

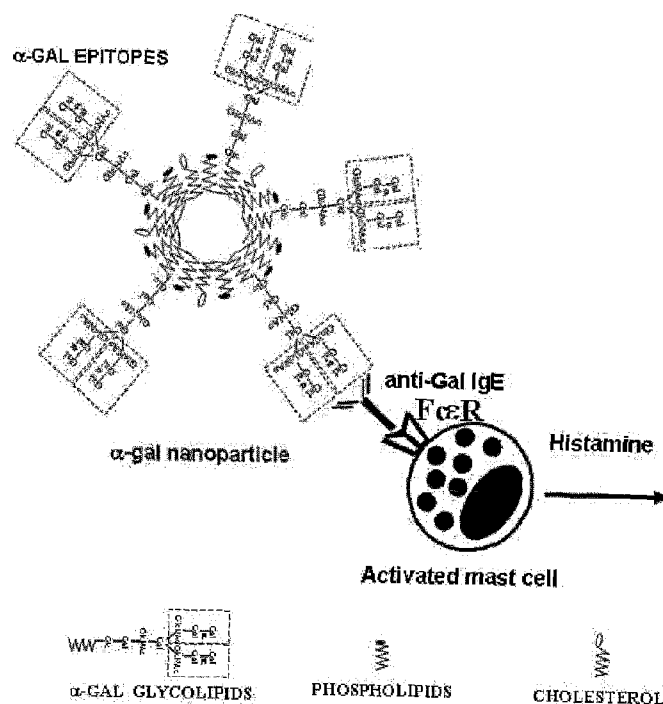


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(54) Title: COMPOSITIONS AND METHODS FOR DETECTING ALLERGY TO A-GAL EPITOPES

Figure 1



(57) **Abstract:** The present invention is related to the method for the identification of individuals allergic to a-gal epitopes by performing an allergy skin test in which glycoconjugates having linked a-gal epitopes are applied to the skin. The glycoconjugates with linked a-gal epitopes are a-gal nanoparticles, or a-gal liposomes. The glycoconjugates could also be glycoproteins, proteoglycans, polymers all of which have linked one, two or multiple a-gal epitopes. The present invention is also related to a method for the identification of anti-Gal-IgE molecules in human serum by ELISA with anti-human IgE as solid-phase antigen.



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COMPOSITIONS AND METHODS FOR DETECTING ALLERGY TO α -GAL EPITOPES

This application claims priority under 35 U.S.C. §119(e) to co-pending U.S. provisional
5 Application Serial No. 61/516,005 filed March 28, 2011, herein incorporated by reference in its
entirety for all purposes.

FIELD OF INVENTION

The present invention is related to the fields of diagnostics and therapeutic glycoproteins.
10 In particular, the present invention provides compositions and methods for identifying subjects
that are allergic to α -gal epitopes. Identification of such individuals will prevent the induction of
allergic reactions and anaphylactic shock which may develop following binding of the natural
anti-Gal antibody to monoclonal antibodies, and to natural or recombinant glycoproteins or to
other molecules that have α -gal epitopes and which are injected for therapeutic purpose into
15 humans, or into other vertebrates producing the anti-Gal antibody. This is achieved by
performing a skin test in which the individual is injected with α -gal nanoparticles, or α -gal
liposomes, or with nonimmunogenic glycoconjugates that have α -gal epitopes. In addition the
invention describes an in vitro assay for identifying individuals that have measurable anti-Gal
IgE antibodies. When glycoconjugates with α -gal epitopes are administered to the skin,
20 individuals who produce anti-Gal IgE antibodies and are allergic to α -gal epitopes, will develop
a characteristic skin reaction of immediate type hypersensitivity, in the form local redness, rush
and/or wheal. Individuals found to be allergic to α -gal epitopes will be treated with anti-allergic
drugs prior to injection of glycoproteins with α -gal epitopes, or not treated with such
glycoproteins.

BACKGROUND OF THE INVENTION

Therapeutic glycoproteins which are injected into the blood stream or into various tissues
range from a variety of monoclonal antibodies, to recombinant enzymes, hormones and various
proteins with biological activities. When produced in nucleated cells, the asparagines (Asn or N)-
30 linked carbohydrate chains of these glycoproteins are synthesized on asparagines (N) which are

part of an amino acids sequence N-X-S/T (glycosylation sites with the sequence of asparagine-any amino acid- serine or threonine). If these carbohydrate chains carry α -gal epitopes (Gal α 1-3Gal β 1-4GlcNAc-R or Gal α 1-3Gal β 1-3GlcNAc-R or Gal α 1-3Gal-R), such therapeutic glycoproteins will interact with the natural anti-Gal antibody in the recipient of the therapeutic glycoprotein. Anti-Gal is produced in humans as ~1% of IgG (Galili et al., J Exp Med 160:1519, 1984), and >1% of IgM, and is readily detected in various body secretions as an IgA antibodies (Hamadeh et al., Clin Diagnos Lab Immunol 2:125, 1995). Recently, the anti-Gal antibody was found to be produced in a proportion of the population as an IgE antibody that mediates allergic reactions (Chung et al., New Engl J Med 358: 1109, 2008). Anti-Gal interacts specifically with α -gal epitopes (Galili et al., J Exp Med. 162:573, 1985; Galili et al., J Exp Med 165:693, 1987). If patients have anti-Gal IgE antibodies and are injected with monoclonal antibodies that have α -gal epitopes on their carbohydrate chains, or injected with recombinant glycoproteins that have α -gal epitopes on their carbohydrate chains, the binding of anti-Gal IgE antibodies to the α -gal epitopes can have adverse effects. Anti-Gal IgE antibodies bound to mast cells and basophils, will bind the α -gal epitopes on the therapeutic glycoprotein and monoclonal antibodies. This interaction will result in cross-linking of the IgE molecules on the mast cells and basophils, thus inducing the activation of these cells to undergo de-granulation as in any allergic reaction. The substances within the released granules will induce an allergic reaction, that ultimately may cause an anaphylactic shock. Thus, the use of a simple assay that can identify patients that are allergic to α -gal epitopes, can help in selecting patients that are in no risk for developing allergic reactions due to anti-Gal IgE activity. Alternatively, patients allergic to α -gal epitopes may be medicated with anti-allergy drugs prior to injection of glycoproteins and monoclonal antibodies with α -gal epitopes. Furthermore, monitoring patients that repeatedly are treated with a monoclonal antibody, or with a recombinant glycoprotein that have α -gal epitopes, will help to determine whether the treated patient develops allergic response to α -gal epitopes.

