (2) selected to correspond to the antigen; (3) not being cytotoxic at an effective dosage; and (4) stimulating a cell-mediated immune response.

[0008] US2006160766 discloses therapeutic compositions for treatment of cancer comprising a glucan composition which is suitable for oral administration and for absorption through the gastrointestinal tract of the mammal, and at least one antibody for the cancer.

[0009] BONALDO ALESSIO et al. ITALIAN JOURNAL OF ANIMAL SCIENCE, vol. 6, no. 2, April 2007 pages 151-164 disclose the influence of dietary beta-glucans on the adaptive and innate immune responses in European sea bass.

[0010] US2005208079 discloses a method for detecting the amount of IgG secreted in response to beta-1,6-glucan, and immunogenic compositions comprising a glucan and a pharmaceutically acceptable carrier.

[0011] RUBIN-BEJERANO et al. CELL HOST MICROBE. vol. 2, no. 1, 12 July 2007, pages 55 - 67 describe the stimulation of neutrophil phagocytosis by beta-1,6-glucan.

[0012] WO2008057501 discloses compositions comprising beta-1,6-glucans for modulating immune responses.

SUMMARY OF THE INVENTION

[0013] This invention provides a diagnostic kit for measuring IgG1, IgG2, IgG3 and IgG4 levels, and thereby determining responsiveness of a subject which has been exposed to environmental glucans to a pharmaceutical composition comprising β-1,6-glucan, comprising: (i) a composition comprising β-1,6-glucan which corresponds to or is a fragment of, or is highly homologous to the glucan in the pharmaceutical composition for which responsiveness is being determined; and (ii) reagents for detecting IgG1, IgG2, IgG3 and IgG4 antibodies

bound to the β-1,6-glucan of step (i) in a blood sample.

[0014] In one embodiment, the β 1-6 glucan is in solution or lyophilized.

[0015] In another embodiment, the β 1-6 glucan is immobilized on a substrate. Said substrate may comprise a material selected from: plastic, glass, gel, celluloid, paper, magnetic resin, polyvinylidene-fluoride, nylon, nitrocellulose, agarose, latex, and polystyrene, or may comprise an ELISA plate, dipstick, microtiter plate, radioimmunoassay plate, beads, agarose beads, plastic beads, latex beads, immunoblot membranes, and immunoblot papers.

[0016] The reagents may be conjugated to a detectable marker. Said detectable marker may be selected from the group consisting of a radioactive label, fluorescent label, chemiluminescent label, chromophoric label, ligand, fluorescein, radioisotope, phosphatase, biotin, biotin-related compound, avidin, avidin-related compound, and peroxidase.

[0017] In another aspect, the invention contemplates the use of the kit of the invention for predicting the robustness of a response to β-1,6-glucans in a subject.

[0018] In another aspect, the invention contemplates the use of the kit of the invention for predicting a subject's responsiveness to glucan-based vaccines or adjuvants.

[0019] Where number ranges are given in this document, endpoints are included within the range.

Furthermore, it is to be understood that unless otherwise indicated or otherwise evident from the context and understanding of one of ordinary skill in the art, values that are expressed as ranges can assume any specific value or subrange within the stated ranges, optionally including or excluding either or both endpoints, in different embodiments of the invention, to the tenth of the unit of the lower limit of the range, unless the context clearly dictates otherwise. Where a percentage is recited in reference to a value that intrinsically has units that are whole numbers, any resulting fraction may be rounded to the nearest whole number.

BRIEF DESCRIPTION OF THE DRAWINGS

[0020]

Figure 1 demonstrates engulfrnent of β-1,6-glucan-coated beads (A) and ROS production (B) are antibody dependent, irrespective of the presence of complement (C).

Figure 2 demonstrates that β-1,6-glucan antibodies are prevalent in normal adult sera.

Figure 3 demonstrates that select IgG isotypes influence responsiveness to β-glucan.

Figure 4 demonstrates that a P- 1,6-glucan-Herceptin conjugate is functional.

Figure 5 demonstrates that a β-1,6-glucan-Herceptin conjugate mediates the killing of cancer cells by complement and neutrophils.

[0021] It will be appreciated that for simplicity and clarity of illustration, elements shown in the figures have not necessarily been drawn to scale. For example, the dimensions of some of the elements may be exaggerated relative to other elements for clarity. Further, where considered appropriate, reference numerals may be repeated among the figures to indicate corresponding or analogous elements.

DETAILED DESCRIPTION OF THE INVENTION

[0022] In the following detailed description, numerous specific details are set forth in order to provide a thorough understanding of the invention. However, it will be understood by those skilled in the art that the present invention may be practiced without these specific details. In other instances, well-known methods, procedures, and components have not been described in detail so as not to obscure the present invention.

[0023] Glucans are polysaccharides found so far in all studied species of lichenized fungi. Partially O-acetylated pustulans are typical of Umbilicariaceae, and have been described for several species of Umbilicaria, such as U. pustulata and U. hirsute, U. angulata, U. caroliniana, and U. polyphylla.

[0024] Responsiveness to β-1,6-glucans, were found by the inventors to be antibody-dependent, and robustness of this response was found to be associated with subjects having particular immunoglobulin G (IgG) isotype expression. Such expression may be useful therefore in predicting a subject's responsiveness to glucan-based vaccines or adjuvants.

[0025] The diagnostic kits of the invention comprise a composition comprising β-1,6-glucan which corresponds to or is a fragment of, or is highly homologous to the glucan in the pharmaceutical composition for which responsiveness is to be determined.

[0026] The composition is, in some embodiments, distinct from compositions such as pustulan or preparations of fungal cell walls. In certain embodiments of the invention at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 98%, or 99% of the glucan contained in the composition by weight is β 1-6 glucan. In certain embodiments between 20% and 50% of the glucan contained in the composition is β 1-6 glucan. In certain embodiments between 50% and 100% of the glucan contained in the composition is β 1-6 glucan. In one embodiment of any of the compositions or methods of the invention, the glucan contains from about 15 % to about 30% by weight β 1-6 glucan. In another embodiment of any of the compositions or methods of the invention, the glucan contains from about 10 % to about 35% by weight β 1-6 glucan, or in another embodiment, from about 20 % to about 50% by weight β 1-6 glucan, or in another embod-

iment, from about 25 % to about 60% by weight β 1-6 glucan, or in another embodiment, from about 35 % to about 80% by weight β 1-6 glucan, or in another embodiment, from about 18 % to about 35% by weight β 1-6 glucan, or in another embodiment, from about 15 % to about 75% by weight β 1-6 glucan. In certain embodiments, said glucan is a mixture of oligomers or polymers, wherein the β-1,6-glucan is greater than about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 98%, or 99% by weight of those oligomers or polymers. In certain embodiments of the invention "weight" refers to "dry weight". In other embodiments "weight" refers to total weight. In certain embodiments of the invention the β 1-6 glucan is processed. Such processing may comprise, for example, deacetylation, treatment with enzymes that digest glucans other than β 1-6 glucan, limited digestion with enzymes that digest β 1-6 glucan, selection of particular molecular weight ranges, etc. In certain embodiments, processing comprises separation from other glucans, e.g., α-glucans, β 1-3 glucans, etc. In certain embodiments the processing comprises removing β 1-6 glucan side chains from β 1-3 glucans and optionally separating the β 1-6 glucans side chains. In certain embodiments the composition comprises processed β 1-6 glucan, wherein the processed β 1-6 glucan exhibits enhanced ability to desirably modulate the immune response relative to unprocessed glucan or relative to unprocessed β 1-6 glucan.

