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Rational Design of Conjugate Vaccines

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ABSTRACT. Whereas bacterial polysaccharides, classified as T-cell-independent antigens, elicit protective antibodies in adults, booster injections fail to produce an augmented response or promote antibody class switching. Because T-cell-dependent antigens, typically proteins, both produce boosted antibody levels and promote antibody class switching, it has been considered highly desirable to attempt to convert the T-cell-independent polysaccharide antigens into T-cell-dependent antigens, particularly for use in high-risk groups. A number of clinical trials now report the efficacy of conjugate vaccines in inducing the production of antibody in response to a number of previously poorly immunogenic—mainly T-cell-independent—antigens. In addition to conjugate vaccines containing bacterial polysaccharides, vaccines containing relevant peptides from a variety of pathogens are also being formulated and investigated. Questions remain, however, regarding their synthesis, use, and efficacy. The best ages for vaccine administration and selection of the optimal protein carrier are still under investigation, as are questions regarding the use of adjuvants, which can greatly affect the vaccine's potency. Spacing and size of epitope and size and composition of the final structure also must be considered; the importance of molecular size and aggregation of antigen in increasing immunogenicity have been well documented. These questions must be addressed for the much-needed development of conjugate vaccines against some common infections worldwide, including malaria, bacterial meningitis, and infections from *Pseudomonas aeruginosa* and *Neisseria gonorrhoeae* because of increasing susceptibility to these infections and resistance of the pathogens to chemotherapeutic agents and/or antibiotics. (*Pediatr Res* 32: 376-385, 1992)

Abbreviations

TI, T-cell independent
TD, T-cell dependent
T_h, T helper
MTOC, microtubule organizing center
MAP, multiple antigen peptide
LPS, lipopolysaccharide
O-PS, O-polysaccharide of LPS
CS, circumsporozoite
GBMP, group B meningococcal polysaccharide
NPr, N-proprionyl
Hib, *Haemophilus influenzae* type b
OMP, outer membrane protein
ISCOM, immunostimulating complex

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Despite the impressive success of vaccination in preventing disease, there still are a substantial number of bacterial diseases for which effective vaccines do not exist, or for which vaccination fails to offer adequate protection, especially for certain groups at high risk. The pathogens most often implicated in these types of diseases include *Neisseria meningitidis*, *Streptococcus pneumoniae*, *Salmonella typhi*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella*, and *Vibrio cholerae*. One outstanding characteristic that distinguishes these groups is their ability to produce specific and unique antigenic surface carbohydrate components. These surface polysaccharides, which include the capsular polysaccharides, may enhance the virulence of these organisms by acting as protective surface molecules. Fortunately, when purified, many of these capsular polysaccharides can be used in vaccines and elicit protective antibodies in healthy adults.

At present, vaccination with purified capsular polysaccharide immunogens protects adults and older children from infections by Hib, *S. pneumoniae*, and some capsular groups of *N. meningitidis*, the major bacterial culprits in cases of meningitis and pneumonia. However, polysaccharide vaccines have two major drawbacks: *1*) children younger than 18 mo of age, some elderly people, and immunocompromised individuals either fail to produce antibodies after vaccination with these polysaccharides or produce antibodies in levels that are too low to be protective; and *2*) a repeat injection of this type of vaccine fails to elicit a booster (enhanced) response.

ENHANCED IMMUNOLOGIC EFFECTIVENESS OF TD ANTIGENS

Bacterial polysaccharides are classified as TI antigens. This type of antigen is polymeric with multiple repeating identical epitopes or haptens. Booster injections will bring falling antibody levels back to original immunization levels, but they will not produce an augmented response, nor will they promote switching to another class of antibody (isotype).

However, TD antigens, typically proteins, will produce boosted antibody levels upon subsequent injection and promote antibody class switching. This phenomenon is believed to be due to the participation of T_h cells, which are activated during the response to the protein. In addition to dividing upon activation, T cells produce lymphokines, resulting in further stimulation and differentiation of B cells, the antibody-producing progenitors. Another phenomenon occurring along with class switching is an increase in affinity of antibody molecules, resulting from somatic mutation in the B-cell Ig genes.

T-cell dependence and conjugate vaccines. Because the high-risk groups mentioned above—children younger than 18 mo of age, the elderly, and immunocompromised individuals—can produce protective levels of antibody to TD antigens, it has been considered highly desirable to convert the TI polysaccharide antigens into TD antigens. This has currently been successfully accomplished by covalently bonding carbohydrate antigens to proteins, resulting in the basic formulation of conjugate vaccines. A number of clinical trials now report the efficacy of conjugate vaccines in inducing the production of antibody in response to

a number of previously poorly immunogenic—mainly TI—bacterial antigens (1–20). The glycoconjugates thus formed not only are TD antigens, but they also usually generate antibody responses to both the carbohydrate and its protein carrier, although the levels of antibody raised against each moiety often differ.

Children aged 2 to 6 y usually can respond to unconjugated polysaccharide vaccines. However, a number of children in this age group have been identified as having a defect in the ability to respond to unconjugated polysaccharide vaccines even though their Ig levels are normal (21). These children seem to respond very well to a conjugate vaccine, as Granoff *et al.* (22) demonstrated in a study of the response of a group of children who had developed invasive Hib disease after they had been vaccinated with the unconjugated Hib polysaccharide vaccine. Other instances of response to conjugate vaccine in children with a defect in response to the unconjugated vaccine have been reported (23, 24).

PROBLEMS IN CONJUGATE VACCINE DESIGN

Although it seems that the problem of insufficient antigenicity of polysaccharides has been solved by the conjugate vaccines, many aspects of their synthesis, use, and efficacy remain unclear. Questions remain as to the selection of the carbohydrate antigen, determination of its optimum size, consideration of the use of oligosaccharides, and selection of the proper protein carrier.

