SYNTHESIS OF TYPHOID FEVER VACCINE FROM A PLANT OR FRUIT POLYSACCHARIDE

SYNTHÈSE EINES TYPHUSIMPFSTOFFES UNTER VERWENDUNG EINES POLYSACCHARIDS AUS EINER PFLANZE ODER EINER FRUCHT

SYNTHESE DU VACCIN ANTITYPHOIDIQUE À PARTIR D’UN POLYSACCHARIDE DE PLANTE OU DE FRUIT


- DATABASE MEDLINE FILE SERVER STN KARLSRUHE ABSTRACT 90270448, QADRI ET AL 'MONOCLONAL ANTIBODIES AGAINST TWO DISCRETE DETERMINANTS ON VI CAPSULAR POLYSACCHARIDE'
- DATABASE MEDLINE FILE SERVER STN KARLSRUHE ABSTRACT 76096589, KWIATKOWSKI ET AL 'DISRUPTION OF VI BACTERIOPHAGE III AND LOCALIZATION OF ITS DEACETYLASE ACTIVITY'

DESCRIPTION

[0001] The present invention relates to immunophylaxis and vaccines. More particularly it relates to modifying a plant, fruit or synthetic polysaccharide such that it is immunogenic and may be used as a vaccine to prevent typhoid fever in infants and young children.

BACKGROUND OF THE INVENTION

[0002] Typhoid fever, caused by Salmonella typhi, remains a common and serious disease in many parts of the world. The capsular polysaccharide (Vi) is both an essential virulence factor and a protective antigen of Salmonella typhi [19]. Tacket et al. in J. Infect. Dis. 154: 342-345 (1986) disclose a vaccine made from the Vi capsular polysaccharide of Salmonella typhi. Field trials in Nepal and in the Republic of South Africa showed that a single injection of Vi conferred about 70% protection against typhoid fever in older children and in adults [1,13]. The mechanism of its protective action, similar to other polysaccharide vaccines, is to elicit a critical level of serum antibodies.

[0003] The immunologic properties of the Vi that limits its use as a vaccine are: 1) only ~70% efficacy in individuals 5 to 45 years of age; 2) an age-dependent serum antibody response. Vi elicited a comparatively short-lived antibody responses in 2 to 5 year old children and only low levels of antibodies in a fraction of children <2 years-old and; 3) reinjection did not elicit a booster antibody response (T-cell independent) [15,19]. To increase its immunogenicity and to induce T-cell dependence, the Vi was conjugated to proteins [22,24,25]. A clinical trial in adults in the United States showed that Vi-protein conjugates elicited significantly higher levels of serum antibodies than the Vi alone [25].

[0004] The Vi is a linear homopolymer of (1→4)-α-D-GalAPNac, variably O-acetylated at C3 (Fig. 1) [19, 23]. Whiteside and Baker in J. Immunol. 86:538-542 (1961) and Landy et al., Am. J. Hyg. 73: 55-65 (1961) disclose that the α-acetyl groups on Vi is essential for its antigenicity. Szu et al. disclose a conjugate scheme for Vi capsular polysaccharide covalent bound to a carrier protein (22, 23, 24). International Publication No. WO 94/03208 published February 17, 1994, U.S. Patent No. 5,204,098 issued April 20, 1993 and International Publication No. WO 93/07178 published April 15, 1993 also disclose Vi capsular polysaccharide-protein carrier conjugates. However, synthesis of Vi-protein conjugates poses several problems. First, the high molecular weight of Vi (~2x10^3 kD) causes conjugates to be poorly soluble. Second, standardization of Vi conjugates has been hindered by a lack of a colorimetric method for quantification of this polysaccharide [21]. Colorimetric methods are not applicable to the Vi because the polyhexosaminuronic acid structure resists acid hydrolysis and does not form a chromophore in the carbazole assay.

[0005] Szewczyk and Taylor in Infect. Immun. 29:539-544 (1980) taught the art of O-acetylated polygalacturonic acid to form a compound that is antigenically indistinguishable from the Vi as determined by immunodiffusion. The O-acetylated pectin, even though antigenic, is not immunogenic in vivo. Avery and Goebel in J. Exp. Med. 50: 531 (1929) and Goebel in J. Exp. Med., 50: 469-520 (1929) showed that the immunogenicity of pneumococcus type 3 polysaccharide could be increased by binding it chemically to a carrier protein. This principle has been applied successfully to increase the immunogenicity of capsular polysaccharides of other pathogens (7, 10, 22, 24).

[0006] Until the present invention, the art has not shown a plant or fruit derived O-acetylated oligo- or polygalacturonic acid that is both antigenic and immunogenic against S. typhi.

SUMMARY OF THE INVENTION

[0007] It is an object of the present invention to overcome deficiencies in the prior art, such as those noted above.

[0008] It is a further object of the present invention to produce an antigen based on a plant, fruit or synthetic oligo- or polysaccharide which is immunologically similar to the Vi antigen of Salmonella typhi. Preferably the oligo- or polysaccharide is based on pectin which has been modified by acetylation at the C2 and/or C3 hydroxyls of its galacturonate subunit.

[0009] It is yet another object of the present invention to provide an immunogen that elicits antibodies that bind Vi of S. typhi in which the immunogen is based on a plant, fruit or synthetic oligo- or polysaccharide conjugated with a carrier.

[0010] It is yet another object of the present invention to provide antibodies against Vi of S. typhi which are elicited by immunization with a plant, fruit or synthetic polysaccharide-carrier conjugate.

[0011] According to the present invention, methods are provided to synthesize a modified plant, fruit or synthetic oligo- or polysaccharide which is structurally similar to the Vi antigen.

[0012] According to the present invention, methods are provided to conjugate the modified plant, fruit or synthetic oligo- or polysaccharide with a carrier.
BRIEF DESCRIPTION OF THE DRAWINGS

[0013] Fig. 1 shows the structure of the repeating unit of the Vi, the pectin and the O-acetylated pectin. For Vi, C₂ (R) is N-acetylated and C₃ (R¹) is O-acetylated; for pectin, C₂ and C₃ are hydroxylated; for OAcPec, C₂ and C₃ are O-acetylated, n = number of subunits.

[0014] Fig. 2 shows the methyl resonances on the O-acetylated pectin by ¹³C NMR spectroscopy.

[0015] Fig. 3 shows the HPLC gel filtration profile of the O-acetylated pectin - TT conjugate through Superose® 6 in 0.01 sodium phosphate, 0.1M Na₂SO₄, pH 7.0. The refractive index is the upper line and the 280nm absorbance is the lower line.

[0016] Fig. 4 shows the antigenicity of the O-acetylated pectin compared with Vi by double immunodiffusion. Center well, B-260 Vi antiserum, 1) Vi, 100 µg/ml; 2) OAcPec K⁺ form; 3) OAcPec Ca²⁺ form; 4) OAcPec C₂H₅N⁺ form.

[0017] Fig. 5 shows the quantitative precipitin analysis of pectin (∆), OAcPec (●), and Vi (○).

[0018] Fig. 6 shows the temperature dependent stability of O-acetals on Vi(---) and O-acetylated pectin (_______) at 4°C (○), 22°C (■), 37°C (▲), and 60°C (∆). The decrease in extent of O-acetylation is depicted as the % remaining after incubation at the various intervals and temperatures compared to the starting material.

DETAILED DESCRIPTION OF THE INVENTION

[0019] The Vi molecule of Salmonella typhi has a simple structure which is a linear polysaccharide having repeating sugar subunits. The antigenicity and immunogenicity of Vi depends on its N-acetyl at C₂ and O-acetyl at C₃ on each galacturonate subunit [19,23]. As shown for Vi and other polysaccharides, removal of the O-acetyl removed most of the antigenicity and all of the immunogenicity of the Vi [23,26]. The precise role of N-acetyl is not known as selective removal of the N-acetyl on Vi has not been accomplished. The present invention mimics the simple structure of Vi by modification of plant, fruit or synthetic saccharides. The modified plant, fruit or synthetic saccharides resemble Vi in antigenic and immunogenic properties and as such they have the capacity to act as an effective vaccines against typhoid fever.

[0020] The present invention relies on the use of a modified plant, fruit or synthetic oligosaccharide or polysaccharide. Oligosaccharide as defined herein is a carbohydrate containing from two to ten simple sugar subunits linked together. A polysaccharide as defined herein is a carbohydrate containing more than ten simple sugar subunits linked together. The present invention preferably makes use of a modified pectin or modified D-galacturonan, oligogalacturonate or polygalacturonate and mixtures thereof. As used herein, modified pectin or modified oligogalacturonate or polygalacturonate refers to native or naturally occurring pectin or synthetic D-galacturonan, oligogalacturonate and polygalacturonate that has been structurally altered. Such structural alterations are any alterations that render the modified pectin or modified D-galacturonan, oligogalacturonate or polygalacturonate antigenically similar to the Vi antigen of Salmonella typhi. The structural alterations substantially approximate the structure of the Vi antigen of S. typhi.

[0021] Preferably, the aforementioned modified pectin, D-galacturonan, oligo- and polygalacturonate is further characterized by its ability to immunologically mimic an epitope (antigenic determinant) expressed by S. typhi. Such a modified pectin, D-galacturonan, oligo- and polygalacturonate is useful herein as a component in an inoculum for producing antibodies that react with the Vi of S. typhi, and preferably immunoreact with the Vi of S. typhi.

