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Bacterial polysaccharide–protein conjugate vaccines*

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Abstract: The age-related and T-cell independent properties of polysaccharides limit their use as vaccines. These limitations are overcome by covalently binding polysaccharides to proteins to form conjugates. Widespread use of *Haemophilus influenzae* type b (Hib) conjugates has virtually eliminated systemic infection caused by this pathogen, notably meningitis, in individuals of all ages. The principles derived from the development and use of Hib conjugates have been applied to other capsulated pathogens including pneumococci and meningococci. We have shown that vaccine-induced serum IgG antibodies to the surface polysaccharides of enteric pathogens confer immunity to typhoid fever (Vi) and to *S. sonnei*. Our preliminary data show that synthetic saccharides provide a method for increasing the immunogenicity of conjugates and permitted more direct characterization of a *S. dysenteriae* type 1 conjugate. Both the chain length and density of the saccharides on the protein were related to the immunogenicity of conjugates in mice. A synthetic approach has also been extended to the LPS types of *Vibrio cholerae* O1.

INTRODUCTION

For many bacterial pathogens, essential virulence factor and protective antigen are their surface polysaccharides. These may be capsular polysaccharide of Gram-positives such as pneumococci, Gram-negatives such as meningococci, or the O-specific polysaccharide domain of Gram-negative lipopolysaccharides (LPS). Because it has been most extensively studied, Hib will be considered in detail [1].

These surface polysaccharides have no demonstrable pharmacologic activity: they exert their virulence by 'shielding' the pathogen from the protective actions of serum complement [2]. The specificity for this virulence activity is shown by epidemiologic and experimental data. Only a few of the many surface polysaccharides of bacteria are associated with systemic infections in humans. Full expression of their surface polysaccharides is essential for virulence: capsulated strains lacking their capsular polysaccharides or Gram-negatives lacking an O-specific polysaccharide are not isolated from patients and are nonvirulent in animals. Although there are six distinct *H. influenzae* capsular polysaccharides, most ($\geq 95\%$) isolates from patients with systemic infections, such as meningitis, osteomyelitis, pneumonia, etc., are of type b. Lastly, most of these pathogens are inhabitants of and pathogens for humans only. Serum IgG capsular polysaccharide antibodies confer immunity in healthy individuals to respiratory pathogens, including *H. influenzae* type b, meningococci and pneumococci, and to *Salmonella typhi*, the causative agent of typhoid fever. In neonates too, capsular polysaccharides of Group B streptococci and *Escherichia coli* serve as virulence factors and protective antigens. There is a lesser, but convincing, body of evidence that serum IgG to O-specific polysaccharides confers immunity to both noncapsulated respiratory and to enteric Gram-negative pathogens. Leive *et al*. showed that the

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comparative virulence of *Salmonella typhimurium* could be explained by the low activity of its O-specific polysaccharide in initiating the alternate complement pathway relative to other nontyphoidal salmonellae: its O-specific polysaccharide shields *S. typhimurium* against the protective actions of complement in the same manner as capsular polysaccharides [3].

We proposed that serum IgG antibodies to surface polysaccharides confer immunity by inactivating the inoculum of the pathogen on the epithelial surface of the affected tissue such as the respiratory or intestinal tracts [4,5]. This inactivation is antibody-initiated complement-mediated bacteriolysis for Gram-negatives or opsonophagocytic killing for Gram-positives. It is not commonly appreciated that *serum* IgG confers immunity to both respiratory and to intestinal pathogens.

SERO-EPIDEMIOLOGY

Diseases caused by capsulated bacterial pathogens are rarely observed in newborns and infants up to age 4–6 months: the peak incidence is in young children gradually tapering in adult life. The age-related incidence of these infections is inversely related to the presence of serum IgG antibodies to their surface polysaccharides [1]. Most IgG antibodies are stimulated by cross-reacting nonpathogenic bacteria of the enteric and respiratory tracts and not by the homologous bacteria [6,7]. That cross-reacting bacteria, and not the homologous organism, stimulate the age-related immunity to these pathogens is illustrated by antibodies to the capsular polysaccharide of group A meningococci [8,9]. For the past 50 years, the age-related development of antibodies to these polysaccharides in the United States occurred in the absence of group A meningococci. These 'natural' IgG antibodies are transmitted to the fetus and confer immunity for up to 3–6 months of age.

