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Vaccines based on the cell surface carbohydrates of pathogenic bacteria

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ABSTRACT

Glycoconjugate vaccines, in which a cell surface carbohydrate from a micro-organism is covalently attached to an appropriate carrier protein are proving to be the most effective means to generate protective immune responses to prevent a wide range of diseases. The technology appears to be generic and applicable to a wide range of pathogens, as long as antibodies against surface carbohydrates help protect against infection. Three such vaccines, against *Haemophilus influenzae* type b, *Neisseria meningitidis* Group C and seven serotypes of *Streptococcus pneumoniae*, have already been licensed and many others are in development.

This article discusses the rationale for the development and use of glycoconjugate vaccines, the mechanisms by which they elicit T cell-dependent immune responses and the implications of this for vaccine development, the role of physicochemical methods in the characterisation and quality control of these vaccines, and the novel products which are under development.

Key words: Glycoconjugate Vaccines, Capsular polysaccharides, lipopolysaccharides.

INTRODUCTION

Bacterial infections remain major killers of infants and children, particularly in developing countries. Several million children die each year due to such infections (Table I). The most important pathogens are *Streptococcus pneumoniae, Haemophilius influenzae* type b, *Neisseria meningitidis, Salmonella entericus* subspecies *typhi, Staphylococcus aureus*, and diarrhoea-causing organisms such as *Shigella, Salmonella* and *Vibrio cholerae*. The disease profile can be endemic or epidemic, as occurs with Group A meningococcal disease in sub-Saharan Africa. Disease and mortality are concentrated amongst children in developing countries. Each of these pathogens possesses a cell surface capsular polysaccharide (CPS) or lipopolysaccharide (LPS) shell, or both, which helps the pathogen to establish an infection. The CPS hides cell surface components of the bacterium from the immune system of the host, preventing complement activation by cell surface proteins (Roitt 1997) and inhibiting phagocytosis. If the bacterium is phagocytosed, the CPS helps prevent bacterial killing. The role of LPS as a virulence factor is less well defined. In some cases it has been demonstrated, and in other cases it is suspected, that antibodies against a CPS or LPS O-chain will protect against infection (Lindberg et al. 1991, Popoff 1991, Waldor et al. 1994, Fulop et al. 2001, Coughlin and Bogard 1987, Brahmbhatt et al. 1992, Bowden et al. 1995).

For most of these pathogens, different strains

TABLE I

Estimated annual number of cases and death from selected pathogens: for under 5s unless indicated otherwise.

Organism (bacterial)	Disease	Death rate or morbidity	Vaccine?	Reference for mortality data
Streptococcus pneumoniae	Acute respiratory infections and meningitis	> 1 000 000 deaths 1-2 million deaths (plus many with neurological damage)	Conjugate vaccines becoming available	Anonymous 2003 Eskola and Anttila 1999
Haemophilus influenzae (mainly type b)	Acute respiratory infections and neonatal meningitis	400 000 to 700 000 deaths	Type b conjugate vaccine available	Anonymous 1998
Neisseria meningitidis	Meningitis and bacteraemia	500 000 cases: 50 000 deaths (plus ca. 60 000 left with neurological damage)	Conjugate vaccines becoming available	Anonymous 2002a
Shigella	Shigellosis – diarrhoea	164.7 million cases, 1 100 000 deaths	Conjugate vaccines shown to be feasible	Kotloff et al. 1999
Salmonella enterica ssp *typhi*	Typhoid (all ages)	21.6 million cases: 216 500 deaths (2004 estimate) 16 million cases: 600 000 deaths (1984 estimate)	Polysaccharide vaccine available: conjugates in development	Crump et al. 2004 Edelman and Levine 1986
Staphylococcus aureus	Sepsis	Important cause of neonatal death, but clear figures not available	Vaccine under development, targeted at hospital-acquired infections in developed countries	http://www.who.int/child-adolescent-health/New_Publications/IMCI/WHO_FCH_CAH_01.10/Young_Infant
Vibrio cholerae	Cholera (all ages)	120 000	Conjugate vaccines in development	Ivanoff and Chaignat 2002
Bordetella pertussis	Whooping cough	20-40 million cases, 200 00 – 400 000 deaths	Traditional vaccines available	http://www.who.int/vaccines/en/neotetanus.shtml
Mycobacterium tuberculosis	Tuberculosis	100 000 children (plus 2–3 million adults)	BCG vaccine available	
Other diseases				
Measles	Measles	30–40 million cases, 777 000 deaths (2000)	Vaccine available	Anonymous 2002b
Rotavirus	Diarrhoea	130 million cases, 600 000 deaths	Vaccine developed and withdrawn	http://www.ivi.int
Plasmodium	Malaria	900 000 (Africa, 2000)	No immediate prospect of vaccine	http://www.who.int/child-adolescent-health/New_Publications/CHILD_HEALTH/EPI/CHERG_Malaria_Mortality.pdf
HIV	AIDS	470 000 children (plus 2.1 million adults) (1999)	No immediate prospect of vaccine	http://www.who.int/health-services-delivery/hiv_aids/English/fact-sheet-2/
Respiratory Syncytial Virus	Lower respiratory tract infections	64 million cases, 160 000 deaths	Under development	http://www.who.int/vaccine_research

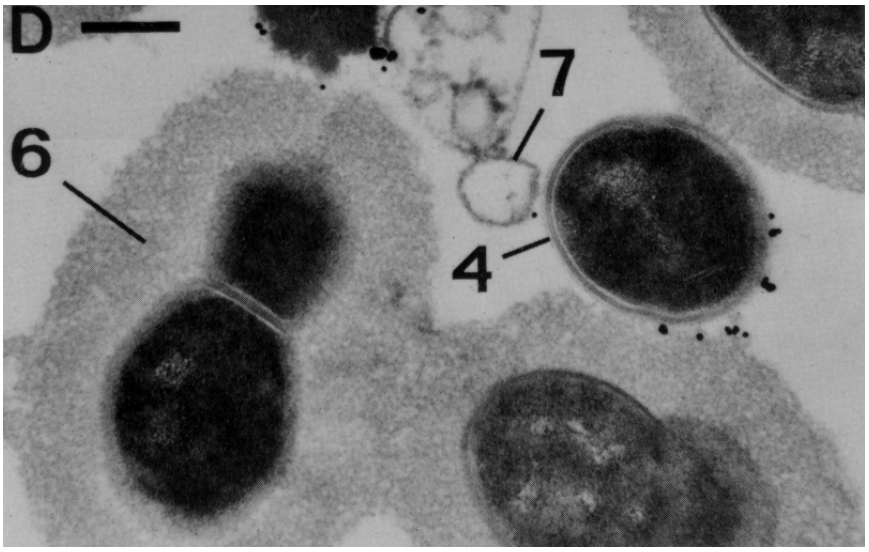

Fig. 1 – Electron micrograph of *Streptococcus pneumoniae* and the associated pneumococcal capsular polysaccharide (labelled 6). The bacteria shows the typical diplococcus morphology of the pneumococcus. Figure reproduced from Skov Sørensen et al. (1988) Infect Immun 56: 1890-1896 (copyright American Society for Microbiology), with permission.

express CPSs or LPSs of different structures, resulting in a number of different serotypes or serogroups. Virulence and pathogenicity may be serotype or serogroup dependent, or there may be geographic differences in the clinically relevant serotypes. Whilst *Haemophilus influenzae* disease is caused overwhelmingly by a single serotype, the type b, pneumococcal disease is caused by a very large number of the ninety known serotypes. Meningococcal disease in developed countries is principally Groups B and C, although Groups W135 and Y are becoming increasingly important, and Group A disease is virtually unknown (Racoosin et al. 1998, Mayer et al. 2002). Meningococcal Group A strains are, however, responsible for the regular meningitis epidemics which plague sub-Saharan Africa. Practically therefore, most of the saccharide-derived vaccines must contain multiple carbohydrates structures to provide adequate coverage against the disease-causing strains. In some cases there is immunological cross reactivity between related structures which can provide partial protection. The optimal choice of polysaccharide to include in the vaccine is therefore a complex epidemiological problem (Robbins et al. 1983). Pneumococcal Types 1 and 5, for example, are important pathogens in South America, but much less important in North America or Europe. For this reason, these serotypes were not included in the first 7-valent glycoconjugate vaccines licensed (Wyeth's Prevenar®), but have been included in subsequent 9- and 11-valent glycoconjugate vaccines under development. A number of these pathogens, including *Neisseria meningitidis* and *Streptococcus pneumoniae*, can leave surviving infants with severe neurological damage. This may affect as many infants as actually die from the infection. This has profound social and economic impacts.