SUMMARY OF THE INVENTION

The present invention is related to the fields of therapeutic glycoproteins and diagnostics. In particular, the present invention provides compositions and methods for identifying individuals allergic to α -gal epitopes by a skin test with α -gal liposomes or α -gal nanoparticles

(referred to as α -gal liposomes/nanoparticles) that present α -gal epitopes or by any other nonimmunogenic glycoconjugates that has multiple α -gal epitopes. α -gal liposomes/nanoparticles are particles of various sizes comprised of phospholipids, cholesterol and α -gal glycolipids (glycolipids with α -gal epitopes on their carbohydrate chains) that are prepared as a sterile suspension at a concentration of 1ng/ml to 0.5gm/ml and a preferable concentration of 1mg/ml or 10mg/ml. When these particles are visible in a microscope and are of a size greater than 0.5 μ m they are referred to as α -gal liposomes. When these particles are of a submicroscopic size smaller than 0.5 μ m they are referred to as α -gal nanoparticles. These α -gal liposomes/nanoparticles are administered into the skin of the tested individual either by injection, scratch or a patch. If that individual has anti-Gal IgE antibodies at high enough titer, the interaction between anti-Gal IgE molecules on mast cells and basophils with the α -gal epitopes on the α -gal liposomes/nanoparticles will activate these cells to degranulate and secrete histamine and other substances that mediate within 30-60 minutes an allergic reaction in the skin (Figure 1). This reaction may be assessed and by a health care worker. Individuals found to be allergic to α -gal epitopes may have to under additional consultation and treatment that diminishes the risks of an allergic response. A similar evaluation of an allergic sensitivity to α -gal epitopes can be performed with any glycoconjugate that carries a number of synthetic or natural α -gal epitopes and which is injected into the skin of the tested individual.

This invention further teaches of a method for performing an ELISA test for specific detection of anti-Gal IgE antibody in the serum. This is performed by capturing IgE antibodies on the surface of an ELISA well coated with anti-IgE antibodies followed by measuring the binding of labeled glycoprotein with linked α -gal epitopes to the IgE antibodies on the well. Such binding will occur only if among the captured IgE molecules there are anti-Gal IgE antibody molecules.

Thus, in one embodiment, the invention provides method for the identification of individuals allergic to α -gal epitopes by performing an allergy skin test in which glycoconjugates having linked α -gal epitopes are applied to the skin. In one embodiment, the glycoconjugates with linked α -gal epitopes are α -gal nanoparticles or α -gal liposomes. In another embodiment, the glycoconjugates with linked α -gal epitopes are glycoproteins, proteoglycans or polymers all of which have linked one, two or multiple α -gal epitopes.

In a further embodiment, the invention provides a method for the identification of anti-Gal IgE molecules in human serum by ELISA with anti-human IgE as solid-phase antigen. In one embodiment, the human IgE captured on ELISA wells is determined for the presence of anti-Gal IgE molecules by binding of biotinylated α -gal BSA followed by avidin coupled peroxidase.

5 In another embodiment, the invention provides a method for capturing human IgE molecules from human serum or plasma specimens by coating ELISA wells with monoclonal anti-human IgE antibody. In a particular embodiment, any labeled glycan with α -gal epitopes is used instead of α -gal BSA and the binding of such glycan may be determined either by direct detection of the label or by the use of the corresponding ligand to the glycan, where said ligand is labeled. In an
10 alternative embodiment, anti-IgE antibody is bound to sepharose bead or other beads with porosity similar to that of sepharose, instead of to ELISA wells, in order to increase the amount of captured human IgE from the tested serum or plasma specimen. In a further embodiment, the labeled α -gal glycoconjugate that binds to anti-Gal IgE captured in beads is detected after washing of the beads and performing the detection analysis with the corresponding method for
15 detecting the label.

BRIEF DESCRIPTION OF THE DRAWING

The following is an illustration of the present invention and is not intended as limiting in any manner.

20 Figure 1 shows a schematic illustration of the method for identification by a skin test individuals with anti-Gal IgE who are allergic to α -gal epitopes. The α -gal liposome/nanoparticles injected into the skin bind anti-Gal IgE molecules that are bound to the surface of mast cells or basophils via Fc ϵ receptors (Fc ϵ R) on the cells. The interaction between α -gal epitopes (marked with broken line rectangles) on the α -gal liposome/nanoparticle and anti-
25 Gal IgE molecules on the mast cells activates the cells to degranulate and release histamine and other allergy mediating substances stored in the granules (small black circles). This results in an allergic reaction in the skin which can be detected within 30-60 minutes. A similar response will occur in basophils that have anti-Gal IgE molecules attached to their Fc ϵ R, that bind α -gal epitopes.

Figure 2 shows α -gal nanoparticles coated with the natural anti-Gal antibody which bind in vitro to cultured macrophages via their Fc γ receptor (Fc γ R) as evaluated by scanning electron microscopy. This figure displays the morphology of α -gal nanoparticles on macrophages. A-E. The α -gal nanoparticles coated with anti-Gal antibody were incubated adherent
 5 macrophages cultured from blood monocytes. After 2h incubation at room temp the plated macrophages were extensively washed to remove nonadherent nanoparticles and subjected to scanning electron microscopy processing and analysis. Some nanoparticles are already internalized, i.e. covered by cell membrane due to activation by Fc/Fc γ R interaction (D). Insets in A and C are magnified in B and D respectively. E. High magnification demonstrating α -gal
 10 nanoparticles with the size of $\sim$ 30nm bound to an adherent macrophage. F. A representative macrophage out of 10 macrophages incubated with uncoated α -gal nanoparticles displays no significant binding of the nanoparticles.

Figure 3 shows the characterization of rabbit RBC extracts by thin layer chromatography (TLC) and immunostaining with the monoclonal anti-Gal, Gal-13. A. TLC separation of rabbit RBC
 15 molecules extracted by chloroform and methanol, as demonstrated by nonspecific orcinol staining. In addition to phospholipids and cholesterol there are various glycolipids including: ceramide trihexoside (CTH) which lacks α -gal epitopes and which is found also in human RBC, ceramide pentahexoside (CPH with 5 carbohydrates), and larger glycolipids. Comparison of nonspecific orcinol staining and immunostaining by monoclonal anti-Gal indicates that
 20 glycolipids with 5-25 carbohydrates, all have α -gal epitopes which bind anti-Gal (CHH-ceramide heptahexoside with 7 carbohydrates). B. Structures of α -gal glycolipids with 5, 7, 10, 15 and 20 carbohydrates in Fig.3A. The α -gal epitope on CPH is marked by the rectangle of broken line and it caps all other glycolipids chains. The structures of the carbohydrate chains are based on several studies: (Eto et al. Arch. Biochem. Biophys. 133: 464, 1973; Dabrowski et al. J
 25 Biol Chem. 259: 7648, 1984; Hanfland et al. Carbohydr Res. 178: 1, 1988).