IMMUNOLOGIC PROPERTIES OF POLYSACCHARIDES

Although they are immunogenic, capsular polysaccharides have limitations as vaccines. Most fail to induce protective levels of antibodies in young children, who suffer the highest incidence of diseases caused by these pathogens, and re-injection of most does not induce a booster response [10]. Rather, injection of a capsular polysaccharide elicits an antibody response that is characteristic of the age of the recipient. Isolated O-specific polysaccharides, probably because of their relatively low M_r, do not induce serum antibodies at any age and are considered as haptens.

Several factors affect the immunological properties of polysaccharides:

1 Epitopes of polysaccharides are repeated regularly throughout the molecule;

2 The M_r of polysaccharides is directly related to their immunogenicity [11,12]. This relation is the basis for standardization of capsular polysaccharide vaccines—their ability to elicit protective levels of antibodies is reliably predicted by estimation of the M_r by gel filtration. Their molecular weights range between 1×10^5 and 5×10^6 Da. O-specific polysaccharides, in contrast, are less than 3×10^4 Da;

3 Almost all capsular polysaccharides are resistant to degradation in mammalian tissues.

There is an age-related antibody responsiveness to bacterial polysaccharides that is likely, in part, to be genetically determined. Some, such as pneumococcus type 3 polysaccharide, elicit protective levels of antibodies in young infants [13]. But most fail to elicit protective antibody levels in children up to the age of two years and are only maximally immunogenic after the age of six years. Although we do not know the mechanism/s involved, capsular polysaccharides that fail to elicit protective levels of antibodies in children are those from organisms that have the highest incidence of disease in this age group.

POLYSACCHARIDE-PROTEIN CONJUGATES

Landsteiner showed that small molecules, including saccharides, could be converted to immunogens by covalently binding them to proteins (haptens). This finding was extended by Avery & Goebel who converted pneumococcus type 3 polysaccharide, not immunogenic in rabbits, to induce biologically active antibodies in that species by binding it to horse globulin [1,10]. They also bound the synthetic disaccharide repeat unit of pneumococcus type 3 to horse globulin and induced antibodies that protected

mice against challenge with this pathogen. Later, investigators prepared conjugates of other polysaccharides but one of their synthetic schemes and routes of vaccination were suitable clinically.

Hib polysaccharide protein conjugates elicited protective levels of antibodies in infants. To-date there are three licensed Hib conjugates used throughout the developed world and are included into the routine immunization of all infants according to the recommendations of the Children's Vaccine Initiative of the World Health Organization: all are safe and highly effective [1].

The synthetic schemes and compositions of the three Hib conjugates are different. PRP-T (Pasteur-Mérieux-Connaught) is prepared by binding a hydrazide derivative of Hib polysaccharide to tetanus toxoid by condensation with a water-soluble carbodiimide [14]. HbOC (Wyeth Lederle Laboratories-Pediatrics) is prepared by limited periodate oxidation of the polysaccharide and removal of saccharides less than 20 repeat units. The 'sized' saccharides, with aldehydes at both ends, are bound to the genetically engineered nontoxic diphtheria toxin (CRM_{197}) by reductive amination [15]. The resultant product has both single and multiple point attachments. Hib-OMP (Merck Sharpe & Dohme) treats the polysaccharide with N,N carbonyldimidazole to form an amino ethane that is bound to a sulfhydryl derivative of outer membrane proteins of group B *Neisseria meningitidis*. The SH of the protein and the amino groups on the polysaccharide are then linked by a thioether bond resulting in multipoint attachment [16].