[0022] As used herein, the phrase "immunologically mimic" in its various grammatical forms refers to the ability of the aforementioned modified pectin, modified D-galacturonan, oligogalacturonate and polygalacturonate to immunoreact with an antibody that recognizes and binds to a native epitope on the Vi of S. typhi as defined herein.

[0023] It should be understood that a subject modified pectin, modified D-galacturonan, oligogalacturonate and polygalacturonate need not be structurally identical to the Vi antigen so long as it includes the required sterical structure and is able to elicit antibodies that react with the Vi antigen on S. typhi.

[0024] A subject modified pectin, modified D-galacturonan, oligogalacturonate and polygalacturonate includes any substituted analog, fragment or chemical derivative of a pectin so long as the modified pectin, modified D-galacturonan, oligogalacturonate and polygalacturonate is capable of reacting with antibodies that react with the Vi antigen. Therefore, a present modified pectin, modified D-galacturonan, oligogalacturonate and polygalacturonate can be subject to various changes that provide for certain advantages in its use.

[0025] The phrase "substitution" includes the use of a chemically derivatized residue in place of a non-derivatized residue provided that such modified pectin, modified D-galacturonan, oligogalacturonate and polygalacturonate display the requisite immunological activity.

[0026] "Chemical derivative" refers to a subject modified pectin, modified D-galacturonan, oligogalacturonate and polygalacturonate having one or more residues chemically derivatized by reaction of a functional side group. Additional residues may also be added for the purpose of providing a "linker" by which the modified pectin, modified D-galacturonan, oligogalacturonate and polygalacturonate can be conveniently affixed to a label or solid matrix or carrier. Labels, solid matrices and carriers that can be used with the subject modified pectin, modified D-galacturonan, oligogalacturon-
The present invention makes use of a modified pectin, modified D-galacturonan, oligogalacturonate and polygalacturonate in which the monosaccharide subunit(s) have one O-acetylated carbon, preferably two O-acetylated carbons. In one embodiment, at least C₂ or C₃ is O-acetylated. In another embodiment, C₃ and C₂ are O-acetylated. In a preferred embodiment, at least 50% of C₂ and C₃ are O-acetylated.

The Vi molecule has N-acetyl groups at position C₂ and O-acetyl groups at position C₃. If all of the C₂ positions have acetyl groups and all the C₃ positions on Vi contain acetyl groups, then by definition, the Vi molecule is theoretically 200% fully acetylated. In most preparations of Vi the percent acetylation varies. The C₂ position is usually about 100% N-acetylated and the C₃ position is from about 60-90% O-acetylated depending on normal variation in preparations of Vi. The modified pectin, D-galacturonan, oligogalacturonate and polygalacturonate employed in the present invention approximates the total percent acetylation of Vi.

The modified pectin, D-galacturonan, oligogalacturonate and polygalacturonate employed in the present invention are from about 50% to about 200% O-acetylated, preferably from about 80% to about 200% O-acetylated, more preferably from about 160% to about 190% O-acetylated.

The modified pectin and the modified D-galacturonan, oligogalacturonate and polygalacturonate employed in the present invention have a molar ratio of O-acetyl groups/mole galacturonan sufficient to elicit antibodies that bind to Vi. The molar ratio may be at least 0.5 mole of O-acetyl/mole galacturonan (Gal A), preferably at least 1.6 moles O-acetyl/mole Gal A, more preferably between about 1.6 and about 1.9 moles O-acetyl/mole Gal A.

In one embodiment, the ratio is about 1.9 moles O-acetyl/mole Gal A. Lower molar ratios may be used for the present invention if the O-acetylated pectin, D-galacturonan, oligogalacturonate and polygalacturonate is shown to be immunogenic by screening techniques described herein.

As with other polysaccharides, the molecular weight of the Vi alone and as a Vi-carrier conjugate is related to its immunogenicity [16, 17, 22]. Thus, the modified pectin and modified D-galacturonan, oligogalacturonate and polygalacturonate may vary in molecular weight in order to enhance its antigenicity or to enhance its immunogenicity when in a conjugate form. The modified pectin and modified D-galacturonan, oligogalacturonate and polygalacturonate may have from about 2 to about 1,000 modified galacturonic subunits, preferably from about 50 to about 800, more preferably from about 200 to about 600 monosaccharide subunits. The molecular weight of the modified pectin may range from about 100 to about 1,000,000, preferably from about 200,000 to about 600,000. In one embodiment the molecular weight of the modified pectin is approximately 400 kD.

In addition to the modifications of the galacturonic acid at position C₂ or C₃, other substitutions or deletions are encompassed, such that the substitutions or deletions result in a modified pectin and modified D-galacturonan, oligogalacturonate and polygalacturonate that is antigenically similar to the Vi antigen of S. typhi.

In one particular embodiment, naturally occurring pectin is modified as to replace the hydroxyl groups at the C₂ and C₃ positions of galacturonic acid with O-acetyl groups. The modified pectin is referred to herein as OAcPec. The characteristics of OAcPec in comparison with Vi of S. typhi is as follows:

1) the Mₘ of Vi (≈2x10⁵ kD) is higher than that of OAcPec (≈400 kD) ; 2) the N-acetyl at C₂ in the Vi is replaced by an O-acetyl in OAcPec and; 3) OAcPec has <5% neutral sugars and Vi had a nondetectable amount. At 3-8°C, the stability of OAcPec as measured by its O-acetyl content and molecular size, is similar to that of Vi. At higher temperatures, the molecular size of Vi is more stable than the OAcPec probably due to the stabilizing effect of a hydrogen bond between the N-acetyl and the carboxyl of the adjacent residue [23]. Since vaccines will be stored at ≤3-8°C, the stability characteristic of OAcPec and Vi can be considered as similar.

OAcPec and Vi are antigenically indistinguishable by immunodiffusion (Fig. 4). However, OAcPec, unlike Vi, is not immunogenic in mice probably due to its lower molecular weight [16].

Another embodiment of the present invention is a modified pectin, and modified D-galacturonan, oligo-, and polygalacturonate-carrier conjugate. The modified pectin and modified D-galacturonan, oligo-, and polygalacturonate-carrier conjugate is immunogenic to Vi in mammals. By immunogenic is meant that the modified pectin-carrier conjugate and modified D-galacturonan, oligo-, and polygalacturonate-carrier conjugate elicit the production of antibodies upon injection into mammals. The antibodies elicited are capable of specifically reacting or binding to S. typhi, are capable of specifically reacting or binding to the Vi of S. typhi and are capable of providing passive protection against S. typhi in humans. The modified pectin and modified D-galacturonan, oligo-, and polygalacturonate-carrier conjugate of the present invention are capable of inducing a statistically significant rise of antibodies that bind to Vi (booster effect) upon reinjection.

Modified pectin, and modified D-galacturonan, oligogalacturonate and modified polygalacturonate have several advantages over the Vi in preparing conjugates for vaccines to prevent typhoid fever. Special P₉ facilities are required to culture pathogens such as S. typhi. This restricts the availability of Vi and presents safety concerns in preparing a Vi vaccine. The present invention of 1) pectin, D-galacturonan, oligo- and polygalacturonate are easy to prepare.
obtain, safe and purification is simpler than extraction of the Vi from S. typhi; 2) modified oligo- and polygalacturonate can be measured during the synthesis of the conjugate and in the final container by a colorimetric reaction and; 3) there is no solubility problem and the yield of modified pectin, D-galacturonan, oligo- and polygalacturonate-carrier conjugates is higher than with Vi; 4) at the 4°C, the standard storage temperature of vaccines, the stability of modified pectin, D-galacturonan, oligo- and polygalacturonate is similar to that of the Vi.

[0038] The present invention provides a method to prepare a synthetic Vi antigen from a plant, fruit or synthetic oligo- or polysaccharide and to conjugate it with a carrier in order to enhance and elicit a booster response against Salmonella typhi capsular polysaccharide.

[0039] In one embodiment of the method, pectin, D-galacturonan, oligogalacturonate, or polygalacturonate is O-acetylated at C2 and C3 positions with acetic anhydride. Through carbodiimide condensation the O-acetylated pectin, D-galacturonan, oligogalacturonate, or polygalacturonate is thiolated with cystamine, or aminated with adipic dihydrazide, diaminoesters, ethyldiamine and the like. Both the thiolated and the aminated O-acetylated pectin, D-galacturonan, oligogalacturonate, or polygalacturonate are stable, may be freeze dried, and stored in cold. The thiolated intermediate may be reduced and covalently linked to a polymeric carrier containing a sulfhydro group, an N-pyridyldithio group. The aminated intermediate may be covalently linked to a polymeric carrier containing a carboxyl group through carbodiimide condensation. The O-acetylated pectin, D-galacturonan, oligogalacturonate, or polygalacturonate covalently linked to a polymeric carrier is immunogenic in mammals and can serve as a typhoid fever vaccine.

[0040] Purification and O-acetylation of a plant or fruit polysaccharide: Pectin extracted and purified from plants or fruits such as, but not exclusive, inner portion of the rind of citrus fruits such as oranges, fruit pomaces as from apples or beets, and the like, can be used as the source of polysaccharide. The pectin may be further purified, for example, by precipitation with ethanol or gel filtration and the like. Pectin can be O-acetylated by treatment with acetic anhydride in formamide and pyridine. The content of O-acetyl groups can be increased by repeating the acetylation process until the desired level of acetylation is achieved.

