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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



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Señora

Jefe de la División de Nuevas Creaciones

Superintendencia de Industria y Comercio

Ministerio de Desarrollo Económico

E. S. D.

Ref.: Expediente No. **03.004.732**

Solicitud de Patente de Invención para: **"VACUNAS PARA PROTECCION DE ESPECTRO AMPLIO CONTRA ENFERMEDADES CAUSADAS POR NEISSERIA MENINGITIDIS"**

Solicitante: **CHILDREN'S HOSPITAL & RESEARCH CENTER AT OAKLAND.**

RESPUESTA AL EXAMEN DE FONDO

MARGARITA CASTELLANOS ABONDANO, mayor de edad, identificada y domiciliada como aparece al pie de mi firma, atentamente me permito presentar respuesta al oficio No. **11363** notificado por estado del 14 de septiembre 2007 correspondiente al examen de fondo y para el cual solicite un prórroga el día 7 de diciembre de 2007, estando dentro del término legal en los siguientes términos:

1. Claridad de la solicitud

El examinador establece, al igual que en el oficio No. 2810 que el capítulo reivindicatorio carece de concisión porque se presentan muchas alternativas posibles que dificultan identificar la materia a proteger. De la misma forma, establece que se usan términos imprecisos que impiden a un tercero determinar con exactitud el alcance de la protección solicitada y que las reivindicaciones carecen de soporte descriptivo.

Definición de la materia a proteger

Con el objeto de superar las objeciones hechas a la claridad de la solicitud, presento un nuevo pliego reivindicatorio en el cual se han introducido las reivindicaciones 18 a 20 (nuevas), dependientes de la reivindicación 1, en donde se definen la primera, segunda y tercera cepas de *Neisseria meningitidis* por su número de depósito. Así entonces, con la anterior modificación del pliego reivindicatorio se da cumplimiento a la sugerencia de ese Despacho en cuanto a la definición de las preparaciones de la invención por medio del número de depósito de las cepas.

Términos imprecisos o relativos

En cuanto a la objeción hecha por ese Despacho con respecto a que la expresión "por lo menos una" (son efectivas para producir una respuesta bactericida del anticuerpo **a por lo menos** una cepa de *Neisseria meningitidis*) dificulta la determinación del alcance de la invención, el solicitante manifiesta su desacuerdo y considera que no se debe eliminar del pliego reivindicatorio. Lo anterior por cuanto, es bien conocido en el estado de la técnica que la actividad bactericida está correlacionada con la eficacia de la vacuna en contra de *Neisseria meningitidis* y, por lo tanto, el alcance de la reivindicación 1 es claro toda vez que allí se establece que las preparaciones definidas son efectivas en la producción de una respuesta bactericida a las cepas de *Neisseria meningitidis*.

Adicionalmente, las características técnicas de la composición de la invención están claramente definidas por una primera y una segunda preparación las cuales, a su vez están bien definidas por los elementos que las componen de tal forma que un técnico medio en la materia podría preparar la composición partiendo de la información proporcionada por la presente solicitud y no se vería limitado en este proceso por el simple hecho que se mencione "por lo menos una" cuando se hace referencia a la actividad bactericida del anticuerpo a las cepas de *Neisseria meningitidis*.

Lo anterior está soportado por el numeral 4.6.3 del Manual Andino de Patentes, referente a términos imprecisos:

".... Se pueden permitir para características técnicas no esenciales cuando esté justificado y siempre y cuando permitan distinguir el estado de la técnica sin ambigüedad (novedad y nivel inventivo)..."

Así entonces, se entiende que, para el presente caso, la expresión "por lo menos una" no limita la determinación del estado de la técnica ya que no define una característica esencial de la invención y, en consecuencia, no debe afectar el estudio de novedad ni de nivel inventivo de la solicitud. Lo anterior por cuanto, como es sabido, dicho estudio de novedad debe basarse en la comparación de las características técnicas de la invención, esto es si existen documentos que contengan todas las características técnicas esenciales de la invención (composición que comprende por una primera preparación y una segunda preparación...) y, el estudio de nivel inventivo debe determinar si las anterioridades llevarían a un técnico medio en la materia a desarrollar una composición tal y como se define en la reivindicación 1.

En conclusión, la reivindicación 1 sí es clara y, contrario a lo afirmado por ese Despacho, la expresión "por lo menos una" no afecta la determinación del alcance de la invención.

Falta de soporte descriptivo.

El solicitante manifiesta su desacuerdo frente a la afirmación de ese Despacho sobre la falta de soporte descriptivo y, aclara que la preparación de la invención se describe en las diferentes modalidades presentadas en la descripción, de tal forma que una persona versada en la materia obtendría la guía adecuada para desarrollar la invención.

Como lo expresé en el memorial de respuesta al oficio No. 2810, las combinaciones de cepas que se ejemplifican en la solicitud son una prueba del principio que, si se inmuniza con preparaciones de antígeno hechas de una primera y una segunda cepa de *N. meningitidis* que difieren en serotipo y serosubtipo, se produce una respuesta bactericida de anticuerpo a por lo menos una cepa de *N. meningitidis* que expresa un epítipo serosubtipo que no está presente en la primera ni en la segunda preparación de antígeno.

Teniendo en cuenta que se conocen y disponen diferentes cepas de *N. meningitidis*, la descripción de la presente solicitud proporciona la guía para que una persona versada en la materia pueda reproducir la invención. Por ejemplo la descripción enseña que las cepas se seleccionan teniendo en cuenta la producción bleb y la producción NspA.

La descripción proporciona datos que muestran que la invención reivindicada es útil para la producción de anticuerpos anti-*N. Meningitidis* que son bactericidas (página 70, línea 23 a página 74 línea 7) y que proporcionan protección contra la infección en el modelo de ratas infantiles (página 74, línea 8 a página 76, línea 1). Las preparaciones de membrana de RM1090, BZ198, y Z1092 fueron usadas como sistemas modelos. A partir de los datos proporcionados, una persona versada en la materia técnica reconocería que las preparaciones

de membrana de cualquier variedad de cepas de N. meningitidis podría usarse en la composición reivindicada, y razonablemente esperaría que dichas preparaciones, cuando se administran a un individuo, fueran efectivas para provocar una respuesta bactericida a por lo menos una cepa de N. meningitidis que expresa un epítipo serosubtipo no incluido en las preparaciones de membrana.

Como se dijo anteriormente, en el estado de la técnica la actividad bactericida está correlacionada con la eficiencia de la vacuna en contra de Neisseria meningitidis. Por ejemplo, las medidas de anticuerpos bactericidas (SBA) son la base para el licenciamiento de vacunas meningococales^{1,2,3,4}. Notablemente, la Organización Mundial de la Salud, y las agencias regulatorias en Europa y Estados Unidos, requieren que se demuestre las respuestas bactericidas de los anticuerpos como un indicador de la eficacia de la vacuna para obtener la licencia de una nueva vacuna meningococal. Para licenciar vacunas de polisacárido meningococal tetravalente en Estados Unidos, la eficacia del grupo Y y de los componentes W135 fue establecida con base en la respuesta bactericida. En el Reino Unido, la eficacia de los datos que soportan la licencia de un nuevo grupo de vacunas de conjugado proteína-polisacárido meningococal C se basó en las respuestas serológicas, siendo la medida bactericida el criterio más importante. Las vacunas mencionadas fueron licenciadas en Europa, Canadá, entre otros, partiendo de los datos de medida bactericida.

Con base en los datos proporcionados por la presente invención, y teniendo en cuenta el hecho que la actividad bactericida es aceptada en el campo técnico como correlacionada con la eficacia de la vacuna en contra de Neisseria Meningitidis, una persona versada en la materia técnica esperaría que las preparaciones de membrana de cualquier variedad de N. meningitidis podría ser usada en la composición reivindicada y esperaría que se produjera una respuesta bactericida a por lo menos una cepa de N. meningitidis que expresa un epítipo serosubtipo no incluido en las preparaciones de membrana.

En conclusión, la presente invención si proporciona la información técnica necesaria para que un técnico medio en la materia desarrolle la composición reivindicada y, por lo tanto el nuevo pliego reivindicatorio cumple con lo estipulado por el artículo 30 de la Decisión 486.

¹ Borrow et al., Serological basis for use of meningococcal serogroup C conjugate vaccines in the United Kingdom: reevaluation of correlates of protection, 2001 Infect Immun. 69:1568.

² Balmer, et al., Serologic correlates of protection for evaluating the response to meningococcal vaccines. Expert Rev Vaccines 2004; 3:89-99.

³ Milagres et al., Immune response of Brazilian children to a Neisseria meningitidis serogroup B outer membrane protein vaccine: comparison with efficacy. Infect Immun 1994; 62: 4419-24.

⁴ Holst et al., Serum bactericidal activity correlates with the vaccine efficacy of outer membrane vesicle vaccines against Neisseria meningitidis serogroup B disease. Vaccine 2003. 21: 734-737 (Exhibit 4).

Materia no patentable

Ese Despacho considera que las reivindicaciones son una adaptación de una reivindicación de método de tratamiento y no a una composición que puede derivarse del estado de la técnica.

Pongo de presente a ese Despacho que el pliego reivindicatorio redirige a una composición definida en los parámetros permitidos, esto es por los elementos que la componen de tal forma que corresponde a una reivindicación de producto y no a un método de tratamiento. Adicionalmente, el examinador debe establecer si la materia reivindicada se deriva del estado de la técnica partiendo del estudio de divulgaciones concretas en el campo de la invención y no por medio de sugerencias sobre la "posible" derivación de las vacunas ya conocidas.

2. Unidad de Invención

Las reivindicaciones 18 y 19 referentes a una composición que puede contener un antígeno aislado fueron eliminadas del nuevo pliego reivindicatorio y, por lo tanto, éste último comprende un único concepto inventivo.

3. Nivel Inventivo

La objeción por posible incumplimiento de los artículos 14 y 15 debe tenerse por superada toda vez que la reivindicación 18 fue eliminada del pliego reivindicatorio.

Cumplido así con lo dispuesto, respetuosamente solicito a Ud., se otorgue el privilegio de patente a la presente solicitud.

4. ANEXOS

- Nuevo pliego reivindicatorio.
- Formulario de Modificaciones
- Recibo por concepto de modificación voluntaria.
- Copia de las referencias citadas

De la Señora Jefe,


Margarita Castellanos Abondano
C.C. No. **39.779.654** de Usaquén
T.P.A. No. **70.819** del C. Sup. de la Judicatura
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Bogotá, D.C., **22 ENE. 2008**

PATENTES/48/4850/MEMORIAL FONDO II

367



Industria y Comercio
SUPERINTENDENCIA

Espacio reservado para el adhesivo de radicación

FORMULARIO ÚNICO DE CORRECCIONES Y MODIFICACIONES

①

SOLICITUD DE:



Corrección de Error Material



Modificación a una solicitud

②

SOLICITANTE

Nombre: CHILDREN'S HOSPITAL & RESEARCH
CENTER AT OAKLAND

Dirección: 5700 Martin Luther King Jr. Way,
Oakland, CA 94609

Nacionalidad o Domicilio: Oakland, CA, US

Lugar de Constitución: California,
ESTADOS UNIDOS DE AMERICA

Teléfono:

Fax:

E-mail:

IDENTIFICACIÓN

C.C.



NIT



C.E.



Otro



Cual

Número

③

REPRESENTANTE
O APODERADO

Nombre: MARGARITA CASTELLANOS A.

Dirección: CALLE 94A No. 7A-09

Teléfono: 610 74 20 Fax: 610 07 06

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IDENTIFICACIÓN

C.C.



NIT



C.E.



Otro



Cual

Número

39'779.654 TPA 70.819

④

Número de Expediente:

03,004,732

⑤

Descripción de la corrección o modificación solicitada:

Presento nuevo pliego reivindicatorio al cual solicito se otorgue el privilegio de patente.

⑥

Comprobante de pago No.

08 - 4,413

Fecha

17/01/2008

⑦

Anexos:



Comprobante de pago de la tasa.



Poderes si fuere el caso, junto con la sustitución de los mismos a mi favor.



Documento que acredita la existencia y representación legal de las partes, cuando sean personas jurídicas



Nuevo capítulo reivindicatorio

⑧

NOMBRE

MARGARITA CASTELLANOS A.

FIRMA

CC 39'779.654 Usaquén - TPA 70.819 CSJ

Instrucciones para el diligenciamiento del presente formulario, ver reverso de esta página - MCA/zca-P4850

22 ENE. 2008

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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

NIT : 800176089-2

RECIBO OFICIAL DE CAJA : 08 - 4,413
FECHA : ENERO 17 DE 2008

***** CONSIGNACION *****

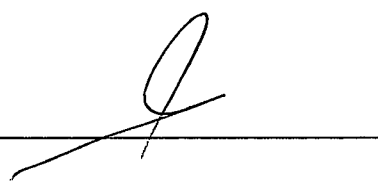
DEPOSITANTE	TIPO PAGO	BANCO	CUENTA	No. PAGO	FECHA PAGO	Vr. PAGO
ALVARO CASTELLANOS	CONSIGNACION	BANCO DE BOGOTA	062754387	1482613	17/01/2008	3,235,000.00

***** 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. _____

REIVINDICACIONES CORREGIDAS

1. Una composición que comprende:

Una primera preparación que comprende un vehículo farmaceuticamente aceptable y vesículas de membrana exterior (OMV), microvesículas (MV), o ambas, OMV y MV de una primera cepa de *Neisseria meningitidis* que es un miembro de un primer serotipo o de un primer serosubtipo; y

Una segunda preparación que comprende un vehículo farmaceuticamente aceptable y vesículas de membrana exterior (OMV), microvesículas (MV), o ambas, OMV y MV de una segunda cepa de *Neisseria meningitidis*, en donde la segunda cepa de *Neisseria meningitidis* es un miembro de un segundo serotipo o de un segundo serosubtipo, en donde el serotipo o serosubtipo de la primera y segunda cepas de *Neisseria meningitidis* es diferente;

En donde la primera y segunda preparación, cuando se administran a un individuo en serie o como mezcla, son efectivas para producir una respuesta bactericida del anticuerpo a por lo menos una cepa de *Neisseria meningitidis* que expresa un epítoto serosubtipo no incluido en la primera o segunda preparación.

2. La composición de la reivindicación 1, en donde la primera preparación comprende MVs y la segunda preparación comprende MVs.

3. La composición según la reivindicación 1 o 2, que comprende además:

una tercera preparación que comprende un vehículo farmaceuticamente aceptable y vesículas de membrana exterior (OMV), microvesículas (MV), o ambas, OMV y MV de una tercera especie de *Neisseria meningitidis*, en donde la tercera especie de *Neisseria*

meningitidis es genéticamente diversa de la primera especie de *Neisseria meningitidis* y, en donde la tercera preparación es formulada para administración en serie o administración como mezcla con la primera y segunda preparaciones.

4. La composición de la reivindicación 3 en donde la primera y segunda especie de *Neisseria Meningitidis* son genéticamente diversas en por lo menos serotipo o serosubtipo.

5. La composición según la reivindicación 3, en donde el serosubtipo de la primera y tercera especies es diferente.

6. La composición según la reivindicación 3, en donde el serosubtipo de la primera, segunda y tercera especies es diferente.

7. La composición de la reivindicaciones 2 a 6, en donde la tercera preparación comprende MVs.

8. La composición de la reivindicaciones 2 a 6, en donde la tercera preparación comprende OMVs.

9. La composición de la reivindicaciones 1 a 8, en donde la primera preparación comprende OMVs y la segunda preparación comprende OMVs.

10. La composición de la reivindicaciones 1 a 8, en donde la primera preparación comprende MVs y la segunda preparación comprende OMVs.

11. La composición de la reivindicaciones 1 a 8, en donde la primera preparación comprende OMVs y la segunda preparación comprende MVs.

12. La composición según la reivindicación 3 a 8, caracterizada

porque:

la primer preparación comprende MV;

la segunda preparación comprende MV; y

la tercer preparación comprende OMV.

13. La composición de cualquiera de las reivindicaciones 1 a 2, caracterizada dichas preparaciones son preparadas por extracción detergente por medio de un detergente diferente al deoxicolato.

14. La composición de cualquiera de las reivindicaciones 2 a 13 caracterizada porque la composición comprende un adyuvante.

15. La composición de la reivindicaciones 14, en donde el adyuvante es fosfato de aluminio, hidróxido de aluminio, alumbre o MF19.

16. La composición de la reivindicaciones 1 a 15, en donde la composición es formulada por administración por inyección, aerosol u oral.

17. La composición de la reivindicaciones 1 a 16, en donde la composición adecuada para administración en humanos.

18. La composición de la reivindicación 1, en donde la primera cepa de *Neisseria meningitidis* es RM1090 (ATCC No. de depósito PTA-3557).

19. La composición de la reivindicación 1, en donde la segunda cepa de *Neisseria meningitidis* es BZ198 (ATCC No. de depósito PTA-3555).

20. La composición de la reivindicación 3, en donde la tercera cepa de *Neisseria meningitidis* es Z1092 (ATCC No. de depósito PTA-3556).

Serological Basis for Use of Meningococcal Serogroup C Conjugate Vaccines in the United Kingdom: Reevaluation of Correlates of Protection

RAY BORROW,^{1*} NICK ANDREWS,² DAVID GOLDBLATT,³ AND ELIZABETH MILLER⁴

PHLS Meningococcal Reference Unit, Withington Hospital, Manchester M20 2LR,¹ PHLS Statistics Unit,² and Immunization Division, PHLS Communicable Disease Surveillance Centre,⁴ London NW9 5EQ, and Immunobiology Unit, Institute of Child Health, London WC1N 1EH,³ United Kingdom

Received 10 October 2000/Returned for modification 1 November 2000/Accepted 7 December 2000

The antibody data supporting the use of meningococcal serogroup C conjugate (MCC) vaccines in the United Kingdom were generated by serum bactericidal assay (SBA) using rabbit complement (rSBA). This may give higher titers than those obtained with human complement (hSBA), for which the "gold standard" correlate of protection for meningococcal C disease is a titer of ≥ 4 . Comparison of rSBA and hSBA titers in sera from unvaccinated adults with an rSBA titer of ≥ 8 showed that for 93% (27 of 29) the titer was ≥ 4 by hSBA, confirming natural protection. Furthermore, sera from MCC vaccinees showed that an rSBA titer of < 8 or ≥ 128 discriminated susceptibility and protection well (85% with rSBA titers of < 8 had hSBA titers of < 4 , and 99% with rSBA titers of ≥ 128 had hSBA titers of ≥ 4). However, discrimination was poor in the rSBA titer range 8 to 64, with only 60% having hSBA titers of ≥ 4 . In such cases we propose that protection can be assumed if there is a fourfold rise in titer between pre- and postvaccination sera or if there is a characteristic booster response to a polysaccharide challenge dose with, if available, evidence of antibody avidity maturation or an hSBA titer of result ≥ 4 . Applying these criteria to toddlers, 10 to 40% of whom had titers in the range 8 to 64 after a single dose of MCC vaccine, showed that 94% had a fourfold rise in titer, including 98% of those in the titer range 8 to 64. In addition, of those with titers of < 128 post-MCC vaccination, 90% had titers of ≥ 128 after a 10- μ g polysaccharide booster dose, compared with only 7% of unprimed age-matched toddlers given a full 50- μ g dose. Furthermore, the increase in geometric mean avidity index pre- and postbooster was independent of post-primary MCC titer. These results indicated that the majority of toddlers with an rSBA titer between 8 and 64, and some of those with an hSBA result of < 4 , have mounted a protective immune response with the induction of immunological memory.

Meningococcal serogroup C (MenC) conjugate (MCC) vaccines have been extensively evaluated in phase I and II trials in the United Kingdom (7, 15, 31–34) and elsewhere (2, 10, 11, 23, 24, 36) and have been shown to be highly immunogenic, generating functional antibodies as measured by serum bactericidal assay (SBA). Studies of military recruits during the 1960s had shown that those with naturally acquired SBA titers of ≥ 4 were protected from MenC disease (20). The United Kingdom Medicines Control Agency therefore took the view that efficacy trials would not be required for MCC vaccines but instead serological correlates utilizing SBA could be relied upon. A similar rationale has also been used to license unconjugated MenC (and MenA) polysaccharide vaccines. Antibody responses to these capsular polysaccharide vaccines have been measured by various serological methods though licensure was gained using data obtained by radioimmunoassay and SBA in the United Kingdom. The original licensure in the United States in the 1970s, however, was based upon classic randomized efficacy trials (4, 16). Serogroup C polysaccharide vaccines have been shown to be efficacious in those over 2 years of age and to reduce carriage of serogroup C meningococci in the

short term (4, 12), although they are poorly immunogenic and not protective in those under 2 years of age (35).

The original serological correlate of protection in military recruits was obtained using an SBA in which human sera was the exogenous complement source (hSBA). However, large volumes of suitable human source complement preserved sera are not available or even practical to obtain due to the occurrence of naturally occurring antimeningococcal antibodies induced by oropharyngeal carriage of meningococci or other neisserial species. In the absence of a commercial source of human complement, the standardization of assays between laboratories, each of whom is using their own in-house source, is difficult.