Countries using any or all of these conjugates for routine vaccination of infants have virtually eliminated Hib disease as well as carriage in the entire population. This technology has been applied to the development of conjugates for other capsulated respiratory pathogens including pneumococci, meningococci, Group B streptococci, *Staphylococcus aureus* and *Salmonella typhi* [1,10].

CLINICAL EXPERIENCE WITH *H. influenzae* TYPE B CONJUGATES

Table 1 shows serum Hib polysaccharide serum antibodies before and after vaccination with the polysaccharide alone or as a conjugate [17–19]. Adults had the highest prevaccination antibody levels (almost all have protective levels) that increased about 20-fold following injection with Hib polysaccharide and about 200-fold with the conjugate. In 2-year-olds, the prevaccination level was one-third that of adults (about 50% have protective levels) and the polysaccharide elicited about a 15-fold rise and the conjugate a 130-fold rise. Reinjection of Hib polysaccharide or its conjugates does not elicit a booster response in individuals > 2 years of age. In most 2-month-olds, maternally acquired antibodies are barely detectable and injection of the Hib polysaccharide does not elicit an antibody response. Three injections of the conjugate, in contrast, elicit booster responses that result in serum antibody levels at least 20 times higher than the protective level: re-injection at about 15 months of age also elicits a booster response [1,19].

Table 1 Age-related responses (Geometric mean μg antibody/mL serum) elicited by *Haemophilus influenzae* type b vaccines [17–19]

	Polysaccharide		Conjugate	
Age group	Pre-	Post-	Pre-	Post-
Adults	0.58	13.0	1.12	220.8
2-years-old	0.18	2.80	0.17	26.8
2-months-old	0.05	0.05	0.05	6.8

Infants in Sweden were injected with Hib conjugate at 3, 5 and 12 months of age [20]. Their Hib antibodies at 6 years of age were only slightly higher than of age-matched nonvaccinated healthy children (2.06 μg/mL compared to 1.32, $P < 0.05$). But 97% of the vaccinees had protective levels of antibodies (≥ 0.15 μg antitype b/mL) in contrast to only 87% of the controls. The continuous stimulation of anti-type b (referred to as 'natural' antibodies) by cross-reacting organisms, explains why there is no need for revaccination of adults against Hib infections.

Infants and young children respond poorly or not at all to capsular polysaccharides following systemic infection [21]. As with the Hib polysaccharide, high-titered serum antibodies following systemic

infection are first observed in 2-year-olds. Conjugates, in contrast, elicit protective levels of serum IgG antibodies in infants.

Initially, it was thought that IgG and IgM antibodies exerted their protective effect by lysing organisms that entered the blood stream through the respiratory epithelium. Experience with Hib conjugates has provided another, possibly a more important mechanism, by which antibodies confer immunity. Hib carriage is found almost exclusively in infants and young children: it is detected in immunocompromised adults or in parents or other close contacts of children with systemic infections [1]. Our explanation for this finding is that 'natural' serum IgG antitype b, found in most healthy adults, inhibits colonization with this pathogen [1,5]. Widespread vaccination with conjugates induces serum IgG anti-type b that prevent colonization and transmission of the pathogen in children and virtually eliminates Hib carriage [22]. A similar effect of vaccination upon meningococcal groups A and C and pneumococci following vaccination has been reported [1,5]. We emphasized that serum IgG is present on all epithelial surfaces and participates in 'mucosal immunity' throughout the body.

T-CELL INDEPENDENT POLYSACCHARIDES AND T-CELL DEPENDENT PROTEINS

We are starting to understand the immunologic properties of polysaccharide-protein conjugates. Bacterial polysaccharides of medical interest are composed of a repeat unit with several epitopes expressed evenly throughout the polymer (multivalent for each epitope): most cannot be enzymatically catabolized by mammals. Proteins considered for carriers, in contrast, have more epitopes than polysaccharides but are monovalent for each epitope: these proteins are cleaved by mammalian enzymes.