Figure 4 shows a schematic illustration of the method for identification of anti-Gal IgE molecules in human serum. The ELISA (Enzyme Linked ImmunoAssay) wells are first coated with goat anti-mouse IgG. Subsequently, mouse monoclonal anti-human IgE antibody (IgG class) is captured by the anti- mouse IgG antibody molecules coating the wells. After removing
 30 the excess of unbound anti-IgE antibody, human serum is placed in the wells. Human IgE

antibodies are captured by the anti-IgE monoclonal antibody in the wells. After washing, biotinylated α -gal BSA (synthetic α -gal epitopes linked via a spacer to bovine serum albumin[BSA]) solution is placed in the well. If some of the captured IgE molecules are anti-Gal IgE antibodies, they will bind the biotinylated α -gal BSA. In this illustration, the schematically drawn most left and most right captured IgE molecules have the anti-Gal specificity, therefore they bind α -gal BSA. After washing to remove the unbound biotinylated α -gal BSA, the biotinylated α -gal BSA bound to the IgE molecules is detected by addition of avidin coupled to horseradish peroxidase (avidin-HRP) which binds to the biotin on α -gal BSA. The extent of avidin-HRP binding is determined after wash by color reaction with ortho-phenylene diamine (OPD) and absorbance (light absorption) is measured at 492nm, as known to those skilled in the art.

Figure 5 demonstrates representative measurements of anti-Gal IgE in serum of a healthy human individual (○) and in the serum of a person with multiple allergies that also has elevated anti-Gal IgE activity (●). The ELISA wells were coated with anti-mouse IgG antibody, then with monoclonal mouse anti-human IgE antibody, as described in Figure 4. The assayed human serum was placed in serial two-fold dilutions in the wells starting at dilution of 1:2. After 4hr incubation at room temperature the wells were washed and biotinylated α -gal BSA was placed in the wells at a concentration of 1 μ g/ml in PBS containing 1% BSA (PBS-BSA). The ELISA plates were incubated for 20hr at 4°C then washed and avidin-HRP (at a dilution of 1:2,500 in PBS-BSA) added to the wells for 1hr. After additional washing, color reaction was developed with OPD. Note that the normal serum had no anti-Gal IgE activity (○), whereas anti-Gal IgE was readily detectable in the serum of the person with multiple allergies, even at a serum dilution of 1:8 (●). Data are of representative of tetraplicates at each serum dilution. The data of the healthy individual are representative of 5 individuals with similar negative results.

DEFINITIONS

To facilitate an understanding of the present invention, a number of terms and phrases are defined below:

The term “therapeutic glycoproteins” as used herein, refers to any protein molecule that carries carbohydrate chains and is used for clinical treatment by injection intravenously or to any

tissue such as intramuscular or intradermal. Therapeutic glycoproteins may originate from blood, tissue extracts, or secretion of various animals such as in milk, or may be produced in natural or recombinant form from various cells grown in cultures. Some non-limiting examples of therapeutic glycoproteins are: monoclonal antibodies, recombinant or natural hormones, recombinant growth factors and cytokines.

The term “ α -gal epitope” as used herein, refers to any molecule, or part of a molecule, with a terminal structure comprising Gal α 1-3Gal β 1-4GlcNAc-R, Gal α 1-3Gal β 1-3GlcNAc-R, or any carbohydrate chain with terminal Gal α 1-3Gal, or terminal α -galactosyl at the non-reducing end, and which are capable of binding natural or elicited anti-Gal antibodies.

The term “glycoproteins with α -gal epitopes” as used herein, refers to any protein with at least one carbohydrate chain which has one or more α -gal epitopes, or any oligopeptide or polypeptide and which has one or more α -gal epitopes.

The term α -gal glycolipid describes a glycolipid in which the carbohydrate chain has one or more α -gal epitopes.

The term α -gal liposomes as used herein, refers to liposomes comprised of α -gal glycolipids, phospholipids and with or without cholesterol which presents multiple α -gal epitopes on its surface. Liposomes presenting multiple α -gal epitopes on glycoconjugates other than glycolipid are also referred to as α -gal liposomes.

The term α -gal nanoparticles refers to submicroscopic particles that present one or more than one α -gal epitopes. A non-limiting example for α -gal nanoparticles is α -gal liposomes that are split into submicroscopic particles by sonication or by any other method known to those skilled in the art.

The term α -gal liposomes/nanoparticles refers to a mixture of α -gal liposomes that can be viewed in a light microscope and α -gal nanoparticles that are of submicroscopic size and thus can not be seen in the light microscope.

GENERAL DESCRIPTION OF THE INVENTION

The present invention teaches a methods for identifying individuals animals who have anti-Gal IgE in the serum and therefore they are allergic to the α -gal epitope and for the identification of anti-Gal IgE in the serum of tested individuals. These identifications are important for preventing injection of recombinant glycoproteins with α -gal epitopes into

individuals that have anti-Gal IgE at a level that can cause the induction of allergic reactions and anaphylactic shock due to the α -gal epitope/anti-Gal IgE interaction.

5 I. Anti-Gal Antibodies and α -Gal Epitopes

Anti-Gal is the most abundant natural antibody in humans constituting ~1% of serum immunoglobulins (Galili et al., J Exp Med, 160:1519, 1984). This antibody interacts specifically with α -gal epitopes (Gal α 1-3Gal β 1-4GlcNAc-R or Gal α 1-3Gal β 1-3GlcNAc-R) on glycolipids and glycoproteins (Galili et al. J Exp Med 162:573, 1985; Galili, Springer Semin Immunopathol, 10 15:155, 1993). Anti-Gal is produced throughout life as a result of antigenic stimulation by bacteria of the gastrointestinal tract (Galili et al., Infect Immun, 56:1730, 1988) and is found in the serum as IgG, IgM and IgA antibodies (Hamadeh et al. Clin. Diagnos. Lab. Immunol. 2:125, 1995). The α -gal epitope is synthesized by the glycosylation enzyme α 1,3galactosyltransferase (α 1,3GT) and expressed in very large amounts on cells of non-primate mammals, prosimians and 15 in New World monkeys (Galili et al., J Biol Chem, 263:17755, 1988). The α 1,3GT gene was inactivated in ancestral Old World primates. Thus humans, apes, and Old World monkeys lack α -gal epitopes and produce high titer anti-Gal antibodies (Galili, Springer Semin Immunopathol, 15:155, 1993). Recently, anti-Gal was reported to be present as IgE antibodies in part of the population (Chung et al. New Engl J Med 358: 1109, 2008) and to mediate allergic reactions to 20 red meat (Jacquenot et al. J Allergy Clin Immunol 124:603, 2009; Commins et al. J Allergy Clin Immunol 123:426, 2009). When therapeutic glycoproteins, such as monoclonal antibodies, that have α -gal epitopes, are administered intravenously into patients with anti-Gal IgE, the binding of this antibody to the α -gal epitopes on such glycoproteins results in the induction of allergic reactions, to the extent of induction of anaphylactic shock (Chung et al. New Engl J Med 358: 25 1109, 2008). Thus, it is clinically important to have reliable assays that detect individuals who are allergic to α -gal epitopes.