What is the best age for vaccine administration? The anticipated efficacy of a vaccine will vary depending on the period during which it is expected to exert its maximum effect. In the case of Hib, the leading cause of bacterial meningitis in the United States, the vaccine should aim to protect infants 2 to 15 mo of age. Fifteen to 20% of all cases of Hib occur in infants 2 to 6 mo of age. Before the age of 2 mo, the infant is probably protected by maternal antibodies, and after 4 to 5 y the child is capable of maintaining a protective level of "natural" antibody independent of immunization, probably in response to nonpathogenic bacterial antigens in the environment that stimulate cross-reactive antibodies (25). The elevated titer of serum antibodies against Hib in older children and adults probably protects against an initial bacteremia, thus preventing subsequent localization in the meninges. In the presence of antibody, the bacteria can be cleared from the blood by lysis resulting from the action of the complement cascade and by the opsonization and subsequent phagocytosis of the organisms. Evidence exists that antipolysaccharide antibody concentration in the blood usually but not always predicts opsonic activity (26, 27) and that the early phase of clearance of organisms from the blood depends more on opsonization than lysis (28).

Protein-carrier selection. In the formulation of conjugate vaccines, should the protein carriers be natural or synthetic? What should be the nature of the linkage and coupling chemistry? The number of proteins that are acceptable for use in humans as potential carrier proteins for polysaccharide or peptide epitopes is restricted. Clinically useful proteins such as diphtheria (1, 7, 17, 20, 22) and tetanus (3–6, 11) toxoids have been used as carriers. In addition, a nontoxic native mutant form of diphtheria toxin, CRM_{197}, has also been successfully used in conjugate vaccines (8, 9). Because most infants will have received their first injection of a diphtheria-tetanus-pertussis (DPT) vaccine by the age of 3 mo, the use of diphtheria and tetanus toxoids as carriers for future conjugate-vaccine administrations avoids the introduction of new components into the immunization protocol for infants. In addition, because periodic booster immunizations are indicated for many immunization procedures, the presence of these toxoids in conjugate vaccines could serve to boost antibody titers to diphtheria and tetanus. However, there is some concern that excessive exposure to these proteins, both as carriers and as part of the DPT regimen, might have the adverse effect of causing tolerance rather than boosting the response. A number of confusing and contradictory reports as to the effect of carrier priming and dose as well as carrier choice have appeared, indicating that additional studies are needed to clarify the issues.

In general, it has been noted that vaccine recipients respond well to both the protein carrier and its conjugated epitope. For example, when 18-mo-old children in Goteborg, Sweden, were given a conjugate of Hib with tetanus toxoid, not only were their IgG levels of anti-Hib polysaccharide 10 times higher than those elicited by Hib alone, but the conjugate also elicited high levels of antibody to tetanus (4). Both components of a conjugate vaccine can generate independent populations of antibodies that may differ not only in isotype, but in antigen specificity as well (5, 6). Some laboratory studies have indicated that preadministration of carrier protein will increase the subsequent antibody response to material conjugated to that carrier (29).

In contrast to the above findings, questions have arisen as to whether previous exposure to the carrier protein suppresses the response to the epitope attached to it (30–32). Recent studies in mice on the effect of carrier priming on the immunogenicity of saccharide-protein conjugates indicate that both the nature of the carrier and the dose used for priming can affect the response to the saccharide (33). Low-dose priming with tetanus toxoid enhanced the response, whereas high-dose carrier priming suppressed the response. Priming with CRM_{197} neither enhanced nor suppressed the subsequent response to the saccharide epitope.

To complicate matters further, a recent study on the immunogenicity of an Hib conjugate vaccine with CRM_{197} as the carrier found that infant monkeys had no response to the Hib unless they were concurrently primed with diphtheria toxoid (34). Thus, in certain cases, previous exposure to a form of the carrier might prove to be not only beneficial, but essential to produce an adequate response to the epitope conjugated to that carrier.

Contradictory findings such as these indicate that studies regarding dose and time of carrier administration, dose and time of conjugate administration, and choice of carrier are required to determine immunologic consequences of carrier preexposure.

PEPTIDE-PROTEIN VACCINE CONJUGATES

In addition to improvements in the immunogenicity of bacterial polysaccharides, improvements in protein or peptide vaccines are being sought. A number of vaccines are now being formulated to contain the relevant immunodominant peptides of pathogenic organisms. The rationale for the use of peptides in vaccines has been based on the finding that relatively short peptides are capable of eliciting an immune response toward the native protein. In general, longer peptides are more easily synthesized by recombinant-DNA technology, whereas synthetic-peptide technology is used for making shorter peptides. Use of synthetic peptides offers the advantage of a highly pure, defined, and safe alternative to the conventional vaccine. Whether a vaccine is a conjugate of a carbohydrate or a peptide, a great need still exists for methods to standardize the new conjugate vaccines and to predict their potency and effectiveness.

PROBLEMS IN ADJUVANT USE

In addition to the actual design of the conjugate vaccine itself, its mode of administration must be considered. Questions about the use of various types of adjuvants also must be addressed. In general, adjuvants are believed to act in two ways. First, they act as repositories of adsorbed antigens at the site of injection. Second, they tend to cause a mild inflammatory response that results in the nonspecific activation of a number of cell types involved in the immune response. Adjuvants can greatly affect vaccine potency. In general, adjuvant preparations have enhanced the immune response, decreasing the amount of conjugate required. Thus far, aluminum hydroxide (alum) is the most widely used adjuvant and the only one currently licensed by the

U.S. Food and Drug Administration for use in human vaccines. Although aluminum hydroxide is both safe and efficacious, some results have been unreliable with certain adjuvant preparations. For example, immunogenicity was shown to decrease when a Hib polysaccharide-tetanus conjugate was adsorbed onto aluminum hydroxide (3). One possible explanation was a loss of vaccine potency upon standing. In another study, no decrease of immunogenicity was noted when the same polysaccharide, conjugated to the outer membrane protein of *N. meningitidis*, was administered with aluminum hydroxide (34a). In the first phase of the latter study, a comparison was made of the safety and immunogenicity of the same lot of vaccine given with either aluminum hydroxide or saline. Both preparations proved to be equally immunogenic. Interestingly, side effects such as discomfort or mild fever were more common after saline injections than after aluminum hydroxide injections. Notable in this study was the fact that the vaccine was stored frozen and lyophilized and was reconstituted in diluent 1–4 h before use.

Liposomes have been considered attractive candidates for use as adjuvants in human vaccines. Liposomes are synthetic phospholipid bilayers made in the form of spherical vesicles that can range in size from 25 nm to 1 μm in diameter, depending on how they are produced. When they are synthesized using naturally occurring lipids, they are biodegradable and do not seem to cause granulomas or other reactions at the site of injection. Some vaccines using multilamellated liposomes as the carrier have proven effective in trials on monkeys, especially when the liposomes are adsorbed onto the adjuvant alum (35). In human clinical trials investigating the use of liposomes in a malaria vaccine formulation, encouraging positive results have been produced (36, 36a).