Once it became clear that antibodies against CPSs protect against infection, it was a logical step to attempt to use these polysaccharides as immunogens. The first attempts were made in the late 1940s (McLeod et al. 1945), but the seemingly miraculous potential of antibiotics to control disease postponed development of this field until the late 1960s, when antibiotic resistance and the potential for neu-

rological damage in "antibiotic-cured" infants became apparent (Cochi et al. 1985). CPS vaccines clearly work, and vaccines of this type are licensed and used in many countries, but this approach has several severe limitations. Repeating polysaccharides are T cell-independent type 2 (TI-2) immunogens: without the involvement of T cells they do not induce immunological memory, avidity maturation and isotype switching do not occur, and the antibodies induced, largely IgM and IgG2 (Musher et al. 1990, Lortan et al. 1993), are not good activators of complement. Crucially, vaccines of this type fail to induce immune responses in infants below the age of about two years, who are the major group at risk for these infections, because this aspect of the immune system develops relatively late. Repeat vaccination does not lead to increased antibody levels, so only one dose is given, but without immunological memory re-vaccination is required at regular intervals as antibody levels decline. This is typically every five years.

Whilst the specificity of the immune response depends upon the structure of the CPS, the magnitude of the response depends critically upon its molecular weight. Only very high molecular weight polysaccharides are immunogenic and product development focussed at first on the isolation of material of sufficiently high molecular weight. For this reason, LPS O-chains and low molecular weight CPSs, such as those from *Staphylococcus aureus*, are not effective as vaccines.

POLYSACCHARIDE STRUCTURE

CPSs and LPS O-chains have strict repeating structure, which may consist of either a single sugar unit or oligosaccharide units, containing as many as seven or eight sugar residues (Kamerling 2000). The repeat units can either be linear or branched and contain non-carbohydrate substituents such as *O*-acetyl, glycerol phosphate, or pyruvate ketals. Structural heterogeneity may occur as a result of the loss of or migration of labile *O*-acetyl groups be-

unusual sugar residues including diamino-, deoxy- and branched chain sugars (Lindberg 1990a). As a general rule, CPSs tend to be anionic in character whilst LPS O-chains are neutral. The structures of some the repeat units of the capsular polysaccharides of clinically important bacteria are shown in Table II. Whilst these vaccines elicit a strong antibody response, it is likely that protection depends upon a relatively small proportion of high avidity antibodies, with those directed against the saccharide backbone perhaps most important. Whilst antibodies against substituents such as *O*-acetyl groups may predominate, they may be of relatively low avidity and not clinically important (Michon et al. 2000).

THE MOLECULAR MECHANISMS OF THE GENERATION OF IMMUNE RESPONSES AGAINST POLYSACCHARIDES

The molecular mechanisms by which TI-2 immunogens with repeating structures, such as bacterial polysaccharides, stimulate an antibody response have been revealed by the work of Snapper and coworkers (Mond et al. 1995, Snapper and Mond 1996, Snapper et al. 1997). In brief, the polysaccharide crosslinks approximately 15-20 surface immunoglobulin molecules (sIg) molecules present on a B cell of appropriate specificity, leading through a series of intermediate protein phosphorylation steps to an increase in free intracellular calcium. Such a cell is primed to secrete antibody, but a second signal is also required. The nature of this second signal has not been well defined, and may be different in the case of a natural infection than when a vaccine is used. When this second signal is received, the B cells mature into plasma cells and secrete antibodies. There appears to be no direct interaction between B cells and T cells. The necessity to crosslink many sIg molecules would seem to be the reason why only high mass CPSs are immunogenic. The mechanism by which glycoconjugate vaccines elicit an immune response is significantly different and is discussed in more detail below, but it is this

TABLE II

Repeating unit structures of important bacterial CPSs involved in vaccine development.

Organism	Structure
Haemophilus influenzae type b Branefors-Helander et al. 1976, Crisel et al. 1975	→ 3)-β-D-Rib*f*-(1→1)-D- Ribitol-(5→OPO_3→
Neisseria meningitidis Group A Bundle et al. 1974, Lemercinier and Jones 1996	→6)-α-D-Man*p*NAc(3/4OAc)-(1→OPO_3→
Neisseria meningitidis Group B Bhattacharjee et al. 1975	→8)-α-D-Neu*p*5Ac-(2→
Neisseria meningitidis Group C Bhattacharjee et al. 1975, Lemercinier and Jones 1996	→9)-α-D-Neu*p*5Ac(7/8OAc)-(2→
Neisseria meningitidis Group W135 Bhattacharjee et al. 1976, Lemercinier and Jones 1996	→6)-α-D-Glc*p*-(1→4)-α-D-Neu*p*5Ac(9OAc)-(2→
Neisseria meningitidis Group Y Bhattacharjee et al. 1976, Lemercinier and Jones 1996	→6)-α-D-Gal*p*-(1→4)-α-D-Neu*p*5Ac(9OAc)-(2→
Salmonella typhi Vi Heyns and Kiessling 1967	→4)-α-D-Gal*p*NAcA(3OAc)-(1→
S. pneumoniae Type 1 Stroop et al. 2002	→3)-D-AAT-α-Gal*p*-(1→4)-α-D-Gal*p*A(2/3OAc)-(1→3)-α-D-Gal*p*A-(1→
S. pneumoniae Type 2 Jansson et al. 1998	→4)-β-D-Glc*p*-(1→3)-[α-D-Glc*p*A-(1→6)-α-D-Glc*p*-(1→2)] -α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→3)β-L-Rha*p*-(1→
S. pneumoniae Type 3 Reeves and Goebel 1941	→3)-β-D-GlcA-(1→4)-β-D-Glc*p*-(1→
S. pneumoniae Type 4 Jones et al. 1991	→3)-β-D-Man*p*NAc-(1→3)-α-L-Fuc*p*NAc-(1→3)-α-D-Gal*p*NAc-(1→4) -α-D-Gal*p*2,3(S)Py-(1→
S. pneumoniae Type 5 Jansson et al. 1985	→4)-β-D-Glc*p*-(1→4)-[α-L-Pne*p*NAc-(1→2)-β-D-Glc*p*A-(1→3)] -α-L-Fuc*p*NAc-(1→3)-β-D-Sug*p*-(1→
S. pneumoniae Type 6B Kenne et al. 1979	→2)-α-D-Gal*p*-(1→3)-α-D-Glc*p*-(1→3)-α-L-Rha*p*-(1→4)-D-Rib-ol-(5→P→
S. pneumoniae Type 9N Jones et al. 1985	→4)-α-D-Glc*p*A-(1→3)-α-D-Glc*p*-(1→3)-β-D-Man*p*NAc-(1→4) -β-D-Glc*p*-(1→4)-α-D-Glc*p*NAc-(1→
S. pneumoniae Type 9V Rutherford et al. 1991	→4)-α-D-Glc*p*A(2/3OAc)-(1→3)-α-D-Gal*p*-(1→3)-β-D-Man*p*NAc(4/6OAc)-(1→4) -β-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→
S. pneumoniae Type 12F Leontein et al. 1981	→4)-[α-D-Gal*p*-(1→3)]α-L-Fuc*p*NAc-(1→3)-β-D-GlcNAc-(1→4)-[α-D-Glc-(1→2) -α-D-Glc-(1→3)]-β-D-ManNAcA-(→
S. pneumoniae Type 14 Lindberg et al. 1977	→4)-β-D-Glc*p*-(1→6)-[β-D-Gal*p*-(1→4)]-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→
S. pneumoniae Type 18C Lindberg 1990b	→4)-β-D-Glc*p*-(1→4)-[α-D-Glc*p*(6OAc) (1→2)][Gro-(1→*P*→3)]-β-D-Gal*p*-(1→4) -α-D-Glc*p*-(1→3)-β-L-Rha*p*-(1→
S. pneumoniae Type 19F Jennings et al. 1980	→4)-β-D-Man*p*NAc-(1→4)-α-D-Glc*p*-(1→2)-α-L-Rha*p*-(1→*P*→
S. pneumoniae Type 23F Richards and Perry 1988	→4)-β-D-Glc*p*-(1→4)-[α-L-Rha*p*-(1→2)]-[Gro-(2→*P*→3)] -β-D-Gal*p*-(1→4)-β-L-Rha*p*-(1→
Staphylococcus aureus Type 5 Moreau et al. 1990, Jones 2005b	→4)-β-D-ManNAcA-(1→4)-α-L-FucNAc(3OAc)-(1→3)-β-D-FucNAc-(1→
Staphylococcus aureus Type 8 Jones 2005b	→3)-β-D-ManNAcA(4OAc)-(1→4)-α-L-FucNAc-(1→3)-α-D-FucNAc-(1→

so much more effective as vaccines, and why they can be used to stimulate an immune response against a much wider variety of carbohydrate immunogens. Processing of zwitterionic capsular polysaccharides by an MHC II pathway has very recently been suggested (Cobb et al. 2004).