Commercially available heterologous complement has the advantage of being manufactured and supplied to a high standard of consistency and offers the only practical way of achieving standardization of SBA results between laboratories. Therefore, 3- to 4-week-old baby rabbit serum is now recommended as an alternative complement source for the SBA (26, 37).

It is generally accepted, however, that serogroup C meningococci are more susceptible to serogroup C-specific antibodies when using baby rabbit complement as opposed to human complement, resulting in higher SBA titers (21). With the reliance on serological correlates of protection for licensure of MCC vaccines, we have evaluated the use of SBA titers gen-

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TABLE 1. Comparison of the numbers of sera positive and negative by human complement stratified by rabbit complement titer for subjects who have received MCC vaccination and for unvaccinated adults

rSBA titer	No. of sera with indicated hSBA titer in:			
	MCC vaccinees		Unvaccinated adults ^a	
	<4	≥4	<4	≥4
<8	34	6		
8	3	0	0	2
16	3	3	0	2
32	2	5	2	4
64	4	10	0	5
128	1	26	0	4
≥256	0	97	0	10
Total	47	147	2	27

^a Note that only those adults with an rSBA titer of ≥8 were tested by the hSBA.

erated with rabbit complement (rSBA) with the aim of defining rSBA responses that predict protection against MenC disease. The objective is to address concerns about specificity without introducing unnecessarily stringent criteria that might obstruct the licensure and use of MCC vaccines elsewhere.

MATERIALS AND METHODS

MCC vaccine. The MCC vaccinees used in this study were the Meningitec vaccine, which contains 10 µg of MenC oligosaccharide coupled to CRM₁₉₇ mutant diphtheria toxin (Wyeth Lederle Vaccine & Pediatrics [WLVP], Pearl River, N.Y.), the Menjugate vaccine, which contains 10 µg of MenC oligosaccharide linked to CRM₁₉₇ mutant diphtheria toxin vaccine (Chiron Vaccines, Siena, Italy) and the Neis-vac, a de-O-acetylated MenC polysaccharide (10 µg) coupled to tetanus toxoid (Baxter Hyland Immuno, formerly North American Vaccines Inc. [NAVA], Beltsville, Md.). Booster doses at 6 months following MCC vaccination comprised a 0.1 ml dose of licensed meningococcal AC polysaccharide (MACP) vaccine [Mengivac (A+C); Pasteur Merieux, Lyon, France] containing 10 µg each of MenA and MenC polysaccharides.

Study groups. The study groups were as previously described, comprising infants (34), toddlers (12 to 14 months) (31), preschool children (3 to 4 years) and school leavers (14 to 17 years) (E. Miller, P. Richmond, R. Borrow, E. Kaczmarek, K. Cartwright, R. Morris, and C. Thornton, Abstr. 11th Int. Pathog. Neisseria Conf., p. 57, 1998), 18 to 26 year olds (33), and laboratory staff (R. Borrow, E. Miller, N. Peake, R. Rahim, N. Andrews, M. Acuna, S. Martin, J. Southern, and E. B. Kaczmarek, submitted for publication) who were participants in trials of MCC vaccinees in the United Kingdom. The postvaccination serological data in this paper are restricted to recipients of the WLVP MCC vaccine with the exception of the toddlers, for whom data for all three manufacturers' vaccines are included. All study sera were assayed by rSBA as part of the respective trials, with selective sera being reassayed by hSBA.

SBA. The SBA for infant, toddler, and 18- to 26-year-old cohorts was performed as described previously (2) using broth culture for growth of target strains prior to the SBA assay. The SBA for preschool and school leaver cohorts was performed as described by Maslanka et al. (26), in which agar culture was utilized for target strain growth. The complement source used was either 3- to 4-week-old baby rabbit complement (Pel-freeze Biologicals, Brown Deer, Wis.) or complement-preserved human serum collected from a subject with no intrinsic SBA activity against strain C11. The target strain used throughout was C11 (C:16:P1.7⁺). SBA titers were expressed as the reciprocal of the final serum dilution causing ≥50% killing at 60 min. For computational purposes and the calculation of fourfold rises, SBA titers of <4 were given a value of 2.

Avidity indices. Serogroup C-specific immunoglobulin G avidity was tested by an elution enzyme-linked immunosorbent assay, using the chaotrophic thiocyanate as described elsewhere (18) and modified for the MenC assay (32).

Statistical methods. rSBA and hSBA titers determined for the same sera were compared by calculating the proportion with protection by hSBA at different rSBA titers with 95% confidence intervals (95% CIs). Specificity within a pop-

ulation of unvaccinated infants and toddlers was also calculated with a 95% CI. 95% CIs were also calculated for the geometric mean avidity index.

RESULTS

Comparison of rSBA and hSBA titers for the same sera. Paired SBA titers generated with both rabbit and human complement were generated on a total of 194 post-MCC vaccination serum samples (Table 1). These comprised sera from infants after single or multiple doses of MCC (34) as well as those from toddlers after a single dose (31). An rSBA titer of <8 or ≥128 discriminated well between susceptibility and protection as defined by the "gold standard" hSBA cutoff, since 34 of 40 (85% [95% CI, 70 to 94]) of sera with an rSBA titer of <8 had an hSBA titer of <4 and 123 of 124 (99% [95% CI, 96 to 99.9%]) with an rSBA titer of ≥128 had an hSBA titer of ≥4. However, discrimination was less good in the rSBA titer range 8 to 64, with only 18 of 30 (60% [95% CI, 41 to 77%]) showing a titer of ≥4 by hSBA. Although all three sera with titers of 8 by rSBA had titers of <4 by hSBA, this titer was included in the equivocal region because only three paired results were available. Sensitivity and specificity are not calculated for the post-MCC vaccination sera because they are highly dependent on the proportion of samples that are in the rSBA titer range 8 to 64, which varies depending on the age and dose (Table 2).

In unvaccinated adults with rSBA titers of ≥8 (Table 1), 27 of 29 (93%) had an hSBA titer of ≥4. Of these 27, only 14 (52%) had an rSBA titer of ≥128. This shows that a high proportion of individuals with natural exposure have an rSBA titer in the equivocal range of 8 to 64. It also shows that in a population of unvaccinated adults, an rSBA of 8 to 64 predicts positivity (natural exposure) well with 13 of 15 also positive by hSBA (87% [95% CI, 60 to 98%]).

Age groups in which equivocal rSBA results occur after MCC vaccine. The rSBA responses in different age groups and with different immunization schedules using the WLVP MCC vaccine are shown in Table 2. Over 90% of infants have rSBA titers of ≥128 after two doses, with a similar proportion being found in 14 to 17 year olds and 18 to 26 year olds after a single

TABLE 2. rSBA titers 4 weeks after vaccination with MCC vaccine (10-µg dose)^a

Vaccine manufacturer and age at first dose	No. of doses	No. tested	GMT ^b	No. (%) of subjects with indicated postvaccination rSBA titer:		
				≤4	8-64	≥128
Wyeth						
2 mo	1	50	33	16 (32)	15 (30)	19 (38)
2 mo	2 (1 mo apart)	56	776	1 (2)	3 (5)	51 (93)
2 mo	3 (1 mo apart)	53	1,011	1 (2)	1 (2)	51 (96)
12-14 mo	1	71	141	6 (8)	17 (24)	48 (68)
3-4 yr	1	87	908	1 (1)	10 (11)	76 (88)
14-17 yr	1	81	3,890	0 (0)	0 (0)	81 (100)
18-26 yr	1	86	1,425	0 (0)	6 (7)	80 (93)
Chiron						
12-14 mo	1	72	123	6 (8)	29 (40)	37 (52)
NAVA						
12-14 mo	1	72	564	0 (0)	7 (10)	65 (90)

^a Data from United Kingdom trials.

^b GMT, geometric mean titer.

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dose. However, a substantial proportion of children under 5 years of age have rSBA titers in the equivocal titer range 8 to 64 after a single dose. The proportions of toddlers given a single dose of Chiron or NAVA MCC vaccine who had rSBA titers in the equivocal range were 40 and 10%, respectively (Table 2).

Resolution of the immune status of children with equivocal titers was essential in order to decide whether a one- or two-dose schedule was required for children of 1 to 4 years in the United Kingdom catch-up MCC immunization program.

Specificity of rabbit complement in the SBA in unvaccinated infants and toddlers. Prevacination sera from 212 toddlers (31) and 54 infants (34) participating in MCC vaccine trials in the United Kingdom were tested by rSBA. None of these children had a history of prior meningococcal infection and should therefore be susceptible to MenC unless protective SBA antibodies were acquired through carriage or, in infants, through transfer of maternal antibodies.

Of the 212 toddler sera, 200 (94%) had rSBA titers of <4, with the remaining 12 distributed as follows: for six sera, titer = 4; for two sera, titer = 16; for three sera, titer = 32; and for one serum, titer = 512. For the 54 prevaccination infant sera, 46 (85%) had rSBA titers of <4; of the remaining 8 sera, six had titers of 4, one had a titer of 8, and one had a titer of 64. Thus, only one prevaccination toddler serum with a titer of 512 would have been categorized as protected based solely on the rSBA result being ≥ 128 . The hSBA titer with human complement for this serum was 64, confirming protection. The specificity of the rSBA test in a population of unvaccinated uninfected individuals is therefore estimated to be 97% (258 of 265 [95% CI, 95 to 99%]) with a cutoff of ≥ 8 and 100% (265 of 265 [95% CI, 98.6 to 100%]) with a cutoff of ≥ 128 . This shows that the rSBA does not measure nonspecific functional antibody in nonimmune subjects.

Fourfold rises. In the United Kingdom toddler study, 1 month after the primary dose of MCC vaccine, 94% of subjects had a rise of four fold or more in rSBA titer compared with their prevaccination titer (for the Chiron vaccine, 91% [60 of 66]; for the WLVP vaccine, 89% [56 of 63]; for the NAVA vaccine, 100% [71 of 71]). For individuals in whom the post-MCC vaccine rSBA titer was between 8 and 64, 98% (50 of 51) had a fourfold rise in rSBA titer compared with prevaccination levels, regardless of the MCC vaccine administered.

Immunological memory responses to plain polysaccharide. In the United Kingdom MCC toddler study (31), the effect of a booster dose with 10 μ g of C polysaccharide in toddlers previously immunized with MCC vaccine was investigated. For recipients of the Chiron, WLVP, and NAVA MCC vaccines, 47, 33, and 10%, respectively, had rSBA titers of <128 1 month after the first dose, but only 5, 6, and 3%, respectively, had titers of <128 postbooster (Table 3). For those with a postprimary SBA titer of <128, 90% had a postbooster titer of ≥ 128 . This compares with 1 of 12 (8%) age-matched children (17 to 28 months) vaccinated with 50 μ g of MACP vaccine as part of an outbreak control measure (8) ($P < 0.0001$).

Avidity indices for assessing immunological memory. Although the United Kingdom toddler study demonstrated a fall in rSBA titer, the avidity index was shown to increase from postprimary to preboost titers (Fig. 1). There was no significant difference in the avidity index when comparing subjects with

TABLE 3. Proportion of toddlers with postbooster rSBA titers of ≥ 128 grouped by postprimary rSBA titer

Vaccine manufacturer	Proportion of toddlers with the indicated postprimary rSBA titer ^a :		
	<8	8-64	≥ 128
Chiron	2/3	22/24	34/34
WLVP	6/6	11/12	41/44
NAVA		6/7	62/63
Total (%)	8/9 (89)	39/43 (91)	137/141 (97)

^a Results are given as number of toddlers with postbooster rSBA titer of ≥ 128 /total number of toddlers.

postprimary SBA titers of <8, 8 to 64, or ≥ 128 , the geometric mean avidity indices (95% CI) being 119 (90 to 158), 128 (118 to 138), and 128 (123 to 134), respectively, for all three vaccines combined.

Fourfold rises, response to boosting and avidity maturation according to post-MCC rSBA titers. In Table 4, the United Kingdom MCC toddler study participants were categorized by three different criteria: (i) by the presence of a fourfold rise in SBA titer pre-MCC to 1 month post-MCC, (ii) by the achievement of an rSBA titer of ≥ 128 after a 10- μ g MACP booster, and (iii) by evidence of avidity maturation (i.e., rise in avidity index from 1 month post-MCC to pre-10- μ g MACP booster 6 months later). There were no differences in the proportions meeting the above criteria between those with post-primary MCC titers of 8 to 64 compared and those with post-primary MCC titers of ≥ 128 .

DISCUSSION

We have demonstrated the high specificity of the rSBA in young children who have not been previously vaccinated with MenC polysaccharide or MCC vaccines and could therefore be presumed to be susceptible to MenC infection. Therefore, rSBA titers of <8 predict susceptibility. This conclusion was corroborated in a recent university outbreak of MenC disease in which rSBA titers in students prior to or at the onset of invasive disease were all <4, suggesting that it is the absence of bactericidal activity is that is important irrespective of the complement source used (22). We have also shown that rSBA titers of ≥ 128 are highly predictive of protection but that many sera with rSBA titers in the range 8 to 64 also come from individuals with protection, particularly for adults with natural exposure. The main issue is therefore the interpretation of rSBA titers in the region of 8 to 64. For groups in whom there is likely to be a large proportion of results in this range, it is important to have other information in order to determine protection. We propose that the other information could be either an hSBA titer (the gold standard), a pre- and postvaccination sample pair that would enable the assessment of fold rises, or a post-polysaccharide booster result that can be compared with the primary response in age-matched controls.

We have shown that evidence of protection, as defined by a fourfold rise in rSBA titer with a characteristic booster response, may be present despite an hSBA titer of <4. We conclude, therefore, that while an hSBA titer of ≥ 4 indicates protection, an hSBA titer of <4 does not always indicate susceptibility. A similar conclusion was reached by Goldschneider

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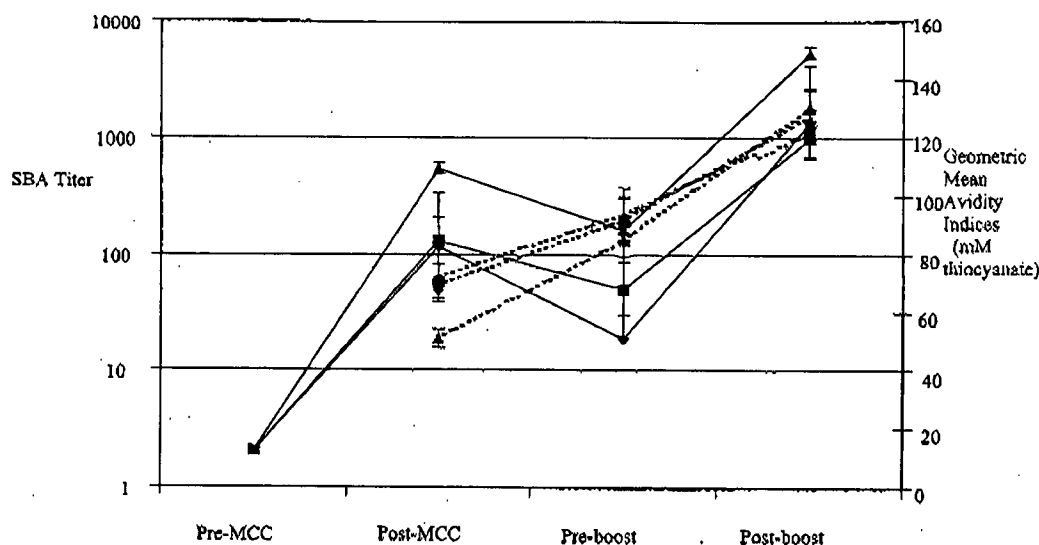


FIG. 1. SBA titers (solid lines) and antibody avidity (dotted lines) in toddlers receiving one dose of MCC followed by a 10- μ g dose of MACP 6 months later. The three vaccines used in the study were manufactured by Chiron (diamonds), WLVP (squares), and NAVA (triangles).

et al. (20), who stated, "The fact that base line sera from cases of meningitis have bactericidal titers of less than 1:4 does not mean that all people with titers below this level are susceptible to infection, although many obviously are."

Fourfold rises in titer from pre- to postvaccination are widely used as measure of a significant response to vaccination, including the meningococcal polysaccharide vaccine (37). The World Health Organization specified the requirements for the meningococcal polysaccharide vaccine, advocating the use of 3- to 4-week-old baby rabbit serum as an exogenous complement source, and stated that "The antibody titers of the sera from at least 90% of the subjects should show a fourfold or greater rise after immunization." It is therefore already accepted that fourfold rises in serum bactericidal titers are indicative of the development of a protective response to unconjugated meningococcal vaccines, and extension of this notion to recipients of MCC would seem appropriate. Using this criterion, we showed that 98% of individuals with post-MCC titers of 8 to 64 should be protected against MenC disease.

Another indicator of long-term protection that is proposed is the SBA response to a polysaccharide booster. For toddlers with a postprimary SBA titer of <128, 90% had a postbooster titer of ≥ 128 , compared with only 8% of age-matched naive controls given a full 50- μ g dose of C polysaccharide (8). This difference in response confirms the induction of immunological memory in the MCC vaccines despite postprimary SBA antibody levels in the equivocal range. Response to a plain polysaccharide boost has also been used to demonstrate immunological memory following *Haemophilus influenzae* type b (Hib) conjugate vaccination, even in the absence of a detectable primary response to the Hib vaccine (14, 19), and it is memory which is thought to contribute to the persistence of protection against Hib in United Kingdom children vaccinated only during infancy (6, 17).

There is now a growing consensus that evidence of successful induction of immunological memory by conjugate vaccines can be obtained by documenting avidity maturation in the months

after primary vaccination with a further increase following a booster dose of plain polysaccharide antigen (1, 14, 18). By examining avidity maturation following a 10- μ g booster dose of meningococcal polysaccharide, we have demonstrated that vaccinated individuals with SBA titers above and below 128 display similar avidity maturation in their antibody response and by this criterion should therefore have immunological memory established. Data now exist which demonstrate the increase in avidity over time following priming of infants or toddlers with Hib (18, 30), *Streptococcus pneumoniae* (3), and *Neisseria meningitidis* serogroup C conjugate vaccines (31). Such data have been generated in the context of boosters of low doses of plain polysaccharide or conjugate vaccine in young infants and are now accepted as indicative that a T-cell-dependent response has been generated.

Concerns have been raised about the relative protection afforded by immunological memory to the different organisms for which conjugate vaccines are available. SBA titers fall dramatically in young children after the primary series, with 25 of 53 (47%) infants with a three-dose schedule (34) and 16 of 65 (25%) toddlers with a one-dose schedule (31) of the WLVP vaccine having SBA titers of <8 6 to 10 months later. This suggests that, as with Hib conjugate vaccines (14), reliance on immunological memory rather than maintenance of SBA titers

TABLE 4. Categorization of United Kingdom MCC toddler sera post-primary MCC rSBA titers into numbers positive by fourfold rises, response to boosting, and avidity maturation

Post-MCC rSBA titer	Vaccine	No. with fourfold rise in titer from pre- to post-MCC/ total no. (%)	No. with rSBA titer of ≥ 128 post-10- μ g MACP booster/ total no. (%)	No. with avidity maturation*/ total no. (%)
<8	All	0/11 (0)	8/9 (89)	3/5 (60)
8-64	All	48/49 (98)	39/43 (91)	25/27 (93)
≥ 128	All	116/117 (99)	137/141 (97)	78/86 (91)

* Rise in avidity from post-MCC to pre-MACP booster.

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above a protective threshold will be necessary if MCC vaccines are to protect over the long term. In general, the pathogenesis of the infections caused by *N. meningitidis* involves invasion following nasopharyngeal colonization (9). Observations during epidemics (13, 20) and in infected laboratory workers (9) indicate that this period is usually short, though a recent case report demonstrated a 7-week interval (28). This suggests that local immunity, induced by carriage, is able to prevent invasion, but in naïve subjects, recent acquisition is associated with invasion because of the delay associated with the mounting of an effective primary response against the relevant strain. The length of this delay is unknown, but is likely to be different in naïve from that in subjects primed by MCC vaccine. For example, with Hib conjugate vaccines, a detectable antibody response post-polysaccharide boosting is present after 4 to 5 days (29), while responses following a primary series take up to 7 days (25). Pneumococcal conjugate vaccines (27), and even MenC polysaccharide vaccines (4, 12), reduce nasopharyngeal carriage of strains contained in the vaccine and therefore clearly provide a degree of mucosal immunity; this is further reinforced by recent pneumococcal conjugate vaccine efficacy data from the United States (5).

Until more data are available it is difficult to correlate the seriousness or speed of an invasive infection with the relative protection afforded by memory. Furthermore, it is likely that some degree of protection at the early stage of infection is mediated by factors other than antibody (such as complement). In addition, data on the seriousness of invasive infection have been correlated with host factors such as cytokine promoter polymorphisms, which may ultimately be more relevant to the outcome of infection. In the context of our current understanding of memory and protection, the theoretical concerns raised above do not justify unnecessarily stringent criteria for licensing and use of the current MCC vaccines. The United Kingdom postlicensure surveillance program will validate these immunological criteria against measures of clinical effectiveness over the coming years.