Immunoglobulins are synthesized by B-cells that have the following properties:

1 B-cells synthesize immunoglobulin of all classes (IgM, IgA, IgG, IgE): only one class is synthesized at a given instant;

2 The B-cell receptor is membrane-bound IgM of one specificity that is the same as the immunoglobulin produced by the cell. The receptor therefore is the very antibody that will be induced by the immunogen [23]. A mature B-cell may be defined as having a critical concentration of IgM receptors. B-cells undergo maturation starting during intrauterine life that differs according to the specificity of the antibody. For example, human B-cells specific for pneumococcus type 3 polysaccharide reach maturation several months after birth whereas, Hib specific B-cells acquire maximal concentration of surface IgM at about 6 years of life [13]. Without specific stimulation, mature B-cells synthesize immunoglobulins at a low level;

3 It is the multivalency of T-cell independent antigens that causes the B-cell receptors to 'cluster'. When this clustering is maximal, the surface IgM receptors induce signal transduction of the adenylate cyclase system followed by immunoglobulin synthesis. Both the multivalency and the critical size of polysaccharides induce clustering, signal transduction and immunoglobulin synthesis of B-cells. In addition to their M_r, Dintzis *et al*. have shown that capping and antibody synthesis require a minimum of 20 epitopes per polymer separated by about 70–100 Å to function as a T-cell independent immunogen [24];

3 Most proteins, in contrast to polysaccharides, are univalent for an epitope and require the participation of T-cells for induction of antibody synthesis (T-cell dependence). Proteins bind to IgM receptors of B-cells by monovalent interaction and do not induce capping. Bound proteins are internalized, hydrolyzed, and the resultant peptides (paratopes) bind to the histocompatibility II surface antigen. It is this peptide-H2 complex that presents the paratope to T-cell receptors. The T-cell receptor consists of three domains one of which is structurally related to immunoglobulins. Stimulation and differentiation induced by a protein can be demonstrated *in-vitro* by the ability of the intact protein or an isolated paratope to induce mitosis of T-cells;

4 Paratope binding induces an intracellular structure, known as a microtubule-organizing center (MTOC), in a subset of T-cells that stimulate B-cells to proliferate and synthesize antibodies (T-helper cells) [25,26]. The MTOC is at the interface between the activated T-cell-B-cell couplet and seems to transport cytokines directly to the B-cell that results in their proliferation and in synthesis of immunoglobulin. The stimulus for this T-B-cell interaction is the protein but it is the polysaccharide

that is synthesized. Subsets of these antigen-specific T-helper cells bind to and transfer combinations of lymphokines such as interferon γ and interleukin 2 or interleukins 4 and 5 to B-cells.

This scheme provides an understanding of the properties of conjugates that induce in synthesis of Hib antibodies in infants. Hib polysaccharide cannot elicit antibodies in infants because there is not a sufficient number of membrane IgM receptors to form a functional cap. It is the polysaccharide of Hib conjugates that bind to the B cells of infants. But it is the protein component that is internalized and hydrolyzed into peptides that bind to the MHC II antigen. This MHC II-paratope of the B-cell attracts, binds to and differentiates a T-helper cell specific for the carrier protein. This interaction results in the direct transmission of lymphokines to B-cells that now proliferate and synthesize Hib antibodies.

THE 'CARRIER' EFFECT UPON THE IMMUNOGENICITY OF CONJUGATES

It is the carrier-specific stimulation of T-cells that initiates in infants or enhances in adults the polysaccharide antibody responses of B cells. This carrier effect may be used to enhance the anti-type b response to conjugates by at least three modes;

1 Prior vaccination with the carrier alone [14,27];

2 Concurrent injection of the carrier with the conjugate [27,28]; and

3 Changing the carrier of the conjugate during the course of vaccination of children [30]. The enhancing effect of Hib polysaccharide conjugates with different carriers permits the prescribed course of vaccination to be completed with any or all of the licensed products.

