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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



No. 03-065841- -00007-0000

Fecha: 2008-02-19 16:28:02 Dep. 2020 NUECREACIONE
Tra 11 PATENTEIYII Eve: 379 FASENACIONALII
Act. 444 RESPUESTAREQUE Folios: 8

Trámite : 011 Evento : 379 Actuación : 444

Referencia : Expediente No. 03.065.841
Asunto : Solicitud de Patente para **VACUNA MULTIVALENTE
CONJUGADA DE POLISACARIDO MENINGOCOCICO-
PROTEINA**
Solicitante : **AVENTIS PASTEUR, INC.**
Clase : A61K 047/048
Fecha de Solicitud : 1 de agosto de 2003

1. Yo, Emilio FERRERO, obrando como apoderado del solicitante en el asunto antes identificado, me refiero al Oficio No. **13067** notificado por fijación en lista del 12 de octubre de 2007.

2. Mediante mi memorial de fecha 10 de enero de 2008 solicité un plazo de 30 días para atender el Oficio No. **13067**.

3. Mediante el Oficio No. **13067** se puso en conocimiento del solicitante el informe que contiene las observaciones hechas por su Despacho sobre la presente solicitud de patente.

Atiendo a las observaciones de la manera siguiente:

3.1 CLARIDAD, UNIDAD DE INVENCION, REIVINDICACIONES DE USO Y EXCEPCION DE PATENTABILIDAD.

Para subsanar las objeciones que sobre claridad, unidad de invención y excepciones de patentabilidad hizo su Despacho, presento nuevo pliego reivindicatorio con

11 reivindicaciones en el cual se han hecho cambios que permiten apreciar claramente el objeto reivindicado en la presente solicitud de patente. Las modificaciones efectuadas fueron las siguientes:

- El pliego reivindicatorio se ha limitado a composiciones conjugadas tetravalentes A, C, W, Y; cuya actividad se encuentra debidamente soportada en la descripción por datos reales de administración clínica a humanos, los cuales sustentan la actividad y eficacia de dichas composiciones tetravalentes como inmunogénicas y seguras (ver ejemplo 9, páginas 45 a 62).
- Las reivindicaciones 4 y 12 han sido eliminadas de este pliego reivindicatorio.
- De la misma forma, las reivindicaciones 9 y 17 que reclamaban métodos de tratamiento han sido eliminadas de éste pliego reivindicatorio.

De esta manera es claro que con las anteriores modificaciones del capítulo descriptivo y reivindicatorio quedan subsanadas las objeciones que por claridad, unidad de invención y excepciones de patentabilidad hizo su Despacho, ya que ahora, dicho pliego trata un único concepto inventivo, a saber, un dispositivo médico; permite determinar el alcance de la protección reivindicada y compararla frente al estado del arte, de acuerdo con las disposiciones de los artículos 20 (d), 25, 28 y 30 de la Decisión 486.

4. Por lo tanto, le solicito se sirva continuar con el trámite de la presente solicitud pues tanto el pliego descriptivo como las reivindicaciones se presentan de una forma clara y en consecuencia, con fundamento en las mismas una persona versada en la materia podría realizar el examen de patentabilidad del producto objeto de esta invención. A pesar que las objeciones sobre claridad, unidad de invención y excepciones de patentabilidad que planteó ese Despacho en el Oficio No. **13067** se deben tener por superadas, en el evento que el Despacho considere que sea necesario, respetuosamente le solicito hacer uso de la facultad que le otorga el artículo 45 inciso 2. de la Decisión 486

de la Comisión de la Comunidad Andina a efecto de requerir nuevamente de nuestra parte cualquier complementación o aclaración que considere necesaria.

5. Puesto que se han atendido a cabalidad las observaciones hechas por su Despacho sobre la presente solicitud de patente, atentamente solicito que se continúe su tramitación.

6. **Anexos:**

- 6.1 Nuevo pliego reivindicatorio con 11 reivindicaciones;
- 6.2 Formulario para modificaciones;
- 6.3 Recibo por valor de \$103.000 por concepto de modificaciones en solicitud pendiente;

Señora Jefe,


EMILIO FERRERO
T.P.A. No. 31.929

COLO-13882-841-001-7
NNJ\NNJ\ID-119470\WPC13882
No. M-2008-119470\NNJ

FORMULARIO UNICO DE CORRECCIONES Y MODIFICACIONES

1	SOLICITUD DE: ☐ Corrección de Error Material ☒ Modificación a una solicitud	
2 SOLICITANTE	Nombre: AVENTIS PASTEUR, INC. Sociedad constituida y existente bajo las leyes del Estado de Delaware, Estados Unidos de América. Dirección: 1 Discovery Drive, Swiftwater, Pennsylvania 18370-0187, Estados Unidos de América Domicilio: Swiftwater, Pennsylvania, Estados Unidos de América Teléfono: 546 09 70 Fax: 249 40 70 E-mail: clientes@cavelier.com	IDENTIFICACION C.C. ☐ NIT ☐ C.E. ☐ Otro ☐ Número: _____
3 REPRESENTANTE O APODERADO	Nombre: Emilio FERRERO Dirección: Carrera 4ª No. 72-35 Teléfono: 347 36 11 Fax: 217 92 11 E-mail: cavelier@cavelier.com	IDENTIFICACION C.C. ☒ NIT ☐ C.E. ☐ Otro ☐ Número: <u>438.019 TP 31.929</u>
4	Número de expediente: <u>03.065.841</u>	
5	Descripción de la corrección o modificación solicitada: <u>Presento nuevo capítulo reivindicatorio compuesto por 11 reivindicaciones con el fin de superar las objeciones hechas por Su Despacho a la presente solicitud y pido que el examen de patentabilidad sea realizado sobre este nuevo capítulo reivindicatorio.</u>	
6	Comprobante de pago No. <u>08-14,165</u> Fecha: <u>18 de febrero de 2008</u>	
7	Anexos ☒ Comprobante de pago de la tasa por \$103.000.00 ☐ Poderes, si fuere el caso ☐ Documento que acredita la existencia y representación legal de las partes, cuando sean personas jurídicas.	
8	NOMBRE: EMILIO FERRERO	FIRMA: 
C.C. 438.019 de Usaquén T.P.A. 31.929		
NNJ		

REIVINDICACIONES

1. Una composición inmunológica que comprende 4 conjugados de polisacárido-proteína distintos, en donde cada uno de los conjugados comprende un polisacárido capsular de 2 o más serogrupos de *N. meningitidis* conjugado, y una o más proteínas portadoras.
2. La composición inmunológica de la reivindicación 1 en donde los polisacáridos capsulares son seleccionados del grupo que consiste de polisacáridos capsulares de los serogrupos A,C,W-135 y Y de la *N.meningitidis*.
3. La composición inmunológica de la reivindicación 1, en donde los polisacáridos capsulares son de los serogrupos A y C de la *N.meningitidis*.
4. La composición inmunológica de la reivindicación 1, en donde la proteína portadora es toxoide de difteria.
5. La composición inmunológica de la reivindicación 1, que comprende adicionalmente un adyuvante.
6. La composición inmunológica de la reivindicación 5, en donde el adyuvante es hidróxido de aluminio o fosfato de aluminio.
7. Una vacuna meningococica multivalente que comprende cantidades inmunológicamente efectivas de 4 conjugados de polisacárido-proteína distintos, en donde cada uno de los conjugados contiene un polisacárido capsular diferente conjugado con una proteína portadora, y en donde cada

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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

NIT : 800176089-2

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



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Act 444 RESPUESTAREQUE Folios: 8

RECIBO OFICIAL DE CAJA : 08 - 14,165
FECHA : FEBRERO 18 DE 2008

***** CONSIGNACION *****

DEPOSITANTE	TIPO PAGO	BANCO	CUENTA	No. PAGO	FECHA PGO	Vr. PAGO
CAVELIER ABOGADOS	CONSIGNACION	BANCO DE BOGOTA	062754387	2808613	18/02/2008	39,393,000.00

***** CONCEPTO *****

CANT.	RENTISTICO	CONCEPTO	TOTAL CONCEPTO
1	50005-01-03	CAMBIO DE MODALIDAD Y MODIFICA	
		12 MARCAS Y LEMAS COMERCIALES	103,000.00
		TOTAL :	\$ 103,000.00

SON: CIENTO TRES MIL PESOS

RESPONSABLE : _____

RECIBO DE CAJA APLICADO AL EXPEDIENTE No. _____

Colo 13882-841-007

Meningococcal Polysaccharide-Protein Conjugate Vaccines

Dan M. Granoff, MD;^{*,†} Bruce Forrest, MD;^{*} and Rino Rappuoli, PhD[†]

ABSTRACT

Meningococcal oligosaccharide- and polysaccharide-protein conjugate vaccines are under investigation in humans for prevention of disease caused by serogroup A and C organisms. These vaccines appear to be safe and well tolerated. Repeated immunization of infants and toddlers with meningococcal C conjugate vaccines, given either alone or in a single formulation with meningococcal A conjugate vaccine, elicits boostable increases in serum meningococcal C bactericidal antibody titer. The C conjugate vaccine also induces memory B cells that are capable of responding to a subsequent immunization with unconjugated meningococcal polysaccharide vaccine. In contrast, immunization with unconjugated meningococcal C polysaccharide vaccine in these age groups is much less immunogenic and does not induce long-term immunologic memory. Less data are available on immune responses to meningococcal A conjugate vaccines. However, toddlers vaccinated in the United States with a meningococcal A vaccine combined with a meningococcal C conjugate vaccine, show much higher meningococcal A bactericidal antibody responses and greater induction of memory B cells than control toddlers given unconjugated meningococcal polysaccharide vaccine. The same conjugate vaccine was given to infants in Gambia, where there were no significant differences in the magnitude of the anti-A antibody responses, or in induction of memory B cells, compared to control infants given unconjugated polysaccharide vaccine. Meningococcal B conjugate vaccines also are under development. In experimental animals, these vaccines are much less immunogenic than A or C conjugates, probably because of immunologic tolerance induced to the B polysaccharide by exposure to cross-reacting polysialic acid expressed in the brain and other tissues of the host. To circumvent tolerance, conjugate vaccines have been prepared from derivatized meningococcal B polysaccharide, where *N*-propionyl groups have been substituted for *N*-acetyl groups. In experimental animals, such conjugates show increased immunogenicity, including development of bactericidal antibodies, but a subset of the antibodies still shows strong auto-antibody activity with host polysialic acid. Although there is no direct evidence that such antibodies are harmful, it will be a difficult task to prove that such a meningococcal B vaccine is safe to use in humans.