II. Prerequisites for effective allergens that detect allergic sensitivity to α -gal epitopes

The most common test for determining the allergic sensitivity (hypersensitivity) to a 30 given allergen is a skin test in which the allergen in question is applied to the skin by a scratch

(also known as puncture or prick), or by intradermal injection, or by a patch placed on the skin. If the individual is allergic to the tested allergen, the IgE specific to that allergen and bound to mast cells or basophils will bind the allergen. This interaction will result in degranulation of the mast cells or basophils and formation of a local allergic reaction by the histamine and other allergy mediating substances in the released granules. This allergic reaction is identified in the skin by a doctor or a nurse, usually within 30min after application of the allergen to the skin. Patch tests may take longer to induce an allergic reaction. The reaction is detected in the skin as a wheal (swelling of the skin) and/or a rush (reddening of the skin) at the application site.

An allergen for testing allergy to α -gal epitopes has to have two characteristics: 1. It should contain a very large number of α -gal epitopes, and 2. It should not elicit an immune response by itself. The reasons for the need for such characteristics are as follows: 1. Since all humans produce the natural anti-Gal IgG and natural anti-Gal IgM antibodies as the most abundant antibodies in human serum, the allergen testing for α -gal epitope allergy has to introduce multiple α -gal epitopes into the skin at the test site. Because of the high concentration of the natural anti-Gal antibody, many of the α -gal epitopes may be masked by anti-Gal IgG and IgM immunoglobulin molecules binding to them, thereby preventing anti-Gal IgE binding. Therefore, a sufficient number of α -gal epitopes has to be available also for IgE binding in order to elicit the allergic reaction in the skin. 2. Patients treated with therapeutic glycoproteins that have α -gal epitopes (e.g. cetuximab) may receive repeated infusions of the therapeutic glycoprotein. Some of these patients may develop an allergy to α -gal epitopes linked to the therapeutic glycoprotein during the therapy course. Therefore, it is important that the allergen will not be immunogenic by itself, i.e. it should not elicit an immune response to the portion of the molecule to which the α -gal epitope is linked. If that molecule is immunogenic (e.g. a foreign protein such as bovine albumin) it would elicit an immune response that upon repeated testing may provide a "false positive" result because of an allergic reaction to that protein or because of an Arthus reaction (i.e. localized complement activation due to antigen/antibody interaction) in the skin. α -Gal liposomes and α -gal nanoparticles serve as optimal allergens in that they present multiple α -gal epitopes and they are not immunogenic. The lack of immunogenicity stems from the fact that α -gal epitopes can not activate T cells and because the phospholipids and cholesterol

of the α -gal liposomes/nanoparticles are not immunogenic (Tanemura et al. J Clin Invest 105: 301, 2000).

III. α -gal nanoparticle as detectors of allergic sensitivity to α -gal epitopes

5 α -Gal liposomes and α -gal nanoparticles are produced by splitting α -gal liposomes into submicroscopic particles, thus α -gal nanoparticles are defined as submicroscopic α -gal liposomes. α -Gal nanoparticles are produced from chloroform:methanol extracts of rabbit red blood cells (RBC) membranes since these RBC have the highest amount of α -gal glycolipids among mammalian RBC (Galili et al. J Immunol 148: 4676, 2007; Galili et al. Burns 36:239, 10 2010). The RBC are lysed by hypotonic shock in water and washed repeatedly by centrifugation and re-suspension of the pellet in water, until the supernatant is clear with no hemoglobin. After extraction in a mixture of chloroform and methanol under constant stirring for a period of several minutes to one or several days, the denatured proteins precipitate and are removed together with residual RBC membrane material by filtration through a Whatman paper placed in a Buchner 15 funnel. The extracting solution contains the phospholipids, cholesterol and glycolipids. The extract is dried in a rotary evaporator and is sonicated in saline to generate a suspension of liposomes. The liposomes formed in this procedure are sterile and are comprised of glycolipids, phospholipids and cholesterol. Subsequently, these liposomes are converted into α -gal nanoparticles by additional sonication with a sonication probe in a sterile laminar flow hood. The 20 suspension of α -gal nanoparticles is further sterilized by filtration through a 0.2 μ m filter. The α -gal nanoparticles are shown in Figure 2, where anti-Gal IgG coated α -gal nanoparticles are attached to the Fc γ receptors of macrophages. The size of these α -gal nanoparticles ranges between 20 and 400nm, but the size is not limited to this range.

25 The glycolipids, comprising of carbohydrate chains linked to ceramide, are anchored via the fatty acid tails of the ceramide into the lipid membrane of the nanoparticle. The hydrophilic carbohydrate chains are protruding out as described in Figure 1. All glycolipids in rabbit RBC membranes with 5 or more sugars are capped with α -gal epitopes (i.e. are α -gal glycolipids) and comprise >90% of glycolipid molecules. As shown in Figure 3, these α -gal glycolipids increase in their size by increments of 5 carbohydrates, each forming an additional branch (called also

antenna), with the exception of ceramide heptahexoside which has 7 carbohydrates and is a non-branched chain. The expression of α -gal epitopes on the various α -gal glycolipids is indicated by the binding of monoclonal anti-Gal as shown in the right lane of Figure 3A. Because of the abundance of α -gal epitopes on these nanoparticles, they are referred to as α -gal nanoparticles.

5 With the exception of α -gal glycolipids, all other components of α -gal nanoparticles are present also in human RBC. These include phospholipids, cholesterol and a glycolipid with 3 carbohydrates called ceramide tri-hexoside (CTH). It is estimated that there are $\sim 10^{15}$ α -gal epitopes on 1mg α -gal nanoparticles.