[0027] The β 1-6 glucan may be enriched for O-acetylated groups. For example, the glucan may contain at least 25 % by weight O-acetylated glucan, e.g. from about 15 % to about 30% by weight O-acetylated glucan. In another embodiment, the glucan contains from about 10 % to about 35% by weight O-acetylated glucan, or in another embodiment, from about 20 % to about 50% by weight O-acetylated glucan, or in another embodiment, from about 25 % to about 60% by weight O-acetylated glucan, or in another embodiment, from about 35 % to about 80% by weight O-acetylated glucan, or in another embodiment, from about 18 % to about 35% by weight O-acetylated glucan, or in another embodiment, from about 15 % to about 75% by weight O-acetylated glucan. In other embodiments, the glucan contains between about 75% and 100% by weight O-acetylated glucan, e.g., between 75% and 90%, or between 90% and 100% by weight O-acetylated glucan. In one embodiment, the glucan contains approximately that percentage of O-acetylated glucose units (by weight or number, in various embodiments of the invention) that would result from digestion of a naturally occurring β 1-6 glucan (e.g., pustulan or any other β 1-6 glucan mentioned herein) with a β 1-6 endoglucanase for a time sufficient to digest at least 90% by weight of the β 1-6 glucan to oligosaccharides comprising 5 or fewer glucose units followed by (i) removal of those oligosaccharides comprising 5 or fewer glucose residues from the composition or (ii) isolation of a portion of the resulting composition having a molecular weight greater than 5 kD, or in some embodiment greater than 10, 20, 30, 50, or 100 kD.

[0028] In some embodiments, the term "enriched for O-acetylated residues" refers to the enhanced % of O-acetylated sites in individual glucose units within the glucan molecule, enhanced % of O-acetylated glucose units within the glucan molecule, or a combination thereof, as compared to a native glucan molecule. In one embodiment, reference to glucan preparations enriched by a particular weight percent for O-acetylated glucan, refers to preparations comprising an enhanced % of O-acetylated sites in individual glucose units within the glucan molecule, an enhanced % of O-acetylated glucose units within the glucan molecule, or a combination thereof, as compared to a glucan molecule.

[0029] Glucans derived from different sources may comprise varying amounts of O-acetylation in terms of O-acetylated sites in individual glucose units, O-acetylated glucose units within the glucan molecule, or a combination thereof. According to this aspect of the invention, the term "enriched for O-acetylated glucan" refers, in some embodiments, to enhanced O-acetylation as described herein, between the reference source from which the glucan is derived, and may not represent an overall enrichment as compared to any glucan source.

[0030] The term "enriched for O-acetylated glucan" may refer to an enrichment of at least 25% by weight of the glucan chains, which are O-acetylated on at least one glucose unit, or at least 25% of the glucose units present in the glucan in the composition are O-acetylated, or a combination thereof. In some embodiments, at least 25% of the glucose units in at least 1%, or in another embodiment, at least 5% of the beta glucan chains are O-acetylated. In other embodiments between 25% and 35%, between 25% and 50%, between 25% and 75%, between 15% and 45%, between 20% and 60%, between 35% and 80%, or others of the glucose units in at least 5% of the beta glucan chains are O-acetylated, etc. In other embodiments, embodiments between 25% and 35%, between 25% and 50%, between 25% and 75%, between 15% and 45%, between 20% and 60%, between 35% and 80%, or others of the glucose units, in at least 10% of the beta glucan chains, or in another embodiment, in at least 15% of the beta glucan chains, or in another embodiment, in at least 20% of the beta glucan chains, are O-acetylated.

[0031] In one embodiment, the glucan is isolated or derived from a lichen, which in one embodiment is from the genus Umbilicariaceae. In one embodiment, the glucan is isolated from a fungus. In one embodiment, the glucan is isolated from yeast, or in another embodiment the glucan is chemically synthesized or acetylated. In another embodiment, the glucan is conjugated to a solid support.

[0032] Glucans are glucose-containing polysaccharides found inter alia in fungal cell walls, α-glucans include one or more α-linkages between glucose subunits and β-glucans include one or more β-linkages between glucose subunits.

[0033] β-1,6-glucans occur frequently in fungi but are rarer outside fungi. The glucan used in accordance with the invention comprises β 1 ,6 glucan. In some embodiments, the β-glucans are derived from Umbilicariaceae, such as *U. pustulata* and *U. hirsute, U. angulata, U. caroliniana,* and *U. polyphylla.*

[0034] In some embodiments, the β-glucans are derived from *Candida,* such as *C. albicans*. Other organisms from which β-glucans may be used include *Coccidioides immitis, Trichophyton verrucosum, Blastomyces dermatidis, Cryptococcus neoformans*, *Histoplasma capsulatum, Saccharomyces cerevisiae, Paracoccidioides brasiliensis*, and *Pythiumn insidiosum.* In some embodiments, the β-glucans are chemically or enzymatically synthesized, as is known in the art, or in other embodiments, the β-glucans are derived from any species producing the same, and chemically or enzymatically altered, for example, to increase O-acetylation of the molecule.

[0035] In some embodiments, the β-glucans are fungal glucans. A 'fungal' glucan will generally be obtained from a fungus but, where a particular glucan structure is found in both fungi and non-fungi (e.g., in bacteria, lower plants or algae) then the non-fungal organism may be used as an alternative source.

[0036] Full-length native β-glucans are insoluble and have a molecular weight in the megadalton range. In some embodiments, this invention provides soluble β-1,6-glucan. In some embodiments, this invention provides soluble O-acetylated β-1,6-glucan. Solubilization may be achieved by fragmenting long insoluble glucans, in some embodiments. This may be achieved by, for example, hydrolysis or, in some embodiments, by digestion with a glucanase (e.g., with a β-1,3 glucanase or limited digestion with a β-1,3 glucanase). In other embodiments, glucans can be prepared synthetically, for example, and in some embodiments, by joining monosaccharide building blocks. O-acetylation of such glucans can readily be accomplished by methods known in the art. Such methods may include chemical and/or enzymatic acetylation, such as are known in the art.