Key Words: conjugate vaccines, meningococcus, polysaccharide

Int J Infect Dis 1997;1:152-157.

Meningococcal polysaccharide vaccines are available for prevention of invasive diseases caused by *Neisseria meningitidis*, serogroups A, C, Y, and W135. Protection correlates with the ability of vaccination to induce serum complement-mediated bactericidal antibody.¹ In general, the antibody responses to these unconjugated polysaccharides are age-dependent: these vaccines are highly effective in adults, but with the exception of meningococcal A polysaccharide, they elicit negligible or incomplete and short-lived protection in infants and preschool children.²⁻⁴ For reasons that are unknown, meningococcal A polysaccharide is immunogenic and protective in infants as young as 3 months of age.^{3,5} However, duration of protection to meningococcal A is age-dependent in infants, declining dramatically within 1 to 2 years.³ Polysaccharide vaccines for prevention of disease caused by serogroup B meningococcal strains are not available. The group B polysaccharide is a poor immunogen at all ages, possibly because of immunologic tolerance induced in the host by the presence of cross-reactive polysialylated glycoproteins in fetal and adult tissues.⁶

Most polysaccharides appear to elicit antibody responses largely without the need for T-cell help (i.e., they are thymic-independent, or TI antigens). Conjugation of a polysaccharide to a protein carrier profoundly alters the immunologic properties of the polysaccharide, converting it from a TI to a thymic-dependent (TD) antigen. In the resulting TD conjugate, the immunogenicity of the polysaccharide is greatly enhanced, giving rise to IgG anticapsular antibodies and memory B cells.

EXPERIENCE

Haemophilus influenzae Type b Conjugate Vaccines

Much has been learned about human immunity to polysaccharide-protein conjugate vaccines from studies of *Haemophilus influenzae* type b (Hib) vaccines. Compared to unconjugated Hib polysaccharide, the immunogenicity of the conjugated Hib polysaccharide in infants is greatly enhanced, and repeated injections elicit IgG booster responses, whereas such responses are not observed after repeated doses of unconjugated Hib polysaccharide.⁷ Serum antibody to the type b capsule confers protection against invasive Hib disease. In addition,

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Hib conjugate vaccination primes for long-term immunologic memory to the Hib polysaccharide, a property not elicited by vaccination with unconjugated Hib polysaccharide, or even after recovery from Hib disease.⁸ The ability to develop memory B cells, which leads to rapid IgG anticapsular antibody responses upon encountering Hib organisms, may be an important alternative mechanism of protection against developing disease in vaccinated individuals who have shown subprotective antibody responses to Hib conjugate vaccination.⁹ Induction of immunologic memory also may be important for conferring protection to children whose serum antibody concentrations have declined to below the protective level.¹⁰

Haemophilus influenzae type b conjugate vaccines are effective in two other important ways: first, the anti-capsular antibody elicited by repeated injections of Hib conjugate vaccines undergoes affinity maturation (Granoff DM. Unpublished). The resulting higher avidity antibodies are more efficient at activating complement-mediated bacteriolysis or opsonization of Hib than lower avidity antibodies.^{11,12} Second, immunization with Hib conjugate vaccines not only protects the individual from developing invasive Hib disease by inducing protective immunity, but also lowers the rate of nasopharyngeal colonization and transmission of Hib in the population.^{13,14} By this mechanism, Hib conjugate vaccination can have a better effect on decreasing the incidence of Hib disease in the population than would be predicted based on vaccine coverage.¹⁵ It seems likely that meningococcal polysaccharide-protein conjugate vaccines will share similar properties, but these areas require further study.

Meningococcal Polysaccharide-Protein Conjugate Vaccines

Meningococcal oligosaccharide- and polysaccharide-protein conjugate vaccines have been prepared for prevention of diseases caused by serogroup A, B, and C organisms.¹⁶⁻²⁰ To date, data from humans are limited to serogroup A and C meningococcal oligosaccharide-protein conjugate vaccines. A first-generation prototype vaccine was prepared at Chiron Vaccines (Emeryville, CA) using meningococcal A and C oligosaccharides that were independently coupled to CRM₁₉₇ carrier protein (a cross-reactive nontoxic mutant diphtheria toxin).²⁰ As summarized in Figure 1, the conjugation method is based on selective end-reducing group activation of oligosaccharides and subsequent coupling to the protein through a six-carbon "spacer" molecule, adipic acid. In adults, the immunogenicity of the first-generation meningococcal A and C conjugate vaccine appeared to be similar to that of a control unconjugated meningococcal polysaccharide vaccine.²¹ A "second-generation" meningococcal C vaccine was prepared using similar chemistry, except that very small oligomers (degree of polymerization [Dp] less than six monomers in length) were removed prior to

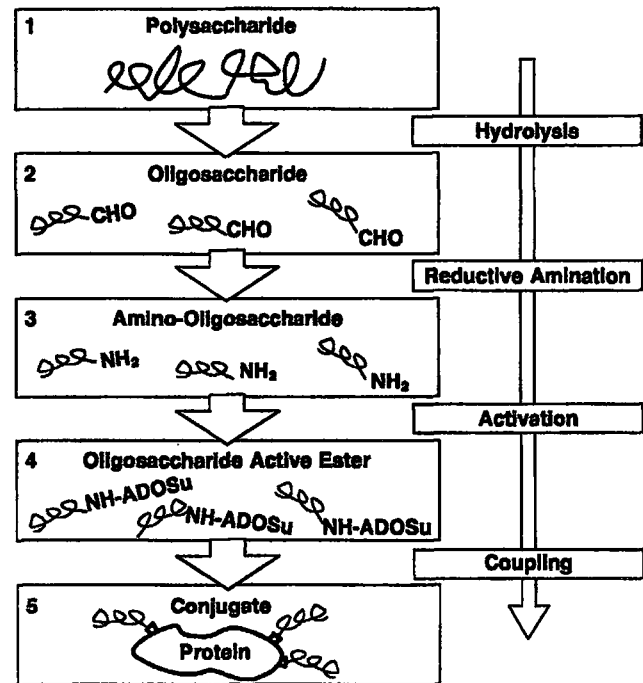


Figure 1. Strategy of conjugation of meningococcal oligosaccharides to CRM₁₉₇, a cross-reactive, nontoxic, mutant diphtheria toxoid, used in the first-generation Chiron meningococcal A and C conjugate vaccine. The method is based on selective end-reducing group activation of oligosaccharides and subsequent coupling to the protein via a six-carbon "spacer" molecule, adipic acid. ADOSu refers to the *N*-hydroxysuccinimide ester derivative of the adipic acid modified oligosaccharide. (Modified after Costantino P, et al.²⁰)

conjugation.²² In phase I and II clinical trials in humans, both vaccines have been shown to be safe in infants, toddlers, and adults.²⁰⁻²⁶ Expanded clinical trials with the second-generation meningococcal C conjugate vaccine are currently in progress.

Immunogenicity

Toddlers

In a study of American toddlers, 18 to 23 months of age, conducted at the University of California, Los Angeles (UCLA), two doses of the Chiron meningococcal A and C conjugate vaccine given 2 months apart elicited 50- to over 100-fold higher bactericidal antibody responses to both group A and group C strains, compared to those observed in control toddlers vaccinated with two doses of licensed unconjugated meningococcal polysaccharide vaccine (Figure 2).²⁴ Interestingly, the relative differences in immunogenicity between the conjugate and unconjugated vaccines in this study were less striking when the antibody responses of the toddlers were measured by an enzyme-linked immunosorbent assay (ELISA), performed using a consensus protocol developed at the Centers For

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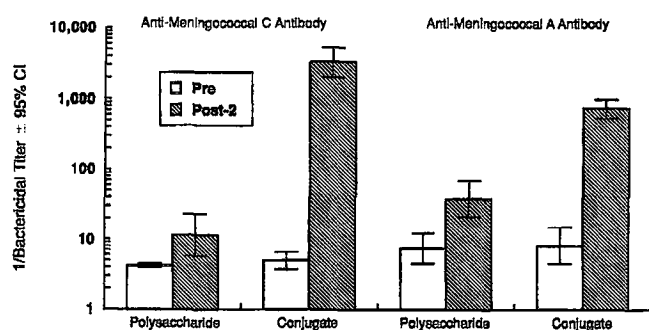


Figure 2. Serum bactericidal antibody responses of American toddlers vaccinated with a meningococcal A and C oligosaccharide-CRM conjugate vaccine. Toddlers were given two doses of either Chiron meningococcal A and C oligosaccharide-CRM₁₉₇ conjugate vaccine, or meningococcal polysaccharide vaccine (Menomune, Connaught Laboratories, Inc., Swiftwater, PA). The injections were separated by 2 months. Complement-mediated bactericidal activity was measured in serum obtained immediately before dose one (pre-) and 1 month after dose two (post-2). (Adapted from Lieberman JM, et al.²⁴)

Disease Control (i.e., relative differences in antibody responses of twofold, instead of 50- to over 100-fold, as determined by the bactericidal assay). These results suggest that the anticapsular antibody elicited by this conjugated meningococcal oligosaccharide vaccine is qualitatively different from that elicited by unconjugated meningococcal polysaccharide vaccine and, on a µg/mL basis, the conjugate-induced antibodies have a higher specific functional activity. Further, the ELISA is insensitive to these qualitative antibody differences, which may be important in protection against developing meningococcal disease.