[0041] Polymeric carriers: Carriers are chosen on the basis of facilitating two functions: 1) to increase the immunogenicity of the polysaccharide and 2) antibodies raised against the carrier are medically beneficial. Carriers that fulfill these criteria are described in the art (7, 10, 22-25). Polymeric carriers can be a natural or a synthetic material containing a primary or and a secondary amino group, an azido group or a carboxyl group. The carrier can be water soluble or insoluble. Examples of water soluble carriers include but are not limited to natural or synthetic peptides or proteins from bacteria or virus, e.g., tetanus toxin/toxoid, diphtheria toxin/toxoid, Pseudomonas aeruginosa exotoxin/toxoid/protein, pertussis toxin/toxoid, Clostridium perfringens exotoxins/toxoid, and hepatitis B surface antigen and core antigen. Example of water insoluble carriers include but are not limited to are aminohexyl-Sepharose®, e.g., aminopropyl or aminohexyl Sepharose®, and aminopropyl glass and the like. Other carriers may be used when an amino or carboxyl group is added through covalent linkage with a linker molecule.

[0042] Synthesis of O-acetylated pectin, D-galacturonan, oligogalacturonate, or polygalacturonate conjugated with a carrier: The O-acetylated pectin, D-galacturonan, oligogalacturonate, or polygalacturonate can be covalently bound to a carrier with or without a linking molecule. To conjugate without a linker, the O-acetylated pectin, D-galacturonan, oligogalacturonate, or polygalacturonate and carrier are mixed in the presence of carbodiimide activation agent, such as carbodiimide in a choice of solvent appropriate for both the pectin, D-galacturonan, oligogalacturonate, or polygalacturonate and the carrier as are known in the art (24).

[0043] The O-acetylated plant, fruit or synthetic D-galacturonan, oligosaccharide or polysaccharide is preferably conjugated to a carrier using a linking molecule. A linker or crosslinking agent, as used in the present invention, is a small linear molecule having a molecular weight of approximately <500 and is non-pyrogenic and non-toxic in the final product form (7, 10, 22-25). To conjugate with a linker or crosslinking agent, either or both of the pectin, D-galacturonan, oligogalacturonate, or polygalacturonate and the carrier are covalently bound to a linker first. The linkers or crosslinking agents are a homobifunctional or heterobifunctional molecules, e.g., adipic dihydrazide, ethylene diamine, cystamine, N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP), N-succinimidyl N-(2-maleimidomethyl)-b-alanine-propionate (SIAP), succinimidyl 4-(N-Maleimido-methyl) cyclohexane-1-carboxylate (SMCC), 3,3'-dithiodipropionic acid and the like. The linkers are bound to the carboxyl groups of the O-acetylated pectin, D-galacturonan, oligogalacturonate, or polygalacturonate or the carrier through carbodiimide condensation. The linkers are bound to the amino groups of the carrier through carbodiimide condensation or N-hydroxysuccinimide ester. The unbound materials are removed by gel filtration or ion exchange column depending on the materials to be separated. The final conjugate consist of the oligo- or polysaccharide and the carrier bound through a linker.

[0044] Clinical evidence has shown that serum antibodies to the Vi antigen confers immunity to typhoid fever. (1,2). The immunogen used to elicit the antibodies was the Vi capsular polysaccharide. Because Vi antibodies have been shown to be protective against typhoid fever and due to the complexity and safety issues that arise from culturing S. typhi, the World Health Organization (WHO) and the U.S. Food and Drug Administration (FDA) no longer require challenge data as criteria for licensing an acellular vaccine against Salmonella typhi (32). WHO and FDA criteria for licensing an acellular vaccine against Salmonella typhi is the demonstration that the preparation elicits Vi antibodies or that the
The modified pectin-carrier conjugates and modified D-galacturonan, oligogalacturonate and polygalacturonate-carrier conjugates of the present invention elicit antibodies that react with or bind to the Vi antigen. The anti-Vi antibody levels elicited by the modified pectin-carrier conjugates were comparable to those elicited by a Vi-Pseudomonas aeruginosa recombinant exoprotein A (rEPA) conjugate as measured by ELISA. Thus, the modified pectin-carrier and modified D-galacturonan, oligogalacturonate and polygalacturonate-carrier conjugate may be used as an effective vaccine against S. typhi to prevent or ameliorate typhoid fever in humans.

The present inoculum contains an effective, immunogenic amount of modified pectin-carrier conjugate and modified D-galacturonan, oligogalacturonate and polygalacturonate-carrier conjugates of this invention. The effective amount of modified pectin-carrier conjugate and modified D-galacturonan, oligogalacturonate and polygalacturonate-carrier conjugate concentrations of oligo- or polysaccharide of about 1 microgram to about 100 milligrams per inoculation (dose), preferably about 25 micrograms to about 50 milligrams per dose.

The term "unit dose" as it pertains to the inocula refers to physically discrete units suitable as unitary dosages for mammals, each unit containing a predetermined quantity of active material (oligo- or polysaccharide) calculated to produce the desired immunogenic effect in association with the required diluent. The specifications for the novel unit dose of an inoculum of this invention are dictated by and are directly dependent on (a) the unique characteristics of the active material and the particular immunologic effect to be achieved, and (b) the limitations inherent in the art of compounding such active material for immunologic use in animals, as disclosed in detail herein, these being features of the present invention.

Inocula are typically prepared as a solution in tolerable (acceptable) diluent such as water, saline or phosphate-buffered saline or other physiologically tolerable diluent such as water and the like to form an aqueous pharmaceutical composition.

The route of inoculation may be intramuscular, sub-cutaneous and the like, which results in eliciting antibodies protective against S. typhi. The dose is administered at least once. In order to increase the antibody level, a second or booster dose may be administered approximately 4 to 6 weeks after the initial injection. Subsequent doses may be administered as indicated.

Testing of the modified pectin-carrier conjugate and modified D-galacturonan, oligogalacturonate, and polygalacturonate-carrier vaccines is conducted as prescribed by the World Health Organization as described in Example 6 or by any equivalent immunological assay. Elicitation of Vi antibodies is predictive of in vivo efficacy of the conjugates in humans. Antibodies elicited by the modified pectin-carrier conjugates and modified D-galacturonan, oligogalacturonate and polygalacturonate-carrier conjugates are useful in providing passive protection to an individual exposed to S. typhi to prevent or ameliorate infection and disease caused by the microorganism.

The term antibody in its various grammatical form is used herein to refer to immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, i.e., molecules that contain an antibody combining site or paratope. Exemplary antibody molecules are intact immunoglobulin molecules, substantially intact immunoglobulin molecules and portions of an immunoglobulin molecule, including those portions known in the art as Fab, Fab', F(ab')\textsubscript{2} and F\textsubscript{v} as well as chimeric antibody molecules.

An antibody combining site or antigen binding fragment is that structural portion of an antibody molecule comprised of a heavy and light chain variable and hypervariable regions that specifically binds (immunoreacts with) an antigen. The term immunoreact in its various forms means specific binding between an antigenic determinant-containing molecule and a molecule containing an antibody combining site such as a whole antibody molecule or a portion thereof.

In one embodiment the antibody is characterized as comprising antibody molecules that immunoreact with: 1) S. typhi, 2) isolated Vi antigen of S. typhi and 3) a modified pectin of the present invention, and being substantially free of antibody molecules that immunoreact with native or naturally occurring pectin.

The aforementioned antibody is typically produced by immunizing a mammal with an inoculum containing a modified pectin-carrier conjugate and modified D-galacturonan, oligogalacturonate and polygalacturonate-carrier conjugates of this invention and thereby induce in the mammal, antibody molecules having immunospecificity for the immunizing conjugate. The antibody molecules are then collected from the mammal. The antibody molecules may be polyclonal or monoclonal antibody. Monoclonal antibodies may be produced by methods known in the art. The antibody may be contained in blood plasma, serum, hydridoma supernatants and the like. Alternatively, the antibody is isolated to the extent desired by well known techniques such as, for example, by using DEAE Sephadex®. The antibodies may be purified so as to obtain specific classes or subclasses of antibody such as IgM, IgG, IgA, IgG\textsubscript{1}, IgG\textsubscript{2}, IgG\textsubscript{3}, IgG\textsubscript{4} and the like. Antibody of the IgG class are preferred for purposes of passive protection.

The aforementioned antibodies have a number of diagnostic and therapeutic uses. The antibodies can be
used as an in vitro diagnostic agent to test for the presence of *S. typhi* in biological samples in standard immunoassay protocols. Such assays include, but are not limited to, radioimmunoassays, EIA, fluorescence assay, Western blot and the like. In one such assay, the biological sample is contacted to said antibodies and a labelled second antibody is used to detect the presence of *S. typhi*, or the Vi antigen of *S. typhi* to which the antibodies are bound.

**[0056]** Such assays may be, for example, of direct format (where the labelled first antibody is reactive with the antigen), an indirect format (where a labelled second antibody is reactive with the first antibody), a competitive format (such as the addition of a labelled antigen), or a sandwich format (where both labelled and unlabelled antibody are utilized), as well as other formats described in the art.