Ideally, conjugates of Hib (1), pneumococci ($n = 7$ to 9), meningococci ($n = 4$ or possibly 5) could be administered to infants in a single formulation along with DTP. Children in developing countries would benefit from inclusion of conjugates for *S. typhi* ($n = 1$), *Shigella* ($n = 3$) and cholera ($n = 2$ and possibly 3). We proposed that the 5 capsular polysaccharides of group B streptococci also be considered for routine infant vaccination [30]. This proposal would have 18–21 polysaccharide protein conjugates for routine vaccination of infants. There is no interference with the immunogenicity of each component of the 23 valent pneumococcal or the 4 valent meningococcal polysaccharide vaccines [31]. This is explained by the specific binding of IgM receptors so that each polysaccharide activates different populations of B-cells. The carriers of conjugates are T-cell dependent and, at the present, only three proteins are in use although several more have been investigated. There is, however, an inhibiting effect upon polysaccharide antibody synthesis when formulations of conjugates have only one carrier [32,33]. This interference may be explained by competition between the carrier for only one T-cell type resulting in a limited amount of T-cell B-cell couplets and of cytokine secretion. Whatever the explanation, it will be necessary to have more carrier proteins for the proposed infant conjugate formulation.

Vi CONJUGATES FOR TYPHOID FEVER

Vi polysaccharide typhoid vaccine is licensed in about 70 countries. As with other polysaccharide vaccines, Vi elicits protective levels of antibodies only in individuals older than 5 years of age (age-related immunogenicity) and reinjection does not induce a booster response (T-cell independence) [34–36]. The principles established for Hib conjugates were applied to the Vi of *S. typhi* [37].

Conjugates, using *Pseudomonas aeruginosa* recombinant exoprotein A (*r*EPA) as the carrier, elicit higher levels of antibodies than Vi alone in individuals older than 5 years of age [37]. In 2–4 years-olds, Vi conjugates induced booster responses with levels that were higher than those of children 5–14 years of age injected with Vi alone [38]. Evaluation of the efficacy of these new vaccines in 2–5-year-olds is underway [39]. Our objective is to introduce Vi conjugates into the routine vaccination schedule of infants in countries where typhoid fever is endemic.

Development of conjugates of Vi has not been easy because of its unusual characteristics. Vi is a linear homopolymer of α(1→4)-*D*-GalA *N*-acetylated at C2 and *O*-acetylated at C3. The *N*-and *O*-acetyls dominate the surface and are essential for both the antigenicity and immunogenicity of Vi [39]. Vi has no vicinal hydroxyls and its carboxyl at C6 is relatively inaccessible. A structurally similar plant polysaccharide, pectin [poly α-(1→4)-*D*-Gal*p*-], differs from Vi in three respects:

1 The M_r of pectin ($\approx 5 \times 10^5$ Da) is lower than that of Vi ($\approx 3 \times 10^6$ Da);

2 The repeat unit of pectin is interrupted by another saccharide about every 10 residues;

3 Pectin is not substituted on its C2 and C3: both can be *O*-acetylated by treatment with acetic anhydride and the resultant di-*O*-acetyl pectin is antigenically identical to Vi [40]. The substitution of an *N*-acetyl with an *O*-acetyl could 'open' the tightly wound structure of Vi created by the hydrogen bond between the *N*-acetyl and the adjacent carboxyl;

4 In contrast to Vi, di-*O*-acetyl-pectin alone does not elicit serum IgG anti-Vi in mice probably due to its comparatively lower M_r.

Conjugates of di-*O*-acetyl-pectin were synthesized by carbodiimide-mediated condensation with a hydrazide derivative of *r*EPA [40]. Vi conjugates were slightly more immunogenic than their O-acetyl pectin analogs but both Vi and O-acetyl pectin conjugates were significantly more immunogenic in mice than Vi alone and induced booster responses. Antibodies elicited by the Vi-*r*EPA and *O*-acetyl pectin-*r*EPA precipitated with an identity reaction with Vi by immunodiffusion.

There are advantages to using pectin for conjugate vaccines for typhoid. Pectin is abundant, has no LPS and requires a simple chemical modification to prepare its di-*O*-acetyl derivative. Our yields of di-*O*-acetyl pectin-*r*EPA were higher than those of Vi-*r*EPA. Clinical evaluation of O-acetyl pectin-*r*EPA is planned.