A second-generation Chiron meningococcal C polysaccharide-protein conjugate vaccine, in which small saccharide oligomers were removed prior to conjugation, was recently evaluated in toddlers 12 to 23 months of age in a multicenter Canadian study.²² This vaccine also elicited much higher serum bactericidal antibody responses after one or two injections than those observed in sera of control toddlers vaccinated with unconjugated polysaccharide vaccine.

Infants

The safety and immunogenicity of Chiron meningococcal conjugate vaccines also have been investigated in infants less than 6 months of age. The first study was performed with the first-generation combined A and C conjugate vaccine in Gambian infants immunized at 2, 3, and 4 months of age, or 2 and 6 months, or 6 months of age.²³ The anticapsular antibody responses of the infants to the A component of the conjugate vaccine were of similar magnitude to those of a control group receiving an unconjugated meningococcal A and C polysaccharide vaccine. In contrast, the conjugate vaccine elicited two- to fourfold higher anticapsular antibody

responses to meningococcal C polysaccharide than those observed in infants given the unconjugated polysaccharide vaccine. However, the elevated anti-C antibody concentrations in the conjugate group began to decline within 3 months after vaccination.

In the Gambian study, only ELISA antibody responses were reported. Based on the experience in the UCLA toddler study described above,²⁴ the ELISA results may have underestimated the relative effectiveness of the conjugate vaccine as compared to unconjugated polysaccharide, had the responses been assessed by serum bactericidal antibody. In a study in the United Kingdom, 58 infants were vaccinated with the combined Chiron meningococcal A and C conjugate vaccine at 2, 3, and 4 months of age.²⁶ To date, bactericidal antibody responses to the group A vaccine have not been evaluated. However, the infants showed excellent anti-meningococcal C bactericidal responses (geometric mean bactericidal titer [GMT] of <1:4 prior to vaccination increasing to >1:3082 after three injections). However, by 14 months of age the GMT had declined to 1:10.

There are two other reports of the use of meningococcal C conjugate vaccines in infants. In one study, the second-generation Chiron meningococcal C polysaccharide-protein conjugate vaccine was given to infants in the United Kingdom at 2, 3, and 4 months of age.²⁵ Preliminary results were limited to assays of sera obtained prior to vaccination and 1 month after the third injection. The infants showed 25-fold increases in anticapsular antibody concentrations, as measured by ELISA, and >50-fold increases in bactericidal titers. In the other study, a meningococcal serogroup C oligosaccharide-CRM₁₉₇ conjugate vaccine was prepared by Lederle Praxis Biologics (Rochester, NY) and administered to American infants at 2, 4, and 6 months of age.¹⁹ This conjugate vaccine appeared to be well tolerated and elicited significant increases in serum anticapsular antibody concentration after two or three injections, as assessed by ELISA.

INDUCTION OF IMMUNOLOGIC MEMORY

In the studies of infants in Gambia or the United Kingdom given the Chiron first-generation combined meningococcal A and C vaccine, serum antibody concentrations began to decline rapidly after conjugate vaccination.²³ An important question, therefore, is whether or not the conjugate vaccination induced memory B cells that might allow the infants to respond rapidly with an increase in serum anticapsular antibody concentration upon encountering group A or C meningococci. To examine this question, participants in the Gambian study were re-vaccinated at 18 to 24 months of age with an unconjugated meningococcal polysaccharide vaccine.²⁷ Prior to the booster injection, the serum antibody concentrations to the A or C polysaccharides in the

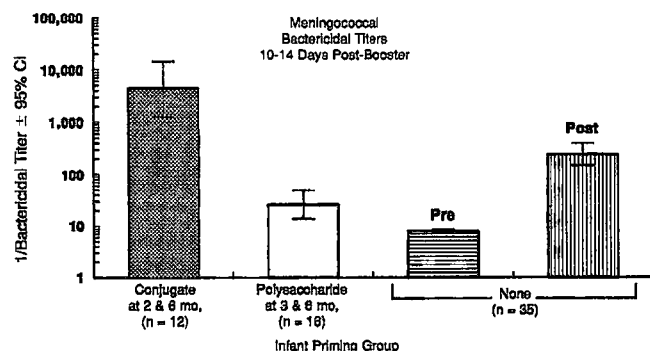


Figure 3. Effect of conjugate vaccine priming on serum meningococcal C bactericidal antibody responses to unconjugated meningococcal polysaccharide vaccine. Toddlers in Gambia were boosted with a dose of unconjugated meningococcal polysaccharide vaccine (Men-povax A plus C, Biocine S.p.A., Siena, Italy) at a mean age of 19.7 months. The subjects had been previously vaccinated either at 3 and 6 months of age with unconjugated meningococcal polysaccharide, or at 2 and 6 months of age with Chiron meningococcal A and C oligosaccharide-CRM conjugate vaccine.²³ A group of control unprimed toddlers from the same study area were vaccinated with unconjugated polysaccharide vaccine for the first time. Data shown are bactericidal titers measured in sera obtained 10 to 14 days after the polysaccharide booster vaccination. (Adapted from Leach A, et al.²⁷)

groups previously given the conjugate vaccine were not significantly different from those of toddlers of similar age who had not been previously vaccinated. However, as shown in Figure 3, the toddlers who had been primed with meningococcal conjugate vaccine at 2 and 6 months of age, and boosted with unconjugated meningococcal A and C polysaccharide vaccine, developed much higher anti-meningococcal C bactericidal titers after the booster vaccination than did Gambian toddlers of similar age immunized with unconjugated meningococcal polysaccharide vaccine for the first time. In contrast, the Gambian toddlers who had been primed with *unconjugated* meningococcal polysaccharide vaccine at 3 and 5 months of age showed significantly *lower* anti-C bactericidal responses to the polysaccharide booster injection than the control toddlers vaccinated for the first time (see Figure 3). This result confirms previous data suggesting that immunization at an early age with unconjugated meningococcal group C polysaccharide vaccine induces immunologic tolerance and impairs the ability to respond to a subsequent immunization with unconjugated meningococcal polysaccharide vaccines. The clinical importance of this finding is unknown. However, immunologic tolerance to meningococcal C polysaccharide could enhance susceptibility to developing invasive meningococcal disease by impairing the ability of the child to develop a protective serum anticapsular antibody response upon encountering the organism.

Although the Gambian toddlers who had been given conjugate vaccine as infants were primed for memory antibody responses to meningococcal C polysaccharide,

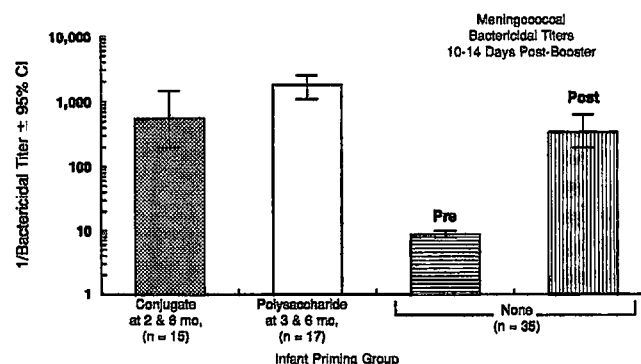


Figure 4. Effect of conjugate vaccine priming on serum meningococcal A bactericidal antibody responses to unconjugated meningococcal polysaccharide vaccine. Methods as described in Figure 3.

similar priming was not observed to the group A polysaccharide: that is, the magnitude of the anti-A antibody response to the booster injection of unconjugated polysaccharide vaccine was not significantly higher in the conjugate-primed group than in Gambian toddlers vaccinated with unconjugated meningococcal polysaccharide for the first time (Figure 4). Further, in contrast to meningococcal C polysaccharide, evidence of immunologic tolerance to meningococcal A polysaccharide was not observed in the toddlers previously given the unconjugated polysaccharide vaccine at 3 and 5 months of age (see Figure 4). Indeed, the toddlers vaccinated with unconjugated polysaccharide vaccine as infants appeared to have shown secondary antibody responses to the serogroup A polysaccharide booster. These data confirm the results of many previous studies indicating that group A and group C meningococcal unconjugated polysaccharide vaccines have very different immunologic properties⁴: specifically, meningococcal A polysaccharide vaccines are both immunogenic and protective in early infancy,³ and may even prime for secondary antibody responses to a subsequent injection.⁵ Meningococcal C polysaccharide vaccine shows none of these properties. The immunologic basis for these differences remains unknown.

Recently, toddlers who participated in the UCLA study of the Chiron first-generation meningococcal A and C conjugate vaccine were also given a booster injection of unconjugated meningococcal polysaccharide vaccine approximately 1 year later.²⁸ The group that had been primed with the conjugate vaccine showed evidence of induction of memory B cells to both the serogroup A and C polysaccharides, as evidenced by high serum bactericidal booster antibody responses. These results are in contrast to those of the Gambian infants given this conjugate vaccine in whom evidence of induction of memory B cells was limited to the serogroup C polysaccharide, and not the serogroup A polysaccharide (see above).²⁷ Further studies of immunologic priming induced by meningo-

coccal A conjugate vaccines at different ages and in different populations are needed to clarify this discrepancy.