[0037] There are various sources of fungal β-glucans. For instance, pure β-glucans are commercially available e.g., pustulan (Calbiochem) is a β-1,6-glucan purified from Umbilicaria papullosa. β-glucans can be purified from fungal cell walls in various ways, for example, as described in Tokunaka et al. [(1999) Carbohydr Res 316:161-172], and the product may be enriched for β-1,6-glucan moieties, or O-acetylated β-1,6-glucan moieties, by methods as are known in the art.

[0038] One of ordinary skill in the art will be able to identify or select appropriate methods to enrich for β-1,6-glucan moieties and/or for O-acetylated β-1,6-glucan. In one embodiment, O-acetylation of beta-glucan is performed chemically. For example, polysaccharides (50mg) are dried in a speed vac centrifuge and resuspended in 1.5 mL of acetic anhydride (Mallindcrockdt). After resuspension, a few crystals of 4-dimethylaminopyridine (Avocado Research Chemist, Ltd) are added as catalyst. The reaction is allowed to proceed at room temperature for 5, 20, or 120 minutes and then stopped with 2 volumes of water. Afterwards the samples are dialyzed overnight against water. It will be appreciated that this process could be varied or scaled up, as evident to one of skill in the art. In other embodiments, methods for separating O-acetylated β-1,6-glucan include one or more of the following steps, which could be performed in various orders: (a) separation based on higher hydrophobicity, such as binding to any hydrophobic matrix/ resin; (b) separation based on digestion with a suitable endo- or exo-glucanase or combination thereof, wherein the O-acetylated β-1,6-glucan is resistant to digestion; (c) affinity separation using antibodies or other moieties that bind to β-1,6-glucan or to O-acetyl groups thereon; (d) separation based on molecular weight. In one embodiment, β-1,6-glucan is digested with an enzyme that digests unacetylated and/or lightly acetylated β-1,6-glucan. The resulting material is separated based on size or molecular weight and a portion comprising heavily acetylated glucan is isolated. In some embodiments, β-1,6-glucan preparations are obtained, digested and O-acetylated oligosaccharides are separated or in another embodiment, isolated, and used in the preparation of new compositions. Such compositions represent embodiments of the P- 1,6-glucan preparations enriched for O-acetylated residues of this invention.

[0039] It is to be understood that the products of any process for preparing enriched O-acetylated β-1,6-glucan preparations are to be considered as appropriate for use in the kits of this invention.

[0040] In some embodiments, the glucans for use in the kits and/or according to the methods of this invention may comprise structural modifications, not present in native glucan preparations. Such modifications may comprise, O-acetylation, as described herein. In other embodiments, such modifications may comprise methylation, alkylation, alkoylation, sulfation, phosphorylation, lipid conjugation or other modifications, as are known to one skilled in the art. In some embodiments the modification comprises modification (e.g., esterification) with an acid such as formic, succinic, citric acid, or other acid known in the art.

[0041] In some embodiments, lipid conjugation to any or all free hydroxyl groups may be accomplished by any number of means known in the art, for example, as described in Drouillat B, et al, Pharm Sci. 1998 Jan;87(1):25-30, B. N. A. Mbadugha, et al, Org. Lett., 5 (22), 4041 -4044, 2003.

[0042] In some embodiments, methylation may be accomplished and verified by any number of means known in the art, for example, as described in Mischnick et al. 1994 Carbohydr. Res., 264, 293-304; Bowie et al. 1984, Carbohydr. Res., 125, 301-307; Sherman and Gray 1992, Carbohydr. Res., 231, 221-235; Stankowski and Zeller 1992, Carbohydr. Res., 234, 337-341; Harris, P.J., et al. (1984) Carbohydr. Res. 127, 59-73; Carpita, N.C.

& Shea, E.M. (1989) Linkage structure of carbohydrates by gas chromatography-mass spectrometry (GC-MS) of partially methylated alditol acetates. In Analysis of Carbohydrates by GLC and MS (Biermann, CJ. & McGinnis, G.D., eds), pp. 157-216. CRC Press, Boca Raton, FL.

[0043] In some embodiments, methylation can be confirmed by GLC of further-derived TMS ethers, acetates or other esters, coupled MS, or digestion to monosaccharides, de-O-methylation and analysis by derivatization and GLC/MS, for example as described in Pazur 1986, Carbohydrate Analysis - A Practical Approach, IRL Press, Oxford, pp. 55-96; Montreuil et al. 1986, Glycoproteins. In M.F. Chaplin and J.F. Kennedy, (eds.), Carbohydrate Analysis - a Practical Approach, IRL Press, Oxford, pp. 143-204; Sellers et al. 1990, Carbohydr. Res., 207, C1-C5; O'Neill et al. 1990, Pectic polysaccharides of primary cell walls. In P.M. Dey (ed.), Methods in Plant Biochemistry, Volume 2, Carbohydrates, Academic Press, London, pp. 415-441; Stephen et al. 1990, Methods in Plant Biochemistry, Volume 2, Carbohydrates, Academic Press, London, pp. 483-522; or Churms 1991, CRC Handbook of Chromatography. Carbohydrates, Volume II, CRC Press, Boca Raton, Florida, USA).

[0044] In some embodiments, phosphorylation, optionally including the introduction of other modifications, and verification of the obtained product may be accomplished by means well known to those skilled in the art, see for example, Brown, D. H., Biochem. Biophys. Acta, 7, 487 (1951); Roseman, S., and Daffner, L, Anal. Chem., 28, 1743 (1956); Romberg, A., and Horecker,. B. L., in Methods in enzymology, Vol. I, Academic Press, New York, 1955, p. 323; United States Patent Number 4,818,752.

[0045] In some embodiments, glucan sulfation and verification of the obtained product may be accomplished by any of the means well known in the art, for example, as described in Alban, S., and Franz, G. (2001), Biomacromolecules 2, 354-361; Alban, et al. (1992) Arzneimittelforschung 42, 1005-1008; or Alban, S., et al. (2001). Carbohydr. Polym. 47, 267-276.

[0046] The term "homology", when in reference to the beta-1,6-glucan comprised in the kits of the invention, indicates a percentage of structural identity or identity in terms of composition or content in the candidate molecule as compared to a corresponding glucan reference molecule.

[0047] In one embodiment, the terms "homology", "homologue" or "homologous", in any instance, indicate that the molecule referred to, exhibits at least 70 % correspondence with the reference molecule. In another embodiment, the glucan molecule exhibits at least 72 % correspondence with the reference molecule. In another embodiment, the glucan molecule exhibits at least 75 % correspondence with the reference molecule. In another embodiment, the glucan molecule exhibits at least 77 % correspondence with the reference molecule. In another embodiment, the glucan molecule exhibits at least 80 % correspondence with the reference molecule. In another embodiment, the glucan molecule exhibits at least 82 % correspondence with the reference molecule. In another embodiment, the glucan molecule exhibits at least 85 % correspondence with the reference molecule. In another embodiment, the glucan molecule exhibits at least 87 % correspondence with the indicated sequence. In another embodiment, the glucan molecule exhibits at least 90 % correspondence with the reference molecule. In another embodiment, the glucan molecule exhibits at least 92 % correspondence with the reference molecule. In another embodiment, the glucan molecule exhibits at least 95 % or more correspondence with the reference molecule. In another embodiment, the glucan molecule exhibits at least 95 % - 100 % correspondence to the reference molecule.