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REFERENCES

- Ahman, H., H. Kayhty, O. Lehtonen, J. Leroy, E. Froeschle, and J. Eskola. 1998. *Streptococcus pneumoniae* capsular polysaccharide-diphtheria toxoid conjugate vaccine is immunogenic in early infancy and able to induce immunologic memory. *Pediatr. Infect. Dis. J.* 17:211-216.
- Anderson, E. L., T. Bowers, C. M. Mink, D. J. Kennedy, R. B. Belshe, H. Harakeh, L. Pals, P. Holder, and G. M. Carlone. 1994. Safety and immunogenicity of meningococcal A and C polysaccharide conjugate vaccine in adults. *Infect. Immun.* 62:3391-3395.
- Anttila, M., J. Eskola, H. Ahman, and H. Kayhty. 1998. Avidity of IgG for *Streptococcus pneumoniae* type 6B and 23F polysaccharides in infants primed with pneumococcal conjugates and boosted with polysaccharide or conjugate vaccines. *J. Infect. Dis.* 177:1614-1621.
- Artenstein, M. S., R. Gold, J. G. Zimmerman, F. A. Wyle, H. Schneider, and C. Harkins. 1970. Prevention of meningococcal disease by group C polysaccharide vaccine. *N. Engl. J. Med.* 282:417-420.
- Black, S., H. Shinefield, B. Fireman, E. Lewis, P. Ray, J. R. Hansen, L. Elvin, K. M. Ensor, J. Hackell, G. Siber, F. Malinoski, D. Madore, I. Chang, R. Kohb, W. Watson, R. Austrian, and K. Edwards. 2000. Efficacy, safety and immunogenicity of heptavalent pneumococcal conjugate vaccine in children. *Pediatr. Infect. Dis. J.* 19:187-195.
- Booy, R., S. Hodgson, L. Carpenter, R. T. Mayon-White, M. P. Slack, J. A. Macfar, E. A. Haworth, M. Kiddle, S. Shribman, J. S. Roberts, and E. R. Moxon. 1994. Efficacy of *Haemophilus influenzae* type b conjugate vaccine PRP-T. *Lancet* 344:362-366.
- Borrow, R., A. J. Fox, P. C. Richmond, S. Clark, F. Sadler, J. Findlow, R. Morris, N. T. Begg, and K. A. V. Cartwright. 2000. Induction of immunological memory in UK infants by a meningococcal A/C conjugate vaccine. *Epidemiol. Infect.* 124:427-432.
- Borrow, R., P. Richmond, E. B. Kaczmarek, A. Iverson, S. L. Martin, J. Findlow, M. Acuna, E. Longworth, R. O'Connor, J. Paul, and E. Miller. 2000. Meningococcal serogroup C-specific IgG antibody responses and serum bactericidal titers in children following vaccination with a meningococcal A/C polysaccharide vaccine. *FEMS Immunol. Med. Microbiol.* 28:79-85.
- Brandtzaeg, P. 1995. Pathogenesis of meningococcal infections, p. 71-114. In K. Cartwright (ed.), *Meningococcal disease*. John Wiley and Sons Ltd. Chichester, United Kingdom.
- Campagne, G., A. Garba, P. Fabre, A. Schuchat, R. Ryall, D. Boulanger, M. Bybel, G. Carlone, P. Briantais, B. Ivanoff, B. Xerri, and J.-P. Chippaux. 2000. Safety and immunogenicity of three doses of a *Neisseria meningitidis* A + C diphtheria conjugate vaccine in infants from Niger. *Pediatr. Infect. Dis. J.* 19:144-150.
- Costantino, P., S. Viti, A. Podda, M. A. Velmonte, L. Nencioni, and R. Rappuoli. 1992. Development and phase 1 clinical testing of a conjugate vaccine against meningococci A and C. *Vaccine* 10:691-698.
- Devine, L. F., W. E. Pierce, T. M. Floyd, S. L. Rhode, E. A. Edwards, E. E. Sless, and R. O. Peckinpaugh. 1970. Evaluation of group C meningococcal polysaccharide vaccine in marine recruits, San Diego, California. *Am. J. Epidemiol.* 92:25-32.
- Edwards, E. A., L. F. Devine, C. H. Sengbusch, and H. W. Ward. 1977. Immunological investigations of meningococcal disease. III. Brevity of group C acquisition prior to disease occurrence. *Scand. J. Infect. Dis.* 9:105-110.
- Eskola, J., J. Ward, R. Dagan, D. Goldblatt, F. Zepp, and C. A. Siegfried. 1999. Combined vaccination of *Haemophilus influenzae* type b conjugate and diphtheria-tetanus-pertussis containing acellular pertussis. *Lancet* 354:2063-2068.
- Fairley, C. K., N. Begg, R. Borrow, A. J. Fox, D. M. Jones, and K. A. V. Cartwright. 1996. Reactogenicity and immunogenicity of conjugate meningococcal serogroup A and C vaccine in UK infants. *J. Infect. Dis.* 174:1360-1363.
- Gold, R., and M. S. Artenstein. 1971. Meningococcal infections. 2. Field trial of group C meningococcal polysaccharide vaccine in 1969-70. *Bull. W. H. O.* 45:279-282.
- Goldblatt, D., E. Miller, N. McCloskey, and K. Cartwright. 1998. Immunological response to conjugate vaccines in infants: follow up study. *Br. Med. J.* 316:1570-1571.
- Goldblatt, D., R. Pinto Vas, and E. Miller. 1998. Antibody avidity as a surrogate marker of successful priming by *Haemophilus influenzae* type b conjugate vaccine following infant immunization. *J. Infect. Dis.* 177:1112-1115.
- Goldblatt, D., P. Richmond, E. Millard, C. Thornton, and E. Miller. 1999. The induction of immunologic memory after vaccination with *Haemophilus influenzae* type b conjugate and acellular pertussis-containing diphtheria, tetanus, and pertussis vaccine combination. *J. Infect. Dis.* 180:538-541.
- Goldschneider, I., E. C. Gotschlich, and M. S. Artenstein. 1969. Human immunity to the meningococcus. I. The role of humoral antibodies. *J. Exp. Med.* 129:1307-1326.
- Griffiss, J. M., and D. K. Goroff. 1983. IgA blocks IgM and IgG-initiated immune lysis by separate molecular mechanisms by separate molecular mechanisms. *J. Immunol.* 130:2882-2885.
- Jones, G. R., J. N. Williams, M. Christodoulides, K. Jolley, and J. E. Heckels. 2000. Lack of immunity in university students before an outbreak of serogroup C meningococcal infection. *J. Infect. Dis.* 181:1172-1175.
- Leach, A., P. A. Twumasi, S. Kumah, W. S. Banya, S. Jaffar, B. D. Forrest, D. M. Granoff, D. E. Libutti, G. M. Carlone, L. B. Pals, C. V. Broome, and B. M. Greenwood. 1997. Induction of immunologic memory in Gambian children by vaccination in infancy with a group A plus group C meningococcal polysaccharide-protein conjugate vaccine. *J. Infect. Dis.* 175:200-204.
- Macdonald, N. E., S. A. Halperin, B. J. Law, B. Forrest, L. E. Danzig, and D. M. Granoff. 1998. Induction of immunologic memory by conjugated vs

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- plain meningococcal C polysaccharide vaccine in toddlers. *JAMA* 280:1685-1689.
25. Madore, D. V., C. L. Johnson-Krines, E. P. Rothstein, and D. H. Smith. 1999. Kinetics of antibody response to *Haemophilus influenzae* type b vaccines. *Pennridge Pediatrics Associates, Curr. Med. Res. Opin.* 15:105-112.
 26. Maslanka, S. E., L. L. Gheesling, D. E. LiButti, K. B. J. Donaldson, H. S. Harakeh, J. K. Dykes, F. F. Arhin, S. J. N. Devi, C. E. Frasch, J. C. Huang, P. Kris-Kuzemenska, R. D. Lemmon, M. Lorange, C. C. A. M. Peeters, S. Quataert, J. Y. Tai, and G. M. Carlone. 1997. Standardization and a multi-laboratory comparison of *Neisseria meningitidis* serogroup A and C serum bactericidal assays. *Clin. Diagn. Lab. Immunol.* 4:156-167.
 27. Mbelle, N., R. E. Huebner, A. D. Wasas, A. Kimura, I. Chang, and K. P. Klugman. 1999. Immunogenicity and impact on nasopharyngeal carriage of a nonavalent pneumococcal conjugate vaccine. *J. Infect. Dis.* 180:1171-1176.
 28. Neal, K. R., J. S. Nguyen-van-Tam, R. C. Slack, E. B. Kaczmarski, A. White, and D. A. Ala'Aldeen. 1999. Seven-week interval between acquisition of a meningococcus and the onset of invasive disease. A case report. *Epidemiol. Infect.* 123:507-509.
 29. Pichichero, M. E., T. Voloshen, and S. Passador. 1999. Kinetics of booster responses to *Haemophilus influenzae* type b conjugate after combined diphtheria-tetanus-acellular pertussis *Haemophilus influenzae* type b vaccination in infants. *Pediatr. Infect. Dis. J.* 18:1106-1108.
 30. Pichichero, M. E., T. Voloshen, D. Zajac, and S. Passador. 1999. Avidity maturation of antibody to *Haemophilus influenzae* type b (Hib) after immunization with diphtheria-tetanus-acellular pertussis-hib-hepatitis B combined vaccine in infants. *J. Infect. Dis.* 180:1390-1393.
 31. Richmond, P., R. Borrow, D. Goldblatt, J. Findlow, S. Martin, R. Morris, K. Cartwright, and E. Miller. 2001. Ability of three different meningococcal C conjugate vaccines to induce immunological memory after a single dose in UK toddlers. *J. Infect. Dis.* 183:160-163.
 32. Richmond, P., D. Goldblatt, P. C. Fusco, J. D. S. Fusco, I. Heron, S. Clark, R. Borrow, and F. Michon. 1999. Safety and immunogenicity of a new *Neisseria meningitidis* serogroup C-tetanus toxoid conjugate vaccine in healthy adults. *Vaccine* 18:641-646.
 33. Richmond, P., E. Kaczmarski, R. Borrow, J. Findlow, S. Clark, R. McCann, J. Hill, M. Barker, and E. Miller. 2000. Meningococcal C polysaccharide vaccine induces immunologic hyporesponsiveness in adults that is overcome by meningococcal C conjugate vaccines. *J. Infect. Dis.* 181:761-764.
 34. Richmond, P. C., E. Miller, R. Borrow, S. Clark, F. Sadler, A. J. Fox, N. T. Begg, R. Morris, and K. A. V. Cartwright. 1999. Meningococcal serogroup C conjugate vaccine is immunogenic in infancy and primes for memory. *J. Infect. Dis.* 179:1569-1572.
 35. Taunay, A. E., P. A. Galvao, J. S. de Moraes, E. C. Gotschlich, and R. A. Feldman. 1974. Disease prevention by meningococcal serogroup C polysaccharide vaccine in pre-school children: results after eleven months in Sao Paulo, Brazil. *Abstr. Pediatr. Res.* 8:429.
 36. Twumasi, P. A., S. Kumah, A. Leach, T. J. O'Dempsey, S. J. Ceesay, J. Todd, C. V. Broome, G. M. Carlone, L. B. Pais, P. K. Holder, B. D. Plikaytis, and B. M. Greenwood. 1995. A trial of group A and group C meningococcal polysaccharide-protein conjugate vaccine in African infants. *J. Infect. Dis.* 171:632-638.
 37. World Health Organization. 1976. Requirements for meningococcal polysaccharide vaccine. World Health Organization technical report series, no. 594. World Health Organization, Geneva, Switzerland.

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Review

Serologic correlates of protection for evaluating the response to meningococcal vaccines

Paul Balmer and Ray Borrow[†]

Meningococci cause serious disease worldwide and the organism remains the most common cause of bacterial meningitis in children and young adults. The only effective means of controlling disease is through vaccination. Although polysaccharide vaccines have been available for serogroup A, C, Y and W135 for many years, serogroup C polysaccharide-protein conjugate vaccines have only recently been licensed in many countries. Conjugate vaccines for combinations of serogroup A, C, Y and W135 are progressing through clinical trials and major efforts are being made to develop a safe and efficacious vaccine against serogroup B. To assess the quality of the immune response after vaccination, laboratory correlates of protection are needed. For serogroups A and C, serum bactericidal antibody is a well established predictor for protection but for serogroup B, other mechanisms besides serum bactericidal antibody may also be involved in conferring protection against disease. The serologic correlates of protection for evaluating the response to meningococcal vaccines are described in this review.

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Meningococcal disease remains a significant global public health problem with approximately 1.2 million cases per year causing an estimated 135,000 deaths (1). Large epidemics occur in sub-Saharan Africa, in a region known as the 'Meningitis Belt' which extends from Senegal to Ethiopia. *Neisseria meningitidis* serogroup A is mainly responsible for the epidemics observed in this region, however in 2002, a large outbreak of serogroup W135 occurred in Burkino Faso. Endemic meningococcal disease due mainly to serogroups B and C is observed in Europe and the USA, with the addition of serogroup Y contributing to a significant proportion of meningococcal disease in the USA. An epidemic of meningococcal serogroup B disease is ongoing in New Zealand and previous epidemics of serogroup B disease have occurred in Brazil, Norway, Chile and Cuba.

The epidemiology of meningococcal disease in Europe is changing to predominantly serogroup B infection due to a decline in serogroup C infection following the introduction

of serogroup C conjugate (MCC) vaccines. Meningococcal serogroup A and C polysaccharide vaccines have been available since the 1970s but have limited use due to the serogroup C components' lack of immunogenicity in children aged less than 2 years and the nature of the T-cell-independent immune response stimulated by vaccination which in general fails to induce immunologic memory. Conjugation of the capsular polysaccharide to a protein carrier converts the vaccine to a T-cell-dependent immunogen and is immunogenic in young children (2-7). Unfortunately no such vaccines exist for serogroup B disease due to the serogroup B capsule being poorly immunogenic (8) and concerns over safety with the possible induction of autoantibodies (9). Therefore, the approach taken has been to utilize subcapsular antigen either as outer membrane vesicles (OMVs) (10-12), an aggregate of outer membrane proteins (13) or more recently individual antigens are being explored (14,15).

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This review details what is known to date about serologic correlates of short-term protection for evaluating meningococcal vaccines. The correlates described below are to date only for short-term efficacy following vaccination. It should be noted that even the performance of efficacy trials, such as the one recently performed for Prevnar® (Wyeth, NJ, USA) in the Kaiser Permanente medical center (CA, USA) [16] only provide information on correlates of short-term protection, in this case for the particular pneumococcal conjugate vaccine. Only post-licensure surveillance can help to provide information on long-term efficacy and the immunologic mechanisms correlated with this. An example of this is *Haemophilus influenzae* type b (Hib) disease in the UK where 10 years following the introduction of the Hib conjugate vaccine, long-term efficacy data are available through post-licensure surveillance. Thus, for MCC vaccines described below we are still many years away from realizing the correlates for long-term protection.

MCC vaccines

The UK was the first country to introduce MCC vaccines [17]. A phased introduction commenced in 1999/2000 with infants receiving three doses at 2, 3 and 4 months of age, infants 5 to 11 months of age received two doses and those aged 1 to 19 years received one dose. The vaccination program was extended in 2001 to include those aged up to 24 years [18]. An enhanced surveillance program was initiated [10] which was designed to investigate any vaccine failures and provide vaccination coverage data. Those data generated have been used to calculate vaccine efficacy estimates and to determine if herd immunity was occurring in unvaccinated individuals.

The impact of MCC vaccines has been significant [17,19-20]. A reduction of 67% in the attack rate in the targeted age groups from 2001-2002 was observed with vaccine efficacy estimates at 94% overall [20]. The success of the vaccination program has resulted in a significant reduction in deaths due to meningococcal serogroup C infection from 84 in 1998/1999 in those aged less than 20 years to nine in 2001/2002. The prevalence of nasopharyngeal carriage of serogroup C meningococci has also reduced by 67% in teenagers following immunization with MCC vaccines [21]. Evidence of a herd immunity effect from the MCC vaccines has been recently published demonstrating a 67% reduction in the attack rate in unvaccinated children within the targeted age groups [20].

The success of the MCC vaccines in the UK has also been observed in other European countries, such as Ireland and the Netherlands and has prompted countries, such as Spain, Australia and Iceland to implement similar vaccination programs.

Meningococcal serogroup C correlates of protection

The MCC vaccines were licensed in the UK and elsewhere without large-scale Phase III efficacy trials. Extensive pre-licensure studies funded by both the Department of Health and vaccine manufacturers demonstrated the safety and immunogenicity of the candidate MCC vaccines in the targeted age groups [17,19]. The MCC vaccines were then licensed using

those data generated from these studies and the known efficacy and correlates of protection of the meningococcal plain polysaccharide vaccine in children aged 2 years and above. The immunogenicity studies required a substantial effort to develop and standardize laboratory assays to provide data on relevant laboratory correlates of protection.

The original correlate of protection for meningococcal serogroup C (MenC) disease was established by Goldschneider and colleagues [22] as a serum bactericidal antibody (SBA) titer with human complement (hSBA) of ≥ 4 for a short-term of duration (approximately 8 weeks). The study involved bleeding army recruits at the initiation of their basic training and the level of SBA determined in those who subsequently acquired a meningococcal infection during the subsequent 8 weeks. It was found that only three out of the 54 prospective meningococcal cases had circulating SBA titers ≥ 4 prior to initiation of training compared with 444 of 540 controls (those who did not acquire a meningococcal infection) who had a SBA titer of ≥ 4 . The correlate of an SBA titer was then used to determine the relationship between the age-related incidence of disease and the presence of bactericidal antibody above the proposed cut-off. Sera from children and military recruits were used to give an age range from 0-26 years. The percentage of individuals with a SBA titer ≥ 4 in defined age groups was observed to have an inverse relationship to the incidence of MenC disease. The low incidence of MenC disease in early infancy correlates with a high proportion of individuals with an SBA titer ≥ 4 due to maternal antibody. As the percentage with a SBA titer ≥ 4 declines from birth, the incidence of MenC disease increases peaking at 1-2 years. From 2 years onwards, except for a small peak in young adults, the incidence of disease is lower, while the percentage with an SBA titer ≥ 4 remains high. The study by Goldschneider and colleagues established that an SBA titer of ≥ 4 was an appropriate individual correlate of protection but also that this translates to a correlate for a population. An interesting observation made in the studies performed by Goldschneider and colleagues was that the bactericidal activity observed was not limited to capsular polysaccharide-specific antibodies because sera from army recruits demonstrated bactericidal activity against serogroups A, B and C [22]. Further work confirmed that antibodies to subcapsular antigens were of importance by the observation that adsorption of sera with serogroup C polysaccharide inhibited bactericidal activity to serogroup C strains but not to serogroups A and B [23]. Hence, it is likely that the correlate of short-term protection established as an SBA titer ≥ 4 included capsular polysaccharide and subcapsular antigen specific bactericidal activity. However, Goldschneider *et al.* [22] also stated that although an SBA titer of ≥ 4 indicates protection, an SBA titer of <4 did not always indicate susceptibility to MenC disease.

The classical studies performed by Goldschneider and colleagues established SBA as the 'gold standard' correlate of protection. However, this was achieved using human complement as the source of exogenous complement in the bactericidal assay which is difficult to obtain and standardize,

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thus preventing significant inter-laboratory comparisons of data. It is now recommended that commercially available baby rabbit complement is used in a standardized bactericidal assay (rSBA) for evaluation of meningococcal polysaccharide vaccines (24). The immunogenicity data from the evaluation studies of the MCC vaccines in the UK, was generated using baby rabbit complement. Meningococci are known to be more susceptible to complement-mediated lysis in the presence of exogenous rabbit complement compared with human complement (25-26). Thus, in the UK, two approaches were undertaken to re-evaluate the SBA and arrive at a protective titer for the rSBA.

First, the inverse relationship between disease incidence and SBA was recently re-assessed in the UK using the rSBA (27). A total of 1689 sera from age-stratified individuals (ranging from 53-267 per age group), collected prior to the introduction of the MCC vaccine (1996-1999) were tested and SBA titers plotted against disease incidence for those age groups (FIGURE 1). Similar plots to the Goldschneider and colleagues study (22) were observed with peak incidence of disease occurring in infants and young children and a secondary peak occurring in older teenagers. It is interesting to note that the percentage of infants with a rSBA titer ≥ 8 that corresponds to the peak incidence of disease in the UK data is similar to the percentage of US infants with a hSBA titer ≥ 4 in the studies of Goldschneider and colleagues (22). There are important differences between the two studies, such as the sample size, which was substantially bigger in the UK study and the epidemiology of the study populations that may account for the differences in magnitude of responses especially in the older age groups. The inverse relationship between disease incidence and the prevalence of protective antibody titers as described previously (22) appears more consistent with a rSBA titer ≥ 8 than ≥ 128 (FIGURE 1). This study provided evidence for the establishment of a correlate of protection for MenC disease using rSBA titers rather than hSBA.