MENINGOCOCCAL B POLYSACCHARIDE-PROTEIN CONJUGATE VACCINES

Investigational polysaccharide-protein conjugate vaccines also have been prepared for prevention of disease caused by serogroup B organisms.^{18,29-31} In general, these conjugates are much less immunogenic than those prepared with serogroup A or C polysaccharides. However, one promising immunogenic meningococcal B vaccine candidate is a conjugate in which the polysaccharide component has been modified by substitution of *N*-propionyl (NPr) groups for *N*-acetyl (NAC) groups (NPr-meningococcal B polysaccharide).³⁰ This vaccine is reported to elicit IgG bactericidal antibody responses in mice, rabbits, and subhuman primates. However, it will be a difficult task to prove that such vaccines are safe in humans, because, in mice, data suggest that NPr-meningococcal B polysaccharide-protein conjugate vaccines induce anti-NPr-meningococcal B polysaccharide antibodies that cross-react with native NAC-meningococcal B polysaccharide and also appear to have autoantibody activity.³² Whether or not the use of alternative substitutions on the polysaccharide, such as *N*-butanoyl,³³ or the administration of the NPr-meningococcal B polysaccharide conjugate vaccine with new adjuvants³⁴ will truly permit an antibody response in humans that is functional, entirely pathogen-specific, and not cross-reactive with host antigens, remains to be ascertained.

CONCLUSIONS AND THE FUTURE

Meningococcal A and C conjugate vaccines hold enormous promise for providing solid long-term protection to infant age groups that are currently poorly protected by licensed unconjugated meningococcal polysaccharide vaccines. Further, third-generation conjugate vaccines are under development in which the acquisition of immunity is accelerated and immunogenicity is enhanced by administration of the conjugate vaccine with novel adjuvants.³⁵ The use of adjuvants suitable for humans holds promise for decreasing the number of doses of conjugate vaccine required for induction of immunity in infants. This approach also may be useful for enhancing or maintaining immunogenicity of future multicomponent conjugate vaccines. For prevention of group B meningococcal disease, adjuvanted polysaccharide-protein conjugate vaccines also may prove to be immunogenic and protective, and help avoid some of the safety issues of inducing anticapsular autoantibody.³⁴ However, for prevention of meningococcal B disease, alternative approaches may be needed to avoid completely the safety

concerns of inducing anticapsular antibodies with autoantibody reactivity.

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Safety and immunogenicity of three doses of a *Neisseria meningitidis* A + C diphtheria conjugate vaccine in infants from Niger

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Background. High rates of endemic disease and recurrent epidemics of serogroup A and C meningococcal meningitis continue to occur in sub-Saharan Africa. A meningococcal A + C polysaccharide diphtheria-toxoid-conjugated vaccine may address this issue.

Methods. In Niger three doses of a bivalent meningococcal A + C diphtheria-toxoid-conjugated vaccine (MenD), containing 1, 4 or 16 µg of each polysaccharide per dose, administered at 6, 10 and 14 weeks of age, were compared with *Haemophilus influenzae* type b-tetanus toxoid-conjugated (PRP-T) vaccine given with the same schedule or with a meningococcal A + C polysaccharide vaccine (MenPS) given at 10 and 14 weeks of age. One blood sample was taken at the time of enrollment (6 weeks of age) and another was taken 4 weeks after the primary series.

Results. All doses of MenD were well-tolerated. After the primary series a higher proportion of infants had detectable serum bactericidal activity against serogroup A for each dose of MenD (from 94% to 100%) than for MenPS (31%) or *H. influenzae* type b-tetanus toxoid-conjugated vaccine (18.9%); $P \leq 0.05$. Significant differences were also observed for serogroup C MenD 4 µg or MenD 16 µg (100%) vs. MenPS (69.7%) or *Haemophilus influenzae* type b-tetanus toxoid-conjugated vaccine (24.3%); $P \leq 0.05$. When MenPS vaccine was given to 11-month-old children, the immune response measured by both enzyme-linked immunosorbent assay and serum

bactericidal assay was greater in those previously immunized with MenD than in those immunized with MenPS vaccine.

Conclusion. MenD was safe among infants in Niger, and immunization led to significantly greater functional antibody activity than with MenPS. The 4-µg dose of MenD for both the A and C serogroups has been selected for further studies.

INTRODUCTION

In Niger, which lies within the "meningitis belt" of sub-Saharan Africa,^{1,2} *Neisseria meningitidis* causes an annual average between epidemics of 30 cases of meningitis per 100 000 population; 85% of meningococcal cases are caused by serogroup A strains, with the remaining cases predominantly caused by serogroup C meningococci.³ Although meningococcal polysaccharide vaccine is currently used to control serogroup A and C epidemics in Niger, even if the epidemic is detected early, vaccination is nearly always started too late to prevent most cases. Conjugation of polysaccharide antigens to protein carriers could potentially overcome the poor immunogenicity of polysaccharide vaccines in infancy by converting a T cell-independent response to a T cell-dependent one and result in efficacious conjugate vaccines that could be incorporated into the Expanded Program of Immunization (EPI).

This study determined the immunogenicity and safety in infants from Niamey, Niger of three formulations (containing 1, 4 or 16 µg of both polysaccharides per dose) of *N. meningitidis* A + C-diphtheria toxoid-conjugated vaccine (MenD) in comparison with two controls: a meningococcal A + C polysaccharide vaccine (MenPS) and a *Haemophilus influenzae* type b-tetanus toxoid-conjugated vaccine (PRP-T). The anamnestic response was assessed 8 months after primary immunization by vaccination with a single dose of MenPS at 11 to 12 months of age in the children already immunized with MenD or MenPS. This study followed preliminary studies conducted in adults, toddlers and infants in the US to evaluate the safety and immunogenicity of the same three formulations of MenD.

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METHODS

The study was conducted between January, 1996, and March, 1997, at Yantala Centre de Santé Maternelle et Infantile in Niamey, Niger. The parents of the infants gave their written consent before inclusion, after being informed about the nature of the trial and any potential risks. If neither parent could read or write, two witnesses had to attest, by signing the consent form, that the parents had been properly informed about the protocol and had agreed to their child's inclusion. Free medical care was provided to the infants during the course of the study. The study protocol was approved by the Ethics Committee of Niger, the WHO Ethics Committee, the Western Institutional Review Board and the CDC's Institutional Review Board. It was also submitted to the Food and Drug Administration and conducted under the IND No. 6201. The study was performed in accordance with the Declaration of Helsinki and the 1996 ICH Good Clinical Practice guideline.

Subjects. Healthy children of either sex between 5 and 9 weeks of age were recruited for the study on presentation for routine EPI administration of the first diphtheria and tetanus toxoid-whole cell pertussis (DTP) vaccine.

Vaccines. The study vaccine was a polysaccharide-protein conjugate vaccine manufactured by Aventis Pasteur (Swiftwater, PA) prepared with serogroup A and C meningococcal polysaccharides, each covalently bound to diphtheria toxoid (MenD). Three vaccine lots were prepared (Lots 950213, 950214 and 950215) that contained, respectively, per 0.5-ml dose, 1, 4 or 16 μ g of each polysaccharide and 5.8, 32.2 or 161.0 μ g of diphtheria toxoid. The investigational vaccine was presented in a prefilled (0.5-ml) ready to use glass syringe.

Infants in the control groups received either meningococcal polysaccharide A + C vaccine (MenPS, Vaccin Polyosidique Meningococcique A + C, Batches L0878 and L0991, containing 50 μ g of group A and 50 μ g of group C meningococcal polysaccharides) or one 0.5-ml

dose of *H. influenzae* type b conjugate vaccine (PRP-T, Act-HIB, Batch L0670), both manufactured by Aventis Pasteur (Lyon, France). The placebo was 0.5 ml of a 0.4% normal saline solution. The study vaccine or control vaccines were administered intramuscularly in the anterolateral aspect of the right thigh.

Infants recruited into the study already had received Calmette-Guérin bacillus and oral polio vaccine at birth according to Niger's EPI schedule. At the ages of 6, 10 and 14 weeks, they received DTP vaccine subcutaneously in the left deltoid region and oral polio vaccine. At 9 months of age measles and yellow fever vaccines were administered. All vaccines were obtained from EPI-approved vaccine manufacturers.

Study design. This was a randomized dose escalation study. A total of 180 children were randomly allocated to 1 of 5 groups (Table 1). The 108 infants allocated to the MenD group received 1 of the 3 formulations of the meningococcal conjugate vaccine at 6, 10 and 14 weeks of age. Dose escalation was sequential. A first subgroup of 36 infants received the 1- μ g polysaccharide dose; if no reactogenicity was observed exceeding that commonly expected for DTP vaccine, then a second group of 36 infants received the 4- μ g polysaccharide dose; a third group of 36 infants received the 16- μ g polysaccharide dose. Two other groups of infants in this joint randomization, ~36 subjects each, enrolled as 3 series of 12 subjects, served as controls at each step. In the preceding year, 1995, a massive serogroup A meningococcal outbreak in Niger had caused more than 40 000 reported cases,⁴ so the study was designed such that all participating infants would be offered a meningococcal vaccine by the end of the study. Thus one control group received the MenPS vaccine (1 dose of saline placebo at 6 weeks and 2 doses of MenPS at 10 and 14 weeks of age), and the other group, the PRP-T vaccine control group (3 doses at 6, 10 and 14 weeks of age), received a dose of meningococcal A + C polysaccharide vaccine at 18 weeks of age, after participation in the initial study was complete.