POLYSACCHARIDE VACCINES IN CLINICAL USE TODAY

Three families of CPS vaccines are in widespread clinical use at present, whilst a fourth against *Haemophilus influenzae* type b (Hib) infection was used as a short term measure before the introduction of Hib conjugates. The simplest CPS vaccine, against typhoid, contains the so-called Vi (for virulence) antigen as its sole component, but with, typically, lactose present as a stabiliser. Clinical trials of the *Salmonella typhi* Vi polysaccharide in Nepal indicated an efficacy of approximately 70%, which is similar to older whole cell vaccines against typhoid but the side effects of the polysaccharide vaccine are much less severe (Acharya et al. 1987, Yang et al. 2001). Vaccines containing two (Groups A and C), three (Groups A, C and W135) or four meningococcal (Groups A, C, Y and W135) CPSs are licensed. In developed countries they are currently used for control of outbreaks, but vaccination is required by Muslims undertaking the Hajj pilgrimage to Mecca. These vaccines are also used to control epidemic Group A meningitis in sub-Saharan Africa (Anonymous 2002a).

The pneumococcal polysaccharide vaccine is a blend of 23 serotype-specific polysaccharides, and is used in developed countries to protect the elderly from pneumonia. There is active discussion about exactly how effective these vaccines are for that purpose: some recent metastudies have cast doubt upon its efficacy (Melegaro and Edmunds 2004, Jackson et al. 2003). It is known that, for genetic reasons, some vaccinees are incapable of generating an immune response against some of the serotypes (Musher et al. 1998). This appears to be linked to the very limited genetic diversity of the immune tion appear to produce antibodies of lower avidity (Romero-Steiner et al. 1999).

Despite their limitations, polysaccharide vaccines are available, have moderate efficacy in appropriate populations, are generally cheap (Fleck 2003, Plans 2002) and have an excellent safety record. Glycoconjugate vaccines against meningococcal Group C and against seven pneumococcal serotypes have been licensed, whilst glycoconjugate vaccines against other meningococcal CPSs, more pneumococcal serotypes and typhoid are in development (see below).

GLYCOCONJUGATE VACCINES

The means to increase the immunogenicity of polysaccharides was first discovered by Avery and Goebel in 1931 (Avery and Goebel 1931) – covalent attachment of the polysaccharide to an appropriate protein carrier, to form a conjugate. Such conjugates provide T cell-dependent immunogenicity against the saccharide hapten. With the involvement of T cells, immunological memory is invoked, avidity maturation and isotypes switching occurs, to generate complement-activating antibody isotypes such as IgG1 (Wuorimaa et al. 2001). The avidity of the antibodies elicited is much higher than those from polysaccharide vaccines. Crucially, since a different arm of the immune system is involved, that used to process protein immunogens, glycoconjugate vaccines are effective in young infants. Multiple immunisations are necessary to provide the required immune response, but not regular revaccination. In the UK the vaccination regime for Hib conjugate vaccines is at two, three and four months, and a booster at 18 months has recently been introduced to ensure long term protection.

The mechanism by which glycoconjugates stimulate an immune response involves an initial binding of the conjugate to the surface immunoglobulin (sIg) of B cell with appropriate specificity for the saccharide hapten (Siber 1994). This complex is internalised and the carrier protein degraded

ported to and displayed by MHC II complexes. The peptide-loaded MHC II complex is recognised by T cells, which then provide appropriate signals through direct interactions of cell surface proteins and through cytokine signalling processes, to induce maturation of the B cell into an antibody secreting plasma cell. The role of dendritic cells in the process is not yet defined, and the process is probably different in adults who have already been exposed to the saccharide immunogens – glycoconjugate vaccines typically invoke an antibody response in adults after a single dose (Baxendale et al. 2000, Hougs et al. 1999). Since crosslinking of surface immunoglobulin molecules is not required, glycoconjugate vaccines can be produced from small saccharide chains. In many cases, the glycans attached in the conjugate are oligosaccharides prepared by degradation of the original polysaccharide (Anderson et al. 1986, Costantino et al. 1999). In addition, glycoconjugates can be produced from relatively low molecular weight oligosaccharides related to the repeating polymers (Mawas et al. 2002, Benaissa-Trouw et al. 2001, Jansen et al. 2001, Jansen and Snippe 2004), or the short glycans of LPS O-chain (Gupta et al. 1998), or low molecular weight capsular polysaccharides such as those expressed by *Staphylococcus aureus* (Fattom et al. 2004). It has been shown possible to make effective glycoconjugate immunogens from low molecular weight oligosaccharides such as those present on the lipo-oligosaccharides of pathogens such as a *Neisseria meningitidis* (Mieszala et al. 2003). The same glycoconjugate technology has been used to prepare immunotherapeutics to slow the redevelopment of cancer following chemotherapy, prepared from the glycan chains of glycolipids overexpressed by tumour cells (Musselli et al. 2001). Further discussion of cancer immunotherapeutics is outside the remit of this review.

The first glycoconjugate vaccines against *Haemophilus influenzae* type b were licensed in the late 1980s. They arose from the academic work of Porter Anderson and of others (Anderson et al. Lugowski 1981, Schneerson et al. 1980). The Anderson approach involved reductive amination of periodate-generated aldehyde-terminated oligosaccharides to a carrier protein. In modern preparations, CRM197, a genetically toxoided variant of diphtheria toxin is used. The resulting glycoconjugate is approximately 90 kDa in size, is approximately 30% carbohydrate and contains an average of six glycan chains per carrier protein. It is similar in size and saccharide content to many serum proteins, and can be termed a "neo-glycoconjugate" vaccine. Another approach, originally developed by Hilleman (Tai et al. 1987) and commercialised by Aventis Pasteur and GSK, involves random activation of the polysaccharide with cyanogen bromide, addition of linker such as 6-aminocaproic acid or adipic acid dihydrazide linker, and attachment to an appropriate carrier protein – typically tetanus toxoid. As there are multiple activation points within each polysaccharide and multiple linkage points on each carrier protein, the resulting conjugate is a crosslinked network of polysaccharide and protein with a molecular weight of, on average, 5×10^6 Da. Such a vaccine can be described as a "crosslinked network". The third approach uses conjugation of size-reduced polysaccharide to LPS-depleted vesicles of outer membrane proteins – a "vesicle vaccine" (Donnelly et al. 1990). Thus there are three fundamentally different structures for these conjugates, which are illustrated as cartoons in Figure 2.

The immune responses elicited by these different structural variants are generally similar – all are T cell-dependent immunogens, although the vesicle-based vaccines seem to be characterised by a stronger antibody response following the first immunisation, a less pronounced booster effect on subsequent immunisations, and that the antibodies produced tend to be of lower avidity to than those produced by the other two structural types (Schlesinger and Granoff 1992), and different light chain V regions are used (Granoff et al. 1993). The time course for the development of an antibody response following administration of different Hib