**[0057]** The antibodies and antigen binding fragments referred to above are useful in prevention and treatment of infections and diseases caused by *S. typhi* and other microorganisms that have structures immunologically similar to the Vi antigen.

**[0058]** In providing a patient with the antibodies or antigen binding fragments referred to above to a recipient mammal, preferably a human, the dosage of administered antibodies or antigen binding fragments will vary depending upon such factors as the mammal's age, weight, height, sex, general medical condition, previous medical history and the like.

**[0059]** In general, it is desirable to provide the recipient with a dosage of antibodies or antigen-binding fragments which is in the range of from about 1 mg/kg to about 10 mg/kg body weight of the mammal, although a lower or higher dose may be administered.

**[0060]** The antibodies or antigen-binding fragments referred to above are intended to be provided to the recipient subject in an amount sufficient to prevent, lessen or attenuate the severity, extent or duration of the infection by *S. typhi*.

**[0061]** The administration of the agents of the invention may be for either "prophylactic" or "therapeutic" purpose. When provided prophylactically, the agents are provided in advance of any symptom. The prophylactic administration of the agent serves to prevent or ameliorate any subsequent infection. When provided therapeutically, the agent is provided at (or shortly after) the onset of a symptom of infection. The agent of the present invention may, thus, be provided either prior to the anticipated exposure to *S. typhi* (so as to attenuate the anticipated severity, duration or extent of an infection and disease symptoms) or after the initiation of the infection.

**[0062]** For all therapeutic, prophylactic and diagnostic uses, the modified pectin, modified D-galacturonan, oligogalacturonate, polygalacturonate, alone or linked to a carrier, as well as antibodies and other necessary reagents and appropriate devices and accessories may be provided in kit form so as to be readily available and easily used.

**Example 1**

**Materials and Methods**

**[0063]** **Reagents:** Pectin (GENU pectin, from Copenhagen, Denmark, type LM-1912CSZ) was extracted from citrus. Pyrogen-free water (PFW) and pyrogen-free saline (PFS) for clinical use were from Baxter, Deerfield, WI; N-succinimidyl 3(2-pyridyldithio) propionate (SPDP) from Pierce, Rockford, IL; formamide, cystamine from Fluka, Ronkonkoma, NY; pyridine, NaOH, HCI from Baker Chemical, Philipsburg, NJ; acetic anhydride, diithiothreitol (DTT), EDTA, 1-ethyl-3(dimethylaminopropyl) carbodiimide (EDAC), acetyl choline, BSA, dithionitrobenzoic acid (Ellman reagent), D-galacturonic acid monohydrate (GalA) and tetrabutylammonium hydroxide from Sigma, St. Louis, MO.; carbazole from Aldrich, Milwaukee, WI.; HEPES from Calbiochem, La Jolla, CA; bicinchoninic acid (BCA) protein reagent, Sephacryl® S-1000, Sephadex® G-50, Superose® 6 from Pharmacia, Piscataway, NJ. Antiserum to tetanus toxoid (TT) was donated by William Habig, CBER, FDA. Pseudomonas aeruginosa exotoxin A (ETA) and goat antiserum to this protein were from List Biological Lab., Campbell, CA, Vi antiserum (B-260) was prepared by multiple intravenous injections of a burro with heat-killed *S. typhi* strain Ty-2 [19]. *Pseudomonas aeruginosa* recombinant exoprotein A (rEPA) was made as described in U.S. Patent No. 5,328,984 and U.S. Patent No. 4,892,827. The Vi-rEPA was made as described in U.S. Patent No. 5,204,098 issued April 20, 1993.

**[0064]** **Analytic:** The molecular sizes of polysaccharides and conjugates were measured with Superose® 6 HPLC column in 0.02M sodium phosphate buffer containing 0.1M Na₂SO₄ a pH 7.0. Carboxyls were measured by the carbazole reaction with pectin as a standard [3,27]. O-acetyl was measured with acetyl choline as a standard and the results expressed as moles/mole Ga1A [12]. The concentration of sulfhydryl was determined by Ellman reaction [8]. Protein was determined with BCA with BSA as a standard [20] and the content of nucleic acids was determined by A₂₆₀ [28]. ¹³C nuclear magnetic resonance spectroscopy (NMR) was performed with a General Electric GN300 spectrometer at room temperature [23].
Example 2

Preparation of O-acetylated pectin from citrus

Pectin was dissolved in PFW (10 mg/mL) at 60°C for 1 hour, cooled to room temperature and adjusted to pH 7.0 with 1M NaOH. The polysaccharide was precipitated twice with 75% ethanol and then freeze-dried. Pectin so treated contained less than 1% of protein and nucleic acid [28]. O-acetylation of pectin was performed as described [6]. Briefly, pectin (1 g) was suspended in formamide (20 mg/mL) at 50°C for 1 hour, 20 mL pyridine added, mixed and cooled to room temperature. Acetic anhydride (15 mL) was added dropwise with mixing at room temperature for 2 hours. The reaction mixture was poured into cold absolute ethanol. The precipitate was filtered, dissolved in PFW and dialyzed at 3 to 8°C against multiple changes of deionized water and freeze-dried. The degree of O-acetylation was about 50%, compared to the maximum possible yield of 200%. In order to reach a higher degree of O-acetylation, the OAcPec was subjected again to the same procedure. The final product was passed through a 2.5 x 50 cm column of Sephadex® G-50 in PFW and the void volume peak was passed through a sterile 0.45 micron membrane and freeze-dried. This preparation contained ~1.6 moles of O-acetyl/mole GalA or 80% yield.

The molar content of the O-acetyl groups are determined by Hestrin reaction (12). The distribution of the O-acetyl groups are studied by the methyl resonances of 13C NMR spectroscopy (Fig. 2).

The antigenicity of the O-acetylated pectin was studied by reaction with the antiserum against Salmonella typhi in 2-dimensional immunodiffusion using Vi polysaccharide as a comparison. Immunodiffusion was performed in 1% agarose in PBS with B-260 antiserum. Quantitative precipitation was performed with 100 µL of B-260 with equal volumes of antigen, containing 1 to 100 µg/mL, at 37°C for 1 hour and at 3-8°C for five days with occasional mixing. The precipitates were washed in cold PBS three times, dissolved in 0.8% SDS and their A280 recorded [23]. Serum Vi antibodies were measured by ELISA using a pooled hyperimmune mouse sera, quantitated by radioimmunoassay, as the standard [1].

The stability of the O-acetyl groups are studied at various temperatures for various periods of time. OAcPec and Vi (1 mg/mL) in PBS, pH 7.0, were incubated at 3-8°C, 25°C, 37°C and 60°C. Aliquots were removed at 1, 2, and 12 wks and analyzed for their content of O-acetyl and molecular size by gel filtration.

Example 3

Preparation of conjugates

The synthesis of conjugates followed that described for Vi [24]. The polysaccharide (5 mg/mL) was dissolved in 0.2 M NaCl. Cystamine (0.1 M) was added and the pH adjusted to 5.0 with 0.1M HCl. The temperature was 37°C for Vi and room temperature for OAcPec. EDAC (0.1 M) was added and the reaction mixture stirred for 4 hours with the pH maintained between 4.9 to 5.1. The reaction mixture was dialyzed against PBS with B-260 antiserum. Quantitative precipitation was performed with 100 µL of B-260 with equal volumes of antigen, containing 1 to 100 µg/mL, at 37°C for 1 hour and at 3-8°C for five days with occasional mixing. The precipitates were washed in cold PBS three times, dissolved in 0.8% SDS and their A280 recorded [23]. Serum Vi antibodies were measured by ELISA using a pooled hyperimmune mouse sera, quantitated by radioimmunoassay, as the standard [1].

The stability of the O-acetyl groups are studied at various temperatures for various periods of time. OAcPec and Vi (1 mg/mL) in PBS, pH 7.0, were incubated at 3-8°C, 25°C, 37°C and 60°C. Aliquots were removed at 1, 2, and 12 wks and analyzed for their content of O-acetyl and molecular size by gel filtration.

Derivatization of proteins with SPDP. SPDP, in absolute ethanol, was added dropwise at room temperature with stirring to protein (5 mg/mL) in 0.15M HEPES, 0.001 M EDTA, pH 7.5 (HE buffer) to a final concentration of 0.04M. The reaction proceeded for 1 hour and dialyzed against the HE buffer overnight. The reaction mixture was passed through a 2.5x35 cm column of P10 in HE buffer and the void volume fractions concentrated to ~10 mg/mL. An aliquot was treated with 0.075 M DTT at room temperature for 2 hours and its A343 used to calculate the molar ratio of SPDP to protein [5].