TABLE 1. Design of a randomized trial in Niamey, Niger, assessing the safety and immunogenicity of three doses of MenD, MenPS or PRP-T

Primary Series							Amendment MenPS Vaccination at 11-12 mo of Age			
Study Groups		Preinjection blood sample	Injections				Postinjection blood sample	Preinjection blood sample	Injection	Postinjection blood sample
Vaccine	N	6 wk	6 wk	10 wk	14 wk	18 wk	11-12 mo	11-12 mo	+4 wk after MenPS	
MenD 1 μ g	36	◆*	MenD 1 μ g	MenD 1 μ g	MenD 1 μ g	◆	◆	MenPS	◆	
MenD 4 μ g	36	◆	MenD 4 μ g	MenD 4 μ g	MenD 4 μ g	◆	◆	MenPS	◆	
MenD 16 μ g	36	◆	MenD 16 μ g	MenD 16 μ g	MenD 16 μ g	◆	◆	MenPS	◆	
MenPS	35	◆	Placebo	MenPS	MenPS	◆	◆	MenPS	◆	
PRP-T†	37	◆	PRP-T	PRP-T	PRP-T	◆				

* ♦, blood sample.

† Infants in the PRP-T control group were offered a dose of meningococcal A + C polysaccharide vaccine at 18 weeks of age, but antibodies were not measured subsequently.

To evaluate the impact of MenD vaccine on memory priming, as compared with the plain polysaccharide vaccine, an amendment to this study was proposed in December, 1996, such that all infants of the MenD and MenPS groups received one dose of MenPS vaccine between 11 and 12 months of age. (Infants of the PRP-T control group were not tested for memory priming.)

Two blood samples were collected by venipuncture from each infant at 6 and 18 weeks of age. For infants in the MenD and MenPS groups included in the study amendment, two additional blood samples were collected, one before the MenPS vaccination dose at 11 months of age and one 4 weeks later.

The parents, the home visitors who assessed local and systemic reactions and the laboratory personnel were blinded to the subjects' study groups.

Reactogenicity. Infants in the study were observed in the clinic for 15 min after each vaccination for immediate reactions. During the dose escalation stage of this study, local and systemic reactions were monitored by home visitors during each of the 5 days after each injection; after MenPS vaccination at 11 months of age, local and systemic events were monitored at 24 and 72 h after injection, respectively.

Laboratory methods. Meningococcal A and C polysaccharide total antibody concentrations (measured as IgG) were determined for all serum samples using a standardized enzyme-linked immunosorbent assay (ELISA) at Aventis Pasteur (US) and expressed as micrograms/ml.^{5,6} Serum bactericidal activity (SBA) was measured for all specimens at the Centers for Disease Control and Prevention Laboratory (Atlanta, GA) by adding group-specific meningococcal bacteria in the presence of baby rabbit origin complement to serial dilutions of a serum sample.⁷ Bactericidal activity was defined as the reciprocal serum dilution yielding $\geq 50\%$ killing as compared with the controls. Seroprotection levels were defined as the percentage of subjects with postimmunization ELISA antibody concentrations ≥ 2 $\mu\text{g/ml}$ and reciprocal SBA titers ≥ 8 .^{8,9}

Data analysis. Safety data were described for each group and each vaccine dose by the percentage of infants with at least one local or systemic reaction. Geometric mean titer (GMT) values of ELISA antibody concentrations and SBA activities, as well as the seroprotection rates, were calculated with their 95% confidence intervals. Groups were compared by analysis of covariance, including the log₁₀-transformed 18-week baseline titer as the dependent variable, the log₁₀-transformed 6-week baseline titer as the independent factor and the group-by-baseline interaction as the covariate. As a result of multiple endpoints (i.e. two serogroups) and multiple comparisons, statistical significance was declared for the global comparison at an alpha risk of 0.025 (two-sided) and of the linear con-

trasts at an alpha risk of 0.00625 (two-sided). Statistical analysis was carried out by the Aventis Pasteur Biometry Department (Lyon, France) on SAS Version 6.08 (SAS Inc., Cary, NC)

RESULTS

A total of 180 infants were enrolled; the male-to-female ratio was 0.97 and the mean age at inclusion was 44.8 ± 5.8 days, with no difference between the study groups for either characteristic. Eight months later 116 infants were recontacted and included in the 11-month MenPS vaccination.

Local and systemic reactions after primary series. No child suffered an immediate reaction during the 15 min after any injection. The most frequently observed local reactions were tenderness and redness at the site of injection (Table 2), which were mild and short lived. The cumulative frequency of tenderness after three injections at the injection site in the three MenD groups combined was 37.6%; for MenPS, it was 30.8%, and for PRP-T, 19.6%. The cumulative frequency of redness after three injections was 18.1% for children in the three MenD groups, 7.5% for MenPS recipients and 11.2% for PRP-T recipients. There was no increase in the incidence of local reactions with subsequent injections during the primary immunization series.

The systemic reactions in the MenD 16- μg vaccine group, ranging from 38.9 to 57.1%, appeared to be more frequent than with either one of the lower doses, which were never more than 37% (Table 2). The rates of systemic reactions during the primary immunization series also did not increase with successive injections in any group.

Local and systemic reactions after MenPS vaccination of the 11-month-old-children. The reactogenicity profile after the MenPS vaccination at 11 months of age in infants who had previously received the MenD vaccine was similar to that observed (Table 2) during the primary series. There appeared to be, once again, a more pronounced local reactogenicity observed among the infants receiving MenD at the lower doses as compared with the MenD 16- μg group. This was particularly noteworthy for local tenderness (Data not shown).

Serious adverse events. Eight serious adverse events requiring hospitalization were reported during the trial, all of which were considered by the investigator either to be unrelated to or have an improbable relationship with the study vaccine. In the 1- μg MenD group, there was one case of diarrhea and dehydration associated with fever, and one infant in the MenPS group developed an intercurrent infection and severe dehydration; both led to the death of the child. For all other events, by the end of documented study follow-up, the infant had at least recovered partially.

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TABLE 2. Percentage of infants experiencing local or systemic reactions during the 5 days after primary immunization at 6, 10 and 14 weeks of age with MenD, MenPS or PRP-T

		Local Reaction (%)					Systemic Reaction (%)				
	N*	>1	Redness	Tenderness	Other†		Fever		Vomiting	Diarrhea	Irritability
						>1	>38°C	>39°C			
Dose 1											
MenD											
1 µg	36	66.7	36.1	55.6	13.9	33.3	5.6	0	8.3	8.3	16.7
4 µg	36	41.7	22.2	41.7	0	27.8	2.8	2.8	8.3	11.1	8.3
16 µg	36	22.2	8.3	22.2	0	38.9	13.9	2.8	8.3	11.1	13.9
MenPS	35	25.7	11.4	25.7	5.7	31.4	5.7	0	17.1	11.4	17.1
PRP-T	37	29.7	16.2	29.7	2.7	21.6	5.4	0	10.8	2.7	10.8
Dose 2											
MenD											
1 µg	35	54.3	25.7	51.4	0	37.1	8.6	0	5.7	17.1	8.6
4 µg	35	42.9	20.0	42.9	0	28.6	2.9	0	0	22.9	2.9
16 µg	35	31.4	8.6	31.4	0	57.1	17.1	0	8.6	11.4	34.3
MenPS	35	28.6	8.6	28.6	5.7	45.7	14.3	0	14.3	14.3	25.7
PRP-T	37	24.3	8.1	24.3	0	37.8	8.1	0	8.1	10.8	24.3
Dose 3											
MenD											
1 µg	35	48.6	20.0	48.6	0	25.7	14.3	2.9	2.9	11.4	5.7
4 µg	34	32.4	11.8	32.4	0	11.8	0	0	0	5.9	5.9
16 µg	32	9.4	9.4	9.4	0	40.6	3.1	0	3.1	15.6	25.0
MenPS	33	9.1	3.0	9.1	0	36.4	6.1	0	0	30.3	9.1
PRP-T	36	25.0	8.3	25.0	0	38.9	5.6	0	5.6	13.9	19.4

* Total number of infants, 180.

† Other local reactions: edema, induration, crying on movement of injected limb.

Immunogenicity: antibody response measured by ELISA. Table 3 shows the immune response expressed in terms of GMT values of antibody measured by ELISA. (In the text that follows, the proportion of infants achieving an antibody titer ≥ 2 µg/ml is given in parentheses.)

For serogroup A, the post-Dose 3 immune responses by ELISA observed with the MenD vaccine were 4.6 µg/ml (88.6%), 5.8 µg/ml (88.2%) and 9.9 µg/ml (96.9%) for the 1-, 4- and 16-µg vaccine doses, respectively. There was a significant difference in dose effect between the 16-µg and the 1-µg dose ($P = 0.0002$). For serogroup C the post-Dose 3 ELISA immune responses

measured with the MenD vaccine were 1.5 µg/ml (34.0%), 2.8 µg/ml (64.5%) and 4.8 µg/ml (87.5%), respectively, for the three dose levels. The MenPS vaccine induced comparable ELISA results for serogroups A, 5.5 µg/ml (87.9%), and C, 5.3 µg/ml (81.8%). After the 11-month MenPS vaccination we observed serum antibody titers ≥ 2 µg/ml in 91.7, 83.3 and 69.2% against serogroup A and 83.3, 83.3 and 92.3% against serogroup C, for the 1-, 4- and 16-µg MenD dose groups, respectively. The same proportion of infants in the MenPS group (58.6%) had seroprotective levels against serogroups A and C after this 11-month MenPS vaccination.