A novel form of adjuvant may prove to be the OMP of *N. meningitidis*, which has been used successfully as a carrier in several conjugate vaccines. As noted above (34a), a conjugate vaccine of Hib-OMP was equally immunogenic whether administered in saline or alum adjuvant, indicating that the OMP itself may have adjuvant properties. In addition, it has been noted that when this carrier is in the form of an OMP complex it has adjuvant properties (37). Proteins in the OMP complex are present in liposomes composed of LPS-containing lipids derived from the outer membranes of the *Neisseria*.

Other novel adjuvants, such as block copolymers and Quil A, used by themselves in squalene-water emulsions or along with nontoxic forms of LPS, have been used successfully in animal experiments and show promise for future use in humans (38). They have been shown to influence antibody isotype as well as antibody titer. Block copolymers are nonionic surface-active agents composed of hydrophilic polyoxyethylene and hydrophobic polyoxypropylene. Their adjuvanticity probably rests on their ability to concentrate antigens and host proteins on the hydrophobic-hydrophilic interface, making presentation to the immune system more effective. The adjuvant, Quil A, is a glycoside obtained as a saponin extract from the bark of a South American tree (*Saponariaquiloya molina*). It is a component of the cage-like adjuvant structure, ISCOM. Antigens attached to ISCOM through hydrophobic interactions have been found to be highly immunogenic, evidently because they are accessible as multimers in a defined submicroscopic particle with Quil A as the inbuilt adjuvant. Toxicologic studies with ISCOM in humans are being carried out; thus far, studies with monkeys indicate little or no toxicity (39).

CELLULAR MECHANISMS INVOLVED IN ANTIBODY PRODUCTION

Although the conjugation of a TD antigen to bacterial carbohydrate moieties acts to elicit a TD type of immune response against the carbohydrate, the cellular mechanisms involved are still not completely understood. The active involvement of T cells and their secretory products in an immune response seems to be necessary for class switching from a predominantly IgM response to other isotypes, with a concomitant increase in antibody affinity and titer. B lymphocytes, with their specific receptors for antigen, are believed to internalize antigen-receptor complexes and partially degrade the protein component of the antigen into peptides. Some of these peptides may then be complexed with class II major histocompatibility antigens and expressed on the B-cell surface. This complex is recognized by the appropriate peptide–MHC-specific T_h cell. The activated T_h cell not only begins to divide, but also secretes lymphokines that activate the B cell, stimulating it to differentiate and give rise to a clone of antibody-secreting plasma cells. Such B cell to T_h cell contact has been demonstrated by electron-microscopic studies by Kupfer and colleagues (40–44) using an antigen-specific T-cell line and a B-cell hybridoma presenting that same antigen.

Reorientation of MTOC during T- and B-cell interaction. Within the cytoplasm of cells, microtubules seem to radiate away from the cell center, or centrosome. The centrosome, the major MTOC in the cell, can be visualized by use of antitubulin antibodies. The MTOC is intimately associated with the Golgi apparatus, the organelle involved in processing secretory proteins and packaging them into secretory granules. These secretory granules are believed to be moved along microtubule tracks to the plasma membrane, and the position of the MTOC in a cell may determine where secretion will occur.

In the case of T- and B-cell interactions, a reorientation of the MTOC may allow the T-cell lymphokines to be secreted directly toward the specific B cell being activated. Thus, in the delivery of lymphokine-containing secretory granules to the surface of a T_h cell in the region of B-cell contact, one might expect to see a reorientation of the MTOC. Using a fluorescent marker for the MTOC, it was shown that during specific interactions between responding T and B cells the MTOC does indeed reorient itself inside the T_h cell to face the contact point between the B and T cell (Fig. 1). In addition, the membrane-associated cytoskeletal protein, talin, becomes concentrated under the contacting T_h cell membrane. The reorientation of the MTOC and change in the cellular distribution of the talin occur only when a specific cell-cell interaction has taken place and not during nonspecific adherence between two cells. Similar reorientations of the MTOC have been demonstrated when cytotoxic T cells make contact with their targets (45).

Micrographs of T cell-B cell joinings taken at various times after antigen exposure illustrate a sequence of stages beginning with blast formation of the activated B cell and ending with its mitosis. After activation of one B cell, T cells could redirect their MTOC toward a second B cell to deliver a new round of lymphokine help. Time sequence studies illustrate polarization of the T-cell lymphokines IL-2 and γ-interferon toward the B cells in the T cell-B cell joinings. Other T cell-B cell interactions demonstrate similar polarization of the lymphokines IL-4 and IL-5. The lymphokines are not delivered continuously but follow a preprogrammed sequence. These micrograph studies illustrate the mechanism whereby a fine antigen specificity of interaction between T and B cells may be achieved even though the factors for activation are themselves not antigen specific.

Synthetic vaccines incorporating B-cell and T-cell isotopes. The increasing recognition of the importance of involving T cells in an immune response to increase immunogenicity has been reflected in vaccine design. One direct and novel approach has been exemplified by the work of Tam (46, 47) to deliberately incorporate separate T-cell and B-cell epitopes into a chemically defined synthetic vaccine. The work was stimulated by the search for an effective antimalarial vaccine after a disappointing response to a previously synthesized conjugate vaccine. When the vaccine—consisting of synthetic peptide of the circumsporozoite protein of *Plasmodium berghei* conjugated to tetanus toxoid—was administered to human volunteers, only a small percentage produced protective antibody titers (48). It was postulated that the immune response to the peptide was suppressed because the