Conjugation reaction. The cystamine-derivatized polysaccharide, 10 mg/mL PBS, pH 7.4, was treated with 0.05M DTT at room temperature for two hours and passed through a 2.5x35 cm column of Sephadex® G-50 in PBS, pH 7.0. An aliquot was taken to determine its sulfhydryl content and the remainder mixed with an equal weight of SPDP-derivatized protein for 4 hours and at 3-8°C overnight. The reaction mixture was passed through a 2.5x95 cm column of Sephacryl® S-1000 in PBS at 3-8°C. For the OAcPec-TT, fractions containing protein and polysaccharide were pooled into two batches: OAcPec-TT1 for the void volume peak and OAcPec-TT2 for the lower molecular weight fractions. Vi-rEPA was passed through a 2.5x95 cm column of Sephacryl® S-1000 in PBS and the void volume fractions pooled.
Example 4

Characterization of O-acetylated pectin

[0072] Physico-chemical characterization of OAcPec: O-acetylation ranged from 0.1 to 1.6 moles/GalA for pectins. Unless specified, the OAcPec described in the following had 1.6 mole O-acetyl/mole GalA. ¹³C N.M.R. of OAcPec showed more than two signals observed with acetyl methyl resonances indicating that mono and diacetylated species are present: non-O-acetylated residues could, however, be present (Figure 2). The stoichiometry of C₂ and C₃ O-acetylation are the same. Therefore, the O-acetyl groups are probably distributed equally between C₂ and C₃ (Fig. 2), at least 60% of the GalA are di-O-acetylated, while 20% are mono-O-acetylated. Neutral sugar content in the pectin is lower than 5%.

[0073] The Mᵣ of OAcPec, similar to that of the pectin, had a broad distribution with the major peak ~400 kD (Fig. 3). Unlike pectin, OAcPec was soluble in 0.15M NaCl and did not form a gel in the presence of Ca++. Molar absorbances in the carboxyze assay were 1.32×10³ for OAcPec, 1.61×10³ for pectin and 1.63×10³ for GalA. The differences between pectin and GalA were <2% and are probably due to neutral sugars in the pectin. Vi, in contrast, did not react in the carboxyze assay.

[0074] Pectin did not react with B-260 serum in double immunodiffusion. OAcPec, in contrast, formed a line of identity with Vi (Fig. 4). Precipitation of OAcPec with Vi antiserum did not change with different counter ions including Na⁺, Ca++, K⁺ or tetrabutylammonium. At lower degrees of O-acetylation (0.4 - 0.9 moles O-acetyl/mole GalA) pectin also yielded a line of identity with the Vi (not shown). No precipitation in double immunodiffusion was observed when the O-acetylation of pectin was ≤0.2 mole/mole GalA. Quantitative precipitation showed that both Vi and OAcPec precipitated 2.6 mg/mL Ab from B-260 antiserum (Fig. 5).

[0075] Stability of OAcPec and Vi O-acetyl The thermostability of the O-acetyls was similar for OAcPec compared to Vi (Fig. 6). Following storage at 3-8°C for 12 weeks, there was no change in the concentration of O-acetyl for Vi and OAcPec to the original level of O-acetyl for Vi and OAcPec prior to storage: at 22°C, O-acetyl declined to 93% for Vi and to 88% for OAcPec and at 60°C, only 12% of the O-acetyl remained on Vi and 10% on OAcPec.

Example 5

Characterization and immunogenicity of the conjugate

[0077] The degree of thiolation was 4% for the O-acetylated pectin. The HPLC profile of OAcPec-TT shows the conjugate and a small portion of OAcPec were eluted in the void volume (Fig. 3). In several experiments (not shown) the final yield of the conjugate was 20-30%. The polysaccharide-protein ratio is ~0.4 -0.8% wt/wt.

<table>
<thead>
<tr>
<th>Table 1</th>
</tr>
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<tbody>
<tr>
<td>Composition of conjugates of O-acetylated pectin (OAcPec) with tetanus toxoid (TT) and Vi with Pseudomonas aeruginosa recombinant Exoprotein A (rEPA).</td>
</tr>
<tr>
<td>Conjugate</td>
</tr>
<tr>
<td>OAcPect-TT₁</td>
</tr>
<tr>
<td>OAcPect-TT₂</td>
</tr>
<tr>
<td>Vi-rEPA</td>
</tr>
</tbody>
</table>

[0078] Immunization: 16-20 g general purpose mice from the NIH colony were injected subcutaneously 1, 2, or 3 times at 2 week intervals with 2.5 µg of the polysaccharide alone or as a conjugate. 10 mice from each group were exsanguinated two weeks after the first injection and one week after the second and third injections. Controls included mice injected with saline, Vi or OAcPec. Vi antibody levels were measured by ELISA with a reference calibrated by RIA.

ELISA Procedure for the Measurement of Vi Antibody

[0079] Reagents: Vi antigen purified from S. typhi, alkaline phosphatase labeled goat anti-mouse or alkaline phos-
Buffers:

[0080]

Coating Buffer: sodium carbonate-sodium bicarbonate buffer solution, pH 9.5 at 20°C, 30 ml 0.1M Na₂CO₃, 70 ml 0.1M NaHCO₃.
Washing Buffer: 0.85% NaCl, 0.1% Brij® 35, 0.02% NaN₃.
Dilution Buffer: 1 x PBS, 0.1% Brij® 35, 2% BSA, prepare fresh each time, filter with 0.45 µm Millipore® filter.
Dilution buffer without BSA (DB) can be prepared as a "stock solution" and BSA added before use.
Substrate Buffer: 1000 ml Tris-HCl 300 ml 1MMgCl₂, adjust pH to 9.8 with HCl.
Conjugate Buffer: is the same as DB.

Procedure:

[0081]

1. Store frozen Vi polysaccharide (0.1 mg/ml) 500 µl aliquots.
2. Coat microtiter plates (96 well, flat bottom, polystyrene Immunolon® microtiter plates) with Vi. Dilute 1 to 2 µg Vi per ml in "coating buffer" use 100 µl/well, shake gently. Incubate plates at 4°C overnight covered with polyester film.
3. Wash plates twice in "washing buffer", shake dry.
4. Dilute antibody samples in dilution buffer DB.BSA. Dilution factor may be 5 to 10.
5. Make serial dilutions in the plate, pipet 100 µl of DB.BSA in all wells except for the first row. Deliver 200 µl of the diluted sample in the first row. Transfer 100 µl subsequently from the top row down, use the multichannel pipet to mix in the wells. Remove the excess 100 µl from the bottom row.
6. Incubate the antigen-antibody mixture at room temperature overnight.
7. Wash plates twice.
8. Add 100 µl/well conjugate diluted 1:500 to 1:1000 in DB and incubate at 37°C for 4 hours.
9. Wash plates twice.
10. Add 100 µl/well phosphatase substrate (1 mg/ml) freshly prepared in substrate buffer. Add it to each vertical row every 6 seconds, 3 minute interval between each plate.
11. Read plates at 410 nm approximately 15-20 minutes after the addition of the substrate. Sometimes the plates should be read at various time durations depending on the concentration and strength of the conjugate. Optimum optical density should be between 1.0 and 1.5.

[0082] Statistical: Logarithms of antibody concentrations were used for all calculations. Antibody concentrations below the sensitivity of the ELISA were assigned one half of that value. Comparisons of geometric means were performed by unpaired t-tests. The Statistical Analysis System (SAS) was used for all data analyses.

[0083] Vi antibodies: As reported, Vi elicited serum antibodies in mice after one injection and reinjection did not elicit a booster response [14,22-25]. Neither the pectin nor the OAcPec elicited Vi antibodies after any injections. After one injection, the Vi and OAcPec conjugates elicited similar levels of antibodies. Following the second injection, the conjugates elicited a booster response (P<0.001) with the geometric mean antibody levels highest for Vi-rEPA (17.1) > OAcPec-TT₂ (7.65) > OAcPec-TT₁ (5.47). These differences, however, were not statistically significant. The third injection of all 3 conjugates did not elicit a booster response. Lastly, there were no statistically significant differences in the geometric mean (GM) Vi antibody levels elicited by OAcPec-TT₁ and OAcPecTT₂ after any of the three injections.

Table 2

<table>
<thead>
<tr>
<th>Vi antibodies (µg Ab/mL serum) in mice immunized with Vi, Vi-rEPA, Pectin, O-acetyl Pectin (OAcPec) and OAcPec-TT conjugates.</th>
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<tbody>
<tr>
<td>Geometric Mean [n=10]</td>
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<tr>
<td>Immunogen</td>
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<td>--------------------</td>
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<tr>
<td>Vi</td>
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</table>
Example 6

WHO Testing Protocol of S. typhi Vaccines

[0084] The modified pectin, modified D-galacturonan, oligogalacturonate and polygalacturonate-carrier conjugates vaccines are tested as per WHO requirements for acellular vaccines against Salmonella typhi (32).

[0085] The following tests are carried out on each lot of modified saccharide-carrier conjugate vaccine as per WHO requirements as briefly outlined:

1) serological testing for immunological identify with a standardized Vi antigen; 2) polysaccharide content; 3) sterility testing; 4) pyrogenicity testing; 5) toxicity testing; 6) preservative content (if added); 7) pH; and 8) stability studies.

[0086] Those lots fulfilling the WHO requirements are suitable for use in humans as a Salmonella typhi vaccine.

Example 7

Human Vaccination Using A Modified Pectin-Carrier Conjugate

[0087] Volunteers between 18 and 45 years of age, who have no antibodies to hepatitis B and to HIV-1 are recruited. Following receipt of their informed consent, volunteers receive 1 injection of Vi (25 µg in 0.5 mL) (1,13) or 1 injection of a modified pectin-carrier conjugate (25 µg polysaccharide in 0.5 mL) of the present invention intramuscularly. Oral temperature is taken and the injection site of each volunteer is inspected 6, 24 and 48 hours after each injection. Volunteers receive a second injection at 6 weeks and are bled 2 weeks later and 26 weeks after the first injection. Antibodies reactive to Vi are determined by ELISA as described herein.