TABLE 3. ELISA antibody titers (micrograms/ml) against meningococcal serogroups A + C before and after both the primary series immunization (6, 10 and 14 weeks of age) and the vaccination with the plain meningococcal A + C polysaccharide vaccine given at 11 to 12 months of age

Vaccine Group	6 wk (Prevaccination)			18 wk (Post-primary Series)		Before the 11-12-mo MenPS Vaccination			1 mo after the 11-12-mo MenPS Vaccination	
	n	GMT	95% CI	GMT	95% CI	n	GMT	95% CI	GMT	95% CI
Serogroup A										
MenD (1 µg)	35	2.7	1.7-4.2	4.6	3.6-5.8	25	0.4	0.2-0.6	10	5.5-18.3
MenD (4 µg)	34	3.4	2.2-5.4	5.8	4.3-8.0	31	0.2	0.1-0.3	7.0	4.0-12.0
MenD (16 µg)	32	3.6	2.4-5.4	9.9*	7.6-12.9	27	0.3	0.2-0.5	6.0	3.3-11.0
MenPS	35	3.0	1.7-5.3	5.5	4.1-7.3	30	0.9	0.5-1.4	3.1	2.0-4.7
PRP-T	36	3.2	2.2-4.5	0.6†	0.4-0.8	NA	NA	NA	NA	NA
Serogroup C										
MenD (1 µg)	35	1.9	1.2-3.0	1.5‡	1.1-2.0	25	0.2	0.1-0.3	8.9	5.3-15.0
MenD (4 µg)	31	1.6	0.9-2.8	2.8	2.0-3.9	31	0.1	0.1-0.2	8.1	4.5-14.6
MenD (16 µg)	32	2.4	1.5-4.0	4.8	3.7-6.3	27	0.3	0.2-0.5	8.3	5.4-12.9
MenPS	35	2.3	1.4-3.9	5.3	3.8-7.4	30	0.6	0.3-1.0	2.8	1.7-4.7
PRP-T	36	2.0	1.2-3.5	0.5†	0.3-0.7	NA	NA	NA	NA	NA

* MenD 1 µg vs. MenD 16 µg: $P = 0.0002$ (ANOVA).

† PRP-T vs. all MenD doses and MenPS: $P = 0.0001$ (ANOVA).

‡ MenD 1 µg vs. MenD 16 µg and MenD 4 µg: $P = 0.001$ (ANOVA).

NA, not applicable; ANOVA, analysis of variance.

Immunogenicity: SBA. Table 4 shows the SBA expressed as GMTs, the reciprocal of the dilution. (In the following text the proportion of infants achieving reciprocal titers ≥ 8 and the *P* value of the 95% confidence intervals (CIs) comparison are given in parentheses.)

For serogroup A the post-Dose 3 immune responses observed with the MenD vaccine were 261 (97.1%), 171 (94%) and 370 (100%) for the three levels of doses, with no statistically significant difference between them. In contrast the SBA values in the MenPS and the PRP-T groups were 7.0 (31%) and 6.7 (18.9%), respectively, each significantly less than for the MenD groups: $P = 0.0001$ ($P \leq 0.05$ when comparing the 95% CIs). For serogroup C the SBA values were 72.1 (94.3%), 189 (100%) and 325 (100%) for the three doses, with a statistically significant dose effect: $P = 0.0001$. The SBA values in the MenPS and the PRP-T groups were 25.4 (69.7%) and 7.3 (24.3%), respectively, both significantly less than for the MenD groups: $P = 0.0001$ ($P \leq 0.01$ when comparing MenD 1 μg vs. both the 4- and 16- μg MenD groups). One month after the 11-month MenPS vaccination, for both serogroups we observed good SBA GMT values and seroprotection rates at least equal to 90% in the MenD groups (Table 4). These immune responses appeared to be inversely correlated with the dose and better than that noted for the MenPS group. The same proportion of infants in the MenPS group appeared to be seroprotected (59.3%) against serogroups A and C after this 11-month MenPS vaccination.

DISCUSSION

The objectives of this study were to investigate the safety and immunogenicity in infants in Africa of a new meningococcal conjugate vaccine and to select a dose

for future studies that might allow inclusion of this vaccine in the EPI.

All doses of MenD vaccines were well-tolerated. This is consistent with previous studies indicating that polysaccharide-protein conjugate vaccines, including those directed against *N. meningitidis*, are moderately reactogenic.¹⁰⁻¹² The local reactogenicity profiles of the different doses of MenD vaccines appear comparable with those of MenPS and PRP-T vaccines, although there appeared to be a slight decrease in the local reaction rates when the dose was increased. This may have been the result of either detection bias¹³ introduced by the sequential approach or a true immunologic effect of the low doses. No difference was observed between the incidences of the systemic reactions observed for the conjugate vaccine and the licensed meningococcal polysaccharide vaccines, with the exception of an apparent increase after three injections of the MenD 16- μg dose, which might be attributable to the greater quantity of diphtheria toxoid.

We used the 2- $\mu\text{g}/\text{ml}$ cutoff in ELISA measurements and set the limit of detection of the SBA assay (i.e. 1:8) to define seroprotection; however, the seroprotective level of meningococcal antibodies measured by ELISA is not known for certain, but the evidence suggests it to be 1 to 2 $\mu\text{g}/\text{ml}$.¹⁴⁻¹⁶ Likewise the functional antibody concentration indicative of protection has not been established^{7, 14} for the SBA assay used in this study.

After primary immunization MenD elicited ELISA antibody concentrations against serogroup A that were comparable with those obtained after immunization with MenPS, whereas concentrations of antibodies against serogroup C tended to be lower after immunization with the MenD vaccine, especially at the 1- μg dose. The ELISA antibody concentration induced by the 1- μg MenD vaccine was significantly lower than

TABLE 4. Serum bactericidal activity titers (reciprocal of dilution) against meningococcal serogroups A + C polysaccharides before and after both the primary series immunization (6, 10 and 14 weeks of age) and the vaccination with the plain meningococcal A + C polysaccharide vaccine given at 11 to 12 months of age

Vaccine Group	6 wk (Prevaccination)			18 wk (Post-primary Series)		Before the 11-12-mo MenPS Vaccination			1 mo after the 11-12-mo MenPS Vaccination	
	n	GMT	95% CI	GMT	95% CI	n	GMT	95% CI	GMT	95% CI
Serogroup A										
MenD (1 μg)	32	7.6	4.9-11.8	261	152-450	22	17.9	8.1-39.4	747	393-1419
MenD (4 μg)	34	11.8	7.2-19.3	177	101-312	24	10.1	5.6-18	373	163-853
MenD (16 μg)	32	10.9	6.7-17.5	370	264-518	21	16.6	8.5-32.4	184	78.6-431
MenPS	32	14.7	8.5-25.4	7.0*	4.7-10.5	26	6.1	3.9-9.5	24.1	11-53
PRP-T	35	11.2	6.8-18.3	6.7*	4.3-10.5	NA	NA	NA	NA	NA
Serogroup C										
MenD (1 μg)	35	37.2	24-107	72.1†	45.5-114	25	5.4	3.7-3.8	264	104-677
MenD (4 μg)	34	50.8	24-107	189	128-278	27	4.6	3.6-5.6	287	96.2-858
MenD (16 μg)	32	61.6	29-131	325	204-517	20	6.9	4.7-11.4	178	63.6-499
MenPS	32	62.7	29-131	25.4*	14.4-44.6	26	4.1	3.9-4.3	14.4	7.9-26.1
PRP-T	36	45.3	21.9-133	7.3*	7.7-11.3	NA	NA	NA	NA	NA

* All MenD doses vs. the two control groups: $P = 0.0001$ (ANOVA).

† MenD 1 μg statistically different from both the 4- and 16- μg MenD groups: $P < 0.01$ (SNK).

NA, not applicable; ANOVA, analysis of variance, SNK, Student-Newman-Keuls test.

that for the MenPS vaccine. In contrast to the response to the primary series, concentrations of antibodies measured by ELISA against serogroup A or C after MenPS vaccination at 11 months of age were higher in infants initially immunized with MenD as compared with MenPS.

Goldschneider¹⁷ and others¹⁸ demonstrated that serum bactericidal assays measure functional antibody activity, whereas the standard ELISA^{5,6} determines serum antibody concentrations even in the absence of detectable bactericidal activity.^{11, 19-21} The results of bactericidal assays reveal major differences between the responses to conjugate and polysaccharide vaccines. SBA values measured against both serogroup A and serogroup C after primary immunization are substantially greater among children who received MenD than among those who had initially received MenPS. After the injection at 11 months of age of a dose of polysaccharide vaccine, the GMT values for the SBA were 10 to 25 times greater for those infants previously immunized with MenD vaccine than for those who had received MenPS vaccine.

The 4- μ g dose for both the A and C serogroups has been selected for further studies of MenD vaccine based on a balance of several factors, including the post-primary and post-11-month-MenPS immunogenicity results (ELISA and SBA) as well as the amount of diphtheria protein carrier that would be administered. For the response to serogroup C the observed difference in SBA, after the primary vaccination series, between the 1- μ g dose and larger doses suggested that at least 4 μ g would be needed for subsequent serogroup C formulations. Either a 1- μ g or a 4- μ g dose of serogroup A seemed reasonable based on SBA activity after the primary series and after polysaccharide vaccination at 11 months of age.

The dramatic antibody increase observed in this study after polysaccharide vaccination at 11 months in the MenD groups, in comparison with the MenPS group, supports the hypothesis that conjugate vaccines are able to induce immune memory at a young age. To evaluate this hypothesis further, an additional study is assessing, in children already primed with one to three doses of MenD vaccine during infancy, the immune response 7 to 10 days after MenPS vaccination at 24 months of age. Both total IgG and immunoglobulin classes, as well as SBA, elicited by this conjugate vaccine, in comparison with the plain polysaccharide vaccine, will be measured.

Some authors, like Goldschneider,¹⁷ Gold,^{22, 23} Twumasi¹⁰ and Leach,²⁴ have called attention to the possibility that multiple doses of serogroup C polysaccharide antigen administered in infancy may lead to immunologic hyporesponsiveness against *N. meningitidis* serogroup C. In these studies the post-second year

of life polysaccharide vaccination titer measured for the serogroup C in the polysaccharide vaccine arm was not as elevated as might have been anticipated if the children had not been vaccinated. Others have since reported similar results both in children²⁵ and in adults.²⁶ On the other hand Peltola,²⁷ in a trial conducted in 1985 in Finland, called into question any suppressive effect when the first dose of group C polysaccharide is given at 3 months of age. The relevance of these observations is unknown.