O-SPECIFIC POLYSACCHARIDE CONJUGATE VACCINES

LPS is a major constituent of the outer membrane of Gram-negatives that include common enteric pathogens of humans: *Salmonella*, *Shigella*, pathogenic *E. coli*, *Vibrio cholerae*, *Campylobacter* and *Helicobacter pylori*. The outer domain of LPS, the O-specific polysaccharide, serves both as a protective antigen and as an essential virulence factor [4,10]. Using experimental and clinical data for several Gram-negative pathogens, we proposed that a critical level of serum IgG anti-O-specific polysaccharide confers immunity by lysing the inoculum on the jejunal epithelium [4,5]. This proposal about enteric infection has been buttressed with epidemiologic, experimental and clinical data.

IMMUNITY TO *Shigella*

The notion that immunity to *Shigella*, as well as to other enteric organisms, is mediated by secretory IgA antibodies is based upon the failure of parenterally administered inactivated bacteria to prevent shigellosis although these vaccines stimulated serum antibodies [4]. Multiple *intravenous* injections of inactivated bacteria induced high levels of antibodies and protection against challenge with Gram-negative enteric bacteria in animal models but this type of vaccination is unacceptable for humans. Clinical evaluations of cellular typhoid, *E. coli* and one or two intramuscular injections of inactivated *Shigella* of *V. cholerae* 01 vaccines did not induce a high or long-lived level of IgG anti-LPS in humans [4]. In fact, most vaccines composed of inactivated bacteria have been replaced by purified products.

The Genus *Shigella* is defined as Gram-negative bacilli that ferment glucose, but not lactose, and are nonmotile (do not have flagella). Shigellae were divided into four groups based upon biochemical properties and the structures of their O-specific polysaccharides. Group A (*dysenteriae*) are defined by their inability to ferment mannitol: 12 O-specific polysaccharide types have been identified. *S. dysenteriae* type 1 (also known as Shiga's bacillus) is the most important type: it is unique in that it produces one or two related protein exotoxins denoted as *Shigella* toxins and causes several extraintestinal complications including hemolytic uremic syndrome. Members of Group B (*flexneri*), the most common cause of shigellosis in developing countries, share a tetrasaccharide repeat unit of their O-specific polysaccharides. To-date, 11 types, created by substitutions of glucose and or O-acetyl along the tetrasaccharide repeat unit, have been identified. *S. flexneri* type 2a, and to a lesser degree, type 1a, are the most common types within Group B associated with disease. Group D (*sonnei*) has only one O-specific polysaccharide type and is the most common cause of shigellosis in developed countries. The O-specific polysaccharide structure and several biochemical characteristics of Group D differ from those of other shigellae [4].

There is information about the pathogenesis of and immunity to *Shigella*. Full expression of LPS is required for the virulence of shigellae. Convalescence from shigellosis confers type (O-specific polysaccharide)-specific immunity, although incomplete and of limited duration [4]. Shigellosis is rare up to 6 months of age and independent of breast feeding. The peak incidence, morbidity and mortality of shigellosis is in young children. Prospective studies of Israeli Armed Forces recruits showed that resistance to shigellosis is predicted by the level of serum IgG anti-O-specific polysaccharide [41]. There is an age-related development of serum IgG anti-LPS of shigellae. The stimulus for these 'natural' and protective antibodies includes nonpathogenic cross-reacting bacteria as shown for capsulated respiratory pathogens. For example, there is an overlap of serum IgG anti-LPS levels to *S. dysenteriae* type 1 in patients and controls in Vietnam and in healthy individuals from Stockholm (*S. dysenteriae* type 1 has not been reported in Sweden in the past 50 years) [42]. These findings indicated that a critical level of serum IgG LPS antibody confers immunity to shigellosis.

Not commonly appreciated is that *Shigella* and *E. coli* should be considered as one Genus. For this reason, we proposed that information about immunity to *Shigella* is applicable to *E. coli* O157 and related organisms [4].