α -Gal nanoparticles are not immunogenic in humans and do not elicit an immune
10 response. This is because phospholipids, cholesterol and ceramide trihexoside are molecules present in humans cells and thus the human immune system is immunotolerant to them. α -Gal glycolipids are also not immunogenic since the α -gal epitope can elicit an immune response only if it is linked to an immunogenic protein but not to a lipid (Tanemura et al. J Clin Invest 105:301, 2001; Galili Transplantation 78:1093, 2004). Therefore, α -gal nanoparticles fulfill the
15 prerequisites for an effective allergen for α -gal epitope allergic sensitivity in that they present multiple α -gal epitopes (10^{15} α -gal epitopes/mg α -gal nanoparticles) and they are not immunogenic. The ability of α -gal epitopes on α -gal liposomes to bind the anti-Gal antibody was previously demonstrated (Galili et al. Burns 36:239, 2010).

For evaluation of allergic hypersensitivity to α -gal epitopes, α -gal nanoparticles in an
20 amount that may range from 1ng to 100mg is applied to the tested individual by methods known to those skilled in the art, including , but not limited to intradermal injection, through a scratch in the skin, or by a patch. The skin area where α -gal nanoparticles were applied is inspected by a doctor, or a nurse or other health care givers for the appearance of allergic reaction, including but not limited to a wealth, redness, or rash of the skin. Such an allergic reaction will occur in
25 individuals that have anti-Gal IgE in titers high enough to mediate an allergic response.

A negative control confirming that the allergic hypersensitivity is specific to the α -gal epitope may be the performance of a similar skin test using nanoparticles that lack α -gal epitopes. Such nanoparticles may be produced by the same method described above for the preparation of α -gal nanoparticles. However, the RBC to be used will be human blood type O

RBC, or RBC from α 1,3galactosyltransferase knockout pigs, or other RBC lacking α -gal epitopes, as the source for the mixture of glycolipids, phospholipids and cholesterol. Allergic reaction with α -gal nanoparticles and no allergic reaction with nanoparticles lacking these epitopes will confirm that the allergic reaction is the result of anti-Gal IgE on mast cells and on
5 basophils interacting with α -gal epitopes on the nanoparticles.

The test for identification of individuals with allergy to α -gal epitopes is not limited to α -gal nanoparticles, α -gal liposomes, or a mixture of α -gal liposomes/nanoparticles. Other glycoconjugates which carry α -gal epitopes may be suitable applying to the skin for detecting allergic hypersensitivity to α -gal epitopes. These include but are not limited to natural or
10 synthetic α -gal nanoparticles and α -gal liposomes, glycoproteins with natural or synthetic α -gal epitopes linked to them, polymers with linked α -gal epitopes and proteoglycans with linked α -gal epitopes.

IV. ELISA for detection of anti-Gal IgE in the serum

The detection of anti-Gal IgE may be performed by "regular" ELISA in which α -gal BSA, the recombinant glycoprotein with α -gal epitopes, or any glycoprotein with α -gal epitopes may be attached to ELISA wells as solid-phase antigen. This will be followed by incubation of the assayed serum in such ELISA wells coated with α -gal glycoconjugates and detection of the IgE molecules bound to the wells by the use of a secondary anti-human IgE antibody coupled with
20 peroxidase or alkaline phosphatase or any other label known to those skilled in the art. This method is unreliable since the concentration of anti-Gal IgG, IgM and IgA in the serum is much higher than that of anti-Gal IgE. Therefore anti-Gal isotypes other than IgE will preferentially bind to the α -gal epitopes on the solid-phase antigen coating the wells and prevent the binding of anti-Gal IgE to the ELISA wells. The problem of competition with other anti-Gal isotypes is
25 avoidable by the use of the assay that the current invention is teaching.

The detection of anti-Gal IgE, while avoiding the use of labeled anti-human IgE antibody, is feasible by the use of labeled α -gal glycoconjugate, i.e. biotin labeled α -gal BSA, or any other glycoconjugates (glycoproteins and other molecules with carbohydrate chains) with α -gal epitopes and which is labeled. The labeling of the glycoconjugate carrying α -gal epitopes
30 can be performed with biotin, radioactive isotope, enzyme such as peroxidase or any label known

to those skilled in the art.

The basic principle in this method is the coating of ELISA wells by monoclonal or polyclonal anti-human IgE antibodies from any source and by any method known to those skilled in the art. The anti-IgE antibodies can be used directly to coat the ELISA wells, or by the use of a secondary antibody such as, but not limited to, anti-mouse IgG antibodies that coat wells in order to bind mouse anti-human IgE antibodies. Alternatively, the ELISA wells can be coated first with protein A or protein G for subsequent capture of anti-IgE antibody. A non-limiting example is schematically illustrated in Figure 4 and is performed as the following: The ELISA wells are coated for several hours with anti-mouse IgG antibodies in order to subsequently capture mouse anti-human monoclonal anti-IgE antibody.

The wells are washed and monoclonal mouse anti-human IgE solution is incubated in the wells for a period less than an hour up to several hours. This enables the anti-human IgE antibodies which by themselves are of the IgG isotype, to be captured by the anti-mouse IgG antibodies coating the wells. After binding of the anti-human IgE antibodies to the wells, the wells are washed and the assayed human serum is placed in aliquots in the wells at serial two fold dilutions. The serum is incubated for few minutes to several hours in the wells in PBS containing 1% BSA (PBS-BSA) or any other appropriate buffer, in order to capture human IgE molecules by the anti-human IgE antibody molecules coating the ELISA wells. The wells are then washed and biotinylated α -gal BSA is placed in the wells. The concentration of biotinylated α -gal BSA may vary, however the preferred concentration is 1 μ g/ml. The biotinylated α -gal BSA is incubated in the wells for any time period, with a preferable incubation period of 20hr at any temperature, preferably at 4°C. This incubation enables the biotinylated α -gal BSA to bind to anti-Gal IgE antibody molecules, if such molecules are among IgE molecules captured in the wells. At the end of incubation, the wells are washed and the biotinylated α -gal BSA molecules bound to the wells are detected by incubation with avidin coupled to peroxidase (Sigma) diluted in PBS-BSA. Other solutions known to those skilled in the art may be used for preparing the avidin-HRP solution. Avidin linked to other labels known to those skilled in the art, may be used instead of peroxidase. After incubation with avidin-peroxidase for a period of several minutes to several hours, the wells are washed and color development as a result of the bound HRP is measured with the color substrate ortho-phenylene diamine (OPD [Sigma]). Color absorbance is measured at 492nm. Other color substrates may be used according to the corresponding label

coupled to avidin.