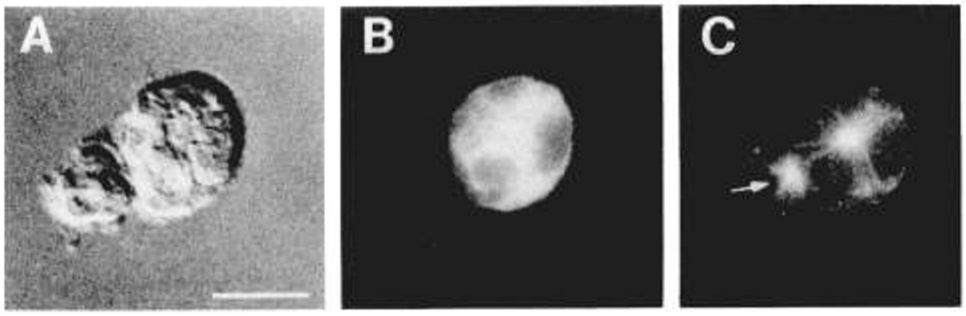

Fig. 1. A cell couple formed between a cloned helper T cell and a hybridoma B cell that had been pulsed with the specific antigen conalbumin. The couple is seen in Nomarski optics in *A*. The same couple was double-labeled immunofluorescently for a cell surface marker to label the B cell (*B*) and for microtubules and the MTOC (*C*). The MTOC in the T cell faces the region of cell-cell contact, but the MTOC in the B cell faces away. Bar = 10 μm. (From Kupfer A, Swain SL, Janeway CA, Singer SJ: The specific direct interaction of helper T cells and antigen-presenting B cells. Proc Natl Acad Sci USA 83:6080–6083, 1986).

volunteers had been previously vaccinated with the carrier (tetanus toxoid). Therefore, a new approach was tried—avoiding the use of a protein carrier altogether.

MAP system. To replace the protein carrier, a scaffold of multilysine matrices was synthesized (46, 47). One scaffold model was an eight-branched structure consisting of a core matrix of three levels of lysine with eight amino ends to which could be anchored suitable peptide antigens (Fig. 2). This system is termed the MAP system. It is especially intriguing because a MAP containing both the carrier and the antigenic peptides can be synthesized in a single manipulation by the solid-phase method (49). The MAP system produces a structure consisting of a small core matrix surrounded by layers of a high density of peptide antigens. Thus, it has the appearance and molecular weight of a small protein. In contrast, in conventional conjugated vaccines, a large protein carrier is conjugated to a low density of peptide or polysaccharide antigen.

B-cell and T-cell epitopes of the circumsporozoite protein of the malaria parasite *P. berghei* were identified and incorporated into a MAP system (50). Vaccines produced in this way were found to be strong immunogens in a number of inbred mouse strains when administered first in complete Freund's adjuvant and boosted two subsequent times in incomplete Freund's adjuvant. The effect of the stoichiometry, orientation, and arrangement of the B and T epitopes on the immunogenicity of the MAP was studied. Ten MAP models were prepared containing various arrangements of the T and B epitopes of the circumsporozoite protein of *P. berghei*. The B epitope was represented by the 16-residue peptide $(PPPPNPND)_2$, and the T epitope was represented by a 12-residue peptide, KQIRDSITEEWS. The latter peptide was identified as a T-cell epitope because it was recognized by several strains of mice and induced helper activity *in vivo* (51). Examples of the types of structures formulated as MAP models are shown in Figure 3. The immunogenicity and protective value of the MAP when these structures were tested in mice are presented in Table 1. The best arrangement of B-

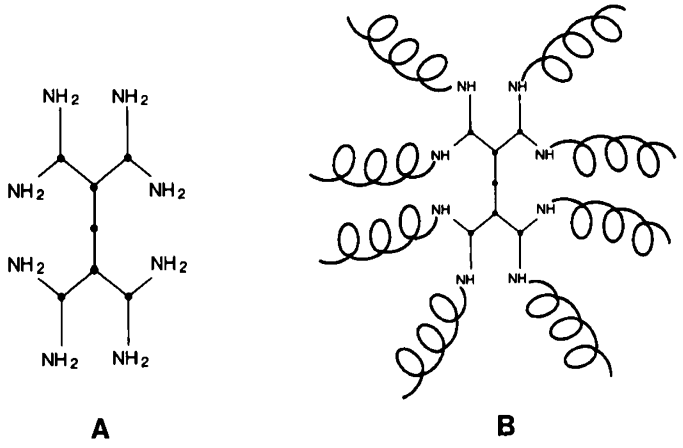

Fig. 2. Schematic representation of the core matrix of the MAP. The lysyl core of the octabranching MAP (*A*) and MAP with eight peptide antigens attached (*B*). (From Tam JP: Multiple antigen peptide system: a novel design for synthetic peptide vaccine and immunoassay. In: Synthetic Peptides: Approaches to Biological Problems. Alan R Liss, New York, pp 3–18, 1989.)

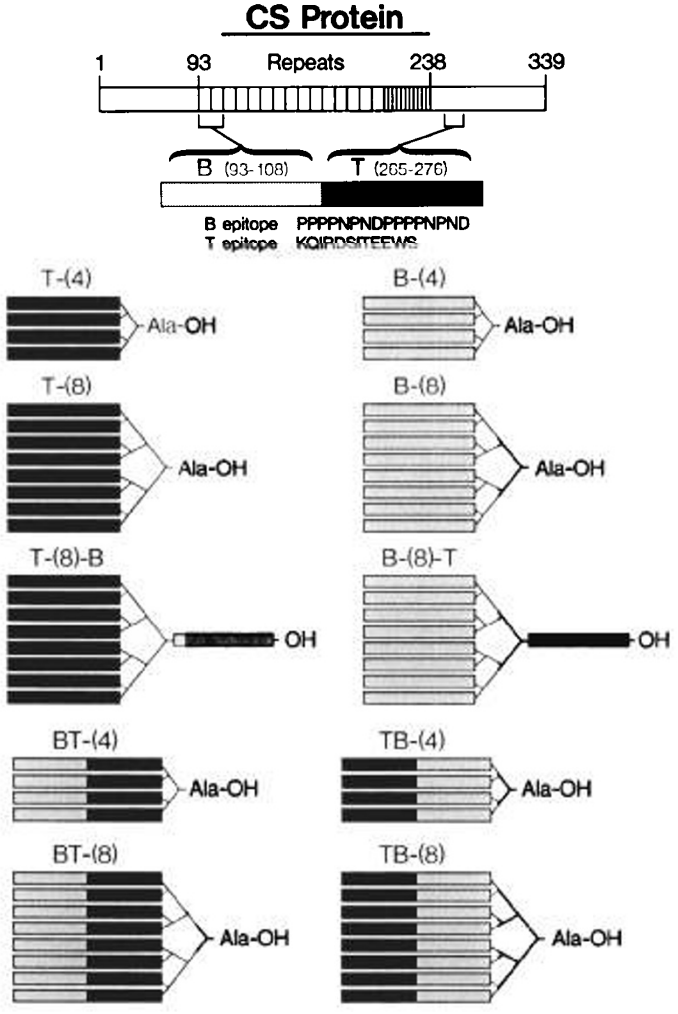

Fig. 3. Schematic representation of the structure of the CS protein of *P. berghei* of the monomeric form of a peptide containing tandem B- and T-cell epitopes and of 10 MAP models. (Reproduced from J Exp Med 171:299–306, 1990, by copyright permission of the Rockefeller University Press.)