REFERENCES

[0088]

31. Kawata, Y. 1970. A study of the molecular types of immunoglobulin. II. Mouse protection study of Vi antibody
Claims

1. A method to prepare an immunogenic modified oligogalacturonate or polygalacturonate-carrier conjugate against *Salmonella typhi* comprising:

   (a) O-acetylating an oligogalacturonate or a polygalacturonate, which are derived from a plant, fruit or a synthetic source, to form an O-acetylated oligogalacturonate or an O-acetylated polygalacturonate,
   
   (b) conjugating the O-acetylated oligogalacturonate or the O-acetylated polygalacturonate to a carrier to form the modified oligogalacturonate or modified polygalacturonate-carrier conjugate which is immunogenic against *Salmonella typhi*.

2. The method according to claim 1 wherein the O-acetylated oligogalacturonate or the O-acetylated polygalacturonate is at least 50% O-acetylated.

3. The method according to claim 1 or 2, wherein the O-acetylated oligogalacturonate or the O-acetylated polygalacturonate is about 80% to about 200% O-acetylated.

4. The method according to any one of claims 1 to 3, wherein the O-acetylated oligogalacturonate or the O-acetylated polygalacturonate is about 160% to about 190% O-acetylated.

5. The method according to any one of claims 1 to 4, wherein the method provides at least 0.5 mole of O-acetyl per mole of galacturonate of the O-acetylated oligogalacturonate or the O-acetylated polygalacturonate.

6. The method according to any one of claim 1 to 5, wherein the method provides at least 1.6 moles of O-acetyl per mole of galacturonate of the O-acetylated oligogalacturonate or the O-acetylated polygalacturonate.

7. The method according to any one of claims 1 to 6, wherein the method provides about 1.6 to about 1.9 moles of O-acetyl per mole of galacturonate of the O-acetylated oligogalacturonate or the O-acetylated polygalacturonate.

8. The method according to any one of claim 1 to 7, wherein the acetylation occurs at position C₂, C₃ or C₂ and C₃ on a galacturonic acid subunit of oligogalacturonate or polygalacturonate.

9. The method of any one of claim 1 to 8, wherein the oligogalacturonate or polygalacturonate is pectin isolated from plants or fruits.

10. The method of any one of claims 1 to 9 wherein the carrier is a protein selected from the group consisting of bacterial protein, viral protein, tetanus toxoid, tetanus toxin, diphtheria toxin, *Pseudomonas aeruginosa* exotoxin, *Pseudomonas aeruginosa* toxoid, pertussis toxin, pertussis toxoid, *Clostridium perfringens* exotoxin, *Clostridium perfringens* toxoid, hepatitis B surface antigen, hepatitis B core antigen and *Pseudomonas* exoprotein A or is a water insoluble carrier selected from the group consisting of aminoalkyl-Sepharose®, amino propyl-Sepharose®, aminohexyl-Sepharose®, and amino propyl glass.

11. The method of any one of claims 1 to 10 wherein the oligogalacturonate or polygalacturonate is O-acetylated in organic solvents with acetic anhydride.

12. The method of any one of claims 1 to 11 wherein the O-acetylated oligogalacturonate or polygalacturonate is derivatized with a homobifunctional or a heterobifunctional cross-linking agent or is thiolated and linked to a carrier containing a sulfhydryl group or is aminated and linked to a carrier containing a carboxyl group.

13. The method of claim 12 wherein the agent is selected from the group consisting of N-succinimidyl 3-(2-pyridyldithio) propionate, adipic dihydrazide, cystamine, 3,3′dithiodipropionic acid, ethylene diamine, N-(2-iodoacetyl)-b-alanine-propionate and succinimidyl 4-(N-Maleimido-methyl) cyclohexane-1-carboxylate.
14. An immunogenic modified oligogalacturonate or polygalacturonate carrier conjugate against *Salmonella typhi* obtainable according to the method of claim 1 or 6.

15. An immunogenic modified oligogalacturonate or polygalacturonate carrier conjugate against *Salmonella typhi* obtainable according to the method of claim 8 wherein the carrier is tetanus toxoid.

16. An immunogenic modified oligogalacturonate or polygalacturonate carrier conjugate against *Salmonella typhi* obtainable according to the method of claim 9 wherein the pectin is at least 50% O-acetylated, at least 80% O-acetylated or about 160% to about 190% O-acetylated.

17. An immunogenic modified oligogalacturonate or polygalacturonate carrier conjugate against *Salmonella typhi* obtainable according to the method of claim 12 wherein the crosslinking agent is cystamine.

18. A pharmaceutical composition comprising the conjugate of any one of claims 14 to 17 and a pharmaceutically acceptable diluent.

19. A modified saccharide-carrier conjugate which is immunogenic against *Salmonella typhi* comprising a saccharide selected from the group consisting of pectin, D-galacturonan, oligogalacturonate, polygalacturonate and mixtures thereof, wherein said saccharide is derived from a plant, fruit or a synthetic source, said saccharide is modified by O-acetylation, and said saccharide is covalently linked to a carrier.

20. The modified saccharide-carrier conjugates of claim 19 wherein the saccharide is at least 50% O-acetylated, at least 80% O-acetylated or about 160% to about 190% O-acetylated.

21. The modified saccharide-carrier conjugate of claim 19 or 20 wherein at least the C₂, C₃, or C₂ and C₃ position on a saccharide subunit is O-acetylated.

22. The modified saccharide-carrier conjugate of any one of claims 19 to 21 wherein the modified saccharide has a molar ratio of O-acetyl groups: mole galacturonate of at least 0.5:1 or of at least 1.6:1.

23. The modified saccharide-carrier conjugate of any one of claims 19 to 22 wherein the modified saccharide has a molar ratio of O-acetyl groups per mole galacturonate of between about 1.6 to about 1.9 moles of O-acetyl per mole of galacturonate.

24. The modified saccharide-carrier conjugate of any one of claims 19 to 23 wherein the carrier is selected from the group consisting of bacterial protein, viral protein, tetanus toxoid, diphtheria toxin, *Pseudomonas aeruginosa* exotoxin, *Pseudomonas aeruginosa* toxoid, pertussis toxin, pertussis toxoid, *Clostridium perfringens* exotoxin, *Clostridium perfringens* toxoid, hepatitis B surface antigen, hepatitis B core antigen and *Pseudomonas* exoprotein A.

25. The modified saccharide-carrier conjugate of any one of claims 19 to 24 wherein the saccharide is linked to the carrier by a homobifunctional or heterobifunctional cross-linking agent.

26. The modified saccharide-carrier conjugate of claim 25 wherein the crosslinking agent is selected from the group consisting of adipic dihydrazide, ethylene diamine, cystamine, N-succinimidyl-3-(2-pyridyldithio) propionate, N-succinimidyl N-(2-iodoacetyl)-β-alanine-propionate, succinimidyl 4-(N-Maleimido-methyl) cyclohexane-1-carboxylate, and 3,3′-dithiodipropionic acid.

27. The modified saccharide-carrier conjugate of any one of claims 19 to 26 wherein the modified saccharide has a subunit structure substantially identical to a subunit structure of Vi from *Salmonella typhi*.

28. An immunogen against *Salmonella typhi* comprising: a plant, fruit or synthetic saccharide modified by O-acetylation covalently linked to a carrier, wherein said modified saccharide-carrier conjugate elicits antibodies in mammals, and said antibodies are specifically immunoreactive against Vi of *Salmonella typhi*.

29. The immunogen of claim 28 wherein the modified plant, fruit or synthetic saccharide has immunological identity with the Vi of *Salmonella typhi* as measured by immunodiffusion.
30. The immunogen of claim 28 or 29 wherein the plant or fruit saccharide is derived from citrus fruit, apples, or beets.

31. The immunogen of any one of claims 28 to 30 wherein the saccharide is pectin or is selected from the group consisting of D-galacturonan, oligogalacturonate, polygalacturonate, and mixtures thereof.

32. The immunogen of any one of claims 28 to 31 wherein the carrier is selected from the group consisting of bacterial protein, viral protein, tetanus toxoid, diphtheria toxin, Pseudomonas aeruginosa exotoxin, Ps. aeruginosa toxoid, pertussis toxin, pertussis toxoid, Clostridium perfringens exotoxin, Clostridium perfringens toxoid, hepatitis B surface antigen, hepatitis B core antigen and Pseudomonas exoprotein A.

33. The immunogen of any one of claims 28 to 32 wherein the saccharide is at least 50% O-acetylated.

34. The immunogen of any one of claims 31 to 33 wherein the pectin is at least 80% O-acetylated.

35. A vaccine against typhoid fever comprising the immunogen of any one of claims 28 to 34 and a pharmaceutically acceptable diluent.

36. Use of an O-acetylated plant, fruit or synthetic saccharide linked to a carrier for the preparation of a medicament for use in a method of actively immunizing a human against typhoid fever comprising: administering in vivo a sufficient amount of an O-acetylated plant, fruit or synthetic saccharide linked to a carrier, wherein said amount is sufficient to elicit antibody that binds to Vi of Salmonella typhi.