Because the described assay does not use labeled anti-IgE antibodies and because the presence of anti-Gal IgE molecules is determined by the binding of its corresponding ligand- the α -gal epitope linked to protein or on any labeled glycan, the background nonspecific binding is marginal and the anti-Gal immunoglobulins of other isotypes (IgG, IgM or IgA) do not compete with anti-Gal IgE since they are not captured in the ELISA wells.

EXPERIMENTAL

The following example is provided in order to demonstrate and further illustrate certain preferred embodiments and aspects of the present invention and it is not to be construed as limiting the scope thereof.

In the experimental disclosure which follows, the following abbreviations apply: mg (milligrams); μ g (micrograms); μ l (microliters); nm (nanometers); C (degrees Centigrade); α 1,3GT (α 1,3galactosyltransferase); BSA (bovine serum albumin); ELISA (enzyme linked immunosorbent assay); HRP (horseradish peroxidase); OD (optical density); OPD (ortho phenylene diamine); PBS (phosphate buffered saline).

EXAMPLE 1: DETECTION OF ANTI-GAL IgE IN THE SERUM OF A PERSON WITH MULTIPLE ALLERGIES

The objective of this study is to have a proof of principle on the detection of anti-Gal IgE by the method described in Figure 4. ELISA wells (Falcon plates #3912) were coated with goat anti-mouse IgG diluted 1:100 in PBS and plated as 50 μ l/well. The plates were incubated for 20h at 4°C. Subsequently, the plates were washed 4 times with PBS and monoclonal mouse anti-human IgE (Sigma, ascetic fluid containing the monoclonal antibody as IgG₂ isotype and diluted 1:10,000 in PBS) was placed in the wells as 50 μ l/well. After 4hr incubation at room temperature to allow for the binding of the anti-human IgE antibody to the anti-mouse IgG antibody coating the wells (Figure 4), the plates were washed 4 times with PBS and serum samples from healthy nonallergic people or from a donor who has multiple allergies, were placed in the wells as serial two fold dilutions in PBS containing 1% BSA (PBS-BSA), as 50 μ l/well and starting at a serum dilution of 1:2. The sera were incubated in the wells for 4hr at room temperature to allow maximum binding of human IgE to the anti-human IgE antibodies coating the wells. The wells

were then washed, biotinylated α -gal BSA (1 μ g/ml in PBS-BSA) was placed into each well (50 μ l/well) and the plates were incubated for 20hr at 4°C. For this purpose α -gal BSA was purchased from Dextra (Reading, UK) and was biotinylated with Pierce biotinylation kit (Pierce Inc. Rockford, IL). After incubated for 20hr at 4°C the ELISA wells were washed 4 times with
5 PBS containing 0.05% Tween (PBS-Tween) and avidin-HRP (Sigma) diluted 1:2,500 in PBS-BSA was added to the wells as 50 μ l/well and the plates were incubated for 1hr at room temperature. After additional 4 washes with PBS-Tween, the color substance OPD (1mg/ml) in pH 5.5 buffer (100 μ l aliquots containing hydrogen peroxide) was added to the wells, color development was allowed for 5min and the reaction was stopped with the addition of 50 μ l
10 sulphuric acid (1M). Absorbance by the generated color was measured at 492nm in an ELISA reader and presented as optical density units (O.D.).

As shown in Figure 5, no significant anti-Gal IgE activity was detected in the serum of nonallergic individuals (a representative donor of 5 with similar results). Thus, even at the low serum dilution of 1:2, no anti-Gal IgE activity was detected above the background level of 0.12
15 O.D. In contrast, in the serum of the donor that has multiple allergies, anti-Gal IgE activity was detected at the level of 0.8 O.D. at serum dilution of 1:2, 0.4 O.D. at serum dilution of 1:4 and 0.25 O.D. at serum dilution of 1:8. These data imply that whereas in nonallergic individuals most isotype switches are from IgM producing cells to IgG or IgA producing cells, in this donor, there is the tendency of isotype switch also from IgM to IgE. Since there are many anti-Gal B cells
20 (~1% of B cells) in humans (Galili et al. Blood 82:2485, 1993), a significant proportion of these B cells underwent isotype switch from IgM to IgE producing cells and therefore anti-Gal IgE is found in the serum.

CLAIMS

We claim

- 5 1. A method for the identification of individuals allergic to α -gal epitopes by performing an allergy skin test in which glycoconjugates having linked α -gal epitopes are applied to the skin.
2. The method in Claim 1 in which the glycoconjugates with linked α -gal epitopes are α -gal nanoparticles or α -gal liposomes.
- 10 3. The method in Claim 1 in which the glycoconjugates with linked α -gal epitopes are glycoproteins, proteoglycans or polymers all of which have linked one, two or multiple α -gal epitopes.
- 15 4. A method for the identification of anti-Gal IgE molecules in human serum by ELISA with anti-human IgE as solid-phase antigen.