STUDIES WITH O-SP PROTEIN CONJUGATES FOR SHIGELLOSIS

LPS is not acceptable as a vaccine because of its toxicity (fever, inflammation, shock). Treatment with organic acids or bases reduces the toxicity of the lipid A to acceptable levels. The resultant product, composed of the O-specific polysaccharide and part of the core, can be linked to proteins by a variety of methods. Vaccination of young adults and of 2–5-year-olds with these conjugates elicited O-specific polysaccharide antibodies of the three immunoglobulin classes to *S. dysenteriae* type 1, *S. flexneri* type 2a and to *S. sonnei* with no cross-reactivity [43,44]. In recruits, the levels of IgG anti-LPS to *S. sonnei* and to *S. flexneri* type 2a elicited by conjugates were similar or higher than those in patients [44]. Efficacy of *Shigella* conjugates was evaluated in Israeli recruits who are at risk for outbreaks of shigellosis during their basic training []. A randomized, blinded, vaccine-controlled trial, involving companies of recruits, were vaccinated with a *S. sonnei* conjugate: controls included an orally administered genetically constructed strain for *S. flexneri* type 2a or with groups A and C meningococcal polysaccharide vaccine [45]. The *S. sonnei* conjugate was effective in preventing shigellosis in three companies that experienced outbreaks at 2 months or more after vaccination (Table 2). One company had cases of *S. sonnei* within 13 days following vaccination. Yet, despite the high attack rate of 20% and short interval (many or most of the recruits were probably infected at the time of vaccination), the *S. sonnei* conjugate conferred statistically significant, though low protection. The speed with which the conjugate acted indicates that

Table 2 Efficacy of *Shigella sonnei* O-specific polysaccharide-*Pseudomonas aeruginosa* recombinant exoprotein A (*r*EPA) conjugate: infection with *S. sonnei* (≥ 3 loose stools/day) in companies of Israel Defense Force recruits [45]

	Vaccine groups					
	*S. sonnei-r*EPA		*EcSf*2A-2 or MAC		Efficacy (%)	
Cohort	*n* =/total	%	*n* =/total	%	*P* =	(95% CI)
A	1/32	3.1	7/48	14.6	0.135	75
B	0/32	0	3/48	6.2	0.271	100
C	3/115	2.6	13/72	7.6	0.073	65
Total	4/179	2.2	23/268	8.6	0.006	74 (28–100)
D	15/127	11.8	40/193	20.7	0.039	43 (4–82)

In a blinded, randomized and vaccine-controlled trial, companies of recruits were administered one injection of *S. sonnei-r*EPA or 1 of 2 controls (three orally administered doses of *EcSf*2A-2, a recombinant *E. coli* with genes including the O-specific polysaccharide of *S. flexneri* type 2a (*EcSf*2A-2), or an injection of groups A and C meningococcal polysaccharide vaccine (MAC).

this type of vaccine could also be used to control epidemics. Lastly, the efficacy was related to the level of IgG anti-LPS induced by the *S. sonnei* conjugate.

SYNTHETIC SACCHARIDES FOR CONJUGATE VACCINES

Synthetic polysaccharides could provide an advantage to products prepared from bacteria for several reasons:

1 The synthetic route provides homogeneous saccharides in contrast to the comparatively heterogenous products prepared by hydrolysis of natural products even when separated by physico-chemical methods;

2 Analogues of the natural product could improve the immunogenicity of the conjugate and allow detailed examination of the interaction of the saccharide with antibodies and with lymphoid cells;