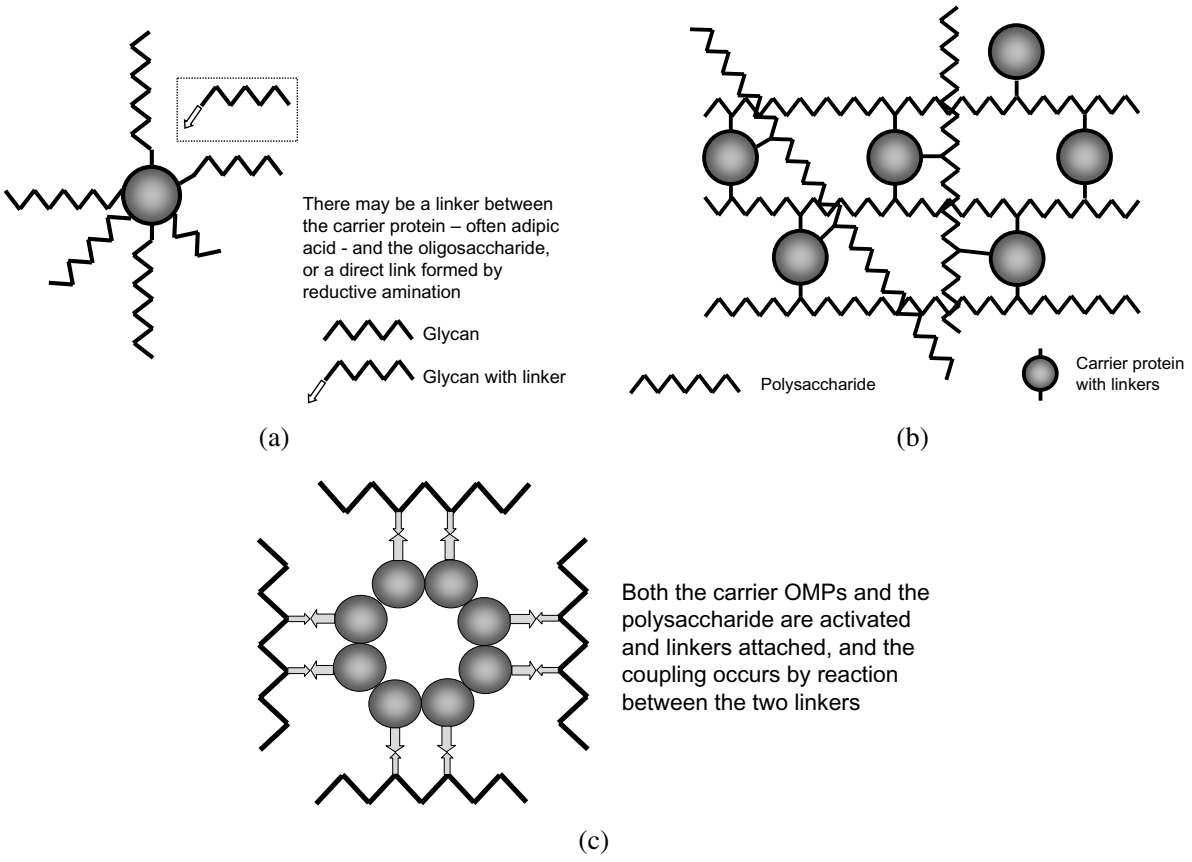

Fig. 2 – Cartoon representations of the different structural types of glycoconjugate vaccines. (a) A neoglycoconjugate vaccine produced by coupling of oligosaccharides to an appropriate carrier protein such as CRM197. Typical CRM197 conjugates contain an average of six chains per carrier protein. Whilst monofunctional activated oligosaccharides such as those produced by active ester chemistry are incapable of crosslinking protein, bifunctional oligosaccharides produced by periodate oxidation may lead to occasional crosslinks. (b) A crosslinked network conjugate vaccine. Random multiple activation of the polysaccharide and coupling to a carrier protein leads to multiple crosslinks between the macromolecules to form a network of very high molecular weight. (c) A vesicle-based vaccine, in which size-reduced polysaccharide is coupled to a LPS-depleted vesicle comprised of outer membrane proteins. There are multiple linkages between the saccharide chain and the "carrier protein". Figure adapted from Ward et al. 1994.

only two doses of the vesicle vaccines and a booster dose are given.

These conjugate vaccines proved extremely effective at preventing disease in those countries which have adopted them as part of mass vaccination programmes, so that Hib meningitis, which had been the most common form of neonatal meningitis in developed countries, has been almost completely eradicated. The startling effectiveness of these vaccines stimulated a demand that their usage be expanded to other countries, with a WHO target of tion of Hib vaccination into some developing countries highlighted the fact that the disease burden due to this organism had been seriously underestimated. It had also become clear that glycoconjugate vaccines were an effective generic technology which could be used to protect against a wide variety of other pathogens, if the conjugates were made.

CHOICE OF CARRIER PROTEIN AND CONJUGATION CHEMISTRIES

Two types of carrier protein are most commonly

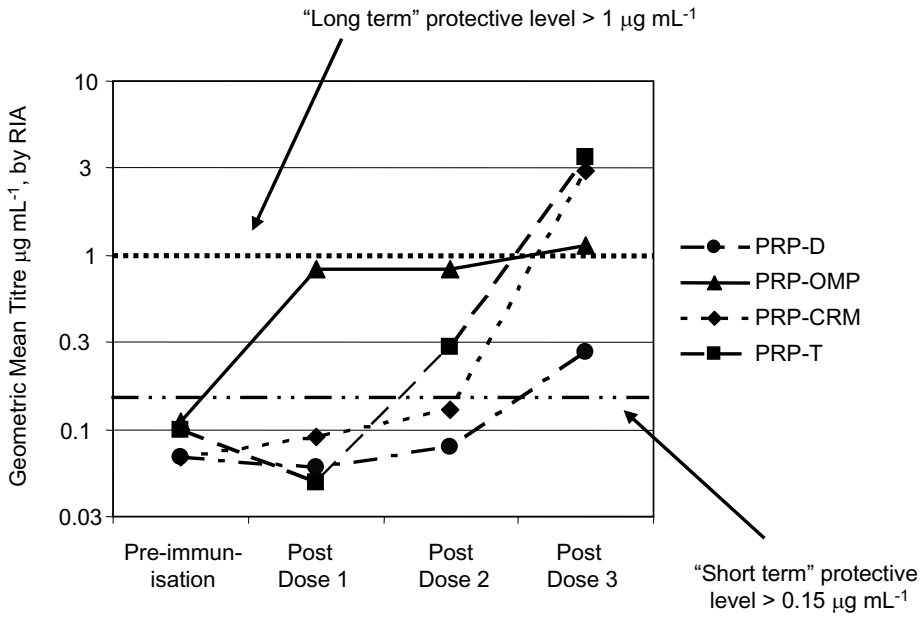

Fig. 3 – Time course of development of antibody responses to Hib conjugate vaccines which differ in the carrier protein. The key difference is that PRP-OMP vaccines stimulate an antibody response after the first immunisation, whilst the other vaccines require at least two immunisations. The accepted protective antibody level for long term protection is 1μg mLi^{-1}. Data replotted from Decker et al. 1992.

been bacterial toxoids, including tetanus and diphtheria toxoid. These proteins were already licensed for human use. Another popular carrier is CRM197, a genetically toxoided variant of diphtheria toxin, as chemical toxoiding is not required and the vaccines are simpler to characterise and control, and there is less variability possible in carrier protein production. The other approach, which has not yet been used in commercial vaccines, is to choose a carrier protein the immune response against which will complement the role of anti-saccharide antibodies. For example, a developmental vaccine using the pneumococcal toxin pneumolysin, in a genetically toxoided form, has been reported (Michon et al. 1998), and the use of Streptococcal proteins (Paoletti et al. 1999) or *Moraxella* OMPs as carriers (Hu et al. 2000). The key factor is that the carrier protein delivers a strong T cell involvement, and carrier proteins to which the infant has had prior exposure, such as tetanus toxoid, are particularly valuable in this respect.

Polysaccharides require activation before attachment to the carrier protein. Sometimes it is necessary to activate to the carrier protein as well. The optimum means to activate the polysaccharide depends upon its structure. This is particularly true if oligosaccharide haptens are to be used. For example, periodate oxidation of the Hib polysaccharide and meningococccal group C polysaccharide results in oligosaccharides with a reactive aldehyde group at either end (Anderson et al. 1986). These can be attached to the free amino groups of lysine residues in the carrier protein by reductive amination. Both the Hib and the meningococccal group C polysaccharides are susceptible to mild acid hydrolysis, generating a protected aldehyde group at the reducing end. This reducing end can be activated, for example by reductive amination, and a linker attached which allows covalently attachment to a carrier protein – this approach has been adopted by Chiron Vaccines (Ravenscroft 2000). The polysac-

aureus, in contrast, have few free hydroxyl groups and are extremely resistant to hydrolysis. These polysaccharides have been activated by the addition of a bifunctional reagent to the uronic acid carboxyl group, which allows conjugation to a suitably activated carrier protein (Szu et al. 1987, Fattom et al. 1988, 1995). Treatment of a polysaccharide with cyanogen bromide, or a cyanogen bromide analogue such as CDAP (Shafer et al. 2000), results in random activation of hydroxyl groups to which a bifunctional linker, such as adipic acid dihydrazide can be attached (Shafer et al. 2000). This linker is then attached to Asp or Glu carboxyl groups on the carrier by treatment with a water-soluble carbodiimide.

Another conjugation chemistry that has been used involves partial de-*N*-acetylation of an *N*-acetylglucosamine residue in the repeat unit of the capsular polysaccharide from *Streptococcus pneumoniae* Type 14. Treatment of material with nitrous acid leads to the formation of an anhydromannose residue with a free aldehyde group, suitable for conjugation by reductive amination to the free *N*ε group of a lysine residue in an appropriate carrier protein (Laferrière et al. 1997, 1998). The effectiveness of this strategy depends on the nature and substitution pattern of the acetamido sugar.

Traditionally, the source of the glycan used for conjugate production has been natural polysaccharide isolated from the pathogen. Whilst the Hib and meningococcal CPSs have been relatively easy to activate, by periodate oxidation or after dilute acid hydrolysis, this becomes more problematic for the pneumococcal CPSs, where the greater structural diversity between serotypes means that a single method may not be suitable. The CPSs from *S. typhi* and *S. aureus* Types 5 and 8 require activation through the carboxylate groups. Total synthesis of oligosaccharides therefore has considerable attractions, as these can be produced with a ready-made activated linker attached, so that a single conjugation chemistry can be used for a wide range of products. This approach has been developed by the Cuban et al. 2004). The amounts of saccharide needed are accessible, if the synthesis is optimised. For example, South America would require ca. 500 g of saccharide for its birth cohort of ca. 14 million, at 10 micrograms of saccharide per dose and a three or four dose regime. These conjugation chemistries are illustrated in Figure 4.

PHYSICOCHEMICAL CHARACTERISATION AND QUALITY CONTROL OF GLYCOCONJUGATE VACCINES

For many of the diseases which glycoconjugate vaccines prevent, there is no useful animal model which correlates with the efficacy in man. For this reason, quality control of these vaccines is heavily dependent upon physicochemical methods, and this, in turn, required careful characterisation of the final vaccine and its individual components. In addition, these relatively simple vaccines are capable of being well characterised by a combination of physical and spectroscopic methods.