[0048] With regard to correspondence to a reference molecule, such correspondence may refer to structural identity or compositional identity, in terms of chemical content. In some embodiments, similarly prepared glucans are utilized in kits of this invention, which are comparable to those utilized in a glucan-based adjuvant or vaccine, however the glucan utilized in the kit may not have been subjected to all processing steps, which comprise the preparation process for a glucan-based vaccine or adjuvant.

[0049] Homology may be determined, in one embodiment, by methods well described in the art, including immunoblot analysis, or via computer algorithm analysis of, utilizing any of a number of software packages available, via established methods.

[0050] In some embodiments, this invention provides kits comprising low molecular weight glucans, having a molecular weight of less than 100 kDa (e.g., less than 80, 70, 60, 50, 40, 30, 25, 20, or 15 kDa). In some embodiments, this invention provides oligosaccharides e.g., containing 85 or fewer (e.g., 85, 84, 83, 82, 81, 80, 79, 78, 77, 76, 75, 74, 73, 72, 71, 70, 69, 68, 67, 66, 65, 64, 63, 62, 61, 60, 59, 58, 57, 56, 55, 54, 53, 52, 51, 50, 49, 48, 47, 46, 45, 44, 43, 42, 41, 40, 39, 38, 37, 36, 35, 34, 33, 32, 31, 30, 29, 28, 27, 26, 25, 24, 23, 22, 21, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4) glucose monosaccharide units.

[0051] In some embodiments the β-1,6-glucan used in the kits of this invention comprises or consists essentially of a low molecular weight glucan. In some embodiments of any method of the invention in which β-1,6-glucan is utilized, the β-1,6-glucan comprises or consists essentially of a low molecular weight glucan. Optionally at least some of the low molecular weight β-1,6-glucan in any embodiment of the invention is enriched for O-acetylated groups.

[0052] A common technique in determining linkage type and structure in glucans is carbon-13 nuclear magnetic resonance spectroscopy (13 C-NMR). The number and relative intensities of ^{13}C signals in a given spectrum can be used to determine linkage configurations and positions in glucan polymers. For example, the chemical shifts (signals) of carbon atoms engaged in the glycosidic

bond are shifted strongly downfield (up to 9 ppm) compared to the corresponding unlinked carbons.

EXAMPLES

Materials and methods

Preparation of IgG-depleted serum

[0053] Protein G-sepharose beads of untreated sepharose beads (control) were washed three times with PBS. Serum was diluted 2-fold in PBS and added to beads. Beads were incubated at room temperature on an end-to-end mixer for 30 min. beads were removed by centrifugation.

[0054] Serum from 10 unimmunized normal healthy donors was pooled

SRBC assay

[0055] SRBC (Accurate chemical and scientific corp.) were washed with gelatin veronal buffer (Sigma) and opsonized with rabbit anti-SRBC antibodies (Accurate chemical and scientific corp.) for 30 minutes at room temperature. IgG-depleted serum (from protein G-sepharose beads) or IgG-containing serum (from untreated sepharose beads) were added to SRBC and incubated at 37 °C for one hour. Water was added as a positive control (complete lysis), and buffer was added as a negative control (no lysis). Lysis was detected by direct microscopic visualization (Fig. 1C a) or by O.D. 414 nm, which detects the heme that is secreted from the lysing SRBC).

Preparation of β-I,6-glucan-coated beads and FACS analysis (phagocytosis and ROS production)

[0056] These methods were performed as described in. Rubin-Bejerano, L, et al, Phagocytosis by human neutrophils is stimulated by a unique fungal cell wall component. Cell Host Microbe, 2007.2(1): p. 55-67.

Cell Culture

[0057] SK-BR-3 cells (ATCC) were cultured in McCoy's 5A Medium (Gibco) supplemented with 10% FBS.

Conjugation

[0058] Herceptin (Genentech, Inc.) or IgG1 isotype control (Sigma) were conjugated to β-1,6-glucan following oxidation with sodium meta periodate (Pierce).

Neutrophils

[0059] Fresh human blood and serum were provided by Research Blood Components (Brighton, MA). Neutrophils were isolated from fresh human blood in accordance with a protocol approved by the MIT Committee on Use of Humans as Experimental Subjects by using Histopaque 1077 and Histopaque 1119 (Sigma).

Opsonization

[0060] Breast cancer cells were opsonized in 40% serum in Phosphate-Buffered Saline without calcium chloride and without magnesium chloride (PBS) (Gibco) for 15 minutes at 37 °C. Cells were then washed three times with cold PBS supplemented with 0.04 mg/ml of the protease inhibitor AEBSF (Sigma).

Antibody binding and C3 deposition

[0061] Fluorescence Activated Cell Sorting (FACS) analysis was used to detect Herceptin binding to breast cancer cells (by using anti-human IgG1 antibodies, Sigma) and C3 deposition (by using anti-human C3 antibodies, Accurate Chem.).

Cytotoxicity

[0062] Cytotoxicity to breast cancer cells following incubation with β-1,6-glucan-conjugated or unconjugated Herceptin and serum was determined by CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega), which detects lactate dehydrogenase released from lysed cells. For neutrophil-dependent killing, above cells were cultured with neutrophils at 37 °C after which cytotoxicity was measured.

EXAMPLE 1

β-I,6-glucan Stimulated Complement Activation is Dependent upon Antibodies

[0063] In order to test whether antibodies are involved in complement activation by β-1,6-glucan, serum used in complement activation assays was depleted of antibodies by using protein G sepharose beads. This IgG-depleted serum was used to opsonize β-1,6-glucan-coated beads. The beads were then tested in a phagocytosis assay conducted with human neutrophils. Phagocytosis and ROS production were reduced when β-1,6-glucan-coated beads were opsonized with IgG-depleted serum (Fig. 1A and B), suggesting that the Classical pathway plays a major role in complement activation by β-1 ,6-glucan.

[0064] The IgG-depleted serum contained functional complement factors. In a sheep red blood cells (SRBC) assay, SRBC opsonized with anti-SRBC antibody were lysed when IgG-depleted serum was utilized (Fig. 1 C).

EXAMPLE 2

Antibodies to β-1,6-glucan are Prevalent in Normal Adults

[0065] Pooled serum from 10 human donors, had high levels of IgG antibodies binding β-1,6-glucan, in contrast to low levels of IgG binding β-1,3-glucan. To test how prevalent is the anti-β-1,6-glucan antibodies in different donors, we collected sera from 12 individuals. The sera were used to opsonize β-1,6-glucan- or β-1,3-glucan-coated beads, and the beads were incubated with human neutrophils. Eleven of the twelve sera had the high response that the pool did, mediating efficient engulfment and ROS production (Fig. 2 Aa, a representative serum of the high responders). One serum mediated a less efficient engulfment and ROS production (Fig. 2 Ab, low responder).