Table 1. *Protective efficacy of different MAP models in mice challenged with 2000 P. berghei sporozoites**

Immunogen†	Antisporozoite (IFA titer × 10^{-3})‡	Number protected/ challenged§	% Protection
BT-(4)	128	4/5	80
TB-(4)	32	3/5	60
TB-(8)	32	3/5	50
BT-(8)	8	2/4	0
T-(4)	<0.2	0/4	0
B-(4)	<0.2	0/5	0
B-(8)	<0.2	0/5	0
BT monomer	<0.2	0/5	0
No immunogen		0/5	0

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† See Figure 4 for pictorial representation of immunogen.

‡ Titer determined by immunofluorescence assay using glutaraldehyde-fixed sporozoites, 34 d after the booster injection of antigen.

§ Mice were challenged by i.v. inoculations of 2000 sporozoites 35 d after the booster injection. Peripheral blood smears were examined daily for parasitized erythrocytes. Protection is defined as absence of parasites from d 3 to 12 after challenge.

and T-cell epitopes was that designated BT-(4), which consists of four B-cell and four T-cell epitopes in tandem. BT-(4) produced approximately 50-fold higher antibody titers than BT-(8), the structure with eight B- and T-cell epitopes in tandem. It is entirely probable that the optimal number and sequence of B-cell and T-cell epitopes may differ for each unique MAP system and testing will be required to determine the best vaccine structure. This engineering of epitope-based vaccines conjugated to nonprotein carriers offers a promising approach to vaccine design.

MOLECULAR PARAMETERS ENHANCING IMMUNOGENICITY

In addition to addressing the problems in determining the best components for the design of optimally immunogenic conjugate vaccines, it is necessary to consider the spacing and size of epitopes and the size and composition of the final structure. Dintzis and colleagues (52-56) have attempted to identify molecular parameters most relevant to immunogenicity. In studies using soluble haptenated polymers as antigens, it was found that such molecules became immunogenic when the molecular mass approached roughly 100 kD. This mass seemed to be necessary in these types of polymeric molecules to achieve a significant immune response in the absence of adjuvant. In addition, they found that the number of haptens or epitopes on the carrier polymer had to exceed 20 to stimulate antihapten or antiepitope antibody production. A variety of polymer carriers—polyacrylamide, dextran, Ficoll, carboxymethyl cellulose, and polyvinyl alcohol—were used successfully. If the mass of the carrier was sufficient, high-density haptens or epitopes yielded the best response. This suggested that, to trigger the onset of an immune response, antigens must bind together several antigen receptors on the surface of the B cell. The optimal molecular mass of the carrier for immunogenicity would have to be determined for each type of polymer used; however, in the Dintzis studies, the optimal molecular mass was about 500 000 D. With molecular masses above 1 million, the response began to fall off.

Net charge was also found to exert an effect. When negatively charged fluorescein was used as hapten, too high a density of the hapten on the carrier seemed to cause a decreased response, possibly due to repulsion by the negatively charged B-cell surface. Thus, the number of positive and negative charges as well as the hydrophobicity that may build up on the carrier molecule as it is derivatized with haptens all contribute to the immunogenicity of the conjugate.

Immunogenicity of multimeric proteins. The aforementioned studies were carried out on TI antigens; however, the importance of molecular aggregates has also been observed in studies with proteins and a TD response. In general, deaggregated or monomeric proteins are not immunogenic by themselves when administered in the absence of adjuvants. A study analogous to the study on nonprotein polymers was conducted to determine the immunogenicity of multimers of proteins (Dintzis HM, unpublished data). As a model protein antigen, BSA was chemically cross-linked; different aggregates produced were then fractionated and assayed for immunogenicity. It was found that monomers as well as oligomers composed of up to six units were barely immunogenic when injected into mice. As the size of the multimeric aggregate increased, the immunogenicity rose sharply. When the aggregates were composed of "50 mers" or "100 mers," they could be described as being viral in size and were extremely immunogenic in mice. These findings were obtained without the use of any adjuvant.

Adjuvants as aggregators. Even adjuvants, however, can provide evidence that aggregation of antigen is important in increasing immunogenicity. Because adjuvants are being used as vehicles for conjugate vaccine administration, knowledge of possible mechanisms of an adjuvant's immunogenicity-enhancing ability would be useful. Traditionally, when aluminum hydroxide (alum) is used as an adjuvant, it is mixed with the antigen to be administered and injected as a slurry. Because the multiplicity of epitopes or haptens in a molecule was found to be crucial for immunogenicity, it was postulated that proteins form arrays on the surface of the alum, thereby presenting themselves to the immune system as aggregates. This could explain alum's immunogenicity-enhancing ability.