However, it was unknown if the established correlate would be appropriate for responses to conjugate vaccines, hence the SBA correlate of protection required further evaluation. An rSBA titer < 8 or ≥ 128 was found to discriminate susceptibility and protection, respectively when compared with the human protective SBA titer ≥ 4 (28). It was proposed that for those individuals with an rSBA titer between 8-64, additional evidence was required, such as a hSBA titer ≥ 4 , a fourfold rise in SBA titer from pre to postvaccination, evidence of antibody avidity maturation or a typical booster response to a polysaccharide challenge (28).

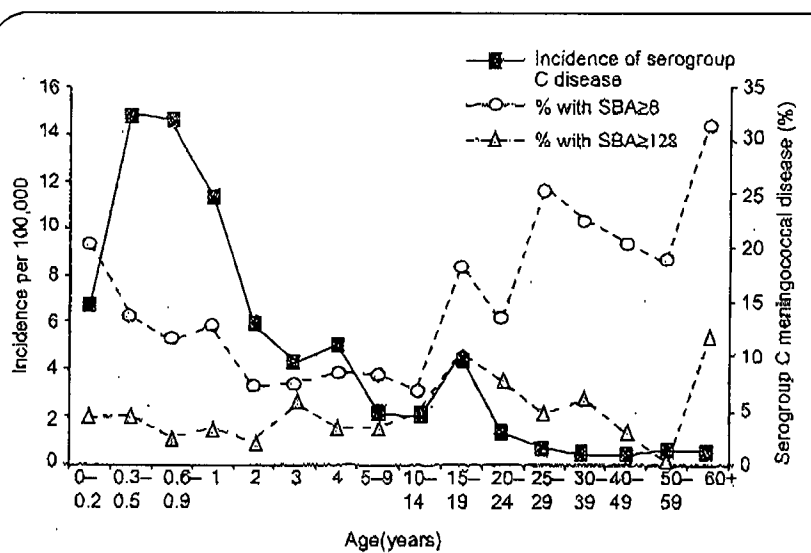


Figure 1. Age-specific incidence of laboratory confirmed serogroup C meningococcal disease in England and Wales 1997/98 and the prevalence of SBA titers ≥ 8 and ≥ 128 (22).

Establishment of an individually-based correlate of protection using rSBA titers would be extremely difficult and require a large cohort (approximately 250,000) to be analyzed postvaccination and followed up for subsequent development of disease (29). Hence, it is deemed more appropriate to rely upon a population-based correlate which may be extrapolated to give a threshold required to protect an individual from disease (30). The post-licensure surveillance data generated in the UK has enabled vaccine efficacy estimates to be calculated. Hence, to establish a population-based correlate of protection, the level of rSBA titer achieved by the majority of a vaccinated group and not achieved by the majority of an unvaccinated group can be compared with the vaccine efficacy in a specific age group. An rSBA of ≥ 8 was found to give strong correlation with the vaccine efficacy estimate for toddlers, 1 month postvaccination indicating this is an appropriate correlate of short-term protection. At 7 months postvaccination, all rSBA titers underestimate the vaccine efficacy observed in the toddler age group suggesting that the individuals are at least reliant upon immunologic memory. The differences between the Goldschneider approach and the UK approach are summarized in TABLE 1.

Long-term protection following MCC vaccination is assumed to be conferred by the induction of immunologic memory as it is known that in infants and toddlers antibody levels decline rapidly (31). The MCC vaccines have been shown to induce memory responses in all targeted age groups including young children (3-5). The traditional method to demonstrate the presence of memory B-cells is to challenge with a plain polysaccharide and observe if a typical booster response is present. However, an alternative laboratory surrogate has been developed which can be used to demonstrate that those

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Table 1. Serologic correlation options for meningococcal serogroup C disease.

	Goldschneider <i>et al</i> (1966 to 1968)	UK (1999 to 2001)
Population	Military recruits	Toddlers (1–2 years)
Number	14,744	Approx 450,000
Intervention	None	Conjugate vaccine
Observation Period	8 weeks	Average 9 months (range 1–18 months)
Serum bactericidal assay	Human complement	Rabbit complement
SBA threshold	1:4	Range
Method	Prospective cohort study	Observational study (post-licensure surveillance)
Sera	Not available	available

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individuals with low levels of circulating SBA have been successfully primed and would be able to mount a rapid memory response if challenged. The generation of immunologic memory requires affinity maturation of humoral responses to T-cell-dependent antigens. This occurs in germinal centers where B-cells undergo somatic mutation, the random mutation of the immunoglobulin (Ig) variable region genes producing B-cell receptors of high affinity for antigen. Competition for survival signals occurs within the germinal center between B-cells of differing affinities with the selection process favoring those with high affinity receptors. Failure to obtain the correct survival signals results in apoptosis. The selected B-cells, following a cognate interaction with T-cells, leave the germinal center and form either memory cells or plasma cells that produce antibodies of a high avidity.

The determination of antibody avidity indices has been utilized as an indicator of affinity maturation and the induction of immunologic memory [3,5,31–33]. Avidity indices have been used to illustrate the induction of memory following MCC vaccination [3,5] and have shown to increase in the presence of declining circulating SBA [5,31]. In children immunized with MCC vaccine 4 years previously, SBA titers had declined to prevaccination levels whereas avidity indices increased following the primary vaccination and remained constant [31]. Following a challenge with plain polysaccharide vaccine, a typical booster response was observed, characterized by the production of high avidity antibody. Hence, determination of avidity indices can be used to assess if successful priming of memory responses have occurred following conjugate vaccination. It seems possible therefore, that it may be necessary to use SBA titer as a correlate of short-term protection and assessment of immunologic memory as a predictor of longer term immunity.

Determination of avidity indices following conjugate vaccination may only be appropriate in children. Adults tend to produce an antibody response of higher avidity, naturally and even following polysaccharide vaccination [34]. Absence of a maturation of antibody avidity has been observed in adults following immunization with pneumococcal plain polysaccharide or conjugate vaccines [35–36]. As shown by Goldschneider and colleagues [22] and more recently, Trotter and colleagues [27], natural immunity to meningococcal disease in adults was associated with a low incidence of disease. It is thought that natural exposure to meningococci or exposure to cross-reactive antigens throughout life results in the development of memory B-cells and that any challenge encountered, such as polysaccharide or conjugate vaccination will stimulate the memory B-cells present. Hence, the use of avidity indices may be limited to the assessment of immunologic priming of young children. However, it is important to note that unlike the correlate of short-term protection, SBA titer, which has been validated using postlicensure efficacy estimates in the UK, no thresholds have been defined for avidity indices.

An alternative method to avidity indices is being investigated in our laboratory. Throughout a primary course of vaccination the level of both SBA titer and serogroup-specific IgG increase. Affinity maturation is reflected by an increase in antibody avidity which is likely to result in a greater proportion of the antibody produced in response to vaccination to be functional i.e., bactericidal. The SBA detected tends to be of high avidity whereas the serogroup-specific IgG is both high and low avidity antibody. Hence, the ratio of SBA titer to serogroup-specific IgG may be a useful method of detecting the induction of memory following vaccination. This approach has been used previously to assess the response to serogroup B vaccines containing outer membrane complexes [37]. The relative bactericidal activity (RBA: bactericidal titer/outer membrane complex specific IgG) was observed to increase following primary vaccination and continued to increase after a second dose of vaccine reflecting the affinity maturation of the response. Preliminary investigations in toddlers and older age groups suggest that those data generated analyzing SBA titer – serogroup-specific IgG ratio – reflects the induction of immunologic memory as indicated by avidity indices for various age groups following MCC vaccination (UNPUBLISHED DATA). This analytical method requires further validation but it may be applicable for analysis of memory responses to other meningococcal conjugate vaccines with the advantage of removing the need for an additional assay to be performed.

Meningococcal serogroup A correlates of protection

For serogroup A disease, the only correlate of protection we have to date is from the Finnish efficacy trials of meningococcal serogroup A polysaccharide vaccine [38–39] and was determined by radioimmunoassay to be 2 µg/mL. This value was the mean level in unimmunized adults [38].

The SBA assay has not been widely used since the early studies of Goldschneider and colleagues [22] and interestingly, the strain A1 Goldschneider used in these studies is no longer considered optimal as it easily lysed even in prevaccination sera in the bacteri-

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cidal assay, therefore tending to very high SBA titers. The Goldschneider studies also used human complement [22]. The serogroup A SBA assay was also used in the Finnish field trials in the 1970s and though the strain used was not quoted, the referenced methodologies eluded to the use of the rSBA [39]. Thus, since the early Goldschneider studies, only rabbit complement has been used in the serogroup A bactericidal assay [40-42]. Most other serogroup A vaccine trials have relied upon solely measuring total serogroup A polysaccharide antibody concentration. With the generation of the rSBA correlate of protection for serogroup C license it is likely that serogroup A conjugate vaccines will be evaluated and licensed on the back of rSBA and appropriate safety data. Extensive trials will be required to confirm the immunogenicity of a candidate serogroup A conjugate vaccine which will provide the opportunity to further validate and standardize laboratory assays, such as SBA and enzyme-linked immunosorbent assay (ELISA).

Immunologic memory responses to meningococcal serogroup A vaccination, both unconjugated and conjugate vaccines, have been evaluated using both the response to a polysaccharide challenge and avidity indices. It is interesting to note that the serogroup A capsular polysaccharide does not appear to be a traditional T-cell-independent antigen as is the serogroup C capsular polysaccharide. First, serogroup A unconjugated vaccine has been shown to be immunogenic in children under 2 years of age [43]. Second, the serogroup A polysaccharide appears to stimulate affinity maturation, as indicated by an increase in avidity indices following vaccination [44]. However, it does appear that there is a difference in the quality of the response induced if the serogroup A polysaccharide is conjugated or unconjugated. A greater level of bactericidal antibody is produced following serogroup A polysaccharide conjugation and the memory response appears to be sustainable [44]. Furthermore, in those aged between 10 and 29 years, serogroup A responses following repeated A/C polysaccharide vaccination were boosted, whereas for serogroup C, immunologic hyporesponsiveness was observed [45].

Meningococcal serogroup B correlates of protection

The poor immunogenicity of the serogroup B capsular polysaccharide means that the conjugation technology applied to vaccines for serogroups A, C, Y and W135 may not be appropriate and alternative vaccine approaches have to be investigated. A number of OMV vaccines have been trialed and utilized to contain epidemics in Norway [10], Brazil [46], Cuba [11] and Chile [47]. Data from the Norwegian serogroup B efficacy trial that took place from 1988-1991 has been recently re-analyzed to evaluate SBA as the appropriate correlate of protection for serogroup B [48]. This randomized double-blind, placebo-controlled efficacy trial involved 172,000 school children aged 13 to 14 years who received two doses of the OMV vaccine (strain B:15:P1.7,16:L3,7,9). The estimated efficacy after 10 months and 29 months was 87 and 57%, respectively. An immunogenicity study using human complement in the SBA, showed that 31% of the vaccinees had SBA titers ≥ 4 before vaccination and 97% reached this SBA titer 6 weeks after the second dose. Titers ≥ 4 persisted in 42% of vaccinees 2 years postvaccination. The percentage of subjects with $\geq$ fourfold rises in

SBA titer 6 weeks post second dose was 80% whilst this percentage decreased to 26% 2 years postvaccination. A separate three dose immunogenicity study was also performed with the cohort receiving a third dose at 10 months. The inclusion of a third dose was observed to improve the clinical performance of the Norwegian OMV vaccine with 96% of individuals achieving an SBA titer ≥ 4 or a $\geq$ fourfold rise in SBA titer 6 weeks after the third dose [48]. The good response observed persisted to 1 year after the third doses with 85% of individuals with a SBA titer ≥ 4 and 81% with a $\geq$ fourfold rise in SBA titer. Good correlation between observed protection and SBA titers was found and a tentative protective SBA titer of ≥ 4 was proposed. Thus, measurements of SBA may be used to evaluate different vaccine formulations against serogroup B disease.

Most studies of serogroup B OMV vaccines have not relied on absolute SBA titers as a cut off, but have used ≥ 4 -fold rises from pre to postvaccination. This approach seems more rational as titers can vary in magnitude between different laboratories due to the inherent variation of the SBA assay, yet the percentage with $\geq$ fourfold increases remains relatively constant [48,49].

Assessment of avidity indices as a marker of antibody maturation following meningococcal OMV vaccination has now been utilized [50,51]. These assays have been of use when investigating avidity maturation in different dose schedules of OMV vaccines and in the study of Longworth and colleagues [50] indicated that avidity maturation was taking place even though SBA titers did not reach putative protective levels in all immunized infants. Further studies using avidity indices as a marker of vaccine responses following OMV vaccination are required to gain full understanding of the usefulness of these assays in predicting both protection and immunologic memory in combination with clinical data.

Additional correlates of protection

SBA titers and assessment of immunologic memory have been generally accepted as appropriate correlates of protection, however, there are other mechanisms of protection involved in immunity to meningococcal disease that might be used as correlates of protection.

The whole blood killing assay is a method that can assess the bactericidal activity of blood incorporating antibody-mediated complement lysis and phagocytosis i.e., both humoral and cellular killing mechanisms [52-54]. It has been suggested that the whole blood assay is more sensitive than the SBA [5,31] with bactericidal activity detected in some individuals by the whole blood assay but not by the SBA assay. However, the whole blood assay is unlikely to be suitable for the evaluation of candidate serogroup B vaccines due to the requirement for large volumes of fresh blood for the assay and the difficulty in standardization of the assay.

Opsonophagocytosis is one mechanism that has been demonstrated to be bactericidal against meningococci [55-57]. *Neisseria meningitidis* can be phagocytosed by polymorphonuclear (PMN) cells [58-60] and monocytes [61,62]. The opsonophagocytic assay (OPA) has provided a useful correlate of protection for

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Streptococcus pneumoniae along with the ELISA (63) and could provide additional information regarding protection against *N. meningitidis*. The OPA can be semi-automated and use beads coated with capsular polysaccharide and can also be multiplexed to analyze four serogroups in the one assay (64). The OPA can also use inactivated bacteria as the target (65) but concerns have been raised that in the treatment of the meningococci, new or hidden epitopes may be exposed that have no functional relevance (66,67). Furthermore, there are issues of standardization between laboratories, particularly regarding the use of cultured cell lines or freshly obtained PMN cells. Further validation of the OPA is required to determine if it is a suitable correlate of protection. A recent study has suggested that SBA is the main immune effector of the vaccine protection against *N. meningitidis* rather than opsonophagocytosis following serogroup C vaccination (68). It is possible that it may be more appropriate for the evaluation of serogroup B vaccine candidates where the protective epitopes are subcapsular and may involve a greater role for cell-mediated immunity unlike serogroups A, C, W135 and Y where the capsular polysaccharide is the focus of the protective mechanisms. An OPA to analyze the level of protection against serogroup B isolates have been developed (65,67,69). A surface labelling technique which detects the initial stage of antibody binding to killed meningococci has also been developed and shows potential as an additional assay for the evaluation of serogroup B vaccines (69).

Another method that has been used to assess immune responses to meningococci is animal models. This approach can provide information on the impact of the host-pathogen relationship on whole tissues and the interaction between both humoral and cellular immune responses. However, there are disadvantages of the animal models, including the fact that the only natural host for *N. meningitidis* is humans and that there are no good models available that mimic the course of human disease.

The murine model has been most extensively used. This involves an intraperitoneal challenge of mice with meningococci but also requires an additional iron source for the infecting bacterial strain (70). The murine intraperitoneal challenge model has been used to assess active protection and passive protection for evaluation of vaccine candidates (71-73). This model was recently utilized to demonstrate protection against different serogroups and strains conferred by *Neisseria lactamica* immunization (74). It is interesting to note that although protection was observed against a lethal challenge, no bactericidal activity was observed in mouse sera.

An infant rat model has also been developed that has the advantage of not requiring an additional iron source and lower inocula are required to produce disease than in the mouse model. However, the rat model does not allow active protection to be assessed, not all strains are virulent and the duration of bacteremia is short and mortality low. It has been used to demonstrate passive protection with antibodies against a number of meningococcal components (75,76) and has been used in evaluating responses to serogroup B meningococci (76-79). One anomaly is that anticapsular antibody has been shown to be protective against a serogroup B challenge in the infant rat model (80) yet the serogroup B capsular

polysaccharide is only poorly immunogenic in humans. Furthermore, the assessment of the protection conferred by a monoclonal antibody specific for inner-core lipopolysaccharide epitope in an infant rat model was observed to correlate with bactericidal and not opsonophagocytic activity (81), illustrating the conflicting data that is currently being generated by animal models. Recently, differences in the response of adults to vaccination with plain polysaccharide serogroup C vaccines or MCC vaccines were investigated using an infant rat model (82). It was observed that at a limiting dose, the immune sera from vaccinees receiving the MCC vaccine was able to confer a greater degree of protection than sera from those receiving the unconjugated polysaccharide vaccine. The infant rat model has also been used to evaluate the potential vaccine candidate, genome-derived *Neisseria* antigen 2132 (GNA2132) (83). Anti-GNA2132 antibody was shown to bind to the surface of serogroups B or C and could passively protect infant rats against meningococcal bacteremia yet lacked bactericidal activity, again highlighting the discrepant data generated between the rat model and SBA assay.

Further data are required to establish both the OPA and animal models as credible correlates of protection. It may be that, as stated, they are more appropriate for use in the evaluation of serogroup B vaccines that are likely to be noncapsular polysaccharide vaccines and induce a wider range of immunologic mechanisms unlike the MCC vaccines which induce mainly bactericidal antibody.

Conclusions

The introduction of the MCC vaccines in the UK has been successful in significantly reducing the level of serogroup C disease incidence. The previously determined correlate of protection, SBA, for natural immunity to serogroup C disease has been re-evaluated for MCC vaccines. An rSBA titer of 8 has been validated using the vaccine efficacy estimates as an appropriate correlate of short-term protection. For long-term, a more appropriate correlate may be the assessment of immunologic memory or a balance of circulating antibody and memory or even an as yet unrealized mechanism. The majority of data have been generated from the determination of avidity indices, which has been shown to reflect affinity maturation and the induction of immunologic memory in infants and young children following MCC vaccination. It is currently unclear if avidity indices are a valid correlate for older age groups due to the production of antibody with a higher avidity in this age group. Additional correlates of protection, such as opsonophagocytic titer, may be of importance for OMV vaccines.

Expert opinion

The introduction of the MCC vaccines into the UK was backed by extensive safety and immunogenicity studies that provided those data required for licensure. Standardization and optimization of laboratory assays were required to evaluate the immunogenicity of MCC vaccines in various age groups and to provide data on appropriate correlates of protection. A combination of immunogenicity data and information gathered through the

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enhanced surveillance program to generate vaccine efficacy estimates, have been used to validate the serum bactericidal assay, and to some degree, avidity indices as correlates of short-term protection. These data have set a precedent for the evaluation of future meningococcal capsular polysaccharide-protein conjugate vaccines that include four serogroups A, C, W-135 and Y, and are likely to be licensed over the coming years. It is expected that these vaccines will induce similar responses, i.e., T-cell-dependent production of SBA, as the MCC vaccines. The serum bactericidal assay for serogroup C has been well characterized but there may be some issues regarding the assays for the remaining serogroups which are not as extensively standardized. Trivalent plain polysaccharide vaccines have been available for several years and successfully induce protective responses in older age groups but the conjugate vaccine(s) should be immunogenic in younger children.

Unlike the tetravalent conjugate vaccines that will be available in the future and are predicted to be as successful as the MCC vaccines, the prospect of a successful universal serogroup B vaccine for global use is less encouraging. The poor immunogenicity and the potential for autoimmunity due to the structural similarities to fetal neural cells means the use of a conjugated serogroup B polysaccharide vaccine will be difficult. Therefore, different approaches are being taken to vaccine formulation with the assessment of immune responses to surface-exposed proteins and noncapsular polysaccharides essential in identifying candidate antigens. Hence, the different composition of the candidate vaccines dictates that additional protective mechanisms are investigated that may be found to be suitable laboratory correlates of protection for serogroup B vaccines. This will include SBA and opsonophagocytic titers, antibody surface labelling, immunologic memory, T-cell responses and protection in animal models. The most substantial data generated for serogroup B vaccines has been from 'wild type' OMV (wtOMV) vaccines based on one representative epidemic bacterial isolate. However, these wtOMV vaccines, tend to induce functional immunity (SBA) to the homologous (vaccine-type) strain and only low levels (approximately 10%) of response to heterologous strains in infants (47). In adults, the heterologous SBA responses have been shown to be about 50–60% (47). Numerous, phenotypically and genotypically diverse serogroup B strains cause invasive disease in the UK (and other countries) and hence it is unlikely that a monovalent vaccine will be suitable. Further work is required to establish correlates of protection for serogroup B to enable the thorough evaluation of the immunogenicity of candidate serogroup B vaccines.

Five-year view

With the recent resurgence of Hib disease and the decline of efficacy in the UK (84), a catch up program of a booster dose of Hib conjugate vaccine commenced in the UK in May 2003 (85,86). Factors which may have contributed to this increase are first, a waning over time of the impact of the catch-up campaign (1–4 years of age), therefore the rate of Hib disease seen now may reflect the true level of protection given by Hib vaccine as part of the routine childhood program alone. Sec-

ondly, a diphtheria-tetanus-acellular pertussis-Hib vaccine used in 2000 and 2001 may not have provided the same high level of protection against Hib disease as the vaccines used before this period, or subsequently. Moreover, as this population of children, immunized with a 3 dose vaccine course were known reliably to induce immune memory (87), this suggests that absolute levels of circulating antibody may be of more importance than previously thought. This may have implications for the MCC vaccine and studies looking at booster doses in preschool children will undoubtedly commence in the near future.