EXAMPLE 3

β-I,6-glucan mediates efficient phagocytosis and production of reactive oxygen species by neutrophils

[0066] In order to assess which isotype of IgG was mediating the β- 1 ,6-glucan recognition, antibodies specific for IgG1, IgG2, IgG3, or IgG4 were used to determine the IgG isotype usage in donor serum with a high or low response to β-1,6-glucan, following exposure to opsonized β-1,6-glucan-coated beads. The high responder produced more IgG1, IgG2, and IgG3, but not IgG4, as compared to the low responder (Fig. 3). Specifically, the level of IgG2 was dramatically higher in the high responder, probably because polysaccharides tend to induce production of the IgG2 isotype.

[0067] The different isotypes differ in their complement activation properties. IgG3 has the highest complement activation properties, then follows IgG1, IgG2 is next, and IgG4 cannot activate complement. IgG2 is a poor activator of the Classical pathway, but it can activate complement through the alternative pathway, and is a good substrate for phagocytosis by neutrophils.

EXAMPLE 4

β-I,6-glucan conjugated to the Herceptin monoclonal antibody mediates recruitment of complement and neutrophils to breast cancer cells

[0068] In order to assess whether conjugated β-1,6-glucan linked to targeting moieties can mediate recruitment of complement and neutrophils to lyse and kill target cells bound by the targeting moieties, a 1,6-glucan linked to a Herceptin monoclonal antibody (mAb) was tested in a system of breast cancer cells (SK-BR-3). The Herceptin mAb is directed against the Her-2/neu protein overexpressed on SK-BR-3 cells. Conjugation of P- 1,6-glucan to Herceptin did not affect its binding to breast cancer cells (Fig. 4 Aa). Furthermore, the conjugate mediated high C3 deposition (Fig. 4 Ab), suggesting that β- 1,6-glucan remained functional. Non-specific isotype control antibodies conjugated to β- 1,6-glucan did not bind breast cancer cells (Fig. 4 Ba), and therefore, did not mediate complement activation and C3 deposition on these cells (Fig. 4 Bb). Mixing β-1,6-glucan with the mAb without chemical conjugation did not mediate C3 deposition on these breast cancer cells (Fig. 4C, compare green to blue). Therefore, it was concluded that the conjugation of P- 1,6-glucan to the targeting moiety was required for directing of complement and consequently neutrophils to target cells. C3 deposition was detected on breast cancer cells treated with Herceptin conjugated to β-1,6-glucan but not β-1,3-glucan (Fig. 4D, compare blue to green), suggesting that indeed β-1,6-glucan was more efficient than β-1,3-glucan in attracting complement.

EXAMPLE 4

β-I,6-glucan conjugated to the Herceptin monoclonal antibody mediates killing of breast cancer cells by complement and neutrophils

[0069] Deposition of the complement protein C3 on breast cancer cells led to lysis of these cancer cells. The Herceptin-β-1,6-glucan conjugate showed a dose-dependent cytotoxic effect on the breast cancer cells, whereas the unconjugated Herceptin lacked any effect (Fig. 5 A). Furthermore, the Herceptin-β-1,6-glucan conjugate showed an increased neutrophil killing of the cancer cells (Fig. 5 B).

Claims

1. A diagnostic kit for measuring IgG1, IgG2, IgG3 and IgG4 levels, and thereby determining responsiveness of a subject which has been exposed to environmental glucans to a pharmaceutical composition comprising β-1,6-glucan, comprising:

   (i) a composition comprising β-1,6-glucan which corresponds to or is a fragment of, or is highly homologous to the glucan in the pharmaceutical composition for which responsiveness is being determined; and
   (ii) reagents for detecting IgG1, IgG2, IgG3 and IgG4 antibodies bound to the β-1,6-glucan of step (i) in a blood sample.

2. The kit of claim 1 for wherein the β 1-6 glucan is in solution or lyophilized.

3. The kit of claim 1 for wherein the β 1-6 glucan is immobilized on a substrate.

4. The kit of claim 3 wherein said substrate comprises a material selected from: plastic, glass, gel, celluloid, paper, magnetic resin, polyvinylidene-fluoride, nylon, nitrocellulose, agarose, latex, and polystyrene.

5. The kit of claim 3 wherein said substrate comprises an ELISA plate, dipstick, microtiter plate, radioimmunoassay plate, beads, agarose beads, plastic beads, latex beads, immunoblot membranes, and immunoblot papers.

6. The kit of any one of the preceding claims wherein said reagents are conjugated to a detectable marker.

7. The kit of claim 6 wherein said detectable marker is selected from the group consisting of a radioactive label, fluorescent label, chemiluminescent label, chromophoric label, ligand, fluorescein, radioisotope, phosphatase, biotin, biotin-related compound, avidin, avidin-related compound, and peroxidase.

8. Use of the kit of any one of the preceding claims for establishing the robustness of a response to β-1,6-glucans in a subject.

9. Use of the kit of any one of the preceding claims for predicting a subject's responsiveness to glucan-based vaccines or adjuvants.

Patentansprüche

1. Diagnostisches Kit zum Messen von IgG1-, IgG2-, IgG3- und IgG4-Spiegeln und dabei Ermittlung der Responsivität eines Subjekts, welches umweltbedingt Glucanen ausgesetzt war, auf eine β-1,6-Glucan enthaltende pharmazeutische Zusammensetzung, umfassend:

   (i) eine Zusammensetzung enthaltend β-1,6-Glucan, das dem Glucan in der pharmazeutischen Zusammensetzung entspricht, für das die Empfindlichkeit bestimmt werden soll, oder das ein Fragment davon oder hoch homolog dazu ist; und
   (ii) Reagenzien zum Nachweisen von an das β-1,6-Glucan aus Schritt (i) gebunden IgG1-, IgG2-, IgG3- und IgG4-Antikörpern in einer Blutprobe.

2. Kit nach Anspruch 1, wobei das β-1,6-Glucan in Lösung oder lyophilisiert vorliegt.

3. Kit nach Anspruch 1, wobei das β-1,6-Glucan auf einem Substrat immobilisiert ist.

4. Kit nach Anspruch 3, wobei das Substrat ein Material enthält, ausgewählt aus der Gruppe: Kunststoff, Glas, Gel, Celluloid, Papier, magnetisches Harz, Polyvinylidenfluorid, Nylon, Nitrocellulose, Agarose, Latex und Polystyrol.