Work initiated to characterise CPSs used as vaccines has carried over to the saccharide components of glycoconjugate vaccines. In our laboratory we have assigned the NMR spectra of all the CPSs used in glycoconjugate production (Jones and Lemercinier 1999). NMR spectroscopy is the sole method used by us to establish the identity and purity of the CPSs, with de-*O*-acetylation *in situ* to determine the degree of *O*-acetylation (Jones and Lemercinier 2002). We also have extensive NMR data to define characteristic resonances arising from CPS degradation (Jones et al. 2000). This in turn defines the predominant degradation pathway. NMR spectra can also be obtained on the activated polysaccharide, which provides information on the average size (where oligosaccharides are used), the average degree of activation and addition of a linker when intact CPSs are used, and whether there is selectivity in the activation of specific hydroxyl groups in the repeat unit (Jones 2005a; Xu et al. 2005). Molecular weight profiles of activated oligosaccharides have been obtained using size exclusion chro-

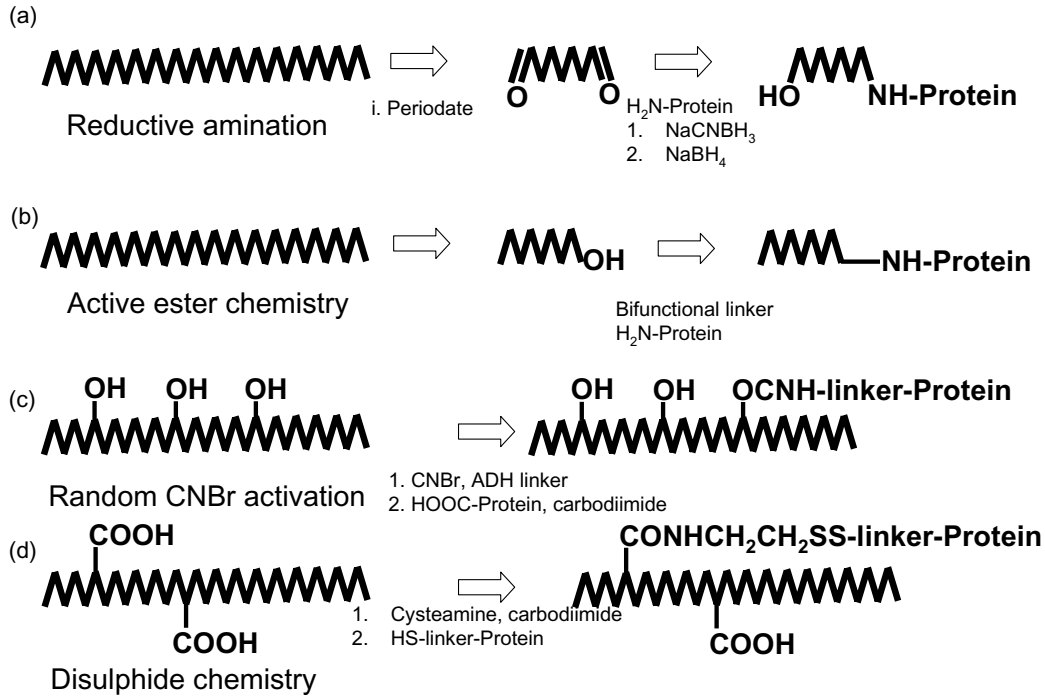

Fig. 4 – Summary of conjugation chemistries. (a) Periodate oxidation with concomitant depolymerisation leads to an oligosaccharide activated at both ends. (b) Acid hydrolysis leads to an oligosaccharide with a single reducing terminus which can be modified by attachment of an appropriately activated linker. Alternative methods to generate a reducing terminus are feasible. (c) Treatment of the CPS with cyanogen bromide, or an analogue, creates random, highly reactive activation sites along the chain, to which an appropriate bifunctional linker can be attached. A separate conjugation step is used to create a covalent bond between the linker and the carrier protein. (d) Treatment of a carboxylate-containing CPS with an amine in the presence of carbodiimide is a means to attach a reactive group – such as a protected thiol – to the CPS. This can be conjugated to a complementary reactive groups pre-attached to the carrier protein to create the polysaccharide-protein complex.

light scattering (MALLS) (D'Ambra et al. 1997) or anion exchange chromatography (Ravenscroft et al. 1999). In most cases it is possible to obtain high quality NMR spectra of the final bulk conjugate, and this is a means to ensure the integrity (identity, degree of *O*-acetylation, absence of degradation) of the saccharide in the final vaccine (Jones 2005a).

When toxoids used as components of other vaccines are employed as carriers in conjugate vaccines they should meet the appropriate pharmacopoeial specifications. In some cases it has proven worthwhile to use more highly purified toxoids for conjugate production. Carrier proteins not been previously used in vaccine manufacture have been subjected to more extensive characterisation, including mass spectrometry, confirmation of the amino acid sequence, size exclusion chromatography, and analysis of secondary structure and stability by optical spectroscopy (Jones et al. 2000). The applicability of different physicochemical methods to the characterisation of glycoconjugate vaccines is summarised in Figure 5.

The extent to which the three major structural types have been characterised differs. The most extensive characterisation has been performed on the neo-glycoconjugate vaccines which use CRM197 as the carrier protein, as the heterogeneity introduced by chemical toxoiding is absent. These studies have included NMR spectroscopy (which reports principally on the glycan chains), optical spectroscopy to

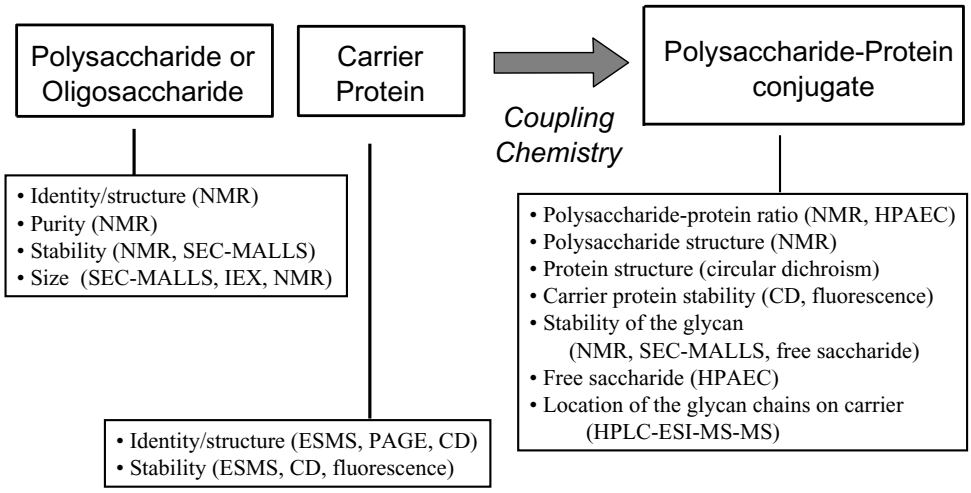

Fig. 5 – Physicochemical methodology appropriate for the characterisation and quality control of glycoconjugate vaccines and their individual components. Abbreviations: NMR – nuclear magnetic resonance spectroscopy: SEC – size exclusion chromatography: MALLS – multiple angle laser light scattering: IEX – ion exchange chromatography: ESMS – electrospray mass spectrometry: PAGE – polyacrylamide gel electrophoresis: CD – circular dichroism: HPAEC - high performance anion exchange chromatography: HPLC-ESI-MS-MS – coupled HPLC-electrospray mass spectrometry with MS-MS detection for peptide sequencing.

assess the integrity and stability of the carrier protein, and molecular sizing to determine the proportion of protein molecules carrying different numbers of glycan chains. In all of the current neoglycoprotein conjugates except the Cuban synthetic vaccine (Verez-Bencomo et al. 2004), the glycan chains are attached to amino groups, either the $N\varepsilon$ of lysine residues or the amino group at the N-terminus. CRM197 contains 39 lysine residues and one N-terminus, of which six are substituted in the average protein. Different conjugation chemistries are likely to have different specificities for the available amines, depending on differences in pKa and steric accessibility. Attempts have been made to define this pattern for vaccines produce using active ester chemistry (the Chiron process) (Jones et al. 2000). Studies of the crosslinked conjugate vaccines have focused principally on the molecular size, which is a simple but valuable means to assess stability, and the integrity of carrier protein (Parisi and von Hunolstein 1999). Toxoids, such as tetanus toxoid, tend to be very robust.

In some respects these vaccines are no longer example, it is generally accepted that a change in the manufacturing process for the polysaccharide component can be validated by detailed physicochemical characterisation of the polysaccharide, and does not require a clinical bridging trial to demonstrate equal immunogenicity with the original product for which clinical trial data was obtained. However, this probably would not be true for a change in the conjugation process (Holliday and Jones 1999). The Guidelines published by the World Health Organisation are a valuable starting point to understand the control tests and characterisation necessary for production of a new vaccine (Anonymous 2000, 2004b, c).

IMPORTANT FACTORS DEFINING THE EFFICACY AND STABILITY OF CONJUGATE VACCINES

The general principles for the quality control of vaccines are well established but there is a growing emphasis on the application of sophisticated physicochemical approaches (Dellepiane et al. 2000). Experience has shown that a very wide range of structural types can function as effective vaccines. In this section I will discuss those factors which are known

of an effective and stable glycoconjugate vaccine. In most cases, there is no direct link between an individual assay and the expected efficacy of the final vaccine: the intention is to demonstrate consistency in the manufacturing process and that successive production batches are essentially the same as the material used in the clinical trial.