A project to introduce a serogroup A conjugate vaccine into Africa has recently been initiated by the World Health Organization (WHO)/Program for Appropriate Technology for Health (PATH) (88). The aim of the project is to alleviate the large epidemics of serogroup A disease that occur in the sub-Saharan meningitis belt in Africa. It is hoped that the vaccine will be produced, validated and ready for introduction within the next 5–10 years. Trials followed by introduction of this vaccine in Africa will assist in the establishment of correlates of protection for serogroup A.

Further information is required on immunity to serogroup B disease and new candidate vaccines will require assessing. Currently New Zealand is experiencing an epidemic of serogroup B disease dominated by a single strain (B:4:P1.7-2.4) which commenced in 1991 and has seen the incidence of meningococcal disease rise from 1.6 per 100,000 prior to the epidemic to 13.9 per 100,000 over the period 1996–2000 (89). Since the epidemic is dominated by a clonal outbreak of a serogroup B meningococci with a single PorA serosubtype, it was decided that a homologous PorA-based OMV vaccine would have a role in controlling the epidemic (89). A candidate vaccine has been developed and is currently undergoing Phase I and II trials in New Zealand (90). Serologic analysis is primarily using bactericidal assays and will provide extensive data, which will help to determine if SBA titer is an appropriate correlate of protection for meningococcal serogroup B OMV-based vaccines.

Information resources

Articles recommended for further reading

- Miller E, Salisbury D, Ramsay M. Planning, registration and implementation of an immunisation campaign against meningococcal serogroup C disease in the UK: a success story. *Vaccine* 20, S58–S67 (2001).
- Goldschneider I, Gotschlich EC, Artenstein MS. Human immunity to the meningococcus: I. The role of humoral antibodies. *J. Exp. Med.* 129, 1307–1326 (1969).
- Borrow R, Andrews N, Goldblatt D, Miller E. Serological basis for use of meningococcal serogroup C conjugate vaccines in the United Kingdom: Re-evaluation of correlates of protection. *Infect. Immun.* 69, 1568–1573 (2001).
- Andrews N, Borrow R, Miller E. Validation of serological correlate of protection for meningococcal C conjugate vaccine using efficacy estimates from post-licensure surveillance in England. *Clin. Diag. Lab. Immunol.* 10, 780–786 (2003).

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The following websites contain additional information on meningococci & meningococcal disease

- Website of the Health Protection Agency: www.hpa.org.uk Accessed 21st December, 2003.
- Meningitis health topics page on the website of the World Health Organization: www.who.int/health_topics/meningitis Accessed 21st December, 2003.
- Website of the central resource for the Neisseria research community: www.neisseria.org Accessed 20th December, 2003.
- Website of the Meningitis Research Foundation: www.meningitis.org Accessed 20th December, 2003.
- Website of the Meningitis Trust: www.meningitis-trust.org.uk Accessed 21st December, 2003.

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Key issues

- The introduction of meningococcal serogroup C vaccines in the UK has been successful in significantly reducing the incidence of serogroup C disease. There is also evidence of a herd immunity effect reducing the incidence in unvaccinated individuals.
- The established correlate of protection for serogroup C, serum bactericidal antibody (SBA), has been validated against the vaccine efficacy estimates demonstrating that a SBA titer of 8 is an appropriate short-term correlate. For the estimation of long-term protection, the assessment of priming of immunologic memory may be a suitable correlate of protection.
- To evaluate future meningococcal conjugate vaccines, it is accepted that determination of SBA titers and immunologic memory in response to vaccination will be crucial in predicting the protective potential of the actual vaccines.
- Correlates of protection need to be established for immunity to serogroup B meningococci to enable the evaluation of candidate vaccines. This may include not only SBA titers and immunologic memory but also additional protective mechanisms.

References

Papers of special note have been highlighted as:

• of interest

•• of considerable interest

- 1 World Health Organization. *Weekly epidemiological record* 37, 281–288 (2001).
- 2 Fairley CK, Begg N, Borrow R *et al.* Conjugate meningococcal serogroup A and C vaccine: reactogenicity and immunogenicity in United Kingdom infants. *J. Infect. Dis.* 174, 1360–1363 (1996).
- 3 Richmond P, Borrow R, Miller E *et al.* Meningococcal serogroup C conjugate vaccine is immunogenic in infancy and primes for memory. *J. Infect. Dis.* 179, 1569–1572 (1999).
- 4 Richmond P, Borrow R, Findlow J *et al.* Evaluation of de-O-acetylated meningococcal C polysaccharide–tetanus toxoid conjugate vaccine in infancy: Reactogenicity, immunogenicity, immunologic priming and bactericidal activity against O-acetylated and de-O-acetylated serogroup C strains. *Infect. Immun.* 69, 2378–2382 (2001).
- 5 Richmond P, Borrow R, Goldblatt D *et al.* Ability of three different meningococcal C conjugate vaccines to induce immunologic memory after a single dose in UK toddlers. *J. Infect. Dis.* 183, 160–163 (2001).
- 6 Bramley JC, Hall T, Finn A *et al.* Safety and immunogenicity of three lots of meningococcal serogroup C conjugate vaccine administered at 2, 3 and 4 months of age. *Vaccine* 19, 2924–2931 (2001).
- 7 Rennels MB, Edwards KM, Keyserling HL *et al.* Safety and immunogenicity of four doses of *Neisseria meningitidis* group C vaccine conjugated to CRM197 in United States infants. *Pediatr. Infect. Dis. J.* 20, 153–159 (2001).
- 8 Wyle FA, Artenstein MS, Brandt BL *et al.* Immunological response of man to group B meningococcal polysaccharide antigens. *J. Infect. Dis.* 126, 514–522 (1972).
- 9 Finne J, Leinonen M, Mäkelä PH. Antigenic similarities between brain components and bacteria causing meningitis. Implications for vaccine development and pathogenesis. *Lancet* 2, 355–357 (1983).
- 10 Bjune G, Heiby EA, Grønnesby JK *et al.* Effect of outer membrane vesicle vaccine against group B meningococcal disease in Norway. *Lancet* 338, 1093–1096 (1991).
- 11 Sierra GVG, Campa HC, Varcacel NW *et al.* Vaccine against group B *Neisseria meningitidis*: protection trial and mass vaccination results in Cuba. *Natl Inst. Public Health Ann.* 14, 195–207 (1991).
- 12 Cassio de Moraes J, Perkins BA, Camargo MC *et al.* Protective efficacy of a serogroup B meningococcal vaccine in Sao Paulo, Brazil. *Lancet* 340, 1074–1078 (1992).
- 13 Boslego J, García J, Cruz C *et al.* Efficacy, safety, and immunogenicity of a meningococcal group B (15:P1.3) outer membrane protein vaccine in Iquique, Chile. Chilean National Committee for Meningococcal Disease. *Vaccine* 13, 821–829 (1995).
- 14 Masignani V, Comanducci M, Giuliani MM *et al.* Vaccination against *Neisseria meningitidis* using three variants of the lipoprotein GNA1870. *J. Exp. Med.* 197, 789–799 (2003).
- 15 Comanducci M, Bambini S, Brunelli B *et al.* NadA, a novel vaccine candidate of *Neisseria meningitidis*. *J. Exp. Med.* 195, 1445–1454 (2002).
- 16 Black S, Shingfield H, Fireman B *et al.* Efficacy, safety and immunogenicity of heptavalent pneumococcal conjugate vaccine in children. Northern California Kaiser Permanente Vaccine Study Center Group. *Pediatr. Infect. Dis. J.* 3, 187–195 (2000).
- 17 Miller E, Salisbury D, Ramsay M. Planning, registration, and implementation of an immunisation campaign against meningococcal serogroup C disease in the UK: a success story. *Vaccine* 20, S58–S67 (2001).

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- Summarizes the UK immunization campaign against serogroup C disease.
- 18 Chief Medical Officer, Chief Nursing Officer, Chief Pharmaceutical Officer. Extending meningitis C vaccine to 20–24 year olds; Pneumococcal vaccine for at-risk under 2 year olds. *PL/CMO/2002/1, PL/CNO/2002/1, PL/CPHO/2002/1* (2002).
- 19 Balmer P, Borrow R, Miller E. Impact of meningococcal C conjugate vaccine in the UK. *J. Med. Microbiol.* 51, 717–722 (2002).
- 20 Ramsay ME, Andrews NJ, Trotter CL, Kaczmarski EB, Miller E. Herd immunity from meningococcal serogroup C conjugate vaccination in England: database analysis. *Br. Med. J.* 326, 365–366 (2003).
- 21 Maiden M, Stuart J and The United Kingdom Meningococcal Carriage Group. Reduced carriage of serogroup C meningococci in teenagers one year after the introduction of meningococcal C conjugate polysaccharide vaccine in the United Kingdom. *Lancet* 359, 1829–1851 (2002).
- Clearly shows the reduction of serogroup C carriage following immunization with conjugate vaccine.
- 22 Goldschneider I, Gotschlich EC, Aronstein MS. Human immunity to the meningococcus: I. The role of humoral antibodies. *J. Exp. Med.* 129, 1307–1326 (1969).
- Pioneering studies that set the original correlate of protection for serogroup C.
- 23 Goldschneider I, Gotschlich EC, Aronstein MS. Human immunity to the meningococcus: II. Development of natural immunity. *J. Exp. Med.* 129, 1327–1348 (1969).
- Pioneering studies that set the original correlate of protection for serogroup C.
- 24 World Health Organisation. Requirements for meningococcal polysaccharide vaccine. World Health Organization technical report series, no. 594. World Health Organization, Geneva, Switzerland (1976).
- 25 Griffiths JM, Goroff DK. IgA blocks IgM and IgG-initiated immune lysis by separate molecular mechanisms. *J. Immunol.* 130, 2882–2885 (1983).
- 26 Zollinger WD, Mandrell RE. Importance of complement source in bactericidal activity of human antibody and murine monoclonal antibody to meningococcal group B polysaccharide. *Infect. Immun.* 40, 257–264 (1983).
- 27 Trotter C, Borrow R, Andrews N, Miller E. Seroprevalence of meningococcal serogroup C bactericidal antibody in England and Wales in the prevaccination era. *Vaccine* 21, 1094–1098 (2003).
- 28 Borrow R, Andrews N, Goldblatt D, Miller E. Serological basis for use of meningococcal serogroup C conjugate vaccines in the United Kingdom: re-evaluation of correlates of protection. *Infect. Immun.* 69, 1568–1573 (2001).
- Reevaluated the correlates of protection for serogroup C.
- 29 Farrington P, Miller E. Meningococcal vaccine trials. In: *Methods in Molecular Medicine: Meningococcal Vaccines*. AJ Pollard, MC Maiden (Eds.), Humana press, NJ, USA 371–393 (2001).
- 30 Andrews N, Borrow R, Miller E. Validation of serological correlate of protection for meningococcal C conjugate vaccine using efficacy estimates from postlicensure surveillance in England. *Clin. Diag. Lab. Immunol.* 10, 780–786 (2003).
- Validates the serological correlate of protection for serogroup C.
- 31 Borrow R, Goldblatt D, Andrews N *et al.* Antibody persistence and immunological memory at age 4 years after meningococcal group C conjugate vaccination in children in the United Kingdom. *J. Infect. Dis.* 186, 1353–1357 (2002).
- 32 Goldblatt D, Pinto Vaz, ARJPM, Miller E. Antibody avidity as a surrogate marker of successful priming by *Haemophilus influenzae* Type b conjugate vaccines following infant immunization. *J. Infect. Dis.* 177, 1112–1115 (1998).
- 33 Joseph H, Miller E, Dawson M *et al.* Meningococcal serogroup A avidity indices as a surrogate marker of priming for the induction of immunological memory after vaccination with a meningococcal A/C conjugate vaccine in infants in the United Kingdom. *J. Infect. Dis.* 184, 661–662 (2001).
- 34 Goldblatt D, Borrow R, Miller E. Natural and vaccine-induced immunity and immunologic memory to *Neisseria meningitidis* serogroup C in young adults. *J. Infect. Dis.* 185, 397–400 (2002).
- 35 Konradsen HB. Quality and avidity of pneumococcal antibodies before and five years after pneumococcal vaccination of elderly persons. *Clin. Infect. Dis.* 21, 616–620 (1995).
- 36 Wuorimaa T, Käyhty H, Leroy O, Eskola J. Tolerability and immunogenicity of an 11-valent pneumococcal conjugate vaccine in adults. *Vaccine* 19, 1863–1869 (2001).
- 37 Rosenqvist E, Hoiby EA. Relative bactericidal activity of IgG antibodies against outer membrane complex from meningococci, as a function of vaccine type, dose and time after vaccination. In: *Neisseriae – 1990*. Achtman M, Kohl P, Marchal C *et al.* (Eds.), Walter de Gruyter, Berlin, Germany and NY, USA 265–269 (1990).
- 38 Petrola H, Mäkelä H, Käyhty H *et al.* Clinical efficacy of meningococcus group A capsular polysaccharide vaccine in children three months to five years of age. *N. Engl. J. Med.* 297, 686–691 (1977).
- Provides the first insight into correlates of protection for serogroup A.
- 39 Mäkelä PH, Käyhty H, Weckström P, Sivonen E, Renkonen O-V. Effect of group A meningococcal vaccine in army recruits in Finland. *Lancet* 2, 883–886 (1975).
- 40 MacLennan J, Obaro S, Deeks J *et al.* Immune response to revaccination with meningococcal A and C polysaccharides in Gambian children following repeated immunisation during early childhood. *Vaccine* 17, 3086–3093 (1999).
- 41 Leach A, Twumasi PA, Kumah S *et al.* Induction of immunologic memory in Gambian children by vaccination in infancy with a group A plus group C meningococcal polysaccharide-protein conjugate vaccine. *J. Infect. Dis.* 175, 200–204 (1997).
- 42 Borrow R, Joseph H, Andrews N *et al.* Reduced antibody response to revaccination with meningococcal serogroup A polysaccharide vaccine in adults. *Vaccine* 19, 1129–1132 (2000).
- 43 Gold R, Lepow ML, Goldschneider I, Draper TL, Gotschlich EC. Clinical evaluation of group A and group C meningococcal polysaccharide vaccines in infants. *J. Clin. Invest.* 56, 1536–1547 (1975).
- 44 Joseph H, Ryall R, Bybel M *et al.* Immunogenicity and immunological priming of the serogroup A portion of a bivalent meningococcal A/C conjugate vaccine in 2-year-old children. *J. Infect. Dis.* 187, 1142–1146 (2003).
- 45 Jokhdar H, Borrow R, Sultan A *et al.* Immunologic hyporesponsiveness to serogroup C but not serogroup A following repeated meningococcal A/C polysaccharide vaccination in Saudi Arabia. *Clin. Diag. Lab. Immunol.* 11, 83–88 (2004).
- 46 Milagres LG, Ramos SR, Sacchi CT *et al.* Immune response of Brazilian children to a *Neisseria meningitidis* serogroup B outer membrane protein vaccine: comparison with efficacy. *Infect. Immun.* 62, 4419–4424 (1994).

26

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Balmer & Borrow

- 47 Tappero JW, Lagos R, Ballesteros AM *et al.* Immunogenicity of two serogroup B outer-membrane protein meningococcal vaccines: a randomized controlled trial in Chile. *JAMA* 28, 1520-1527 (1999).
- 48 Molst J, Feiring B, Fuglesang JE *et al.* Serum bactericidal activity correlates with the vaccine efficacy of outer membrane vesicle vaccines against *Neisseria meningitidis* serogroup B disease. *Vaccine* 21, 734-737 (2003).
- 49 Martin D, Glennie A, McCallum L *et al.* Evaluates data from an outer membrane vesicle vaccine trial to further understand correlates of protection for serogroup B. Validating the serum bactericidal assay for group B meningococci for use in clinical trials. Seventh meeting of the European Monitoring Group for Meningococci. Lanzarote, Spain. P55 (2003).
- 50 Longworth E, Borrow R, Goldblatt D *et al.* Avidity maturation following vaccination with a meningococcal recombinant hexavalent PorA OMV vaccine in UK infants. *Vaccine* 20, 2592-2596 (2002).
- 51 Vermont CL, van Dijken HH, van Limpt CJ *et al.* Antibody avidity and immunoglobulin G isotype distribution following immunization with a monovalent meningococcal B outer membrane vesicle vaccine. *Infect. Immun.* 70, 584-590 (2002).
- 52 Ison CA, Heyderman RS, Klein NJ, Peakman M, Levin M. Whole blood model for exploring host-bacterial interactions. *Microb. Pathog.* 18, 97-107 (1995).
- 53 Ison CA, Anwar N, Cole MJ *et al.* Assessment of immune response to meningococcal disease: comparison of a whole-blood assay and the serum bactericidal assay. *Microb. Pathog.* 27, 207-214 (1999).
- 54 Ison CA, Anwar N, Cole MJ *et al.* Age dependence of in vitro survival of meningococci in whole blood during childhood. *Pediatr. Infect. Dis. J.* 22, 868-873 (2003).
- 55 Brodus RG, Derix BH, Fijen CA *et al.* Fcγ receptor IIa (CD32) polymorphism in fulminant meningococcal shock in children. *J. Infect. Dis.* 170, 848-853 (1994).
- 56 Estabrook MM, Christopher NC, Griffiss JM, Baker CJ, Mandrell RE. Sialylation and human neutrophil killing of group C *Neisseria meningitidis*. *J. Infect. Dis.* 166, 1079-1088 (1994).
- 57 Schlesinger M, Greenberg R, Levy J, Käyhty M, Levy R. Killing of meningococci by neutrophils: effect of vaccination on patients with complement deficiency. *J. Infect. Dis.* 170, 449-453 (1994).
- 58 Fredlund A, Olcen P, Danielsson D. A reference procedure to study chemiluminescence induced in polymorphonuclear leukocytes by *Neisseria meningitidis*. *Acta Pathol. Microbiol. Immunol. Scand. Sect. 96*, 941-949 (1988).
- 59 Gomez-Lus ML, Aguilar L, Vazquez J *et al.* Ex vivo bactericidal activity against group C *Neisseria meningitidis* in seronegative subjects. *Infection* 28, 305-308 (2000).
- 60 Halstensen A, Sjørsten H, Vollset SE *et al.* Serum opsonins to serogroup B meningococci in meningococcal disease. *J. Infect. Dis.* 21, 267-276 (1989).
- 61 Lowell GH, Smith LE, Arntstein MS, Nash GS, MacDermott Jr RP. Antibody dependent cell mediated antibacterial activity of human mononuclear cells. I. K lymphocytes and monocytes are effective against meningococci in cooperation with human immune sera. *J. Exp. Med.* 150, 127-137 (1979).
- 62 Smith LE, Lowell GH. Antibody-dependent cell mediated antibacterial activity of human mononuclear cells. II. Immune specificity of antimeningococcal activity. *J. Infect. Dis.* 141, 748-751 (1980).
- 63 Martinez JE, Romero-Steiner S, Pilishvili T *et al.* A flow cytometric opsonophagocytic assay for the measurement of functional antibodies elicited after vaccination with the 23-valent pneumococcal polysaccharide vaccine. *Clin. Diagn. Lab. Immunol.* 6, 581-586 (1999).
- 64 Martinez J, Pilishvili T, Barnard S *et al.* Opsonophagocytosis of fluorescent polystyrene beads coupled to *Neisseria meningitidis* serogroup A, C, Y or W-135 polysaccharide correlates with serum bactericidal activity. *Clin. Diagn. Lab. Immunol.* 9, 485-488 (2002).
- 65 Aase A, Holby EA, Michaelsen TE. Opsonophagocytic and bactericidal activity mediated by purified IgG subclass antibodies after vaccination with the Norwegian group B meningococcal vaccine. *Scand. J. Immunol.* 47, 388-396 (1998).
- 66 Michaelsen TE, Aase A, Kolberg J, Wedge E, Rosenqvist E. PorB3 outer membrane protein on *Neisseria meningitidis* is poorly accessible for antibody binding on live bacteria. *Vaccine* 19, 1526-1533 (2001).
- 67 Aase A, Naess LM, Sandin RH *et al.* Comparison of functional immune responses in humans after intranasal and intramuscular immunizations with outer membrane vesicle vaccines against group B meningococcal disease. *Vaccine* 21, 2042-2051 (2003).
- 68 Gomez-Lus ML, Gimenez MJ, Vazquez JA *et al.* Opsonophagocytosis versus complement bactericidal killing as effectors following *Neisseria meningitidis* group C vaccination. *Infection* 31, 51-54 (2003).
- 69 Funnell SGP, Taylor S, Pryer H *et al.* Developing of meningococcal whole cell antibody surface labeling and opsonophagocytic assays for assessment of vaccines containing proteins. 13th International Pathogenic *Neisseria* Conference, Oslo, Norway. P251 (2002).
- 70 Holbein BE. Iron-controlled infection with *Neisseria meningitidis* in mice. *Infect. Immun.* 29, 886-891 (1980).
- 71 Huer M, Sture A. An animal model for testing the activity of meningococcal polysaccharide vaccine. *J. Biol. Stand.* 9, 67-74 (1981).
- 72 Martin D, Cadieux N, Hamel J, Brodeur BR. Highly conserved *Neisseria meningitidis* surface protein confers protection against experimental infection. *J. Exp. Med.* 185, 1173-1183 (1997).
- 73 Brodeur BR, Larose Y, Tsang P, Hamel J, Ryan A. Protection against infection with *Neisseria meningitidis* group B serotype 2b by passive immunization with serotype-specific monoclonal antibody. *Infect. Immun.* 50, 510-516 (1985).
- 74 Oliver KJ, Reddin KM, Bracegirdle P *et al.* *Neisseria lactamica* protects against experimental meningococcal infection. *Infect. Immun.* 70, 3621-3626 (2002).
- 75 Saulskonen K, Leinonen M, Abdillahi H, Poolman JT. Comparative evaluation of potential components for group B meningococcal vaccine by passive protection in the infant rat and in vitro bactericidal assay. *Vaccine* 7, 325-328 (1989).
- 76 Toropainen M, Käyhty H, Saarinen L *et al.* The infant rat model adapted to evaluate human sera for protective immunity to group B meningococci. *Vaccine* 7, 2677-2689 (1999).
- 77 Toropainen M, Saarinen L, van der Ley P, Kuipers B, Käyhty H. Murine monoclonal antibodies to PorA of *Neisseria meningitidis* show reduced protective activity in vivo against B:15:P1.7,16 subtype variants in an infant rat infection model. *Microb. Pathol.* 30, 139-148 (2001).
- 78 Schmidt S, Zhu D, Barniak V *et al.* Passive immunization with *Neisseria meningitidis* PorA specific immune sera reduces nasopharyngeal colonization of group B meningococcus in an infant rat nasal challenge model. *Vaccine* 19, 4851-4858 (2001).