5. Kit nach Anspruch 3, wobei das Substrat eine ELISA-Platte, einen Teststreifen, eine Mikrotiterplatte, eine Radioimmunoassay-Platte, Kügelchen, Agarose-Kügelchen, Kunststoffkügelchen, Latex-kügelchen, Immunoblot-Membranen und Immunoblot-Papiere umfasst.

6. Kit nach einem der vorhergehenden Ansprüche, wobei die Reagenzien mit einem nachweisbaren Marker konjugiert sind.

7. Kit nach Anspruch 6, wobei der nachweisbare Marker ausgewählt ist aus der Gruppe bestehend aus einer radioaktiven Markierung, einer fluoreszierenden Markierung, einer chemilumineszierenden Markierung, einer chromophoren Markierung, einem Liganden, Fluorescein, einem Radioisotop, einer Phosphatase, Biotin, einer Biotinverwandte Verbindung, Avidin, einer Avidin-verwandte Verbindung und Peroxidase.

8. Verwendung des Kits nach einem der vorhergehenden Ansprüche zum Etablieren der Robustheit einer Antwort auf β-1,6-Glucan in einem Subjekt.

9. Verwendung des Kits nach einem der vorhergehenden Ansprüche zum Vorhersagen der Empfindlichkeit eines Subjekts auf Glucan-basierte Impfstoffe oder Adjuvantien.

Revendications

1. Trousse de diagnostic pour mesurer des taux d'IgG1, d'IgG2, d'IgG3 et d'IgG4, et déterminer ainsi la sensibilité d'un sujet qui a été exposé à des glucanes environnementaux à une composition pharmaceutique comprenant du β-1,6-glucane, comprenant :

   (i) une composition comprenant du β-1,6-glucane qui correspond à ou est un fragment de, ou est fortement homologue, au glucane dans la composition pharmaceutique pour laquelle la sensibilité est déterminée ; et
   (ii) des réactifs pour détecter des anticorps IgG1, IgG2, IgG3 et IgG4 liés au β-1,6-glucane de l'étape (i) dans un échantillon de sang.

2. Trousse selon la revendication 1, dans laquelle le β-1,6-glucane est en solution ou lyophilisé.

3. Trousse selon la revendication 1, dans laquelle le β-1,6-glucane est immobilisé sur un substrat.

4. Trousse selon la revendication 3, dans laquelle ledit substrat comprend un matériau choisi parmi : le plastique, le verre, le gel, le celluloïd, le papier, la résine magnétique, le poly(fluorure de vinylidène), le nylon, la nitrocellulose, l'agarose, le latex et le poly(styrène).

5. Trousse selon la revendication 3, dans laquelle ledit substrat comprend une plaque ELISA, une bande réactive, une plaque de micro-titration, une plaque de dosage radio-immunologique, des billes, des billes d'agarose, des billes de plastique, des billes de latex, des membranes d'immunotransfert et des papiers d'immunotransfert.

6. Trousse selon l'une quelconque des revendications précédentes, dans laquelle lesdits réactifs sont conjugués à un marqueur détectable.

7. Trousse selon la revendication 6, dans laquelle ledit marqueur détectable est choisi dans le groupe consistant en un traceur radioactif, un traceur fluorescent, un traceur chimioluminescent, un traceur chromophore, un ligand, une fluorescéine, un radioisotope, la phosphatase, la biotine, un composé apparenté à la biotine, l'avidine, un composé apparenté à l'avidine et la peroxydase.

8. Utilisation de la trousse selon l'une quelconque des revendications précédentes pour établir la robustesse d'une réponse aux β-1,6-glucans chez un sujet.

9. Utilisation de la trousse selon l'une quelconque des revendications précédentes pour prédire la sensibilité d'un sujet à des vaccins ou des adjuvants à base de glucane.

Fig. 1

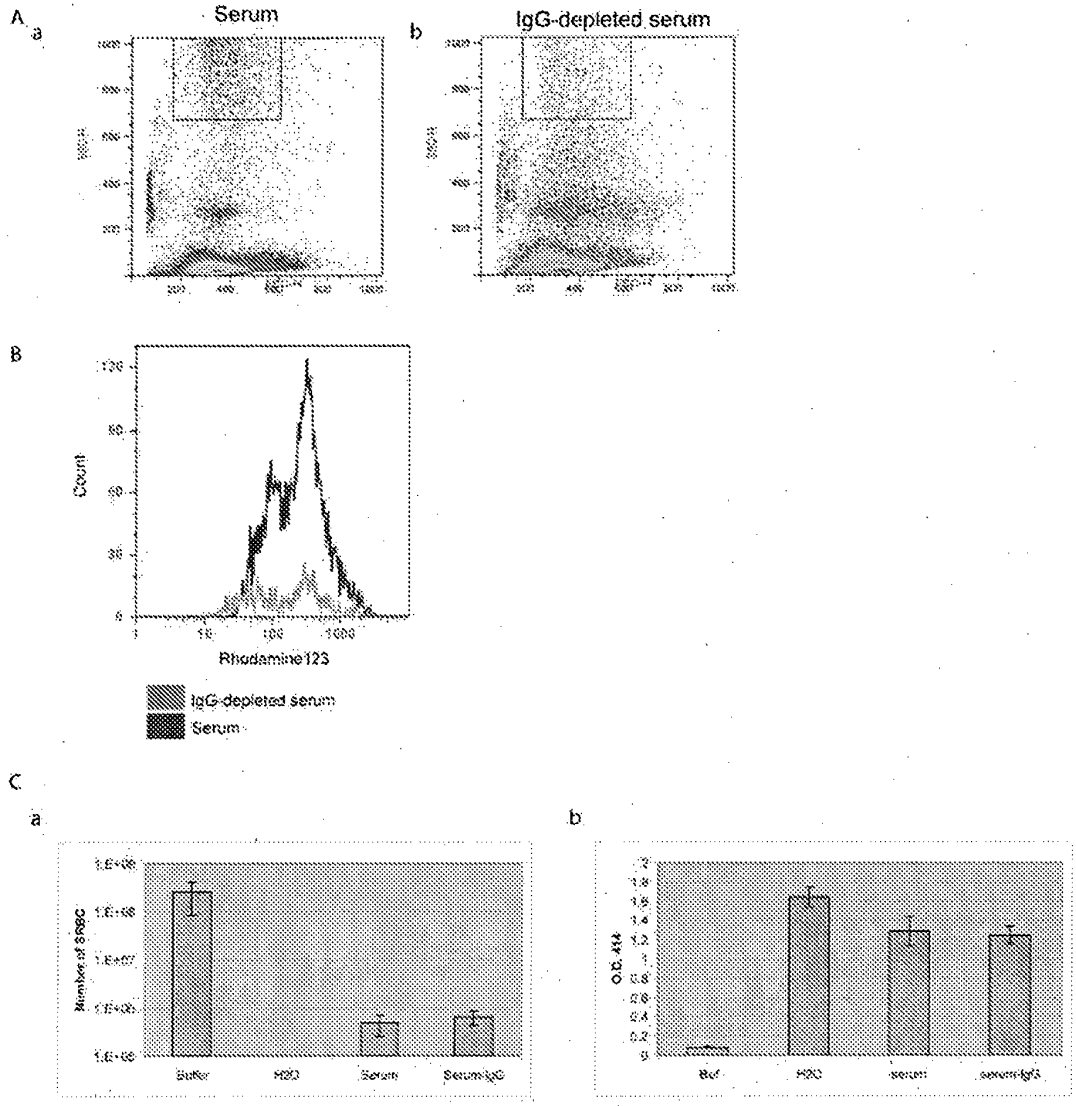

A
a
Serum
b
IgG-depleted serum
B
Count
Rhodamine123
IgG-depleted serum
Serum
C
a
b
Buffer
H2O
Serum
Serum IgG
serum
serum-IgG