- The identity of the polysaccharide used to make the vaccine and confirmation of its structure in the final vaccine. This can be achieved using immunological methods or NMR spectroscopy of the bulk conjugate. Experience with the meningococcal Groups C and Y and *Staphylococcus aureus* conjugate vaccines seems to indicate that the absence of *O*-acetyl groups on the glycan chain does not result in an ineffective vaccine, consistent with the idea that an antibody population recognising the saccharide backbone is sufficient for protection (Michon et al. 2000, Doares and Cowell 2001, Fattom et al. 1998).
- The identity of the carrier protein used to manufacture the vaccine and confirmation of its structure in the final vaccine. If the carrier protein is chemically toxoided, confirmation is required that reversion to toxicity has not occurred.
- The polysaccharide-protein ratio. The desired immune response is against the saccharide, and low polysaccharide-protein ratios require immunisation of the infant with larger than necessary amounts of protein. On the other hand, proteolysis of the carrier protein is required to generate peptides for display by MHC II, and high polysaccharide-protein ratios can interfere with this process. Such vaccines have reduced immunogenicity. The optimal ratio has to be determined by experiment in preclinical studies or clinical trials. The mean polysaccharide-protein ratio may not be sufficient to fully define the vaccine if a single population is not present (Egan et al. 1995). A termine this parameter, principally separate determination of the polysaccharide and protein contents. These have been conjugate specific, reflecting the differences in the chemistry of the glycan chains. NMR spectroscopy provides a more generic approach (Figure 6).
- The presence of free polysaccharide. Free (unconjugated) polysaccharide reduces the immunogenicity of the vaccine, possibly by competing for the sIg molecules. The amount of free saccharide (and unconjugated carrier protein) in the vaccine are key quality control specifications (Peeters et al. 1992).
- Degradation and depolymerisation of the saccharide chain is the principal mechanism by which some glycoconjugate vaccines lose immunogenicity. Coincidentally, the first vaccines against Hib and MenC employed some of the least stable CPSs. Degradation of the saccharide chains can be monitored by an increase in free saccharide, by changes in molecular size, or by the appearance of characteristic resonances in the NMR spectrum of bulk conjugate (Jones et al. 2000: Figure 7). With a neoglycoconjugate vaccine, cleavage of the glycan chain inevitably gives rise to an increase in free saccharide, whilst in a crosslinked network vaccine two cleavages in close proximity are required. Consequently there is a lag phase between the beginning of glycan depolymerisation and the appearance of free saccharide. Aluminium hydroxide adjuvants have been shown to catalyse the depolymerisation of the Hib CPS (Sturgess et al. 1999).
- The size of the glycan chains. Small glycan chains can be used (Jansen and Snippe 2004), but all manufacturers maintain specifications of the glycans conjugated as an indication that they are producing a consistent product (Costantino et al. 1999).
- Molecular sizing of the conjugates is a simple

the final conjugate (Parisi and von Hunolstein 1999).

- Mild denaturation of the carrier protein almost certainly does not reduce the immunogenicity of the conjugate, since the role of the carrier is to be degraded and to provide peptides for display by the MHC II (Siber 1994). More extensive denaturation may result in aggregation, which may influence availability of the conjugate, although such conditions are also likely to cause depolymerisation of the glycan chains.
- The stability of the linkage between the polysaccharide and the carrier protein.
- Limit specifications exist for reagents used in the conjugation process and for uncapped activation sites (Anonymous 2002a, 2004b, c).

CLINICAL EFFICACY OF GLYCOCONJUGATE VACCINES

The clinical trials of 4 different Hib conjugate vaccines (called PRP-D, PRP-T, PRP-OMP and HbOC) manufactured using different sized glycan chains, different carrier proteins and different conjugation chemistries showed high efficacy, a reduction in the incidence of disease, in each case (Heath 1998, Booy et al. 1994, Black et al. 1991, Santosham et al. 1991, Eskola et al. 1987). Of these vaccines, PRP-D, in which the carrier protein is diphtheria toxoid, was not demonstrated to be effective in populations with high levels of endemic disease. On the other hand, PRP-OMP (which used a complex of meningococcal outer membrane proteins as the carrier) proved particularly effective in this population, perhaps because a protective antibody response is elicited after the first dose (Figure 3). Whilst different target populations can react in quantitatively different ways (Santosham et al. 1992), the clear conclusion was that the Hib conjugate vaccines produced by a variety of routes are efficacious in the prevention of disease. Because of the low incidence of meningococccal disease in the UK and the and protection (Goldschneider et al. 1969a, b), licensing of meningococccal group C conjugate vaccines in the UK was on basis of immunogenicity, and particularly functional antibody levels as measured by a bacteriocidal assay, rather than protection against disease. Subsequent surveillance of disease incidence in the UK has indicated an efficacy for these vaccines of approximately 93% (Balmer et al. 2002). Clinical trials of the heptavalent pneumococcal conjugate vaccine in California were so clear cut that the trial was stopped at an early stage (Shinefield et al. 1999, Black et al. 2001). However, clinical trials in Soweto, South Africa (Huebner et al. 2002), and in a Navajo Indian population in the US have indicated lower clinical efficacy (O'Brien et al. 2003). It is not clear what factors influence this – poorer nutrition, or environmental or genetic factors.

Licensing of new pneumococcal conjugate vaccines may prove complicated due to a requirement from the FDA that a new vaccine is "non-inferior" to existing vaccines (Jodar et al. 2003, Lee et al. 2003). For example, is a vaccine which induces a slightly lower immune response to one serotype but contains several additional serotype immunogens "inferior" to an existing vaccine? Production and quality control factors for the development of pneumococcal conjugate vaccines have been codified by WHO (Anonymous 2004c).

GLYCOCONJUGATE VACCINES UNDER DEVELOPMENT

There are a large number of novel glycoconjugate vaccines under development by academic laboratories, by established vaccine manufacturers and smaller start-up biotechnology companies and by public non-governmental institutions. Table III contained details of the novel glycoconjugate vaccines reported to be in development or in clinical trials. Whilst most of these vaccines continue to use high molecular weight repeating polysaccharides as the saccharide hapten, in some lower molecular weight glycans such as the short saccharide chains present

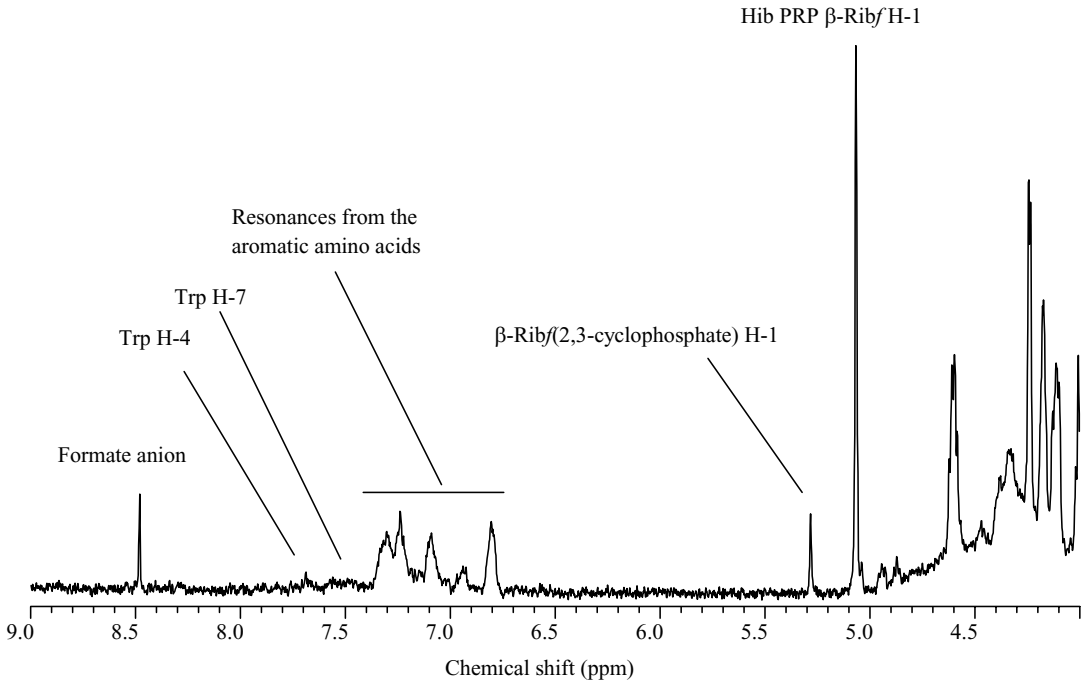

Fig. 6 – Lowfield region of the 500MHz proton NMR spectrum of a Hib-CRM197 conjugate vaccine, obtained at 50°C and with the sample dissolved in 5 M deuterium-exchanged guanidinium hydrochloride to denature the carrier protein. The resonances between 6.7 and 7.7 ppm arise from the sidechains of aromatic amino acids, whilst the sharp resonance at 5.06 ppm arises from the Hib PRP. Comparison of the integrals of these two resonances gives a direct indication of the polysaccharide-protein ratio. The minor resonance at 5.3 ppm arises from ribofuranose-2,3-cyclophosphate residues, the main end group formed from degradation of the PRP chain.

developmental conjugate vaccine against the encapsulated fungal pathogen *Cryptococcus neoformans* shows that this technology is not limited to protection against bacterial infection (Devi 1996).