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Patentansprüche

1. Verfahren zur Herstellung eines immunogenen modifizierten Oligogalacturonat- oder Polygalacturonat-Träger-Konjugats gegen Salmonella typhi, umfassend:
   a) O-Acetylierung eines Oligogalacturonats oder Polygalacturonats, gewonnen aus Pflanzen, Früchten oder aus einer synthetischen Quelle, zur Erzeugung eines O-acetylierten Oligogalacturonats oder O-acetylierten Polygalacturonats;
   b) Konjugation des O-acetylierten Oligogalacturonats oder des O-acetylierten Polygalacturonats an einen Träger zur Erzeugung des modifizierten Oligogalacturonat- oder modifizierten Polygalacturonat-Träger-Konjugats, welches immunogen gegen Salmonella typhi ist.

2. Verfahren nach Anspruch 1, wobei das O-acetylierte Oligogalacturonat oder das O-acetylierte Polygalacturonat zu mindestens 50% O-acetyliert ist.

3. Verfahren nach Anspruch 1 oder 2, wobei das O-acetylierte Oligogalacturonat oder das O-acetylierte Polygalacturonat zu etwa 80% bis etwa 200% O-acetyliert ist.

4. Verfahren nach einem der Ansprüche 1 bis 3, wobei das O-acetylierte Oligogalacturonat oder das O-acetylierte Polygalacturonat zu etwa 160% bis etwa 190% O-acetyliert ist.

5. Verfahren nach einem der Ansprüche 1 bis 4, wobei das Verfahren 0,5 Mol O-Acetyl pro Mol Galacturonat des O-acetylierten Oligogalacturonats oder des O-acetylierten Polygalacturonats bereitstellt.

6. Verfahren nach einem der Ansprüche 1 bis 5, wobei das Verfahren mindestens 1,6 Mole O-Acetyl pro Mol Galacturonat des O-acetylierten Oligogalacturonats oder des O-acetylierten Polygalacturonats bereitstellt.

7. Verfahren nach einem der Ansprüche 1 bis 6, wobei das Verfahren etwa 1,6 bis etwa 1,9 Mole O-Acetyl pro Mol Galacturonat des O-acetylierten Oligogalacturonats oder des O-acetylierten Polygalacturonats bereitstellt.

8. Verfahren nach einem der Ansprüche 1 bis 7, wobei die Positionen C2, C3 oder C2 und C3 einer Galacturonsäure-Untereinheit des Oligogalacturonats oder Polygalacturonats acetyliert werden.

9. Verfahren nach einem der Ansprüche 1 bis 8, wobei das Oligogalacturonat oder das Polygalacturonat ein Pektin ist, isoliert aus Pflanzen oder Früchten.

11. Verfahren nach einem der Ansprüche 1 bis 10, wobei das Oligogalacturonat oder Polygalacturonat in organischen Lösungsmitteln mit Essigsäureanhydrid O-acetyliert wird.

12. Verfahren nach einem der Ansprüche 1 bis 11, wobei das O-acetylierte Oligogalacturonat oder Polygalacturonat mit einem homobifunktionalen oder heterobifunktionalen Vernetzungsmittel derivatisiert wird oder thioliiert und mit einem eine Sulfohydrylgruppe enthaltenden Träger verbunden wird oder aminoliert und mit einem eine Carboxylgruppe enthaltenden Träger verbunden wird.


15. Immunogenes modifiziertes Oligogalacturonat- oder Polygalacturonat-Träger-Konjugat gegen *Salmonella typhi*, erhältlich gemäß dem Verfahren nach Anspruch 8, wobei der Träger Tetanus-Toxoid ist.

16. Immunogenes modifiziertes Oligogalacturonat- oder Polygalacturonat-Träger-Konjugat gegen *Salmonella typhi*, erhältlich gemäß dem Verfahren nach Anspruch 9, wobei das Pektin zu mindestens 50% O-acetyliert, zu mindestens 80% O-acetyliert oder zu etwa 160% bis etwa 190% O-acetyliert ist.

17. Immunogenes modifiziertes Oligogalacturonat- oder Polygalacturonat-Träger-Konjugat gegen *Salmonella typhi*, erhältlich gemäß dem Verfahren nach Anspruch 12, wobei das Vernetzungsmittel Cystamin ist.

18. Arzneimittel, enthaltend das Konjugat nach einem der Ansprüche 14 bis 17 und ein pharmazeutisch verträgliches Lösungsmittel.

19. Modifiziertes Saccharid-Träger-Konjugat welches immunogen gegen *Salmonella typhi* ist, enthaltend ein Saccharid ausgewählt aus der Gruppe bestehend aus Pektin, D-Galacturonan, Oligogalacturonat, Polygalacturonat und Gemischen davon, wobei das Saccharid gewonnen ist aus Pflanzen, Früchten oder synthetischen Quellen, das Saccharid modifiziert ist durch O-Acetylierung und das Saccharid kovalent an einen Träger gebunden ist.

20. Modifizierte Saccharid-Träger-Konjugate nach Anspruch 19, wobei das Saccharid zu mindestens 50% O-acetyliert, zu mindestens 80% O-acetyliert oder zu etwa 160% bis etwa 190% O-acetyliert ist.


25. Modified saccharide-carrier conjugate according to one of the claims 19 to 24, wherein the saccharide is coupled to the carrier through a homobifunctional or heterobifunctional crosslinking agent.

26. Modified saccharide-carrier conjugate according to claim 25, wherein the crosslinking agent is selected from the group consisting of adipindihydrazide, ethylenediamine, cystamine, N-succinimidyl-3-(2-pyridyldithio)-propionan, N-succinimidyl-N-(2-iodacetyl)-b-alaninat-propionan, succinimidyl-4-(N-maleimido-methyl)-cyclohexan-1-carboxylate and 3,3′-dithiodipropionic acid.

27. Modified saccharide-carrier conjugate according to one of the claims 19 to 26, wherein the modified saccharide has a subunit structure essentially identical with the structure of a subunit of Vi of Salmonella typhi.

28. Immunogen against Salmonella typhi comprising:
- a plant, fruit or synthetic plant-derived saccharide modified by O-acetylation, covalently coupled to a carrier, wherein the modified saccharide-carrier conjugate induces the production of antibodies in mammals that specifically react with Vi of Salmonella typhi.

29. Immunogen according to claim 28, wherein the modified plant-derived, fruit-derived or synthetic saccharide is immunologically identical to Vi of Salmonella typhi, wherein the identity is determined by immunodiffusion.

30. Immunogen according to claim 28 or 29, wherein the plant or fruit-derived saccharide is derived from citrus fruits, apples or carrots.

31. Immunogen according to one of the claims 28 to 30, wherein the saccharide is pectin or selected from the group consisting of D-galacturonic acid, oligogalacturonate, polygalacturonate and mixtures thereof.

32. Immunogen according to one of the claims 28 to 31, wherein the carrier is selected from the group consisting of bacterial protein, viral protein, tetanus toxin, diphtheria toxin, pseudomonas aeruginosa exotoxin, pseudomonas aeruginosa toxoid, pertussis toxin, pertussis toxoid, clostridium perfringens exotoxin, clostridium perfringens toxoid, hepatitis B surface antigen, hepatitis B core antigen and pseudomonas exoprotein A.

33. Immunogen according to one of the claims 28 to 32, wherein the saccharide is at least 50% O-acetylated.

34. Immunogen according to one of the claims 31 to 33, wherein the pectin is at least 80% O-acetylated.

35. Vaccine against typhoid, comprising the immunogen according to one of the claims 28 to 34 and a pharmaceutically compatible solution.

36. Use of an O-acetylated plant, fruit-derived or synthetic saccharide, coupled to a carrier, for the manufacture of a medicament for use in an active immunization process against typhoid, comprising:
- in vivo administration of an adequate amount of O-acetylated, plant-derived, fruit-derived or synthetic saccharide, coupled to a carrier, wherein the amount is sufficient to induce the production of antibodies that bind to Vi of Salmonella typhi.

Revendications

1. Procédé pour préparer un conjugué immunogène d'oligogalacturonate ou de polygalacturonate modifié et de support contre Salmonella typhi, comprenant :

(a) l'O-acétylation d'un oligogalacturonate ou d'un polygalacturonate, qui proviennent d'une plante, d'un fruit ou d'une source de synthèse, pour former un oligogalacturonate O-acétylé ou un polygalacturonate O-acétylé,

(b) la conjugaison de l'oligogalacturonate O-acétylé ou du polygalacturonate O-acétylé, avec un support pour former le conjugué d'oligogalacturonate modifié ou de polygalacturonate modifié et de support, qui est immunogène contre Salmonella typhi.
2. Procédé suivant la revendication 1, dans lequel l'oligogalacturonate O-acétylé où le polygalacturonate O-acétylé est au moins O-acétylé à 50%.

3. Procédé suivant les revendications 1 ou 2, dans lequel l'oligogalacturonate O-acétylé ou le polygalacturonate O-acétylé est O-acétylé à raison d'environ 80% jusqu'à environ 200%.

4. Procédé suivant l'une quelconque des revendications 1 à 3, dans lequel l'oligogalacturonate O-acétylé ou le polygalacturonate O-acétylé est O-acétylé à raison d'environ 160% jusqu'à environ 190%.

5. Procédé suivant l'une quelconque des revendications 1 à 4, dans lequel le procédé fournit au moins 0,5 mole de groupe O-acétylé par mole de galacturonate de l'oligogalacturonate O-acétylé ou du polygalacturonate O-acétylé.