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25

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Figure 1

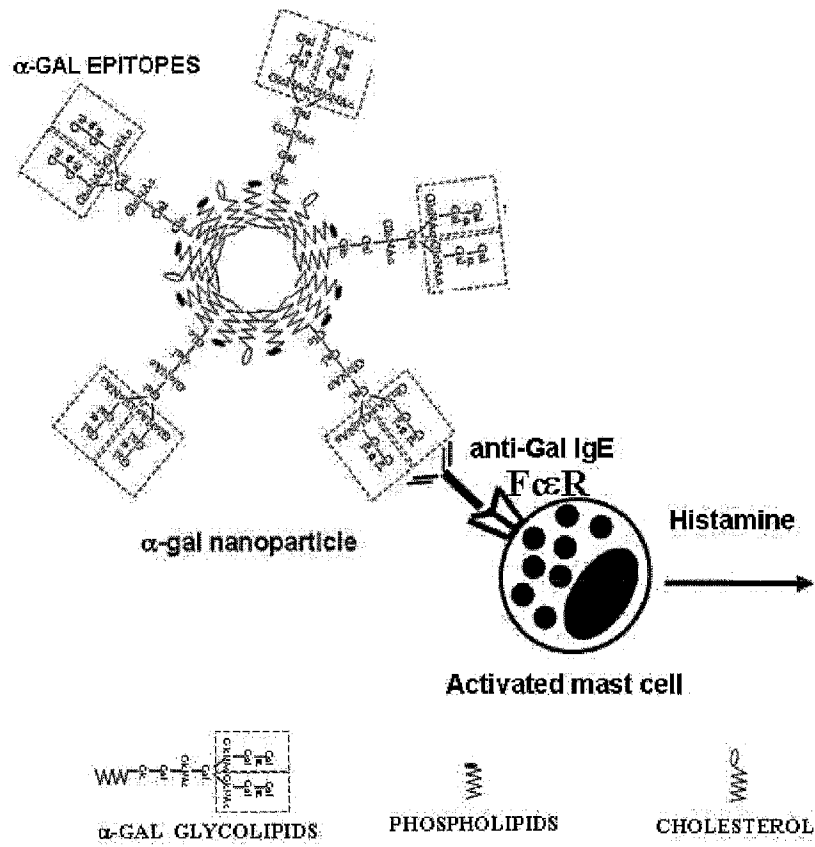


Figure 2

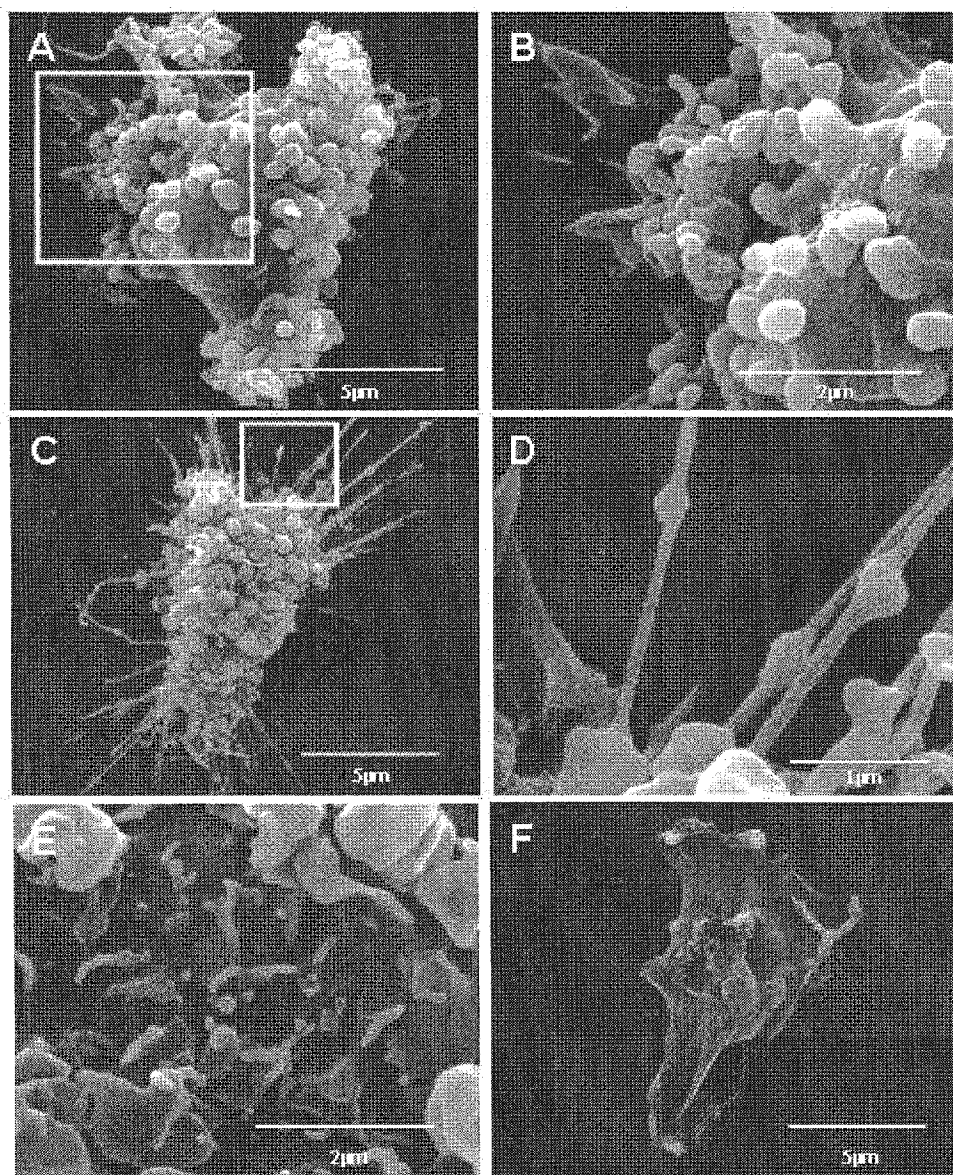


Figure 3

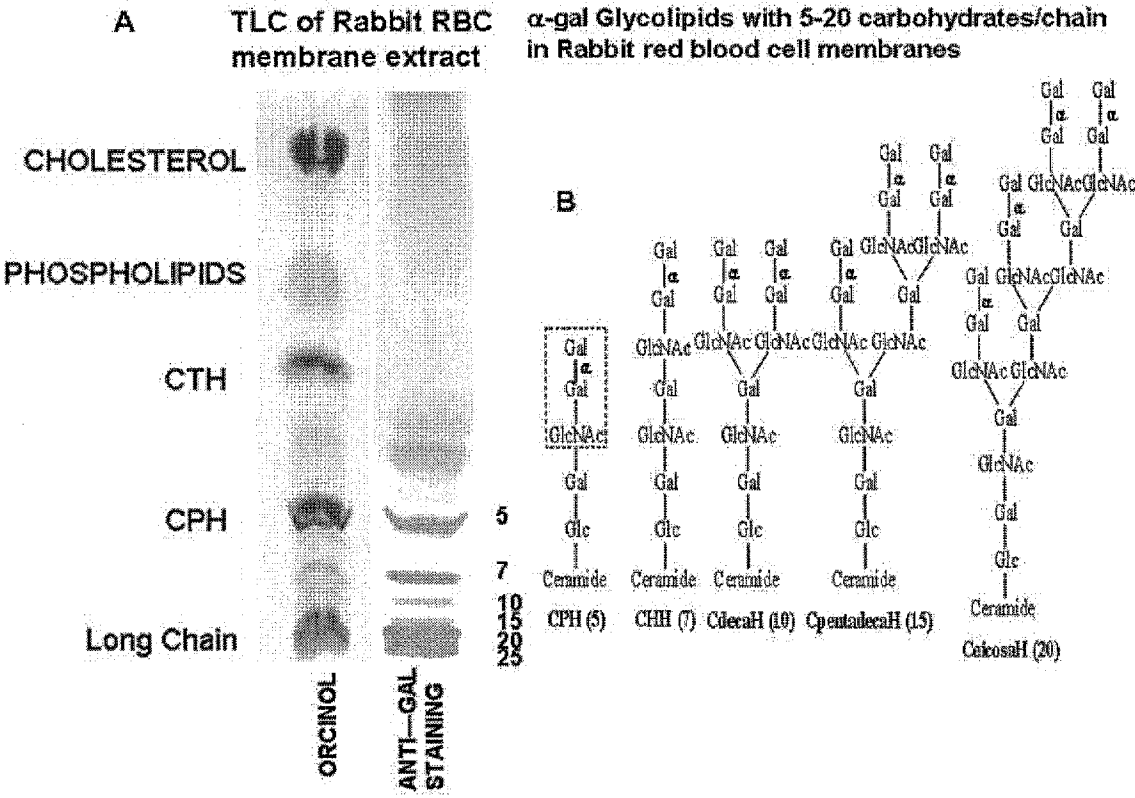
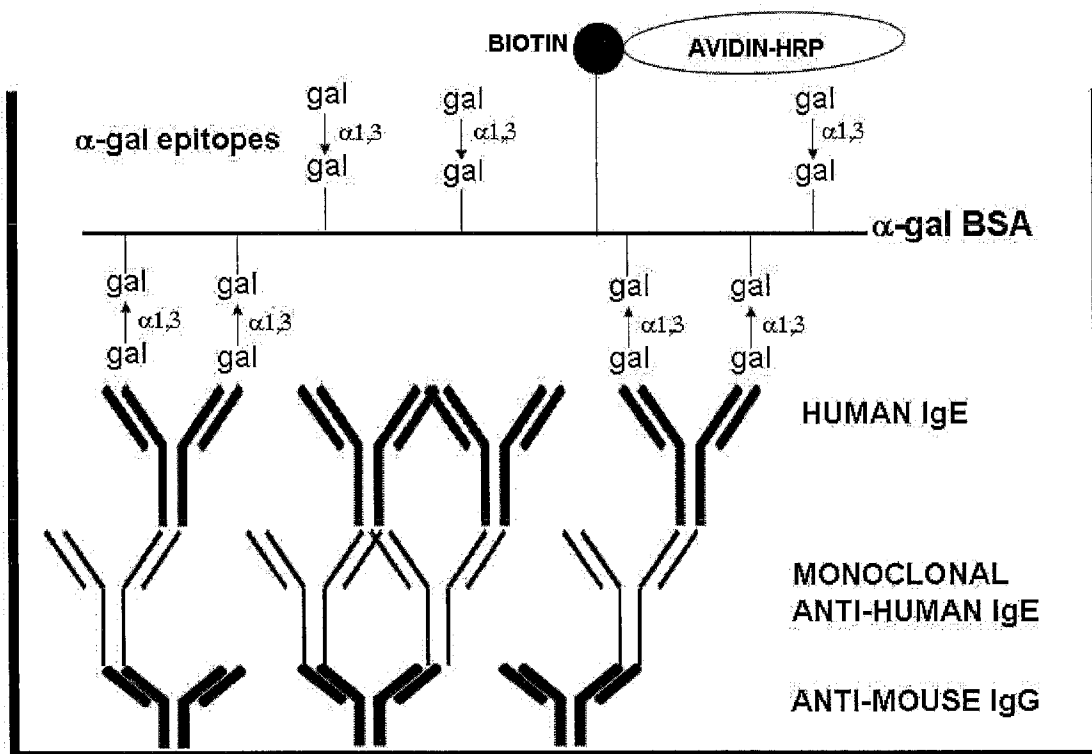
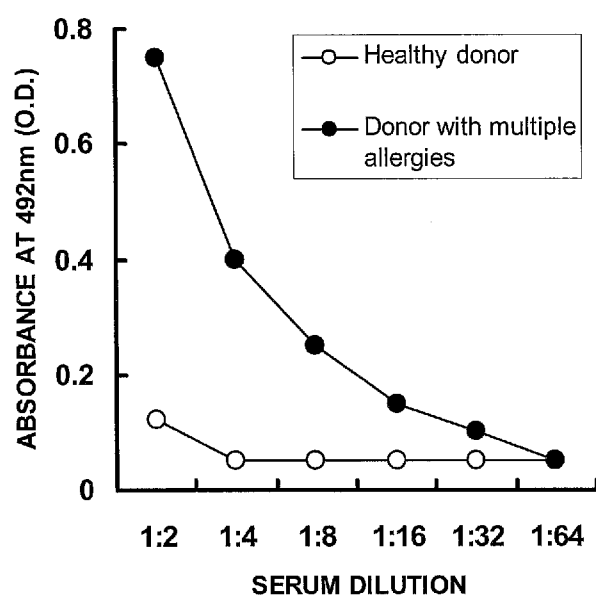


Figure 4



ELISA WELL FOR ANALYSIS OF ANTI-GAL IgE IN HUMAN SERUM

Figure 5



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 12/30890

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 39/385 (2012.01)

USPC - 424/193.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC(8) - A61K 39/385 (2012.01)

USPC - 424/193.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC - 424/193.1, 185.1; 530/300,350; 435/69.1, 69.7

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

PubWest (DB=PGPB,USPT,EPAB,JPAB,DWPI); Free Patents Online (USPT, PGPB, WIPO/PCT, EP,JP, German, NPL, keyword);

DialogWeb (65,2, 35,315,155, 440); Google (Google Scholar)

Search terms: Glycoconjugate, nanoparticle, liposome, glycoprotein, proteoglycan, epitope, Gal, IgE, serum, ELISA, solid-phase, antigen