In developed countries, the costs of vaccine delivery to the target population usually exceed the costs of the vaccine, and there is considerable pressure to reduce the number of injections that a baby receives. These pressures, and commercial concerns, favour the development of combination vaccines, such a hexavalent DTP-Hib-HB-IPV combinations. However, the immune response to one or more of the components in such combination vaccines is frequently reduced (Granoff 2001, Redhead et al. 1994), although this is not generally considered to be clinically significant. Clinical assessment and quality control of these complex combination vaccines can be extremely difficult, especially are already in place and disease incidence may be extremely low.

VACCINE DEMAND AND VACCINE PRICES

Most vaccines are commercial products, and to be widely used demands that they be available to purchasers at prices that they can afford. The major burden of bacterial disease is in amongst the young in developing countries. Whilst novel vaccines may be expensive when initially introduced, the expectation is that mass production, amortisation of capital investment and the emergence of multiple producers will drive prices down, expanding vaccine usage into less wealthy countries, leading to further reduction in prices. A number of non-governmental organisations and charities are working to speed the introduction of novel vaccines into

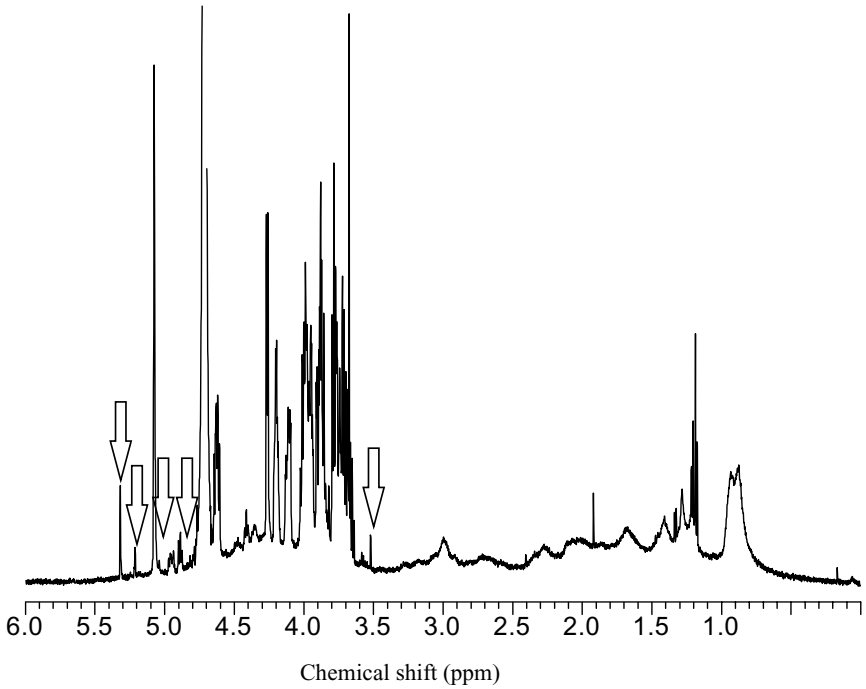

Fig. 7 – Partial 500 MHz proton NMR spectrum obtained at 30°C of a Hib-CRM197 conjugate vaccine that has been deliberately degraded by storage at elevated temperature. This causes cleavage of the glycan chain and the formation of novel end groups, with characteristic resonances in the NMR spectrum. These resonances are indicated by arrows. The broad resonances between 3.5 and 0.5 ppm arise from the carrier protein.

cine, global use of which is a WHO aim, is a example. The global birth cohort is nearly 130 million (http: // www.census.gov/ipc/prod/wp02/tabA-03.pdf). Vaccination of these infants with three or four dose regimes will require between 400 million and 500 million doses of vaccine per year. Production in developed countries is currently of the order of 100 million doses per year. According to published figures, the US government pays approximately $8 per dose for Hib conjugate vaccines in single dose vials (Jacobson et al. 2003). Hib conjugate vaccine is available to South American governments through the PAHO Revolving Fund for $3.50 per dose (http: // www.paho.org/English/AD/FCH/IM/sne2601.pdf). The World Health Organisation believes that global uptake of Hib conjugate vaccine requires a target price of approximately $1 per dose. A number of manufacturers in developing countries are attempting to fill the production gap using a variety of different techniques. The Cuban government have recently licensed a Hib conjugate vaccines produced from synthetic oligosaccharides linked to tetanus toxoid (Verez-Bencomo et al. 2004). Manufacturers in Indonesia, Brazil, India and Mexico are developing crosslinked network vaccines, sometimes in collaboration with other organisations.

Introduction of the other conjugate vaccines has been more recent. Meningococccal Group C conjugates were first introduced into the UK in 1998 (Miller et al. 2001) whilst pneumococcal conjugate vaccines are even more recent. Current prices for these vaccines are higher than for the Hib conjugate vaccine. A recent study assessing cost-effectiveness of Men C conjugate in Canada was based on a price of CDN$50 per dose (De Wals et al. 2004). The prices of complex and multivalent conjugates, such

TABLE III
Existing and developmental polysaccharide and conjugate vaccines.
(Adapted and updated from the ''Jordan Report 2000: Accelerated Development of Vaccines'', available at http:/www.niaid.nih.gov/publications/pdf/Jordan.pdf.)

Organism	Saccharide components	Carrier protein	Development	References
Candida albicans	Cell surface oligomannan	Not reported	Preclinical	Han et al. 1999, Bystricky et al. 2003
Cryptococcus neoformans	Serogroup A glucuronoxylomannan	Tetanus toxoid	Phase 1	Devi et al. 1991, Devi 1996, Casadevall et al. 1992, Pirofski et al. 1995, Nussbaum et al. 1999
Enterococcus faecalis	Teichoic acid-like CPS	Not reported	Research	http:/www.nabi.com/pipeline.
Enterococcus faecium	Teichoic acid-like CPS		Research	Huebner et al. 2000, Wang et al. 1999
Escherichia coli	Detoxified O157 LPS O-chain	*Pseudomonas* exoprotein A	Phase 1	Konadu et al. 1994, 1998, Conlan et al. 1999
	Detoxified O157 LPS O-chain	Shiga toxin 1 B subunit	Research	Konadu et al. 1999
	Detoxified O18 LPS O-chain	*Pseudomonas* exoprotein A	Phase 1	Cryz et al. 1991
	Detoxified O111 LPS O-chain	Tetanus toxoid	Preclinical	Gupta et al. 1995
		Meningococcal OMPs	Phase 1	Cross et al. 2003
	12 valent O-chain		Research	Cross et al. 1990
Francisella tularensis	B strain LPS O-chain	Bovine serum albumin	Research	Conlan et al. 2002
Group A *Streptococcus*	Group-specific CPS	Tetanus toxoid	Preclinical	http://www.niaid.nih.gov/dmid/vaccines/jordan20/jordan20_2002.pdf
Group B *Streptococcus*	Types Ia, Ib, II, III, and V CPS	Tetanus toxoid	Phase II	Lagergard et al. 1990, Wessels et al. 1990, 1998, Paoletti et al. 1990, 1992a,b, 1996, Kasper et al. 1996, Baker et al. 1999, 2000, Paoletti et al. 1999
	Types VI and VIII	Alpha C protein	Preclinical	Gravekamp et al. 1999
	Types III CPS	Cholera toxin b subunit	Research	Shen et al. 2000a,b
	Type III CPS		Research	
Haemophilus influenzae	Type b CPS (high MW)	Tetanus toxoid	Licensed	ActHib®
	Type b CPS (synthetic oligos)	Tetanus toxoid	Licensed (Cuba)	QuimiHib®, Verez-Bencomo et al. 2004
	Type b CPS-derived 12-mers	CRM197	Licensed	HibTiter®, VaxemHib®
	Type b CPS ''size-reduced''	Meningococcal OMPs	Licensed	PedvaxHIB®
	Intact CPS	*Haemophilus* protein D	Preclinical	Akkoyunlu et al. 1997
Haemophilus influenzae	Detoxified LOS from non-typable strains	Tetanus toxoid	Preclinical	Wu and Gu 1999
		Haemophilus OMP	Preclinical	Gu et al. 1996, 1997, Sun et al. 2000
Klebsiella	24 valent CPS mix	None	Phase 1	Cross et al. 1990
Moraxella catarrhalis	Detoxified LOS	Tetanus toxoid	Preclinical	Gu et al. 1998
		CRM197	Preclinical	Jiao et al. 2002
		Haemophilus OMP	Preclinical	Hu et al. 2000
Mycobacterium tuberculosis	Arabinomannan	rEPA	Preclinical	Hamasur et al. 2003
Neisseria meningitidis				
Groups A, C	Groups A and C CPSs	None	Licensed	AC Vax®, Mengivac(A+C)®
Groups A, C, W135 and Y	Groups A, C, W135 and Y CPSs	None	Licensed	ACWY Vax®

as a pneumococcal conjugates, are particularly high, with the heptavalent pneumococcal conjugate being more than $50 per dose, and a four dose regime is required (Pai et al. 2002). Thus the conjugate vaccines are relatively expensive products. Prices for polysaccharide vaccines are of the order of a few cents per dose for *S. typhi* Vi polysaccharide, while trivalent meningococccal A,C,W135 polysaccharide