6. Procédé suivant l'une quelconque des revendications 1 à 5, dans lequel le procédé fournit au moins 1,6 mole de groupe O-acétylé par mole de galacturonate de l'oligogalacturonate O-acétylé ou du polygalacturonate O-acétylé.

7. Procédé suivant l'une quelconque des revendications 1 à 6, dans lequel le procédé fournit environ 1,6 mole à environ 1,9 mole de groupe O-acétylé par mole de galacturonate de l'oligogalacturonate O-acétylé ou du polygalacturonate O-acétylé.

8. Procédé suivant l'une quelconque des revendications 1 à 7, dans lequel l'acétylation a lieu aux positions C2, C3 ou C2 et C3 sur une sous-unité d'acide galacturonique d'oligogalacturonate ou de polygalacturonate.

9. Procédé suivant l'une quelconque des revendications 1 à 8, dans lequel l'oligogalacturonate ou le polygalacturonate est une pectine isolée à partir de plantes ou de fruits.

10. Procédé suivant l'une quelconque des revendications 1 à 9, dans lequel le support est une protéine choisie dans le groupe consistant en protéine bactérienne, protéine virale, toxoïde de tétanos, toxine de tétanos, toxine de diphtérie, exotoxine de *Pseudomonas aeruginosa*, toxoïde de *Pseudomonas aeruginosa*, toxine de coqueluche, exotoxine de *Clostridium perfringens*, toxoïde de *Clostridium perfringens*, antigène de surface d'hépatite B, antigène de noyau d'hépatite B et exoprotéine A de *Pseudomonas*, ou est un support insoluble dans l'eau choisi dans le groupe consistant en aminométhyl-Sepharose®, aminopropyl-Sepharose®, aminohexyl-Sepharose® et amino propyl verre.

11. Procédé suivant l'une quelconque des revendications 1 à 10, dans lequel l'oligogalacturonate ou le polygalacturonate est O-acétylé dans des solvants organiques avec de l'anhydride acétique.

12. Procédé suivant l'une quelconque des revendications 1 à 11, dans lequel l'oligogalacturonate ou le polygalacturonate O-acétylé est traité avec un agent de réticulation homobifonctionnel ou hétérobifonctionnel ou est thiolé et relié à un support contenant un groupe sulfhydro ou est aminolé et relié à un support contenant un groupe carboxy.

13. Procédé suivant la revendication 12, dans lequel l'agent est choisi dans le groupe consistant en 3-(2-pyridylthio)-propionate de N-succinimidyle, dihydrazide adipique, cystamine, acide 3,3'-dithiodipropionique, éthylène diamine, N-(2-iodoacétyl)-b-alaninate-propionate et 4-(N-maléimido-méthyl)-cyclohexane-1-carboxylate de succinimidyle.

14. Conjugué immunogène d'oligogalacturonate ou de polygalacturonate modifié et de support contre *Salmonella typhi* pouvant être obtenu suivant le procédé des revendications 1 ou 6.

15. Conjugué immunogène d'oligogalacturonate ou de polygalacturonate modifié et de support contre *Salmonella typhi* pouvant être obtenu suivant le procédé de la revendication 8 dans lequel le support est un toxoïde de tétanos.

16. Conjugué immunogène d'oligogalacturonate ou de polygalacturonate modifié et de support contre *Salmonella typhi* pouvant être obtenu suivant le procédé de la revendication 9, dans lequel la pectine est au moins O-acétylée à 50%, au moins O-acétylée à 80% ou O-acétylée à raison d'environ 160% jusqu'à 190%.

17. Conjugué immunogène d'oligogalacturonate ou de polygalacturonate modifié et de support contre *Salmonella typhi* pouvant être obtenu suivant le procédé de la revendication 12 dans lequel l'agent de réticulation est la cystamine.
18. Composition pharmaceutique comprenant le conjugué suivant l'une quelconque des revendications 14 à 17 et un diluant acceptable du point de vue pharmaceutique.

19. Conjugué de saccharide modifié et de support qui est immunogène contre *Salmonella typhi* comprenant un saccharide choisi dans le groupe consistant en pectine, D-galacturonane, oligogalacturonate, polygalacturonate et leurs mélanges, dans lequel ledit saccharide provient d'une plante, d'un fruit ou d'une source de synthèse, ledit saccharide est modifié par O-acétylation et ledit saccharide est lié par covalence à un support.

20. Conjugués de saccharide modifié et de support suivant la revendication 19, dans lesquels le saccharide est au moins O-acétylé à 50%, au moins O-acétylé à 80% ou O-acétylé à raison d'environ 160% jusqu'à environ 190%.

21. Conjugué de saccharide modifié et de support suivant les revendications 19 ou 20, dans lequel au moins la position C₂ ou C₃ ou les positions C₂ et C₃ sur une sous-unité saccharide sont O-acétylées.

22. Conjugué de saccharide modifié et de support suivant l'une quelconque des revendications 19 à 21, dans lequel le saccharide modifié a un rapport molaire de groupes O-acétyle : mole de galacturonate d'au moins 0,5 : 1 ou d'au moins 1,6 : 1.

23. Conjugué de saccharide modifié et de support suivant l'une quelconque des revendications 19 à 22, dans lequel le saccharide modifié a un rapport molaire de groupes O-acétyle par mole de galacturonate compris entre environ 1,6 et environ 1,9 mole de groupe O-acétylé par mole de galacturonate.

24. Conjugué de saccharide modifié et de support suivant l'une quelconque des revendications 19 à 23, dans lequel le support est choisi dans le groupe consistant en protéine bactérienne, protéine virale, toxoïde de tétanos, toxine de diphtérie, exotoxine de *Pseudomonas aeruginosa*, toxoïde de *Pseudomonas aeruginosa*, toxine de coqueluche, toxoïde de *Clostridium perfringens*, toxoïde de *Clostridium perfringens*, antigène de surface d'hépatite B, antigène de noyau d'hépatite B et exoprotéine A de *Pseudomonas*.

25. Conjugué de saccharide modifié et de support suivant l'une quelconque des revendications 19 à 24, dans lequel le saccharide est relié au support par un agent de réticulation homobifonctionnel ou hétérobifonctionnel.

26. Conjugué de saccharide modifié et de support suivant la revendication 25, dans lequel l'agent de réticulation est choisi dans le groupe consistant en dihydrazide adipique, éthylène diamine, cystamine, 3-(2-pyridyl)dithio)-propionate de N-succinimidyle, N-(2-iodoacétyl)-b-alaninate-propionate de N-succinimidyle, 4-(N-maléimido-méthyl)-cy-clohexane-1-carboxylate de succinimidyle et acide 3,3'-dithio-dipropionique.

27. Conjugué de saccharide modifié et de support suivant l'une quelconque des revendications 19 à 26, dans lequel le saccharide modifié a une structure sous-unitaire pratiquement identique à une structure sous-unitaire de Vi provenant de *Salmonella typhi*.

28. Immunogène contre *Salmonella typhi* comprenant un saccharide de plante, de fruit ou de synthèse modifié par O-acétylation lié par covalence à un support, dans lequel ledit conjugué de saccharide modifié et de support produit des anticorps chez des mammifères, et lesdits anticorps sont immunoréactifs spécifiquement contre Vi de *Salmonella typhi*.

29. Immunogène suivant la revendication 28, dans lequel le saccharide modifié de plante, de fruit ou de synthèse a une identité immunologique avec le Vi de *Salmonella typhi* telle que mesurée par immunodiffusion.

30. Immunogène suivant les revendications 28 ou 29, dans lequel le saccharide de plante ou de fruit provient d'agrumes, de pommes ou de betteraves.

31. Immunogène suivant l'une quelconque des revendications 28 à 30 dans lequel le saccharide est une pectine ou est choisi dans le groupe consistant en D-galacturonane, oligogalacturonate, polygalacturonate et leurs mélanges.

32. Immunogène suivant l'une quelconque des revendications 28 à 31 dans lequel le support est choisi dans le groupe consistant en protéine bactérienne, protéine virale, toxoïde de tétanos, toxine de diphtérie, exotoxine de *Pseudomonas aeruginosa*, toxoïde de *Pseudomonas aeruginosa*, toxine de coqueluche, toxoïde de coqueluche, exotoxine de *Clostridium perfringens*, toxoïde de *Clostridium perfringens*, antigène de surface d'hépatite B, antigène de...
noyau d'hépatite B et exoprotéine A de *Pseudomonas*.

33. Immunogène suivant l'une quelconque des revendications 28 à 32, dans lequel le saccharide est au moins O-acétylé à 50%.

34. Immunogène suivant l'une quelconque des revendications 31 à 33, dans lequel la pectine est au moins O-acétylée à 80%.

35. Vaccin contre la fièvre typhoïde comprenant l'immunogène suivant l'une quelconque des revendications 28 à 34 et un diluant acceptable du point de vue pharmaceutique.

36. Utilisation d'un saccharide O-acétylé de plante, de fruit ou de synthèse lié à un support pour la préparation d'un médicament utile dans un procédé pour immuniser activement un humain contre la fièvre typhoïde, comprenant l'administration *in vivo* d'une quantité suffisante d'un saccharide O-acétylé de plante, de fruit ou de synthèse lié à un support, dans laquelle ladite quantité est suffisante pour produire un anticorps qui se lie au Vi de *Salmonella typhi*. 
FIG. 3

OAc Pec

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A\text{280} or Refractive Index

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