Fig. 2

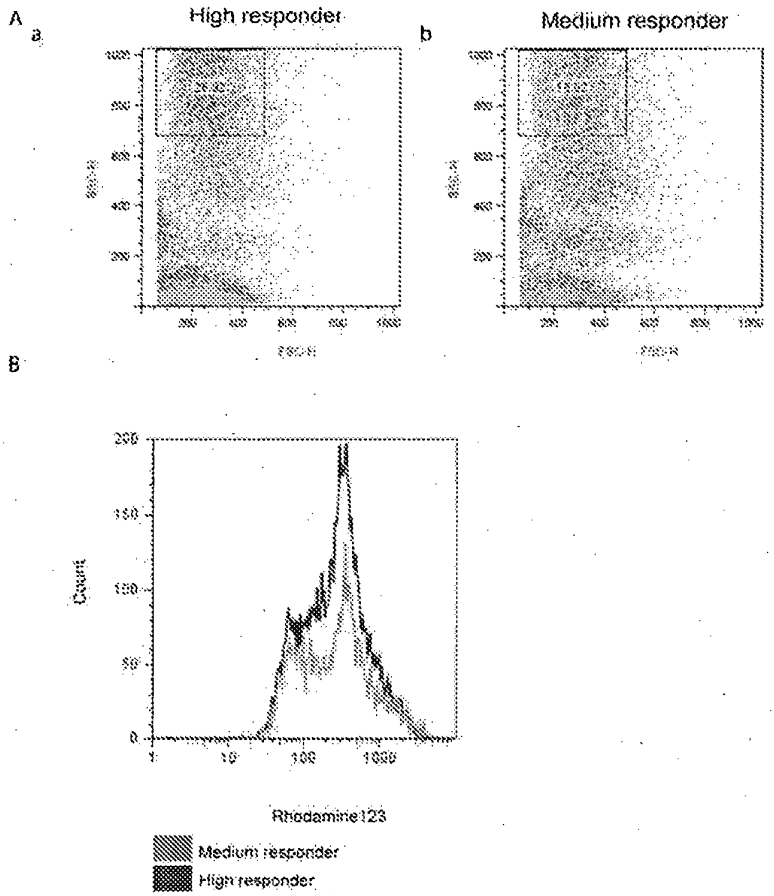

A
a
High responder
b
Medium responder
B
Count
Rhodamine123
Medium responder
High responder

Fig. 3

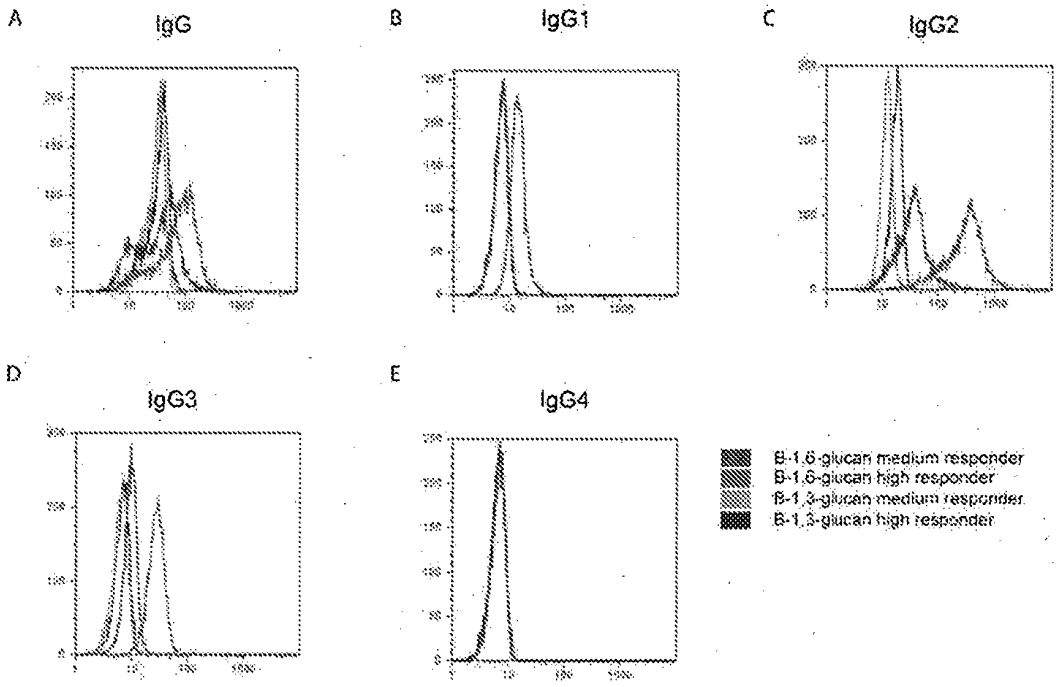

A
IgG
B
IgG1
C
IgG2
D
IgG3
E
IgG4
B-1,6-glucan medium responder
B-1,6-glucan high responder
B-1,3-glucan medium responder
B-1,3-glucan high responder