Electron-microscopy studies of array formation of proteins on the surface of aluminum hydroxide were undertaken (Dintzis HM, unpublished data). Ferritin was used as the model protein because it has a dense core of iron hydroxide that contains several thousand iron atoms, rendering it directly visible in the electron microscope. Commercial fractions of ferritin were further fractionated on gel columns to prepare homogeneous monomer solutions. These monomers were not immunogenic in mice, even when doses were quite high, whereas the same preparation of monomers was intensely immunogenic when adsorbed on aluminum hydroxide (Fig. 4). The small response to the highest dose of ferritin in saline may have been due to a small amount of aggregated ferritin still present. Under conditions commonly used to precipitate proteins with alum, ferritin strongly adhered to the alum particles. In the electron microscope, the ferritin was clearly visible as round black dots (iron

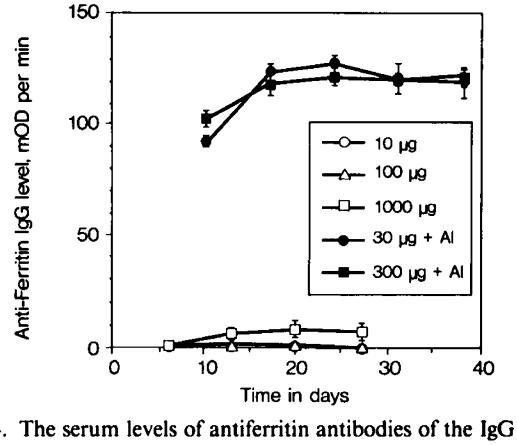

Fig. 4. The serum levels of antiferritin antibodies of the IgG class at various times after intraperitoneal injection of antigen. The *open symbols* show the responses to three doses of monomeric ferritin injected in saline. The *solid symbols* show the responses to two doses of monomeric ferritin adsorbed on 1 mg of aluminum hydroxide adjuvant. Sera were diluted 1000-fold for assay. Duplicate assays were done on three mice for each point, with SD shown.

cores of ferritin) of variable densities on the irregularly shaped gray background of the aluminum hydroxide particle (Fig. 5). The number of black dots correlated with the density of ferritin molecules on the alum and also with the immunogenic response when the alum-protein slurry was injected into mice.

The importance of molecular size and aggregation has been demonstrated in studies on the immunogenicity of dextran, a polysaccharide made by *Leuconostoc mesenteroides*. Here, it was found that molecules with a molecular mass below 70 kD were not immunogenic, whereas those of greater molecular mass were immunogenic (57, 58).

Trials of conjugate vaccines using oligosaccharides coupled to diphtheria toxoid or to CRM_{197}, a nontoxic mutant protein of diphtheria toxoid, demonstrated that epitope valence (number of epitopes per unit of carrier) is also a critical parameter in immunogenicity (8, 9). A higher multiplicity of saccharides per protein carrier was found to give greater immunogenicity.

These and the Dintzis studies demonstrated that multiplicity of antigenic epitopes is important and that immunogenicity is related to presentation of antigen in clusters. These are reasonable observations, inasmuch as the antigenic molecules on the surface of microorganisms and viruses are usually presented to the immune system in dense, highly repetitious, multiple arrays and not as single molecules.

VACCINATION AGAINST SOME COMMON INFECTIONS

Development of conjugate vaccine against P. aeruginosa. In the last 30 years, there has been a dramatic increase in the number of patients susceptible to infections from *P. aeruginosa*, which can be life threatening. This increase has been due in part to the increased use of broad-spectrum antibiotics and also to the increased number of patients who are immunocompromised after toxic chemotherapy and other drastic supportive procedures. In addition, *P. aeruginosa* is responsible for a large number of the pulmonary infections in patients with cystic fibrosis. The need for a vaccine against this organism is, therefore, substantial.

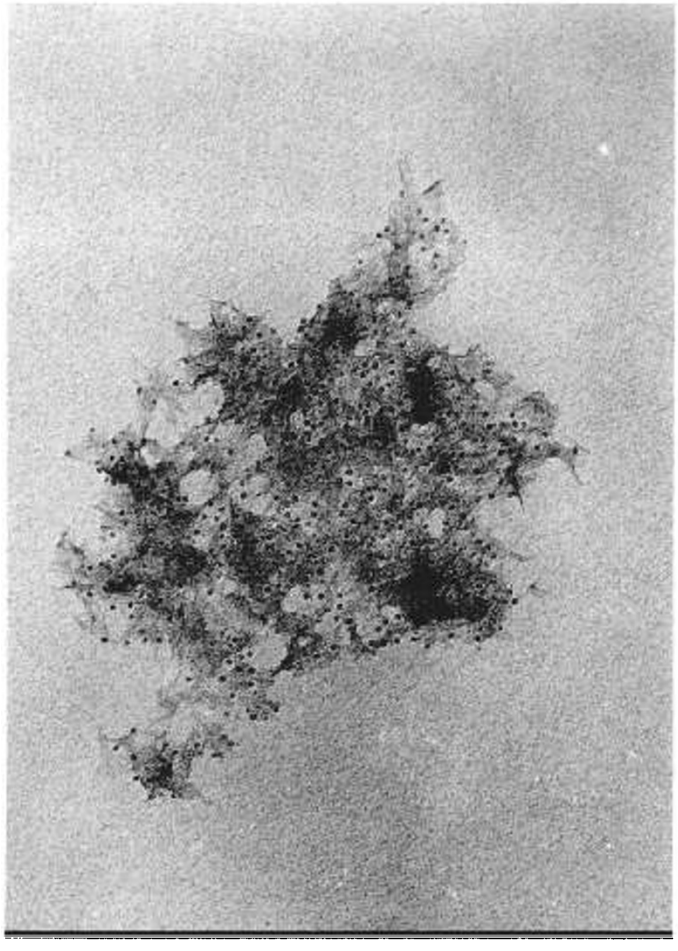

Fig. 5. A transmission electron micrograph of ferritin molecules adsorbed on the surface of an aluminum hydroxide particle. The ferritin was adsorbed at a ratio of 300 μg of ferritin per mg of aluminum hydroxide. The sample was deposited on a collodion membrane, enlarged by electron microscope 50 000×. The final enlargement after photography is 135 000×.

The LPS of the outer membrane of this gram-negative organism is the major surface antigenic component and consists of a lipid portion, termed lipid A, a core polysaccharide, and a specific polysaccharide region, the O-specific chains. Antibodies to the entire LPS as well as those to the lipid A portion are protective against bacteremia caused by *P. aeruginosa*. Unfortunately, both substances are toxic when unmodified; LPS-based vaccines have been associated with high rates of adverse reactions. To circumvent this toxicity problem, two major approaches have been taken. The first has been to detoxify the LPS by treatment with sodium hydroxide under anhydrous conditions. However, conjugates of the detoxified LPS with protein have proven to be poorly immunogenic. A more fruitful approach has been to use the nontoxic O-PS as part of a conjugate construct. By itself, O-PS is nonimmunogenic, but when conjugated to a protein carrier it becomes immunogenic. Use of tetanus toxoid as the protein carrier in the conjugate resulted in a vaccine that was satisfactory. However, there was concern that people previously immunized against tetanus would tend to suppress the immune response to the O-PS part of the conjugate (12). Therefore, a protein carrier to which people are not ordinarily preimmunized was sought. A successful candidate proved to be detoxified toxin A from the *P. aeruginosa* itself. Toxin A, a 71-kD protein, is an extracellular factor made by most *P. aeruginosa* isolates. Antibodies to it had been previously found to be protective against infection by *P. aeruginosa*.