27

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Meningococcal vaccines

- 79 Toropainen M, Saarinen L, Käyhkö H, Mäkelä PH. The Icelandic Study Group. Passive protection in the infant rat protection assay (IRPA) by sera taken after vaccination of young adults by two serogroup B meningococcal outer membrane vesicle vaccines in Iceland. *13th International Pathogenic Neisseria Conference*, Oslo, P302 (2002).
- 80 Toropainen M, Saarinen L, Bolstad K et al. Are IgM antibodies to meningococcal serogroup B capsular polysaccharide responsible for passive protection by pre-vaccination sera in the infant rat protection assay (IRPA)? *13th International Pathogenic Neisseria Conference*, Oslo, P303 (2002).
- 81 Pledes JS, Harris SL, Wright JC et al. Highly conserved *Neisseria meningitidis* inner-core lipopolysaccharide epitope confers protection against experimental meningococcal bacteremia. *J. Infect. Dis.* 187, 1223–1234 (2003).
- 82 Harris SL, Finn A, Granoff DM. Disparity in functional activity between serum anticapsular antibodies induced in adults by immunization with an investigational group A and C *Neisseria meningitidis* – diphtheria toxoid conjugate vaccine and by a polysaccharide vaccine. *Infect. Immun.* 71, 3402–3408 (2003).
- 83 Welsch JA, Moe GR, Rossi R et al. Antibody to genome-derived *Neisseria meningitidis* antigen 2132, a *Neisseria meningitidis* candidate vaccine, confers protection against bacteremia in the absence of complement-mediated bactericidal activity. *J. Infect. Dis.* 188, 1730–1740 (2003).
- 84 Ramsay ME, McVernon J, Andrews NJ, Heath PT, Slade MP. Estimating *Haemophilus influenzae* type b vaccine effectiveness in England and Wales by use of the screening method. *J. Infect. Dis.* 188, 481–485 (2003).
- 85 Chief Medical Officer, Chief Nursing Officer, Chief Pharmaceutical Officer. Planned Hib Vaccination Catch-up Campaign. PL/CMO/2003/1, PL/CNO/2003/1, PL/CPMO/2003/1 (2003).
- 86 Chief Medical Officer, Chief Nursing Officer, Chief Pharmaceutical Officer. Planned Hib Vaccination Catch-up Campaign – further information. PL/CMO/2003/2, PL/CNO/2003/2, PL/CPHO/2003/2 (2003).
- 87 Goldblatt D, Richmond R, Millard E, Thornton C, Miller E. The induction of immunologic memory after vaccination with *Haemophilus influenzae* type b conjugate and acellular pertussis-containing diphtheria, tetanus and pertussis vaccine combination. *J. Infect. Dis.* 180, 538–541 (1999).
- 88 Jodar L, LaForce FM, Ceccarini C, Aguado T, Granoff DM. Meningococcal conjugate vaccine for Africa: a model for development of new vaccines for the poorest countries. *Lancet* 361, 1902–1904 (2003).
- 89 Baker MG, Martin DR, Kieft CEM, Lennon D. A 10-year serogroup B meningococcal disease epidemic in New Zealand: Descriptive epidemiology, 1991–2000. *J. Paediatr. Child Health* 37, S13–S19 (2001).
- 90 Holst J, Aaberge IS, Oster P et al. A 'tailor made' vaccine trialled as part of public health response to group B meningococcal epidemic in New Zealand. *Eurosurveillance Weekly* 7, 030724 (2003).

Website

- 101 Surveillance of impact of meningococcal serogroup C conjugate vaccination program in England and Wales and protocol for investigation of vaccine failures: joint document from the Health Protection Agency and the Institute of Child Health, London
www.hpa.org.uk/infections/topics_az/advice/mensurvw99.pdf

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Immune Response of Brazilian Children to a *Neisseria meningitidis* Serogroup B Outer Membrane Protein Vaccine: Comparison with Efficacy

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Since 1986, serogroup B *Neisseria meningitidis* has caused approximately 80% of the meningococcal disease in Brazil. In 1988, an epidemic caused by *N. meningitidis* B:4:P1.15 was recognized in the greater São Paulo area of Brazil. The São Paulo state government decided to vaccinate children from 3 to 83 months of age with a vaccine consisting of serotype 4 outer membrane protein and group C meningococcal polysaccharide that was produced in Cuba. About 2.7 million children were vaccinated during two immunization campaigns conducted in 1989 and 1990. Because of this, a case-control study was designed to determine vaccine efficacy against group B meningococcal disease. The purpose of our study was to compare the antibody response with the protection from disease estimated from the case-control study. We measured the immune responses of vaccinees by enzyme-linked immunosorbent assay (ELISA), immunoblot, and bactericidal assay. The development of bactericidal antibodies was age dependent and in good agreement with the results of the case-control study. Only 40% of vaccinees showed fourfold or greater increases in bactericidal antibody titers after vaccination. A poor correlation between antibody levels detected by ELISA and those by bactericidal assay was found. Immunoblot analysis showed that about 50% of the serum samples with bactericidal titers higher than 1:4 were reactive with class 1 outer membrane protein. We conclude that the bactericidal assay is a good, laboratory-based, functional assay for the study of vaccine immunogenicity and that an effective solution to group B meningococcal disease remains to be demonstrated.

Since 1986, serogroup B *Neisseria meningitidis* has been responsible for approximately 80% of the meningococcal disease in Brazil (29). In 1988, an epidemic situation in the greater São Paulo area of Brazil, which extends up to the present, was recognized (6). In 1990, greater São Paulo included 17.8 million inhabitants in 38 municipal regions. A single serogroup B *N. meningitidis* clone, being of the ET-5 complex and having serotype 4:P1.15, has been the prevalent strain in the region (8, 29).

Effective polysaccharide vaccines against *N. meningitidis* serogroups A, C, Y, and W135 are available (10, 15, 17). In contrast, the serogroup B polysaccharide is poorly immunogenic and antibodies to this polysaccharide do not appear to be protective (21). Most of the efforts to develop an effective serogroup B vaccine have therefore focused on lipooligosaccharide-depleted outer membrane proteins (OMPs) (2, 14, 27, 36). Efficacy trials with such vaccines have recently been conducted in Chile, Cuba, and Norway (3, 5, 32). Efficacy levels of approximately 50% were found in the Chile and Norway studies. Better protection was reported for the vaccine produced in Cuba, with an efficacy around 80% against disease caused by a B:4:P1.15 strain (32). This was the first clear demonstration that antibodies induced to noncapsular antigens can protect against meningococcal disease. In view of the epidemic situation in greater São Paulo, i.e., 4.06 cases per 100,000 inhabitants in 1988, and recent acquisition of the Cuban serogroup BC vaccine by the São Paulo state govern-

ment, it was decided to vaccinate all children from 3 months to 7 years of age. Two immunization campaigns were conducted. In the first (1989), about 300,000 children attending day care centers were vaccinated (12% of the estimated 2.7 million children in the target age range), and in the second (1990), about 2.4 million children (92% of children in the target age range) were vaccinated. Both campaigns were carried out in regional health clinics where children received two doses of the vaccine (6, 7). The vaccination campaigns in São Paulo were the first use of the Cuban-produced vaccine outside of Cuba. Analysis of the immune responses of vaccinated children was considered fundamental to an evaluation of vaccine effectiveness in Brazil. The purpose of this study was to measure the antibody responses of vaccinees by quantitative and functional methods such as enzyme-linked immunosorbent assay (ELISA) and bactericidal assay and to correlate these responses with the protection against disease by using the results of the case-control study (24), which was designed to determine vaccine efficacy against group B meningococcal disease.

MATERIALS AND METHODS

Vaccine. The vaccine was produced in Cuba (it is often referred to as Cuban BC vaccine) from strain Cu385/83 (B:4:P1.15), consisted of lipooligosaccharide-depleted OMPs and group C polysaccharide, and was enriched with envelope proteins from 65 to 95 kDa (32). The group C polysaccharide was present to improve the solubility of the OMPs and to provide protection against group C meningococcal disease. The vaccine was adsorbed onto aluminum hydroxide. Each dose contains 50 µg of group B proteins, 50 µg of C polysac-

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TABLE 1. Antibody levels as measured by ELISA pre- and postvaccination with the Cuban BC vaccine

Age group (mo)	No. of children	Strain of ELISA antigen	Antibody levels (U/ml) ^a		Fold increase	% >Twofold ^b	% >Fourfold ^b
			Prevaccination	Postvaccination			
3-23	275	Cu385/83	25.5	111.2	4.4	81	51
	276	N.150/88	21.7	59.0	2.7	63	35
	120	N.44/89	36.1	156.7	4.3	84	56
	120	N.577/89	37.2	136.3	3.7	85	51
	120	N.614/89	23.9	71.0	3.0	80	42
24-47	144	Cu385/83	23.3	110.5	4.8	85	54
	144	N.150/88	18.6	73.9	4.0	76	45
	20	N.44/89	20.8	97.0	4.7	100	60
	21	N.577/89	20.9	83.9	4.0	90	52
	21	N.614/89	17.0	53.6	3.2	95	38
48-83	141	Cu385/83	27.1	148.0	5.5	87	56
	141	N.150/88	17.6	82.4	4.7	86	56
	19	N.44/89	31.1	173.1	5.6	95	58
	19	N.577/89	23.9	108.9	4.6	89	63
	19	N.614/89	26.3	100.4	3.8	84	58

^a Geometric mean units per milliliter.^b Percentages of vaccinees showing greater than two- and fourfold increases in antibody levels, respectively.

charide, and 2 mg of aluminum hydroxide with 0.01% thimerosal as a preservative (32). Two intramuscular doses of 0.5 ml were administered to children between the ages of 3 and 83 months at an interval of 6 to 8 weeks.

Serum samples from vaccinees. During the first campaign, blood samples were collected before the first dose and 4 weeks after the second vaccination. Serum samples were stored at -20°C. For serological studies, children were classified in the three age groups used for the case-control study. The age groups and numbers of individuals studied can be seen in Tables 1 and 2.

Meningococcal strains. Brazilian strains designated N.44/89 (B:4:P1.15), N.150/88 (B:4:P1.15), N.577/89 (B:4:nt), and N.614/89 (B:NT:P1.15) and a Cuban strain [Cu385/83 (B:4:P1.15)] were used for the preparation of ELISA solid-phase outer membrane antigens. Strains Cu385/83 and N.150/88 were used in immunoblot analysis and as the target strain for the bactericidal assay, respectively. These strains were serogrouped by slide agglutination and serotyped by immunoblot analysis with monoclonal antibodies (29).

ELISA and immunoblot antigens. Cultures of *N. meningitidis* were grown overnight at 36°C in tryptic soy broth (TSB) (Difco Laboratories, Detroit, Mich.) on a rotatory shaker at 120 rpm. Outer membrane vesicles (OMV) were prepared by extraction of the wet cell pellet for 2.5 h at 50°C with 5 ml of 0.2 M lithium chloride in a 0.1 M sodium acetate buffer (pH 5.8) per g of cells (33). Protein concentrations were determined by the method of Lowry et al. (22), and sodium dodecyl

sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to analyze the OMV proteins. Sialic acid and 2-keto-3-deoxyoctulosonic acid were analyzed by the resorcinol and thiobarbituric acid techniques, respectively (26, 34). Additionally, strain Cu385/83 was grown in TSB containing 42 µM EDDA [ethylenediamine di(*o*-hydroxyphenylacetic acid); Sigma Chemical Company, St. Louis, Mo.] to chelate free ferric iron and induce the formation of iron-regulated proteins (IRPs). OMVs containing IRPs were used only for immunoblot analysis.

ELISA technique. A standardized ELISA was performed in triplicate in microdilution plates (Difco) as described by Harthug et al. (19) with an alkaline phosphatase-conjugated goat anti-human immunoglobulin G (IgG) (Sigma) detection system. As an internal antibody standard, a twofold dilution series of a positive postvaccination serum sample was used in all experiments. The mean value of the observed optical density was transformed to arbitrary units per milliliter by a sigmoidal standard curve (log-logit transformation) calculated from the values of the reference serum sample with a computer program provided by Carl E. Frasch. Initially, all serum samples were analyzed at a 1:200 dilution. Samples with optical density values of ≥90% of the maximum optical density of the standard were further diluted and reanalyzed.

Bactericidal assay. A modification of the assay described by Frasch and Robbins was used for determining bactericidal activity (12). Serum samples were titrated in flat-bottom plates (Linbro; Flow) with Hanks' balanced salt solution containing

TABLE 2. Geometric mean bactericidal titers against *N. meningitidis* N.150/88 (B:4:P1.15) in serum samples from vaccinees

Age group (mo)	No. of children	GMT ^a		% >1:4 ^b		% ≥Fourfold increase ^c	% Point estimate of vaccine efficacy (95% CI) ^d
		Prevaccination	Postvaccination	Prevaccination	Postvaccination		
3-23	122	1.1	2.0	2.5	13.1	22	~37 (~100 to 73%)
24-47	44	1.5	4.9	20.4	43.2	45	47 (-72 to 84%)
48-83	44	2.0	9.2	25.0	52.3	52	74 (16 to 92%)
Total	210	1.5	4.6	10.9	36.2	40	54 (Not given)

^a GMT, geometric mean bactericidal titer.^b Percentages of vaccinees with bactericidal titers of greater than 1:4.^c Percentages of vaccinees with bactericidal titer increases of at least fourfold.^d From the case-control study (24), with the 95% CI also given.

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0.1% bovine serum albumin (HBSS-BSA) as diluent. Meningococci were grown to log phase (2 h) in Mueller-Hinton broth (Difco), and then an equal volume of 10% milk (Oxoid) was added. The culture was then aliquoted and frozen at -70°C . An aliquot was thawed and diluted to yield approximately 2,000 CFU/ml, with the final dilution in HBSS-BSA. By this method, a uniform number of organisms could be used for each assay and a comparison with freshly grown cells gave nearly identical results. The source of complement was human serum from a single donor that lacked bactericidal activity against the target strain and by immunoblot showed no reactivity against the class 1 through class 5 OMPs of strain N.44/89. Microtitration plates were incubated at 37°C for 30 min, and approximately 150 μl of Mueller-Hinton agar containing 10% horse serum and VCN inhibitor (BBL Microbiological Systems, Cockeysville, Md.) was added to each well. Plates were further incubated for 24 h at 37°C with 5% CO_2 . The CFU per well were determined with the aid of a stereoscopic microscope ($\times 40$). The bactericidal titer was determined with the reciprocal serum dilution yielding a $\geq 50\%$ reduction in the viable count. A positive serum sample and bacterial inoculum plus complement source controls were included in each experiment.

Immunoblot. SDS-PAGE and the detection of antibodies by immunoblot were performed as described by Wedege and Frøholm (35), except that Tris-glycine transfer buffer without methanol was used. Monoclonal antibodies against type 4 (2303C5), subtype P1.15 (2731C6), and class 4 (AE3) and class 5 (AG10) OMPs were used to identify the class 1 to 5 proteins. The monoclonal antibodies 2303C5 and 2731C6 were produced by one of us (C.E.F.), and the monoclonal antibodies AE3 and AG10 were provided by Biomanguinhos Institute, Rio de Janeiro, Brazil. For class 3 monoclonal antibody reaction, Empigen BB 0.25% (Albright and Wilson, Whitehaven, United Kingdom) was added to the primary antibody solution (23). The binding of human IgG and mouse IgG was detected with goat anti-human and anti-mouse IgG antibodies conjugated with horseradish peroxidase. The pre- and postvaccination serum samples of 15 individuals with postvaccination titers greater than 1:4 were tested.

Statistical methods. The ELISA and bactericidal assay results were transformed to logarithmic values to calculate the geometric means. This gave a normal distribution of data and allowed us to use standard statistical tests (1). The significance levels of differences between groups were examined by the Student *t* test, Wilcoxon scores, or the Kruskal-Wallis test on the log-transformed data. Fisher's exact test was used to analyze the differences among percentages.

RESULTS

Antigenic analysis. SDS-PAGE analysis of the antigens used for ELISA showed a predominance of class 1, 3, 4, and 5 OMPs. In strain N.150/88, class 4 and 5 OMPs were present in small amounts (data not shown).

The antigens used for immunoblot analysis can be seen in the first lane of Fig. 1. These nitrocellulose strips were stained with amido black. Class 1, 3, 4, and 5 OMPs predominated for both antigens. Also, some high-molecular-weight IRPs can be seen with antigens from strains grown in TSB with EDDA (Fig. 1B).

For all antigen preparations, the ratio of protein to lipooligosaccharide was approximately 1:1 and only traces of sialic acid could be detected.

Antibody responses to OMPs. Table 1 shows the ELISA antibody levels against different *N. meningitidis* group B strains

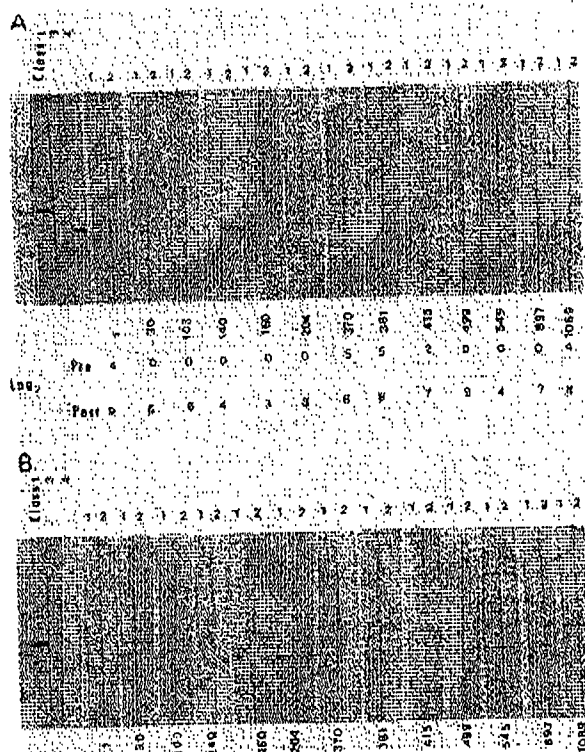


FIG. 1. Immunoblots showing IgG binding to OMPs of *N. meningitidis* Cu385/83 (B:4:P1.15) of pre- (lanes 1) and postimmunization (lanes 2) serum samples from children older than 24 months of age. IgG binding to antigens extracted from the strain grown in normal TSB (A) and TSB containing added EDDA (B) is shown. Amido black-stained strips are shown in the lane at the far left. The next three lanes represent monoclonal antibodies binding to class 1, 3, and 4 proteins, respectively. The bactericidal titers (expressed as $\log_2$ of the reciprocal titers) for pre- and postimmunization serum samples are also shown. The numbers 1, 30, 103, etc., represent vaccinees.

for the age groups examined. The observed differences between the pre- and postimmunization serum samples for all age groups were statistically significant ($P < 0.05$). There were no significant differences in the antibody levels or percent responders among vaccinees among the three age groups studied, except for children from 3 to 23 months of age who showed lower responses against strain N.150/88 ($P < 0.05$). Of all vaccinees studied, 85 and 52% showed at least two- or fourfold increases in antibody levels after vaccination, respectively.