In our study in Niger we observed a decline in SBA against serogroup C in the MenPS vaccine group from the pre- (62.7) to the post-primary series serum level (25.4); however, we have noted a higher SBA GMT value 1 month after the primary series of MenPS vaccine (25.4) in comparison with the PRP-T vaccine group (7.3). After boosting the SBA increased from 4 (pre-) to 14 (post-) in the MenPS vaccine group.

We have also observed in the MenPS vaccine group, with either immunogenicity assay, that 1 month after the 11-month MenPS vaccination the same proportion of children were seroprotected against both serogroups (58.6% against A and C by ELISA, 59.3% against A and C by SBA), suggesting that this MenPS-induced hyporesponsiveness is comparable for both serogroups. It would be useful to evaluate the meningococcal serogroup A and C immune status of the children who participated in this study several years later.

This study shows that the MenD vaccine is safe and that it is superior to currently licensed polysaccharide vaccines in terms of induction of functional antibodies in infants. The greater antibody GMT values, measured by ELISA or SBA, after MenPS vaccination in 11-month-old children already primed with the MenD vaccine, as compared with those primed with MenPS vaccine, suggest that the polysaccharide-protein conjugate vaccine is capable of inducing a measurable degree of immunologic memory at least up to 11 months of age.

Additional data on the number of MenD vaccine doses needed, as well as the duration of protective immunity, will help to determine a schedule by which infant immunization with meningococcal A + C conjugate vaccine can best provide long term protection against recurrent meningococcal meningitis epidemics in Africa.

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(21) International Application Number: PCT/US98/06946 (22) International Filing Date: 9 April 1998 (09.04.98) (30) Priority Data: 60/069,885 9 April 1997 (09.04.97) US 08/936,107 23 September 1997 (23.09.97) US (71) Applicant: EMORY UNIVERSITY [US/US]; 2009 Ridgewood Drive, Atlanta, GA 30322 (US). (72) Inventors: STEPHENS, David, S.; 5221 Gauley River Drive, Stone Mountain, GA 30087 (US). SWARTLEY, John, S.; 7 Wendy Lane, Westport, CT 06880 (US). (74) Agents: FERBER, Donna, M. et al.; Greenlee, Winner and Sullivan, P.C., Suite 201, 5370 Manhattan Circle, Boulder, CO 80303 (US).		(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: SEROGROUP-SPECIFIC NUCLEOTIDE SEQUENCES IN THE MOLECULAR TYPING OF BACTERIAL ISOLATES AND THE PREPARATION OF VACCINES THERETO (57) Abstract The present disclosure provides specific nucleotide sequences and diagnostic methods for prototype serogroup A, B, C, Y and W-135 strains of <i>Neisseria meningitidis</i> . Due to capsule switching <i>in vivo</i> , closely related virulent meningococcal clones may not be recognized by traditional serogroup-based surveillance, and these strains can escape vaccine-induced or natural protective immunity by capsule switching. The invention provides recombinant meningococcal strains, recombinant DNA constructs and immunological preparations useful as diagnostic probes for detection and diagnosis of meningococcal diseases, screening for specific meningococcal serogroups and broad based immunizations with multivalent capsular polysaccharide conjugate vaccines.		

SEROGROUP-SPECIFIC NUCLEOTIDE SEQUENCES
IN THE MOLECULAR TYPING OF BACTERIAL ISOLATES
AND THE PREPARATION OF VACCINES THERETO

This invention was made, at least in part, with funding from the United States
5 National Institute of Allergy and Infectious Diseases, Grant No. AI40247-01. Accordingly,
the United States government may have certain rights in this invention.

BACKGROUND OF THE INVENTION

This invention relates generally to the nucleotide sequences of serogroup-specific
capsular polysaccharides genes and their use in a method for typing of serogroups of
10 pathogenic bacteria, in particular *Neisseria meningitidis*, and further, relates to capsule gene
switching in recombinant strains and the detection thereof.

Contagious outbreaks of epidemic diseases constitute public health emergencies
requiring rapid treatment and chemoprophylaxis of contacts. Vaccination of the population at
risk can be considered if disease cases continue to occur. However, asymptomatic carriage of
15 pathogens in humans is common and some of the adult population may be immunized from
previous outbreaks. The factors leading from acquisition of the organism to invasive disease
point to a clonal origin of the outbreaks and to an enhanced virulence or altered antigenicity
of a particular clone.

Neisseria meningitidis is a leading worldwide cause of meningitis and rapidly fatal
20 sepsis in otherwise health individuals [Apicella, M.A. (1995) in *Principles and Practice of
Infectious Diseases*, eds. Mandell, G.L., Douglas, R.G., and Bennett, J.E., Churchill
Livingstone, New York, pp. 1896-1909]. In excess of 350,000 cases of meningococcal
disease were estimated to have occurred in 1995 [WHO Report (1996) WHO, Geneva, ISBN
92 4 1561823]. The problem of meningococcal disease is emphasized by the recurrence of
25 major epidemics due to serogroups A, B, and C *N. meningitidis* over the last 20 years, such
as: the devastating serogroup A outbreak in sub-Saharan Africa in 1996 [WHO (1996)
Meningitis in Africa. The constant challenge of epidemics. WHO 21:15 March]; the recent
dramatic increases in the incidence of serogroup B and C meningococcal disease in parts of

North America [CDC (1995) *MMWR* 44:121-134; Jackson, L.A. et al. (1995) *JAMA* 273:390-394; Wahlen, C.M. et al. (1995) *JAMA* 273:383-389]; and the emergence in Europe and elsewhere of meningococci with decreased susceptibility to antibiotics [Campos, J. et al. (1992) *J. Infect. Dis.* 166:173-177].

5 Differences in capsular polysaccharide structure determine the meningococcal serogroups [Liu, T.Y. et al. (1971) *J. Biol. Chem.* 246:2849-58; Liu, T.Y. et al. (1971) *J. Biol. Chem.* 246:4703-12]. Meningococci of serogroups B, C, Y, and W-135 express capsules composed entirely of polysialic acid or sialic acid linked to glucose or galactose [Liu, T.Y. et al. (1971) *J. Biol. Chem.* 246:4703-12; Bhattacharjee, A.K. et al. (1976) *Can. J. Biochem.* 54:1-8], while the capsule of group A *N. meningitidis* is composed of *N*-acetyl mannosamine-1-phosphate [Liu, T.Y. et al. (1971) *J. Biol. Chem.* 246:2849-58]. The currently available capsular polysaccharide vaccines for serogroups A, C, Y, or W-135 *N. meningitidis* are effective for control of meningococcal outbreaks in older children and adults. However, because of poor immunogenicity in young children and short-lived immunity [Zollinger, 10 W.D. and Moran, E. (1991) *Trans. R. Soc. Trop. Med. Hyg.* 85:37-43], these vaccines are not routinely used for long-term prevention of meningococcal disease. In the case of group B *N. meningitidis*, whose (α 2-8)-linked polysialic capsule is an immunotolerized self antigen, a reliable polysaccharide vaccine is not yet available. However, rapid progress is being made in development of polysaccharide-protein conjugate vaccines.

20 In some epidemic settings, simultaneous or closely-linked meningococcal outbreaks have occurred in the same population due to different serogroups [Sacchi, C.T. et al. (1994) *J. Clin. Microbiol.* 32:1783-1787; CDC (1995) *MMWR* 44:121-134; Krizova, P. and Musilek, M. (1994) *Centr. Eur. J. Publ. Hlth* 3:189-194]. Further, Caugant et al. [Caugant, D.A. et al. (1986) *Proc. Natl. Acad. Sci. USA* 83:4927-4931; Caugant, D. A. et al. (1987) *J. Bacteriol.* 169:2781-2792] and others have noted that meningococcal isolates of different serogroups may be members of the same enzyme type (ET)-5, ET-37 or ET-4 clonal complexes.

Since 1993, the number of cases of serogroup B meningococcal disease in Oregon and adjacent counties in Washington State has doubled. The U.S. serogroup B meningococcal strains are closely related to the ET-5 complex. ET-5 complex strains have been responsible 30 for major epidemics in Norway, Iceland, Cuba and South America over the last twenty years [Caugant, D.A. et al. (1986) *Proc. Natl. Acad. Sci. USA* 83:4927-4931; Sierra, G.V. et al.

(1991) *NIPH Annals* 14:195-207; Sacchi, C.T. et al. (1992) *J. Clin. Microbiol.* 30:1734-1738]. Since 1994, cases of serogroup C meningococcal disease due to ET-5 complex strains were also noted in Oregon and Washington State. There exists an urgent need to understand the genetic basis for meningococcal capsule expression and to rapidly diagnose strains of serogroup A and other serogroups of *N. meningitidis*.

SUMMARY OF THE INVENTION

The present invention provides the nucleotide sequence of the intergenic region separating *ctrA* from the biosynthesis operon (*synA-D,E,F,G*) of a serogroup A *N. meningitidis*. Whereas in serogroups B, C, Y and W-135 *N. meningitidis*, the intergenic region separating *ctrA* from the biosynthesis operon (*synA-D,E,F,G*) is 134 bp and contains the *ctrA-D* promoter as well as the divergent biosynthesis operon promoter and other transcriptional regulatory elements, in serogroup A *N. meningitidis* the intergenic region is 218 bp in length and does not share any homology with the 134 bp region found in the sialic acid capsular serogroups.

This invention also provides evidence that the DNA located between *ctrA* and *galE* in serogroup A *N. meningitidis* is a cassette containing four genes ORF1-ORF4 responsible for the production of serogroup A capsule from UDP-N-acetylglucosamine. The predicted amino acid sequences encoded by ORF1-ORF4 are also provided by the present invention.