Toxin A was first detoxified by introducing adipic acid dihydrazide into the molecule, which was then coupled to periodate-oxidized O-PS. This conjugate has been tested in humans and seems to be quite effective (12–15). When the same conjugation procedure was attempted using the LPS from *E. coli* rather than from *P. aeruginosa*, it was necessary to reduce considerably the periodate oxidation time to avoid destroying the O-side chains of the *E. coli* LPS. This demonstrates the importance of carefully controlling conjugation procedures when any new conjugation variant is introduced (59).

Specific hyperimmune globulin against *P. aeruginosa* and *Klebsiella* has been produced by plasmaphoresing volunteers who had been immunized with the conjugate vaccine to *P. aeruginosa* and a *Klebsiella* polysaccharide vaccine. This hyperimmune globulin is currently being used in a clinical prophylactic efficacy trial in intensive care units (Sadoff JC, personal communication).

Malaria conjugate vaccines. Malaria is the most common infectious disease in humans. Because of the increasing frequency of plasmodia resistant to chemotherapeutic agents, as well as the development of insecticide resistance in anophelene mosquitoes, there has been a resurgence of interest in malarial vaccines. Most vaccine development has been directed at attempts to elicit antibodies against sporozoite antigens on the surface membrane of *Plasmodium falciparum* sporozoites (60). A specific CS protein has been identified as a good candidate for vaccine development. The central portion of the CS protein contains many tandem repeats of two tetrapeptides. One of these, Asn-Ala-Asn-Pro (NANP), has been identified as an immunodominant region of the CS protein and, therefore, has been used as part of a synthetic peptide epitope in conjugate vaccine synthesis. Investigations have been made as to whether there is a critical size of repeat peptides. Peptides containing three and six repeats of NANP, as well as a recombinant protein R32 containing 15 NANP repeats, were conjugated to protein carriers, administered with alum, and assayed for immunogenicity. The R32 conjugate was found to be the most immunogenic of the group. On a molecular basis,

the weight of the peptide was almost as important for immunogenicity as the ratio of peptide to carrier. Six or seven copies of the 15-repeat peptide per protein carrier molecule were found to be more effective than 25 copies of the smaller three-repeat peptide (61).

The above systems used toxoids from various bacteria as carriers for the peptide or saccharide epitope. Another novel carrier system that is showing much promise is that of multimeric protein preparations of meningococcal OMP. These preparations have been named "proteosomes." The immunogenicity of synthetic peptides of CS peptides was greatly increased by complexing them to proteosomes via hydrophobic moieties added to their amino termini (62). It is evident that epitope-to-carrier ratio, type of carrier, and type of adjuvant all will be extremely important factors in the development of an effective vaccine for humans. The ultimate goal is to develop a malaria vaccine formulation capable of inducing protective levels of antibody in humans after a single injection (60). A malaria peptide conjugate vaccine has been used in a preliminary efficacy trial on volunteers in Thailand and has been found to be protective when specific antibody levels had reached > 8 μg/mL. An encouraging fraction (40%) of the volunteers had achieved this level at different times after vaccination (Sadoff JC, personal communication).

Vaccines for bacterial meningitis. Hib is the leading cause of bacterial meningitis in children under 4 y of age. At birth, maternal antibodies seem to confer protection, but as the maternal antibody level in the infant decreases, infant susceptibility to infection increases. Unfortunately, young infants fail to respond to the Hib capsular polysaccharide, which is a TI type of antigen. However, inasmuch as they are capable of responding to TD antigens, several groups have attempted to produce an efficacious vaccine against Hib by covalently linking the polysaccharide (or an oligosaccharide derived from the larger molecule) to a protein carrier, thereby creating a TD antigen. The carrier proteins used have been derived from different bacteria: tetanus toxoid, diphtheria toxoid, CRM_{197}, which is a nontoxic form of diphtheria toxin, and the OMP of group B *N. meningitidis.* Conjugate vaccines of all four types are now being studied clinically, and side-by-side comparisons of efficacy are being conducted in infants and in older children (reviewed in 63, 64).

Group B *N. meningitidis* and *E. coli* K1, although not as common as Hib as causes of meningitis, are responsible for a considerable number of cases throughout the world. Although there are effective polysaccharide vaccines for groups A and C strains of *N. meningitidis*, there is as yet no uniformly effective vaccine against group B. Group B meningococci and *E. coli* K1 have identical capsular polysaccharides that are composed of a linear arrangement of sialic acid residues (65). The GBMP is poorly immunogenic in humans, even when it is conjugated to a protein. This may be due to a natural immune tolerance to GBMP, inasmuch as it has been shown to be structurally homologous to a number of human tissue antigens (66, 67). The aim in the synthesis of an effective vaccine against these bacteria is to induce antibodies that confer protection against meningitis without binding to GBMP. This would avoid induction of cross-reactive antibodies, which have the potential for inducing an autoimmune condition.

It was found that chemical modification of the GBMP greatly increased its immunogenicity in mice, especially when the polysaccharide was conjugated to tetanus toxoid (68, 69). The most successful modification made was that in which the N-acetyl groups of the sialic acid residues of the GBMP were removed by treatment with a strong base and replaced with NPr groups. A large proportion of the antibodies produced were of the IgG isotype and were GBMP specific. Mice injected with anti-NPr-GBMP serum had greatly reduced bacteremia when challenged with either group B meningococci or *E. coli* K1 (Table 2). Specificity was indicated by the failure of the group B antiserum to protect against a challenge from meningococcus group C (*N. meningitidis* 87147 in Table 2). These properties would qualify the NPr-GBMP-tetanus toxoid as a good candidate for a conjugate vaccine against meningitis caused by either group B *N. meningitidis* or *E. coli* K1.