Bactericidal antibodies. Table 2 shows the bactericidal antibody titers against *N. meningitidis* strain N.150/88 (B:4:P1.15) in serum samples obtained before and after vaccination. Some of the serum samples were also tested against two additional B:4:P1.15 strains (N.44/89 and N.131/88), with comparable titers. For each age group, the increase in antibody titers after vaccination was statistically significant ($P < 0.05$). Also, the percentages of children with bactericidal titers of $> 1:4$ pre- and postvaccination are shown. The postimmunization antibody levels in children less than 24 months of age were significantly lower than those of older children ($P < 0.05$). No differences in bactericidal titers or fold increases in children

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TABLE 3. Geometric mean bactericidal titers against *N. meningitidis* N.150/88 (B:4:P1.15) in individuals with and without detectable prevaccination bactericidal activity

Age group (mo)	Log ₂ bactericidal titer ^a					
	Without activity			With activity		
	Prevaccination ^b	Postvaccination	% $\geq 1:4^c$	Prevaccination ^b	Postvaccination	% $\geq 1:4^c$
3-23	0 (76)	1.7	19.7	1.3 (46)	2.5	26.1
24-47	0 (24)	4.3	54.0	2.5 (20)	6.1	45.0
48-83	0 (23)	4.6	43.5	4.6 (21)	19.7	67.0
Total	0 (123)	3.2	39.1	2.5 (29)	6.5	46.0

^a A value of 1 was assigned to each titer of $<1:2$; thus, log₂ of 1 = 0.

^b Values in parentheses are numbers of individuals.

^c See Table 2, footnote c.

less than 1 year old compared with children 1 to 2 years of age could be seen (data not shown).

The influence of preimmunization antibody levels on bactericidal activity is shown in Table 3. For all age groups, except 24 to 47 months, there were significantly higher bactericidal titers after vaccination for children with demonstrable antibodies before vaccination than for those without such antibodies ($P < 0.05$). No significant differences in the percentages of children showing at least a fourfold increase in antibody titer among the three age groups were found when those with and without previous bactericidal antibodies were compared.

An analysis of the correlation between ELISA and bactericidal assay results is shown in Fig. 2. Poor correlation between the fold increases in antibody levels measured by these two assay techniques was evident. The correlation coefficient (r) varied from 0.2 to 0.4 among the three age groups.

Immunoblot studies. Figure 1 shows the binding profiles of IgG antibodies reactive with different OMPs in the serum samples of 13 vaccinees with bactericidal titers greater than 1:4. The antibodies bound most intensely to class 1, 4, and 5 OMPs. Postvaccination serum samples from individuals 30, 160, 370, 381, 415, 545, and 1069 showed distinct IgG binding to class 1 protein. High-molecular-weight IRPs were recognized in postvaccination serum samples from individuals 30 and 545. Two postvaccination serum samples with bactericidal titers of 1:256 and 1:128 showed no reactions with OMPs (data not shown).

DISCUSSION

Bactericidal antibody against meningococcal group C polysaccharide has been shown to correlate with protection against meningococcal disease (10, 16). Some OMPs from group B meningococci induce bactericidal antibodies and have been used as alternative vaccines (4, 11). Several immunologic tests have been used in the evaluation of meningococcal group B protective immunity. These include ELISA studies, serum bactericidal and opsonic assays, and immunoblot studies (18, 19, 28, 35). The vaccination campaign of 2.4 million children from 3 months through 6 years of age in greater São Paulo provided an important and useful opportunity to study the correlation between immunogenicity and vaccine efficacy as estimated by the case-control study.

Our results showed that the functional immune responses of vaccinated children against group B meningococci are age dependent. There were significantly lower bactericidal titers for children less than 24 months of age and also a significantly lower percentage of these children with bactericidal antibodies

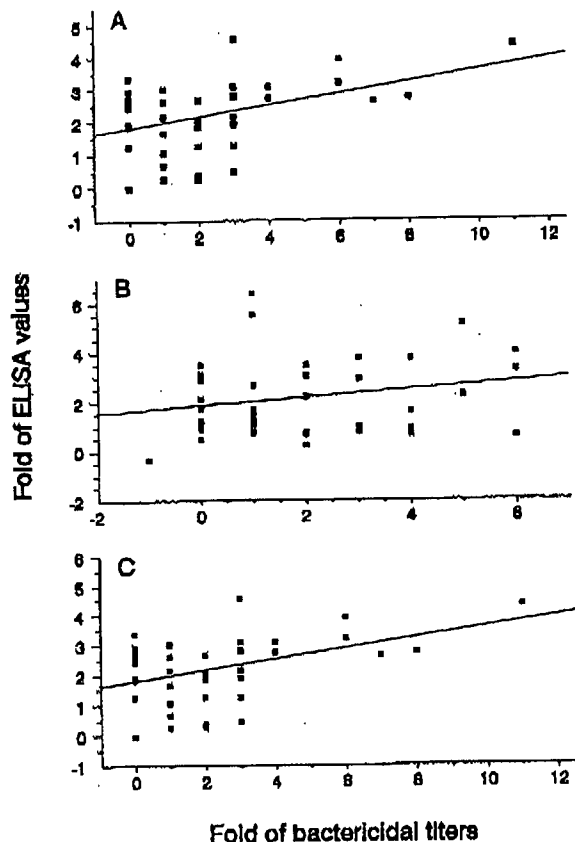


FIG. 2. Correlations between the fold increases of ELISA IgG antibody levels and bactericidal titers against *N. meningitidis* N.150/88 (B:4:P1.15) for children less than 24 months old ($r = 0.20$) (A), from 24 to 47 months old ($r = 0.20$) (B), and from 48 to 83 months old ($r = 0.41$) (C). Least-square regression lines are shown.

(titer, $\geq 1:4$) after vaccination compared with the percentages of older children. The percentage of children under 24 months of age with postvaccination titers of greater than 1:4 was only 13% compared with 47.7% for those over 24 months of age. It is interesting to note that the postvaccination seroconversion rate of children under 24 months of age (13%) was less than the naturally acquired levels of those over 24 months of age (Table 2). The age-specific incidence of group B meningococcal disease is much higher in children under 2 years of age, with a peak incidence in children under 1 year old. Our results are similar to those described by Frasch et al. (11), who studied the antibody responses of children to serotype 2a and 2b OMP vaccines combined with group B meningococcal polysaccharide but without adjuvant.

In agreement with our bactericidal assay results, the São Paulo case-control study showed that the estimated vaccine efficacy varied by age. The efficacy was 74% (95% confidence interval [95% CI], 16 to 92%) for children over 4 years old and 47% (95% CI, -72 to 48%) for those 24 to 47 months of age. The vaccine showed no protection for children less than 24 months of age (-37% and a 95% CI of -100 to 73%), and only 13% of the children showed postvaccination bactericidal titers of greater than 1:4 (24).

Greater increases in bactericidal titers after vaccination

were seen for children with preexisting antibodies than for those without such antibodies. Prior exposure to *Neisseria* proteins may explain the more vigorous response to vaccination. It is known that vaccination with a group B meningococcal outer membrane vaccine primes for a later anamnestic response (32). Although the immune responses in subjects without measurable preexisting antibodies were significantly lower than in those with demonstrable antibodies preimmunization, we found similar seroconversion rates as indicated by a minimum of fourfold increases in antibody titers after vaccination. The increase in antibody levels before vaccination was age dependent ($P < 0.05$). These observations support the role of naturally acquired immunity in protection against meningococcal disease.

On the basis of the estimated antibody levels to noncapsular surface antigens as measured by ELISA, the vaccine induced significant immune responses against group B *N. meningitidis* in vaccinees of all age groups. There were, however, some variations in response against the three B:4:P1.15 strains studied. The increase in antibody levels to OMV from strain N.150/88 after vaccination for children less than 24 months of age was significantly smaller ($P < 0.05$) than those for the other two age groups. The OMV from this strain showed smaller amounts of class 4 and 5 proteins in relation to class 1 protein. Perhaps there are important antigenic differences between strains of the same serotype that are isolated in different regions of the country.

The ELISA results with different group B meningococcal serotypes demonstrated more-intense antibody reactivity with 4:nt than with NT:P1.15 OMV. However, no differences in reactivity against these antigens could be seen by immunoblot studies (data not shown). This is not surprising because conformational differences and associations among major OMPs may account for the differences between ELISA and immunoblot reactivities.

The poor correlation between the overall antibody levels to various surface antigens estimated by ELISA and the bactericidal titers can be explained by the fact that the bactericidal assay measures only the subset of ELISA antibodies that are functionally important. Among important factors for bactericidal activity are the isotype and affinity of antibody molecules, as well as the ability of antibody molecules to activate complement (28, 31). Further studies are required to determine the predominant class of immunoglobulins induced by vaccination since they may have different functional activities.

The immunoblot studies showed considerable individual heterogeneity in antibody responses to vaccination. Considering that the primary antigenic epitopes detected by immunoblot are linear epitopes, class 1 and class 4 OMPs appeared to be the most reactive. An important finding was the correlation of IgG antibody binding to class 1 OMP in serum samples with high bactericidal activities (Fig. 1). This was observed for 7 of the 15 serum samples with titers of greater than 1:4. In this regard, others have correlated the antibodies induced to class 1 protein with protection (20, 30). The ability of antibodies to class 4 OMP to block otherwise bactericidal antibodies to specific cell surface antigens has not been well investigated (25). In general, pre- and postimmunization serum samples tested in our study showed weak reactivities with this protein. Serum samples from individuals 140 and 204, whose bactericidal titers postvaccination were 1:16 and 1:8, respectively, were reactive with class 4 OMP. The preimmunization serum sample from individual 204 clearly showed binding to this protein but did not have bactericidal activity. Weak IgG reactivities of 10 serum sample pairs with bactericidal titers of <1:4 were seen on immunoblots (data not shown).

Although the Cuban BC vaccine does not contain increased amounts of IRPs, one of 30 individuals (number 30) showed an increase in IgG binding to IRPs after vaccination. Also, the pre- and postimmunization serum samples from individual 545 showed reactivity with a high-molecular-weight protein. The human immune response to infection indicates that IRPs are expressed and immunogenic in vivo (4). There is considerable interest in exploring the use of one or more of these IRPs as vaccines (2, 9, 13).

These results demonstrate that the bactericidal assay is a good functional assay for the study of group B meningococcal vaccine immunogenicity, which appears to correlate with vaccine-induced protection. It is, nevertheless, also important to include the role of phagocytic killing in protection against meningococcal disease in future studies.

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REFERENCES

1. Armitage, P., and G. Berry. 1987. Statistical methods in medical research, 2nd ed. Blackwell Scientific Publishers, Boston.
2. Banerjee-Bhatnagar, N., and C. E. Frasch. 1990. Expression of *Neisseria meningitidis* iron-regulated outer membrane proteins, including a 70-kilodalton transferrin receptor, and their potential for use as vaccines. *Infect. Immun.* 58:2875-2881.
3. Bjune, G., E. E. Holby, J. K. Gronnesby, O. Arnesen, J. H. O. Fredriksen, A. Haltstensen, E. Holten, A. K. Lindbak, H. Nøkleby, E. Rosenqvist, L. K. Solberg, O. Closs, L. O. Froholm, A. Lystad, L. S. Bakketeg, and B. Hareid. 1991. Effect of outer membrane vesicle vaccine against group B meningococcal disease in Norway. *Lancet* 338:1093-1096.
4. Black, J. R., D. W. Dyer, M. K. Thompson, and P. F. Sparling. 1986. Human immune response to iron-repressible outer membrane proteins of *Neisseria meningitidis*. *Infect. Immun.* 54:710-713.
5. Boslego, J., W. Zollinger, J. Garcia, C. Cruz, S. Ruiz, B. Brandt, M. Martinez, J. Arthur, P. Underwood, W. Hankins, J. Mays, J. Gilly, and Chilean National Committee for Meningococcal Disease. 1990. Efficacy trial of a meningococcal group B (15:P1.3) outer membrane protein vaccine in Iquique, Chile, abstr. 23. Program Abstr. 7th Int. Pathog. *Neisseria* Conf.
6. Camargo, M. C. C., and N. T. R. Hidalgo. 1989. Doença meningocócica: a vacina contra o meningococo B e a situação atual na Grande São Paulo. *Imunizações* 2:161-167.
7. Camargo, M. C. C., and N. T. R. Hidalgo. 1990. A doença meningocócica na Grande São Paulo. *Imunizações* 3:4-7.
8. Caugant, D. A., C. T. Sacchi, L. O. Froholm, and R. K. Selander. 1991. Genetic structure and epidemiology of serogroup B *Neisseria meningitidis*, p. 37-42. In M. Achtman et al. (ed.), *Neisseria* 1990. Walter de Gruyter, Berlin.
9. Dauve, B., L. Lissolo, M. Mignon, P. Dumas, S. Colombani, A. B. Schryvers, and M.-J. Quentin-Millet. 1993. Transferrin-binding proteins isolated from *Neisseria meningitidis* elicit protective and bactericidal antibodies in laboratory animals. *Vaccine* 11:1214-1220.
10. Frasch, C. E. 1989. Vaccines for prevention of meningococcal disease. *Clin. Microbiol. Rev.* 2(Suppl.):134-138.
11. Frasch, C. E., L. Cortz, L. Wu, L. Y. Wang, and E. Rosenqvist. 1987. Immune responses of adults and children to group B *Neisseria meningitidis* outer membrane protein vaccines, p. 263-272. In J. B. Robbins (ed.), *Bacterial vaccines—1987*. Praeger Publications, New York.
12. Frasch, C. E., and J. D. Robbins. 1978. Protection against group B meningococcal disease. III. Immunogenicity of serotype 2 vaccines and specificity of protection in a guinea pig model. *J. Exp. Med.* 147:629-644.

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13. Frasch, C. E., C. T. Sacchi, M. C. C. Brandileone, V. S. D. Vieira, and L. C. C. Leite. 1991. Development of a second generation group B meningococcal vaccine. *NIPH Ann. (Oslo)* 14:225-231.
14. Frasch, C. E., J. M. Zahradnik, L. Y. Wang, L. F. Mocca, and C. M. Tsai. 1988. Antibody response of adults to an aluminum hydroxide-adsorbed *Neisseria meningitidis* serotype 2b protein-group B polysaccharide vaccine. *J. Infect. Dis.* 158:710-718.
15. Gold, R., M. L. Lepow, I. Goldschneider, and E. C. Gotschlich. 1977. Immune response of human infants to polysaccharide vaccines of groups A and C *Neisseria meningitidis*. *J. Infect. Dis.* 136(Suppl.):31-35.
16. Goldschneider, I., E. C. Gotschlich, and M. S. Artenstein. 1969. Human immunity to the meningococcus. I. The role of humoral antibodies. *J. Exp. Med.* 129:1307-1326.
17. Griffiss, J. M., B. L. Brandt, P. L. Altieri, G. B. Pier, and S. L. Berman. 1981. Safety and immunogenicity of group Y and group W135 meningococcal capsular polysaccharide vaccines in adults. *Infect. Immun.* 34:725-732.
18. Halstensen, A., A. K. Lehmann, H. K. Guttermsen, S. E. Vollset, G. Bjune, and A. Naess. 1991. Serum opsonins to serogroup B meningococci after disease and vaccination. *NIPH Ann. (Oslo)* 14:157-165.
19. Harthug, S., E. Rosenqvist, E. A. Holby, T. W. Gedde-Dahl, and L. O. Froholm. 1986. Antibody response in group B meningococcal disease determined by enzyme-linked immunosorbent assay with serotype 15 outer membrane antigen. *J. Clin. Microbiol.* 24:947-953.
20. Holby, E. A., E. Rosenqvist, L. O. Froholm, G. Bjune, B. Feiring, H. Nøkleby, and R. Ronnild. 1991. Bactericidal antibodies after vaccination with the Norwegian meningococcal serogroup B outer membrane vesicles vaccine. *NIPH Ann. (Oslo)* 14:147-156.
21. Lively, M. R., C. Moreno, and J. C. Linden. 1987. An integrated molecular and immunological approach towards a meningococcal group B vaccine. *Vaccine* 5:11-26.
22. Lowry, O. H., J. N. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265-275.
23. Macland, J. A., and E. Wedege. 1989. Serum antibodies to cross-reactive *Neisseria* outer membrane antigens in healthy persons and patients with meningococcal disease. *Acta Pathol. Microbiol. Immunol. Scand.* 97:774-780.
24. Moraes, J. C., B. A. Perkins, M. C. C. Camargo, N. T. R. Hidalgo, H. A. Barbosa, C. T. Sacchi, I. M. L. Gral, V. L. Gattas, H. G. Vasconcelos, B. D. Pilkaytis, J. D. Wenger, and C. V. Broome. 1992. Protective efficacy of a serogroup B meningococcal vaccine in Sao Paulo, Brazil. *Lancet* 340:1074-1078.
25. Munkley, A., C. R. Tinsley, M. Virji, and J. E. Heckels. 1991. Blocking of bactericidal killing of *Neisseria meningitidis* by antibodies directed against class 4 outer membrane protein. *Microb. Pathog.* 11:447-452.
26. Osborn, M. J., J. E. Gauder, E. Parisi, and J. Carson. 1972. Mechanism of assembly of the outer membrane of *Salmonella typhimurium*. *J. Biol. Chem.* 247:3962-3972.
27. Poolman, J. T. 1990. Polysaccharides and membrane vaccines, p. 57-86. In A. Nizrahi (ed.), *Bacterial vaccines—1990*. Wiley-Liss, New York.
28. Rosenqvist, E., and E. A. Holby. 1990. Relative bactericidal activity of IgG antibodies against outer membrane complexes from meningococci, as a function of vaccine type, dose and time after vaccination, p. 265-269. In M. Atchman et al. (ed.), *Neisseria* 1990. Walter de Gruyter, Berlin.
29. Sacchi, C. T., L. L. Pessoa, S. R. Ramos, L. G. Milagres, M. C. C. Camargo, N. T. R. Hidalgo, C. E. A. Melles, D. A. Caugant, and C. E. Frasch. 1992. Ongoing group B *Neisseria meningitidis* epidemic in São Paulo, Brazil, due to increased prevalence of a single clone of the ET-5 complex. *J. Clin. Microbiol.* 30:1734-1738.
30. Saukkonen, K., M. Lelonen, H. Abdillahi, and J. T. Poolman. 1989. Comparative evaluation of potential components for group B meningococcal vaccine by passive protection in the infant rat and in vitro bactericidal assay. *Vaccine* 7:325-328.
31. Schlesinger, Y., and D. M. Granoff. 1992. Avidity and bactericidal activity of antibody elicited by different *Haemophilus influenzae* type b conjugate vaccines. *JAMA* 267:1489-1494.
32. Sierra, G. V. G., H. C. C. Campa, I. L. Garcia, P. F. Sotolongo, P. L. Izquierdo, N. M. Valcarcel, G. V. Casanueva, M. S. Baro, C. F. Leguen, C. R. Rodriguez, and M. H. Terry. 1991. Efficacy evaluation of the Cuban vaccine VA-MENGOC-BC^R against disease caused by serogroup B *Neisseria meningitidis*, p. 129-134. In M. Atchman et al. (ed.), *Neisseria* 1990. Walter de Gruyter, Berlin.
33. Tsai, C.-M., and C. E. Frasch. 1980. Chemical analysis of major outer membrane proteins of *Neisseria meningitidis*: comparison of serotypes 2 and 11. *J. Bacteriol.* 141:169-176.
34. Warren, L. 1959. The thiobarbituric acid assay of sialic acids. *J. Biol. Chem.* 234:1971-1975.
35. Wedege, E., and L. O. Froholm. 1986. Human antibody response to a group B serotype 2a meningococcal vaccine determined by immunoblotting. *Infect. Immun.* 51:571-578.
36. Zollinger, W. D., R. E. Mandrell, J. M. Griffiss, P. Altieri, and S. Berman. 1979. Complex of meningococcal group B polysaccharide and type 2 outer membrane protein immunogenic in man. *J. Clin. Invest.* 63:836-848.

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Serum bactericidal activity correlates with the vaccine efficacy of outer membrane vesicle vaccines against *Neisseria meningitidis* serogroup B disease

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Abstract

For evaluation of serum bactericidal activity (SBA) as surrogate for the efficacy of outer membrane vesicle (OMV) vaccines against *Neisseria meningitidis* serogroup B disease, we have reanalyzed data from a randomized double blind placebo-controlled efficacy trial involving 172,000 secondary school students (aged 13–14 years) in Norway (1988–1991). A cohort of the efficacy trial consisting of 880 individuals was selected for immunogenicity studies. An efficacy of 87% was calculated for a 10-month observation period. However, after an observation period of 29 months, the estimated efficacy against group B disease induced by vaccination was 57%. The immunogenicity study showed that the SBA geometric mean titer (GMT) for the vaccinees was 2.4 before vaccination and 19.0 six weeks after the second vaccine dose. One year after vaccination the GMT was reduced to 2.8. A separate three-dose study with 304 adolescents showed that with a third dose at 10 months after the second dose (i.e. when cases of disease started to appear) a strong booster response was induced. Ten months after the second dose the SBA was reduced to near pre-immunization level. Following the third dose the SBA geometric mean titer of 2.7 increased to 62.3. One year after the third dose, the GMT was markedly higher than 6 weeks after the second dose (12.6 versus 8.8). Thus, protection after vaccination corresponds with the level of SBA. In order to reach lasting protective levels of SBA in a population, three vaccine doses are probably required. Measurements of SBA are likely to be useful for evaluating various upcoming formulations and improvements of immunization regimens for OMV vaccines.