TABLE III (continuation)

Organism	Saccharide components	Carrier protein	Development	References
Neisseria meningitidis Group C	Group C CPS-derived oligos De-O-Ac CPS-derived oligos	CRM197 Tetanus toxoid	Licensed Licensed	Meningitec®, Menjugate® NeisVac C®
Group A	Group A CPS	CRM197 BSA	Phase II Preclinical	Rosenstein et al. 2001, Twumasi et al. 1995 Jin et al. 2003
Groups W135 and Y	Group Y and W135 CPSs Y and W135 oligos	Diphtheria toxoid CRM197	Preclinical Preclinical	Rosenstein et al. 2001 Rosenstein et al. 2001, Doares and Cowell 2001
Group B	N-propionylated Group B CPS	Tetanus toxoid meningococcal porins Por B	Phase III expected	Fusco et al. 1997, Bruge et al. 2004, Fusco et al. 1997, Devi et al. 1997
All serogroups	LOS	Tetanus toxoid Outer membrane proteins	Basic R& D Basic R& D	http://www.niaid.nih.gov/dmid/vaccines/jordan20/jordan20_2002.pdf, Mieszala et al. 2003 Gu and Tsai 1993, Sun and Hu 1999
Porphyromonas gingivalis	Capsular polysaccharide	Fimbriae	Preclinical	Choi et al. 1998
Pseudomonas aeruginosa	LPS O-chain Detoxified LPS O-chains (7 types) Mucoid exopolysaccharide	rEPA none KLH	Phase I Preclinical Preclinical	Cryz et al. 1997, Pier 2003, Hataro et al. 1994 Theilacker et al. 2003
Burkholderia cepacia	LPS		Basic R& D	http://www.niaid.nih.gov/dmid/vaccines/jordan20/jordan20_2002.pdf
Salmonella typhi	Vi CPS Vi CPS Vi CPS Vi CPS Pectin analogues	None Cholera toxin b subunit Tetanus toxoid Porins BSA, rEPA	Licensed Phase II 	TyphimVi®, Typherix® http://www.niaid.nih.gov/dmid/vaccines/jordan20/jordan20_2002.pdf Szu and Schneerson 1989, Szu et al. 1987, 1994, Kossaczka et al. 1999, Lin et al. 2001 Singh et al. 1999 Kossaczka et al. 1997
Salmonella typhi *Salmonella typhimurium* *Salmonella paratyphi*	Detoxified LPS O-chain Detoxified LPS O-chain Detoxified LPS O-chain Detoxified LPS O-chains	 Linear PADRE epitope Tetanus toxoid	 	Saxena and Di Fabio 1994 Jorbeck et al. 1981, Watson et al. 1992 Alexander et al. 2000 Konadu et al. 1996, 2000
Shigella dysenteriae *Shigella flexneri* *Shigella sonnei*	LPS O-chain LPS O-chain LPS O-chain	rEPA rEPA rEPA	Phase II Phase II Phase II	Robbins et al. 1991, 1992, Chu et al. 1991, Pozsgay et al. 1999 Robbins et al. 1991, 1992, Chu et al. 1991, Pozsgay et al. 1999, Polotsky et al. 1994a,b, Ashkenazi et al. 1999, Passwell et al. 2001, 2003 Cohen et al. 1997, Robin et al. 1999
Staphylococcus aureus	Types 5 and 8 CPSs Types 5 and 8 CPSs	rEPA HSA	Phase III Phase I (veterinary)	Fattom et al. 1990, 1993, 1995, 2004, Welch et al. 1996 Gilbert et al. 1994, Tollersrud et al. 2001
Staphylococcus epidermidis	Capsular polysaccharide Poly(β1,6-*N*-succinylGlcN)	Not reported	Preclinical	Takeda et al. 1991, Kojima et al. 1990, McKenney et al. 2000

TABLE III (continuation)

Organism	Saccharide components	Carrier protein	Development	References
Streptococcus pneumoniae	23 CPSs	None	Licensed	Pneumovax® and Pnu-Immune®
	7 CPSs	CRM197	Licensed	Prevenar®
	9 CPSs	CRM197	Phase III	Combolite®
	9 CPSs	Meningococcal OMPs	Phase III	Blum et al. 2000
	7 CPSs	DTx or TTx	Phase II	Puumalainen et al. 2002
	11 CPSs	DTx or TTx	Phase II	Korkeila et al. 2000
	Type 18C	Pneumolysin	Basic R& D	Kuo et al. 1995, Michon et al. 1998
Vibrio cholerae	O1 Inaba LPS	Cholera toxin b subunit	Phase 1	Gupta et al. 1998
	Synthetic O1 Ogawa O-chain	Bovine serum albumin	Preclinical	Chernyak et al. 2002
	O139 capsular polysaccharide	Diphtheria toxin mutantCRMH21G	Preclinical	Kossaczka et al. 2000
	Detoxified O139 LPS + core	Tetanus toxoid		Boutonnier et al. 2001

''None'' means a pure CPS vaccine. rEPA is genetically detoxified recombinant *Pseudomonas aeruginosa* exotoxin A, HSA is human serum albumin, BSA is bovine serum albumin.

vaccine cost 1 euro per dose (Anonymous 2004a). Other Revolving Fund prices for other vaccines are often of the order of cents per dose or a few US$s per dose (http: // www.paho.org/English/AD/FCH/IM/sne2601.pdf). There is an acknowledged fragmentation of the global vaccine market, with the use of complex, expensive combination vaccine in industrialised countries, whilst developing countries require simpler monovalent vaccines. A key factor in this is the introduction of expensive acellular pertussis and inactivated polio components into combinations for industrialised countries, replacing the cheaper whole cell pertussis vaccines. This provides opportunities for manufacturers in developing countries/emerging economies such as Brazil, Indonesia or India to supply the mass markets through contracts with PAHO and UNICEF (http://www.who.int/ vaccines-access / supply / Divergence_vaccines.pdf). In other cases conjugate vaccines are not the object of commercial development due to low incidence of disease in rich developed countries and the low income of a target population in developing countries. The Programme for Appropriate Technologies in Health (PATH) are, for example, coordinating a project funded by the Gates Foundation, the Rockefeller Foundation, and the World Bank to produce a cheap – US$0.40 per dose tar-cine appropriate for use in sub-Saharan Africa (http://www.meningvax.org/timeline.htm). The International Vaccine Institute in Korea, through its Diseases of the Most Impoverished (DOMI) programme is working to introduce affordable vaccines against typhoid, shigellosis, and cholera (http://www.ivi.int).

THE FUTURE

Existing glycoconjugate vaccines represent the first examples of the use of a generic technology which offers the possibility to reduce the death toll taken on young infants by many other infectious diseases. Unlike, for example, DNA vaccines, they have been proven to work in mass paediatric vaccine campaigns and there would seem to be no major technological hurdles to the development of many other highly effective vaccines of this type. There is a now an extensive body of academic work demonstrating proof-of-principle for a wide range of vaccines. However, conjugates are expensive and the limitation to the development of this technology is likely to be commercial. To some extent, this is being addressed by the involvement of philanthropic foundations and non-governmental organisations, but there is clearly much more to be done in

RESUMO

Vacinas glicoconjugadas, cujo carboidrato da superfície de um microrganismo está covalentemente ligado a uma proteína carreadora, vêm sendo consideradas como efetivas para gerar respostas imunes que previnem um grande número de doenças. A tecnologia é genérica e aplicável a vários patógenos, se os anticorpos contra os carboidratos de superfície forem capazes de proteger contra a infecção. Três vacinas contra *Haemophilus influenzae* tipo b, *Neissseria meningitidis* Grupo C e sete sorotipos de *Streptococcus pneumoniae* já foram licenciadas e muitas outras estão em desenvolvimento.

Este artigo discute o racional para o desenvolvimento e uso de vacinas glicoconjugadas; os mecanismos pelos quais elas induzem respostas imune dependentes de célula T e suas implicações para o seu desenvolvimento; o papel dos métodos físico-químicos na caracterização e no controle de qualidade dessas vacinas; e os produtos novos que estão em desenvolvimento.

Palavras-chave: vacinas glicoconjugadas, polissacarídeos capsulares, lipopolissacarídeos.

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