3 The toxicity of synthetic products can be more effectively controlled. *S. dysenteriae* type 1 was chosen for the following:

a It is the only organism of this Genus that excretes one or two related toxins (*Shigella* toxins) [4]. In about 10% of patients, *S. dysenteriae* type 1 causes the extraintestinal complication of hemolytic uremic syndrome that has a high mortality and may cause chronic renal failure. It is thought that the *Shigella* toxins contributes to the development of this serious complication;

b *S. dysenteriae* type 1 induces enteric disease ranging from watery diarrhea to dysentery (fever, cramps, blood and/or mucus in the stool). It is dysentery, not watery diarrhea, that causes growth retardation probably because this disease is caused by a diffuse inflammation of the intestine simulating a burn [4];

c In many parts of the world, especially Africa, this pathogen is resistant to almost all antibiotics. Further, even effective antibiotic treatment does not alter the severity of the enteric infection or reduces the incidence of hemolytic uremic syndrome [46];

d *S. dysenteriae* type 1 causes both endemic and epidemic shigellosis. In recent times, this pathogen has been a major cause of morbidity and mortality in refugee camps [4];

e *S. dysenteriae* type 1 and *E. coli* O157 are similar from the view point of pathogenesis and immunity. Accordingly, if our approach to induce IgG anti-LPS for prevention of infection with *S. dysenteriae* type 1 is successful, it can be predicted that conjugates will prevent disease by *E. coli* O157 and related organisms [4,47].

The O-specific polysaccharide of *S. dysenteriae* type 1 is composed of a tetrasaccharide repeat unit[3)-α-L-Rha*p*-(1→2)-α-D-Gal*p*-(1→3)-α-D-Glc*p*NAc-(1→3)-α-L-Rha*p*-)1→]^n. This repeat unit was synthesized by a protecting group scheme that facilitated blockwise synthesis of a octa, dodeca and a hexadeca-saccharide each of these polymers were prepared with a hydrazide at the reducing end [48,49]. The saccharides were bound to human serum albumin (HSA) through a spacer by reductive amination. The molar ratios of the saccharide to the protein, including the tetra, octa, dodeca, and hexadeca, ranged from about 4 to 24. The composition of the saccharide-HSA conjugates was calculated from the spectra obtained by matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry. The M_r of HSA was 66.35 kDa and the dodecamer HSA conjugate showed the the major peak centered around 124 kDa.

Mice were injected subcutaneously three times 2 weeks apart with 2.5 μg of saccharide and bled 7 days after the last injection. Serum IgG anti-LPS was measured by ELISA. A conjugate containing 12 chains of the tetramer elicited low levels of IgG anti-LPS. All the conjugates prepared with the synthetic saccharides, excluding the tetramer, elicited anti-LPS after the second injection and a statistically significant rise (booster) after the third. The dodecamers and hexadecamers were slightly more immunogenic than the octamers at comparable molar ratios of chains per HSA. Conjugates containing about nine chains of either the dodecamer or hexadecamer per HSA elicited the highest levels of IgG anti-LPS in mice.

Conversion of O-specific polysaccharides to an immunogen when it is part of a conjugate may be explained two factors: (i) the increase in M_r that allows the O-specific polysaccharide to adhere to a

greater number of membrane-bound IgM and induce signal transduction to the B-cell; and (ii) their protein that is catabolized by the polysaccharide-stimulated B cell resulting in a peptide-histocompatibility II antigen signal to T cells [50,51]. The optimal density for the octamer was 20 chains, nine for both the dodecamer and the hexadecamer. There were only slight differences in the geometric mean IgG anti-LPS levels elicited by the optimal configurations of the octamer, dodecamer and hexadecamer. These experiments in mice showed that both M_r of the saccharide and the number of saccharides per protein were determinants of the conjugates' immunogenicity. The greater precision achieved with synthetic saccharides could explain inconclusive data about the relation of M_r and saccharide length to immunogenicity obtained with conjugates prepared with hydrolytic fragments of natural products [52,53].

Since the immunogenicity of polysaccharide-based vaccines in experimental animals may not always be related to those obtained in human infants for which these *S. dysenteriae* type 1 conjugates are planned, we plan to evaluate clinically evaluate our synthetic polysaccharides bound to medically useful toxoids.

CONJUGATE VACCINES FOR OTHER ENTERIC PATHOGENS

Our approach to induce serum IgG anti-LPS has been extended to other bacterial pathogens including nontyphoidal salmonellae, *Vibrio cholerae* O1 [54–56] and *Escherichia coli* O157 [57] and O111 [58].

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