Fig. 4

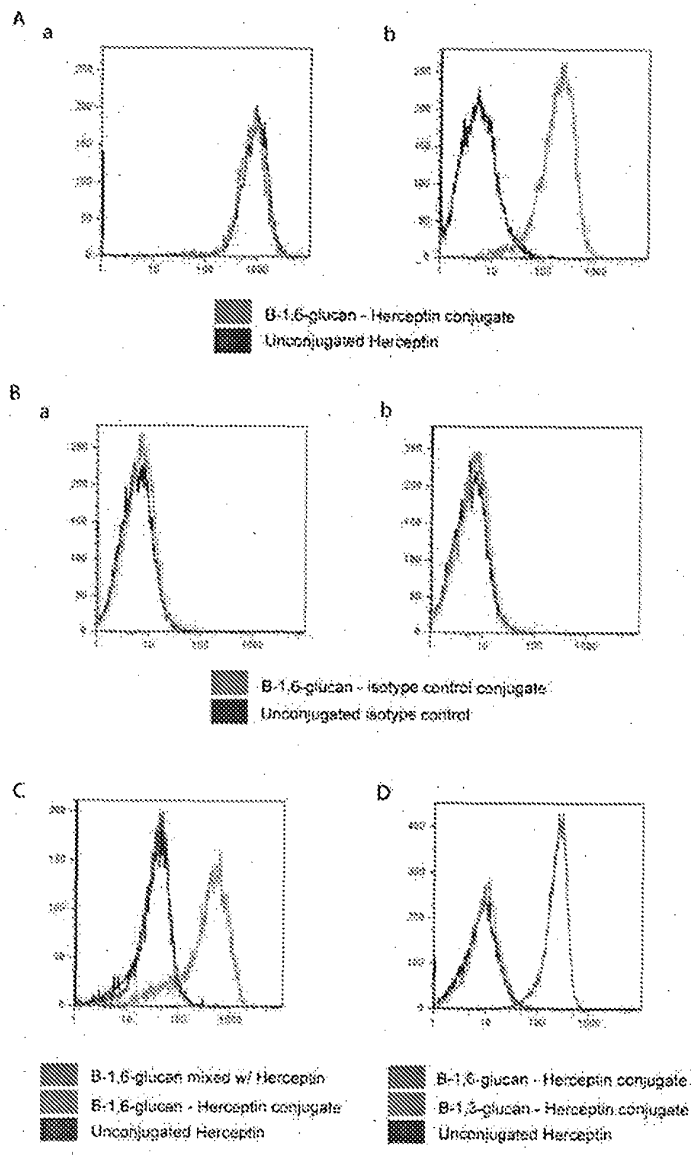
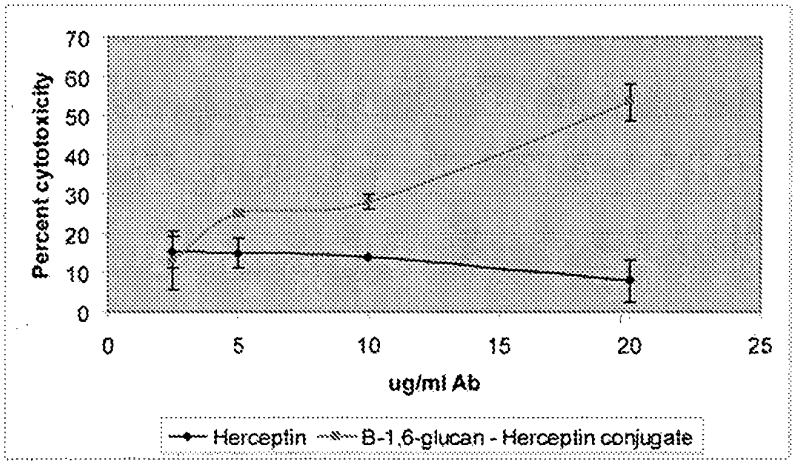
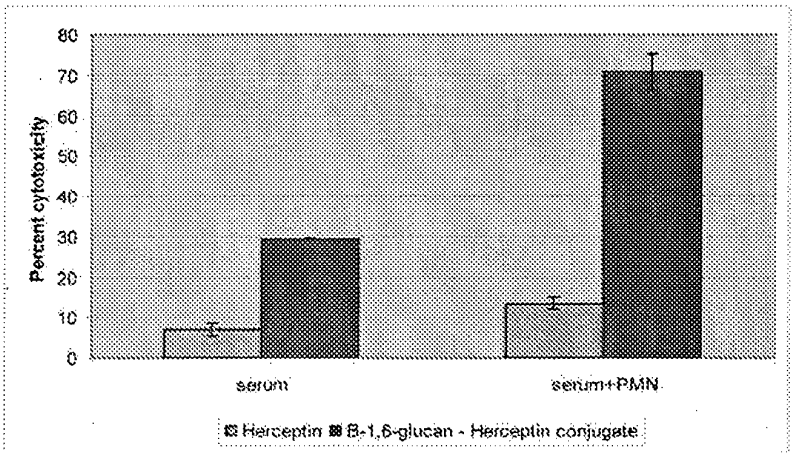

A
a
b
B-1,6-glucan - Herceptin conjugate
Unconjugated Herceptin
B
a
b
B-1,6-glucan - isotype control conjugate
Unconjugated isotype control
C
D
B-1,6-glucan mixed w/ Herceptin
B-1,6-glucan - Herceptin conjugate
Unconjugated Herceptin
B-1,6-glucan - Herceptin conjugate
B-1,3-glucan - Herceptin conjugate
Unconjugated Herceptin

Fig. 5

A


Percent cytotoxicity
70
60
50
40
30
20
10
0
0
5
10
15
20
25
ug/ml Ab
Herceptin
B-1,6-glucan - Herceptin conjugate

B


Percent cytotoxicity
80
70
60
50
40
30
20
10
0
serum
serum+PMN
Herceptin
B-1,6-glucan - Herceptin conjugate

REFERENCES CITED IN THE DESCRIPTION

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专利名称(译)	免疫调节组合物及其使用方法		
公开(公告)号	EP2283358A4	公开(公告)日	2011-09-07
申请号	EP2009739693	申请日	2009-04-29
[标]申请(专利权)人(译)	免疫刺激公司		
申请(专利权)人(译)	IMMUNEXCITE INC.		
当前申请(专利权)人(译)	IMMUNEXCITE INC.		
[标]发明人	RUBIN BEJERANO IFAT FINK GERALD R KOHANE DANIEL S		
发明人	RUBIN-BEJERANO, IFAT FINK, GERALD, R. KOHANE, DANIEL, S.		
IPC分类号	G01N33/53 G01N33/563 C07K16/18 A61K47/48 C07K16/44 G01N33/68		
CPC分类号	A61K47/61 A61P29/00 A61P31/00 A61P31/04 A61P31/10 A61P31/12 A61P33/00 A61P37/02 A61P37/04 A61P37/06 A61P43/00 A61K47/6849 C07K16/44 G01N33/6854 G01N2333/415 G01N2400/02 A61K39/39 A61K45/06 A61K2039/55583		
优先权	61/071437 2008-04-29 US		
其他公开文献	EP2283358B1 EP2283358A2		
外部链接	Espacenet		

摘要(译)

本发明涉及β1-6葡聚糖，包含其的组合物，诊断试剂盒和装置，及其在调节免疫应答和治疗，延缓其进展，降低其发病率或严重性，降低感染，发炎和降低其发生率和严重性中的用途。 自身免疫性疾病。 本发明某些实施方案的β1-6葡聚糖富含O-乙酰化基团和/或缀合至固体支持物或连接至靶向部分。 本发明某些实施方案的β1-6葡聚糖募集免疫球蛋白G抗体以介导补体和中性粒细胞杀伤。 本发明某些实施方案的缀合的β1-6葡聚糖被靶向细胞以通过激活补体介导的嗜中性粒细胞的裂解和募集而刺激靶位置处的免疫应答。