However, by inducing antibodies that might be cross-reactive with self-saccharides, it was thought possible that such a vaccine might also induce an autoimmune response. Interestingly, only a portion of the GBMP-specific antiserum was precipitable by GBMP. The nonprecipitable part was apparently not cross-reactive with GBMP, and it was that nonprecipitable portion that was bactericidal. Presumably, this portion of the antiserum would not be cross-reactive with human tissue antigens either. The bactericidal activity of the NPr-GBMP-specific mouse antisera could be specifically removed by viable group B meningococcal or *E. coli* K1 organisms or both. It has been proposed that the NPr-GBMP probably mimics a complex intermolecular epitope on the surface of group B meningococci and that the antibodies against the NPr-GBMP recognize inner residues of the GBMP associated with another molecule (70). Promising results such as these indicate that it may indeed be possible to design an antimeningitis vaccine that will induce bactericidal antibodies with minimal cross-reactivity with self-antigens.

Genetic engineering, vaccines against N. gonorrhoeae, and OMP of N. meningitidis as vaccine carrier. Because of the increasing incidence of antibiotic-resistant gonococci, as well as the high incidence of reinfection after treatment, an effective vaccine against *N. gonorrhoeae* infections is being sought. Although infected patients may show some degree of immunologic response by developing complement-fixing antibodies, the failure to develop effective immunity is probably related to the antigenic heterogeneity of different strains of gonococci. Complement-fixing antibodies are especially effective against strains of gonococci that cause locally invasive disease (*e.g.* pelvic inflammatory disease). The sensitivity of the bacteria is directly related to the efficiency of insertion of the membrane attack complex of complement. Effective vaccine design, therefore, would aim at stimulating the production of complement-fixing antibodies against a relatively invariant membrane component of the gonococci.

Like other gram-negative bacteria, the gonococcus has an LPS as part of its outer membrane; however, because of the inherent toxicity of LPS, vaccines that contain boiled organisms or membrane vesicles are of limited usefulness. Other membrane components, therefore, have been considered. One of the three OMP of *N. gonorrhoeae* is a porin designated as PI. Porins are channel-forming proteins (usually ion channels) in the outer membrane of gram-negative bacteria. Because PI is antigenically conserved, it has been a prime candidate for use in a gonococcal vaccine, and antibodies toward PI exhibit bactericidal and opsonic activity against the gonococcus. However, PI preparations are almost inevitably contaminated with another highly conserved membrane protein, PIII. Unfortunately, antibodies to PIII have been shown to block human serum killing of the gonococci and thus are implicated in the formation of blocking antibodies (71).

Blocking antibodies, the natural antibodies of the IgG class that are present in some human sera, are believed to inhibit or block the action of complement. The mechanism by which they do this is unknown, but it is proposed that when these antibodies bind to particular surface antigens of the gonococcus they inhibit the necessary localization of complement on the surface of the bacteria (72). Such blocking antibodies can, therefore, seriously interfere with the protective action of antibodies raised against gonococci as the result of vaccine administration. Vaccination with gonococcal PI contaminated by <10% PIII caused a decrease in the bactericidal activity of the serum in volunteers who had a previous gonococcal infection (73). This decrease was associated with the development of anti-PIII antibodies, and these were assumed to be blocking antibodies. Therefore, in making an effective PI gonococcal vaccine, it is important to purify it of any PIII contamination. To bypass the laborious process of chemical purification of PI, a more sophisticated way of eliminating PIII contamination of PI has been developed. PIII

Table 2. *Bactericidal titers and passive protective efficiency of anti-NPr-GBMP serum and normal mouse serum against N. meningitidis B and C strains and E. coli K1**†*

Challenge strain	Bactericidal titer‡	Anti-NPr-GBMP serum§		Normal serum§		% Reduction in viable bacterial counts	
		3 h	5 h	3 h	5 h	3 h	5 h
N. meningitidis 80165 (B:2b:P1.H)	Exp. 1 512	24	16	1064	2144	98	99
	Exp. 2 512	440	0	1850	2700	76	100
N. meningitidis 86370 (B:15:P1.16)	256	760	200	2360	350	68	84
N. meningitidis 87147 (C:2b:P1.2)	4	2000	2920	2180	2736	0	−6
E. coli 871743 (018:K1:H7)		80	24	4658	368	98	93

* Modified from Ashton PE, Ryan JA, Michon F, Jennings HA: Microb Pathog 6:455–458, 1989.

† Mice were injected i.v. with serum and challenged intraperitoneally 1 h later with *N. meningitidis* or *E. coli*. Normal serum was from mice immunized only with adjuvant.

‡ The highest serum dilution (expressed as the reciprocal) causing an approximately 90% reduction in viable bacterial count.

§ Viable bacterial counts/mL of blood 3 or 5 h after challenge with approximately 3×10^3 viable bacteria.

deletion mutants have been genetically engineered, and the resulting organisms can be used to obtain gonococcal proteins in large batches for vaccine use without any PIII contamination (74).

Inasmuch as blocking antibody has been shown to exist in the normal sera of people who have not been exposed to *N. gonorrhoeae* (75), the question arose as to whether there exist other bacterial proteins with enough structural homology to PIII to induce cross-reactive antibodies with blocking activity. To approach this problem, the DNA sequence of the structural gene of gonococcal PIII was determined, and it was found to have homology both with enterobacterial OMP (OmpA) and with a serologically related class 4 OMP of *N. meningitidis* (76, 77). Thus, contact with these microorganisms could be the implicating factor in the development of detrimental blocking antibodies. The structural gene of the class 4 OMP of *N. meningitidis* has been cloned and sequenced, and strains of meningococci that lack class 4 protein have also been genetically engineered (77). Because the OMP preparations of *N. meningitidis* have been used in the conjugate formulation of a malarial CS vaccine (62, 78, 79), as well as in a *Haemophilus influenzae* vaccine (80–82) it would be important to eliminate the possibility of bringing up blocking antibodies when using OMP preparations as carriers in conjugate vaccines (83).

The heightened interest and productivity in the field of conjugate vaccine design reflects the progress being made in understanding the many facets of the immune response. Manipulations at the molecular and cellular levels, as well as new techniques in molecular genetics, have contributed to novel and innovative approaches to vaccine synthesis and design. Clinical trials of several conjugate vaccines are now being conducted, and it is hoped that a number of them will be approved and released for widespread use in the not-too-distant future.

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