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2008/152040 A1 (Altmann et al.) 18 December 2008 (18.12. 2008) pg 7, 12-13, claim13	1-4
A	EP 0 994 353 B1 (Van Ree et al.) 19 April 2000 (19.04.2000) para [0009], [0012]	1-4
A	US 6399578 B1 (Jack et al.) 04 June 2002 (04.06.2002) (col5, ln4-10), (col21 ln 1-8), (col11 ln 23-28),	1, 3-4
A	WO 1997/11963 (Sandrin et al.) 03 April 1997 (03.04.1997) entire document	1, 3-4
A	EP 1 939 628 A1 (Sampson et al.) 02 July 2008 (02.07.2008) entire document	1,3
A	US6361775 (Galili et al.) 26 March 2002 (26.03.2002) entire document	1,3
A	US 2010/0145015 A1 (Galili) 10 June 2010 (10.06.2010) entire document	1,3
A	US 2011/0071043 A1 (Sampson et al.) 24.03.2011 (24.03.2011) entire document	1,3
A	US2010/0008973 A1 (Galili) 14 January 2010 (14.01.2010) entire document	1,3
A	Dotan et al. 'Anti-glycan antibodies as biomarkers for diagnosis and prognosis'. Lupus, 2006, 15, pp 1-10	1,3

☐ Further documents are listed in the continuation of Box C.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

08 June 2012 (08.06.2012)

Date of mailing of the international search report

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