Further, according to this invention, the ORF1-ORF4 genes are divergently co-transcribed from overlapping promoters located in a short intergenic region separating the capsule biosynthetic and transport operons. Mutagenesis of these genes demonstrates the involvement of these genes in serogroup A capsule production.

The invention also provides a model in which meningococcal capsular serogroups are determined by specific genetic biosynthesis cassettes that insert between the *ctrA* operon and *galE*. In specific embodiments, it is demonstrated for serogroup A meningococci that the cassettes determining specificity of serogroups can recombine to switch the type of capsule and serogroup expressed. Such information is critical to the design of improved group A and other meningococcal vaccines and to the understanding of the molecular basis of serogroup A pathogenesis.

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It is an object of the present invention to provide strains of *N. meningitidis* of a particular serogroup transformed *in vitro* to express a capsule polysaccharide marker of a different meningococcal strain serogroup. In a particular embodiment are provided prototype serogroup C, Y and W-135 meningococcal strains transformed *in vitro* with DNA comprising the *synD* of the serogroup B strain NMB. According to the present invention, conversion from one sialic acid expressing capsule serogroup to another can be accomplished by homologous recombination of the sequences encoding the serogroup-specific capsule polymerase. Such recombinant *N. meningitidis* strains are provided according to the invention as genetically engineered *in vitro* recombinations.

Also provided by the present invention are *Neisseria meningitidis* mutant serogroup strains which express different non-isogeneic capsular polysaccharides due to homologous recombination of the sequences encoding the serogroup-specific capsule polymerase. Specifically exemplified herein is a mutant *N. meningitidis* strain 1070 (serogroup B, ET-301) in which genetic markers are isogeneic to serogroup B except for the capsule polysaccharide, which is a serogroup C marker. Such meningococcal isolates comprise a recombinant or switched capsule gene and, in a particular embodiment, a switching or recombination event occurred from a serogroup B to a serogroup C capsule biosynthetic gene. Such recombinant *N. meningitidis* strains are provided according to the invention as naturally-occurring *in vivo* recombinant isolates.

It is also an object of the invention to provide meningococcal serogroup-specific capsule genes encoding characteristic capsular polysaccharide virulence determinants. In specific embodiments of the invention are provided capsule biosynthetic gene preparations of prototype serogroups A, B, C, Y and W-135, each serogroup-specific gene encoding a biosynthetic enzyme for specific and distinguishing capsular polysaccharide.

It is an additional object of the invention to provide cloned DNA molecules which can be used to introduce an additional non-isogeneic capsular polysaccharide virulence determinant into strains of *N. meningitidis*. In a particular embodiment, the cloned DNA fragment containing the stable Tn916 insertion in the *synD* of the serogroup B *N. meningitidis* strain NMB was used to introduce the gene for the serogroup B (α 2-8)-linked capsule polysialyltransferase into other meningococcal strains to produce novel immunotypes. More generally, a cloned DNA fragment containing a stable insertion of a polysialyltransferase gene

of a specific serogroup strain can be used to introduce the corresponding capsular polysaccharide determinant into serologically different strains to produce novel immunotypes. This invention also contemplates that multiple non-isogenic capsular polysaccharide virulence determinants can be introduced into serologically different meningococcal strains.

5 It is an added object of the present invention to provide protective immunity from virulent meningococcal strains that can escape vaccine-induced or natural protective immunity by capsule switching. In particular embodiments, the invention provides multivalent vaccines which effectively anticipate capsule switching events. According to the invention, broad immunization with capsular polysaccharide vaccines effective against all
10 major capsular serogroups can be used to control epidemics and endemic disease or vaccines for particular serogroups can be used in geographic areas (or for travelers to areas) where the cognate serogroup is endemic.

It is yet another object of the invention to provide a method for diagnostic detection and serogroup typing of *N. meningitidis* strains, especially those of Group A. This method is
15 a nucleic acid amplification (e.g., PCR) method or nucleic acid hybridization method based on (a) the specific nucleotide and encoded amino acid sequences of serogroup-specific capsular polysaccharide determinants and (b) oligonucleotide primers designed to anneal to specific capsule polymerase sequences. This method of the invention was particularly exemplified in the typing of *N. meningitidis* serogroups A,B,C, Y and W-135. This nucleic
20 acid amplification method of the invention, based on the use of discriminatory primers derived from serogroup-specific nucleotide sequences (Sequo-grouping), offers advantages over current methods of diagnostic detection of serogroup typing in (a) being independent of the need to grow pathogenic organisms for immunological analyses, (b) being capable of being performed directly on clinical specimens, e.g., blood cerebrospinal fluid, with the need
25 to isolate pathogenic organisms, (c) being capable of detecting nucleotide sequences in not only living but also nonliving or nonviable organisms, (d) reducing the exposure of personnel to large volumes of pathogenic bacteria, (e) reducing the cost per serogroup analysis, and (f) improving significantly the accuracy of the serotyping method. This method is particularly preferred as an easy, convenient and rapid screening method for the presence of virulent
30 strains of encapsulated pathogens.

Also provided are compositions and immunogenic preparations including but not limited to vaccines, as specifically exemplified, comprising at least one capsular polysaccharide derived from one serogroup strain of *N. meningitidis* and at least one capsular polysaccharide from a different meningococcal serogroup strain, and a suitable carrier therefor are provided. Alternatively, the immunogenic composition can comprise cells of at least two different serotype strains of the specifically exemplified *N. meningitidis* strains and a suitable carrier.

BRIEF DESCRIPTION OF THE FIGURES

Figs. 1A-1D present schematically molecular analysis of capsule biosynthesis and membrane transport genes in prototype isolates of serogroup A, B, C, Y and W-135 *N. meningitidis*. Fig. 1A illustrates the genetic basis for serogroup B meningococcal capsular polysaccharide. Meningococcal capsules are produced by genes encoded by the 24 kb *cps* gene complex comprising five regions: E, C, A, D, and B. In serogroup B, four capsular biosynthetic genes (*synX-D*) are found in region A and are transcribed as an operon. Region C, adjacent to region A, contains 4 polycistronic genes, *ctrA-D*, encoding proteins which transport the phospholipid-substituted polysialic acid across the inner and outer membranes. The *ctr* genes are transcribed in the opposite orientation from the *syn* biosynthetic genes of region A, but utilize the same 134 bp promoter region [Swartley et al. (1996) *J. Bacteriol.* 178:4052-4059].

Fig. 1B illustrates the biosynthetic pathway for the production of serogroup B capsule; *SynX* is either the N-acetyl-D-glucosamine-6-phosphate 2-epimerase which produces N-acetyl-D-mannosamine-6-phosphate or a specific phosphatase which converts N-acetyl-D-mannosamine-6-phosphate into N-acetyl-D-mannosamine; *SynB* is the CMP-N-acetylneuraminic acid (NANA) synthetase; *SynC* is the NANA synthetase; and *SynD* is the polysialyltransferase responsible for (α 2-8)-linked polysialic acid chain polymerization and elongation.

Fig. 1C illustrates Southern DNA hybridization showing *ctrA* homology in serogroups A (strains F8229, F8239), B (strains NMB, 1070 [B-301*]), C (strains FAM 18, 1205 [C-301*], 1843 [C-301]), Y (strain GA0929), and W-135 (strain 6083) of *N. meningitidis*.

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Pneumococcal capsular polysaccharide-meningococcal outer membrane protein complex conjugate vaccines: Immunogenicity and efficacy in experimental pneumococcal otitis media.

Giebink GS, Koskela M, Vella PP, Harris M, Le CT.

Otitis Media Research Center, University of Minnesota, Minneapolis.

Vaccines composed of pneumococcal capsular polysaccharides (PS) conjugated to outer membrane protein complex (OMPC) from *Neisseria meningitidis* group B bacteria were tested in the chinchilla otitis media model. Monovalent (types 6B and 23F), bivalent (6B+23F), and tetravalent (6B+14+19F+23F) PS-OMPC conjugate vaccines elicited significant total serum antibody responses against all four PS. Type 6B vaccine elicited IgG, IgM, and IgA antibodies after a single dose and an anamnestic IgG response after a second vaccine dose on day 28. Type 6B and 19F vaccines prevented or greatly attenuated pneumococcal otitis media after direct middle ear challenge with the immunizing serotype, type 14 vaccine was not protective by this challenge route, and type 23F pneumococci were not sufficiently virulent in chinchillas to test vaccine effectiveness. The promising results with two serotypes suggest the PS-OMPC conjugates may be useful in human infants.

PMID: 8421168 [PubMed - indexed for MEDLINE]

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Pneumococcal polysaccharide-meningococcal outer membrane protein complex conjugate vaccine is immunogenic in infants and children.

Käyhkö H, Ahman H, Rönkä PR, Tiliakainen R, Eskola J.

KTL, National Public Health Institute, Helsinki, Finland.

Sixty-two infants and 31 toddlers were vaccinated with the tetravalent pneumococcal conjugate vaccine PncOMPC consisting of the capsular polysaccharide of pneumococcal types 6B, 14, 19F, and 23F conjugated to the outer membrane protein complex of *Neisseria meningitidis*. Infants were vaccinated at 2, 4, and 6 months (group A) or at 4, 6, and 14 months (group B); toddlers were vaccinated at 24 or at 24 and 26 months of age. The IgG responses to the four pneumococcal polysaccharide types were measured by EIA. In infants, types 14 and 19F induced a significant response after the first dose and types 6B and 23F after the second dose. A clearcut booster response was seen to the booster dose given at 14 months, indicating immunologic priming by the primary series at 2-6 months of age. The responses of the toddlers to one or two doses of the vaccine were very similar to the responses in infants.

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