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Keywords: Serum bactericidal activity (SBA); Outer membrane vesicle (OMV); Geometric mean titer (GMT)

1. Introduction

In the UK the serum bactericidal activity (SBA) is considered sufficient for licensure of meningococcal conjugate vaccines against serogroup C disease without the classic randomized efficacy trial [1]. The rationale for this decision is based on a long history of empiric observations and laboratory studies. Already in 1918 Matsunami and Kolmer [2] used a whole-blood bactericidal assay to show that the bactericidal activity of the blood of various animals was related to the resistance to infection with *Neisseria meningitidis* of these animals. They also provided the first evidence of age-dependent rise in immunity to meningococci in humans, demonstrating less bactericidal activity of serum from children compared to the activity of sera from adults. Early in the 1920s, Heist et al. showed that blood from more than

95% of adults was bactericidal to meningococcal carrier isolates [3]. This evidence for individual variation in immune responses and individual susceptibility to infection led Heist to postulate that most cases of meningitis occur among those few individuals who lack bactericidal activity [4].

The importance of bactericidal antibodies for protection against meningococcal disease has been demonstrated indirectly by the inverse relation between anti-meningococcal antibody titers or serum bactericidal activity and the age-related incidence of meningococcal disease [5]. Also the observation that individuals with defects in the terminal complement pathway are highly susceptible to meningococcal disease indicates that complement-dependent killing of the bacteria is correlated with protection [6]. More directly the correlation between SBA titers and protection (or rather clinical estimated efficacy) has been shown in various vaccine trials. Most clearly Goldschneider et al. [5,7] and Gotschlich et al. [8,9] showed this for the group C polysaccharide vaccine.

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2. Materials and methods

2.1. Vaccine and vaccinees

The vaccine, vaccinees and immunization schedule for the efficacy trial have been described previously [10,11]. Briefly, the immunogen was deoxycholate-extracted outer membrane vesicles (OMVs) from meningococcal strain 44/76 (B:15:P1.7,16:L3,7,9). The OMVs were adsorbed to aluminum hydroxide, and each dose contained 25 µg of protein. The placebo was Al(OH)₃ in 0.5 ml buffer. The vaccine, MenBvac, or placebo was injected as 0.5 ml into the deltoid muscle. The efficacy trial, lasting from 1988 until 1991 (29 months), included 172,000 secondary school students (aged 13–14 years), receiving two doses 6 weeks apart. The randomization (1:1) was done at school level. The corresponding two-dose immunogenicity study included 880 individuals, a cohort of the exact same group as those participating in the efficacy trial. The randomization in this trial was done on individual level in the proportion vaccine:placebo, 2:1. A separate three-dose immunogenicity study, giving two doses 6 weeks apart and a third dose 10 months after the second injection, was done in the same age group from 1993 to 1995. In this study, 311 received MenBvac and 62 in the control group received Meningovax A + C from "Pasteur Merieux". The Norwegian Medicines Control Authority and the Regional Committee for Medical Research Ethics in Norway approved all three studies.

2.2. Serum bactericidal activity

The standardized meningococcal variant 44/76-SL (B:15:P1.7,16:L3,7,9), was used as a target strain in the bactericidal test. Other strains, e.g. for analyzing the heterologous immune response, might be used after appropriate characterization. This test for functional immunity is based on two-fold dilution steps of serum in microplates using the agar overlay method, as previously described [12,13]. The bacterial inoculum was adjusted to 70–90 colony-forming units per well, and the assay was performed in presence of 25% human plasma as an exogenous complement source. The mixture of diluted test serum, bacteria and complement serum was incubated for 60 min (alternatively 30 min; see Sections 3 and 4) at 37 °C before the agar was added and the incubation continued overnight at 37 °C in a 5% CO₂ atmosphere. The titers are given as the reciprocal of the last serum dilution giving >50% killing of the inoculum.

3. Results

3.1. Efficacy trial for MenBvac

The total observation period, for the efficacy trial, was 29 months and the estimated efficacy was calculated to 57%

Table 1

The efficacy trial: number of cases and estimated efficacy at different time-points after two doses of MenBvac^a

Observation time	Number of cases		Estimated efficacy (%)
	Vaccine	Placebo	
0–2 weeks	0	(2) ^b	–
0.5–10 months	1	7	87
11–20 months	4	9	59
21–29 months	6	8	30
0.5–29 months ^c	11	24	57

^a Vaccine group: 690 schools (89,000 students); placebo group: 645 schools (83,000 students). Total observation period was 29 months.

^b Two cases of MenB disease occurred in the placebo group during the first 2 weeks. According to the protocol, these two cases were not included in the efficacy calculation.

^c [11].

(95% CI = 21–87%). Nearly all cases among vaccinees appeared later than 10 months after immunization (except for one case which occurred after 6 months). In the placebo group, however, seven cases appeared within the first 10 months of the study (Fig. 1). Retrospective calculations at various time points show that the observed protection rate decreased with the increase of time after vaccination. During the first 10 months the estimated efficacy was 87% (95% CI = 62–100%), whereas it was as low as 30% for the last 10 months [14] (see Table 1 and the right part of Fig. 1).

3.2. Immunogenicity following two doses

As can be seen from Table 2, the increase in SBA 6 weeks after the second dose, dropped rapidly after the second dose. One year after the second dose the functional immunity was almost reduced to pre-vaccination level (in contrast to antibodies measured in ELISA that persisted at substantial levels up to 52 weeks [15]). After 2 years the SBA level increased somewhat, probably due to natural immunization.

3.3. Immunogenicity following three doses

Data from this study can be seen in Table 3 and the immunogenicity part of Fig. 1. In this study the pre-immunization titers appeared to be lower than in the protection trial. However, this is perhaps due to 30 min incubation time as opposed to 60 min (now used as standard). The latter is thought to increase the sensitivity. Six weeks

Table 2

The two-dose immunogenicity study: SBA in a cohort of the efficacy trial

Time	GMT	Percentage with SBA titer ≥ 4	Percentage with ≥ 4-fold rise in SBA
Before first dose	2.4	31	–
6 weeks after first dose	8.5	72	46
6 weeks after second dose	19.0	97	80
1 year after second dose	2.8	32	19
2 years after second dose	3.5	42	26

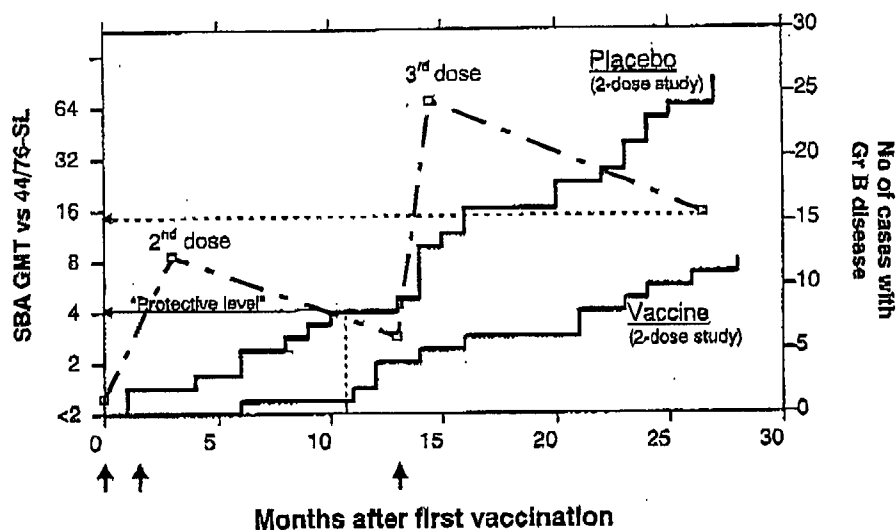


Fig. 1. Bactericidal activity in a three-dose study (left Y-axis) as compared to number of cases (cumulative) in vaccine and placebo groups in the original two-dose efficacy trial (right Y-axis). All cases with systemic group B meningococcal disease, occurring from week 0 are included.

Table 3

The three-dose immunogenicity study: SBA responses^a

Time	GMT	Percentage with SBA titer ≥ 4	Percentage with ≥ 4 -fold rise in SBA
Before first dose	1.2	4	–
6 weeks after second dose	8.8	77	73
10 months after second dose	2.7	35	28
6 weeks after third dose	62.3	96	96
1 year after third dose	12.6	85	81

^a A 30-min incubation was used. This serology was done prior to the "reanalysis" of the cohort from the efficacy trial. At present 60 min is chosen as standard (see Sections 2 and 3).

after the second dose the SBA level reached a similar height as seen in the two-dose study. The same, rather rapid decline in SBA after two doses was seen in both studies. Ten months after the second dose the geometric mean titer (GMT) was reduced to 2.7. Six weeks after the third dose, however, the GMT increased to 62.3, and one year after this third dose it was 12.6 (more than five times above the level, one year after two doses).

4. Discussion

The immunogenicity measured by the SBA test following two doses of MenBvac reflects what is seen in the efficacy trial: a rather short duration of protective immunity. A third dose at 10 months after the second gave a markedly increase in SBA, which persisted higher than after two doses, even a year after. Fig. 1 is a condensed illustration of key performance-data for MenBvac, with the mean SBA titers of the vaccinees in the three-dose study. It is important to note, that the first part of Fig. 1, i.e. immunokinetics following the first two doses, is similar to the results found in the cohort

of the efficacy study (two doses). The immunogenicity data from the three-dose study is compared to the cumulated number of cases appearing with time in the original two-dose efficacy trial. The graph illustrates the point that MenBvac was highly protective up to about 9 months after the second dose. At this time the SBA GMT for the vaccinated population was estimated to be about 4. Ten months after the second vaccination the GMT decreased to 2.7 and "vaccine failures" started to appear. Related to the experimental conditions used, a "mean protective SBA titer" of 4 can be suggested. This value corresponds well with the assumed protective SBA titer for serogroup C disease (8 when baby rabbit complement is used [1]). Furthermore, it can be seen from the figure that a third dose given 10 months after the second dose induced higher and longer-lasting SBA levels. Even 1 year after the third dose, the GMT was about four times higher than the assumed "protective level", which is likely to indicate an increased protection in the vaccinated population.

Bactericidal antibodies against meningococcal group C polysaccharide have been shown to correlate with protection against meningococcal disease [5,16]. For protection against serogroup B meningococcal disease, less clear and general statements have been given (i.e. for all strains, versions of SBA tests and different MenB vaccines [16]). However, for MenBvac all data available show a consistent good correlation between SBA and efficacy. Two comparative international studies, involving the Norwegian vaccine as one of the products tested, have also been consistent with this [17–19].

The SBA test is a biological assay, which may be variable [12,20]. Thus, standardization is of substantial importance. Several factors can influence the SBA titers. These include the choice of bacterial strain, growth conditions of the bacteria, the time of incubation with serum and the source of exogenous complement (human serum or sera from heterologous species such as rabbits or cows). Exogenous

complement from a person with no SBA antibodies and normal complement activity is the procedure chosen as standard. The use of e.g. baby rabbit complement gives substantially higher titers [1].

The target strain in SBA for evaluation of vaccine response of MenBvac is a standardized version of the seed-lot strain 44/76-SL. Among the different technical variables mentioned above, the presence or the degree of expression for some key antigens is most important. One of the key variable outer membrane antigens is the Opc protein (some others are capsule and degree of LPS sialylation) [21]. Human antibodies to Opc are bactericidal, but only bacteria expressing high amounts of Opc are killed in the bactericidal test. The level of surface expressed Opc protein may vary by transcriptional regulation of the *opc* gene, e.g. due to variable growth conditions, and the observed titers may differ depending on the expressed amounts of Opc [12,21]. This illustrates that it is crucial to standardize the inoculum for the target strain in SBA and to include control sera when performing the test. Using defined conditions, the SBA results have proven to be reproducible in both inter- and intra-laboratory studies [17–19].

Results presented in this report demonstrate that the bactericidal test performed according to standardized conditions is a good functional assay for immunogenicity studies of group B meningococcal vaccine. Based on the evaluation of data from three clinical studies with the MenBvac, we conclude that protection after vaccination corresponds with SBA, suggesting that for this vaccine and its improved formulations the SBA test is a good surrogate assay for protection. In order to reach lasting protective levels of SBA, three doses of MenBvac are required.

For different epidemiological situations and other vaccine formulations the SBA test might also be a valid surrogate for protection. However, this needs to be evaluated case by case. The procedure for establishing such a surrogate assay should be through a panel of sera from vaccinees receiving the actual formulation, thorough selection of a suitable target strain and standardized conditions for cultivation of the inoculum and performance of the SBA test.

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References

- [1] Borrow R, Andrews N, Goldblatt D, Miller E. Serological basis for use of meningococcal serogroup C conjugate vaccines in the United Kingdom: reevaluation of correlates of protection. *Infect Immun* 2001;69:1568–73.
- [2] Matsunami T, Kolmer JA. The relation of the meningococcal activity of the blood to resistance to virulent meningococci. *J Immunol* 1918;III:201–12.
- [3] Heist GD, Solis-Choen S, Solis-Choen M. A study of the virulence of meningococci for man and of human susceptibility to meningococcal infection. *J Immunol* 1922;VII:1–33.
- [4] Pollard AJ, Frasch C. Development of natural immunity to *Neisseria meningitidis*. *Vaccine* 2001;19:1327–46.
- [5] Goldschneider I, Gotschlich EC, Artenstein MS. Human immunity to the meningococcus. I. The role of humoral antibodies. *J Exp Med* 1969;129:1307–26.
- [6] Figueroa JE, Densen P. Infectious diseases associated with complement deficiencies. *Clin Microbiol Rev* 1991;4:359–95.
- [7] Goldschneider I, Gotschlich EC, Artenstein MS. Human immunity to the meningococcus. II. Development of natural immunity to meningococcus. *J Exp Med* 1969;129:1327–48.
- [8] Gotschlich EC, Liu TY, Artenstein MS. Human immunity to the meningococcus. III. Preparation and immunochemical properties of the group A, group B and group C meningococcal polysaccharides. *J Exp Med* 1969;129:1348–67.
- [9] Gotschlich EC, Goldschneider I, Artenstein MS. Human immunity to the meningococcus. IV. Immunogenicity of group A and group C meningococcal polysaccharides. *J Exp Med* 1969;129:1367–84.
- [10] Holst Fredriksen J, Rosenqvist E, Wedege E, Bryn K, Bjune G, Frøholm LO, et al. Production, characterization and control of MenB-vaccine "Folkehelsen": an outer membrane vesicle vaccine against group B meningococcal disease. *NIPH Ann* 1991;14:67–79.
- [11] Bjune G, Høiby EA, Grønnesby JK, Arnesen O, Holst Fredriksen J, Halstensen A, et al. Effect of outer membrane vesicle vaccine against group B meningococcal disease in Norway. *Lancet* 1991;338:1093–6.
- [12] Høiby EA, Rosenqvist E, Frøholm LO, Bjune G, Feiring B, Nøkleby H, et al. Bactericidal antibodies after vaccination with the Norwegian meningococcal serogroup B outer membrane vesicle vaccine: a brief survey. *NIPH Ann* 1991;14:147–55.
- [13] Rosenqvist E, Høiby EA, Wedege E, Bryn K, Kolberg J, Klem A, et al. Human antibody responses to meningococcal outer membrane antigens after three doses of the Norwegian group B meningococcal vaccine. *Infect Immun* 1995;63:4642–52.
- [14] Rosenqvist E, Høiby EA, Bjune G, Bryn K, Caugant DA, Closs O, et al. Meningococcal group B outer membrane vesicle vaccine trial among teen-agers in Norway. *Adv Mod Biotechnol (Hayana)* 1992;1:5.2.
- [15] Rosenqvist E, Høiby EA, Bjune G, Bryn K, Closs O, Feiring B, et al. Human antibody responses after vaccination with the Norwegian serogroup B meningococcal outer membrane vesicle vaccine: results from ELISA studies. *NIPH Ann* 1991;14:169–79.
- [16] Frasch CE. Vaccines for prevention of meningococcal disease. *Clin Microbiol Rev* 1989;2(Suppl):S134–8.
- [17] Perkins BA, Jónsdóttir K, Briem H, Griffiths E, Plukaytis BD, Høiby EA, et al. Immunogenicity of two efficacious outer membrane protein-based serogroup B meningococcal vaccines among young adults in Iceland. *J Infect Dis* 1998;177:683–91.
- [18] Tappero JW, Lagos R, Ballesteros AM, Plukaytis B, Williams D, Dykes J, et al. Immunogenicity of 2 serogroup B outer-membrane protein meningococcal vaccines: a randomized controlled trial in Chile. *JAMA* 1999;281:1520–7.
- [19] Carlone GM, Høiby EA, Sotolongo F, Williams D, Dykes J, Kapczynski D, et al. Quality control of serum bactericidal assay and other laboratory protocols used in a *Neisseria meningitidis* group B vaccine immunogenicity study in Iceland. In: Evans JS, Yost SE, Maiden MCJ, Feavers IM, editors. *Proceedings of the Ninth International Pathogenic Neisseria Conference*. Winchester; 1994. p. 473–4.
- [20] Rosenqvist E, Høiby EA, Wedege E, Kusecek B, Achtman M. The 5C protein of *Neisseria meningitidis* is highly immunogenic in humans and induces bactericidal antibodies. *J Infect Dis* 1993;167:1065–73.
- [21] Kahler CM, Martin LE, Shih GC, Rahman MM, Carlson RW, Stephens DS. The (α 2–8)-linked polysialic acid capsule and lipooligosaccharide structure both contribute to the ability of serogroup B *Neisseria meningitidis* to resist the bactericidal activity of normal human serum. *Infect Immun* 1998;12:5939–47.

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Annex 2

REQUIREMENTS FOR MENINGOCOCCAL POLYSACCHARIDE VACCINE

(Requirements for Biological Substances No. 23)

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INTRODUCTION

There have been numerous attempts to develop vaccines against meningococcal meningitis employing whole-cell suspensions or autolysates. Unfortunately, however, the data from trials using these vaccines were often difficult to interpret because the trials were not adequately controlled.

Over the last few years considerable progress has been made in the development of vaccines containing purified capsular polysaccharides of Group A and Group C meningococci. Controlled field trials of

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the Group A vaccine in Egypt, Finland and the Sudan have demonstrated excellent protection against the clinical disease, and similar studies in the USA with the Group C vaccine have shown it to be 90% effective. The protection is group-specific. As these vaccines are already in use in public health practice in several countries their standardization is necessary.

GENERAL CONSIDERATIONS

Meningococcal polysaccharide vaccines differ substantially from commonly used whole-cell bacterial vaccines and the requirements therefore differ in many particulars from those applied to other vaccines. The most important difference is that these products are made from highly purified polysaccharides that are partially chemically defined so that they can be substantially characterized by their composition and molecular weight.

The evidence suggests that the antibody responses of animals to the purified polysaccharides differ from those observed in man. This factor necessitates an approach different from that applied to the biological standardization of other bacterial vaccines in common use. With meningococcal vaccines it is important that the initial batches from a new manufacturer and even batches prepared by an existing manufacturer using a new process be evaluated in man for immunogenicity by monitoring antibody responses.

Unfortunately the lack of susceptibility of experimental animals to meningococcal infections also makes it impossible to estimate the efficacy of these vaccines for man on the basis of studies in animals. Efficacy can be evaluated at present only by clinical studies. The field trials concluded to date have provided a considerable body of data. However, there is still a lack of definitive data on the efficacy of Group C vaccines in infants and young children. It would therefore be desirable to keep appropriate groups of vaccinated infants and young children under surveillance to obtain such data.

Group A polysaccharide consists of repeating units of *N,O*-diacetyl-mannosamine phosphate linked with 1-6-phosphodiester bonds, and these components, together with calcium ions and residual moisture, account for over 90% of the dry weight of the preparation. Unfortunately this polysaccharide is intrinsically unstable and readily depolymerizes at ambient temperature, but the rate of degradation becomes negligible at temperatures of -20°C or lower. The molecular weight

is thus not readily identifiable, because the molecular size distribution is polydispersed, with components ranging from less than 50 000 to well over 200 000. The distribution of the molecular weight is best estimated by gel filtration on Sepharose 4B, and this procedure has been included as a requirement. It is known that preparations degraded by a prolonged exposure to ambient temperatures are significantly less immunogenic; however, the requirements concerning molecular size are such that they are met relatively easily in production yet allow a margin of safety for changes that may occur under non-ideal but practical conditions.

The Group C polysaccharide is a polymer of *N,O*-diacetylmuramic acid, and these components, together with calcium ions and residual moisture, account for over 90% of the weight of the material. Unlike Group A polysaccharide this polymer is not susceptible to degradation at ambient temperatures but in all other respects the properties of the two polysaccharides are similar.

Another unusual feature is that purification of the polysaccharides is carried out under chemically clean but not aseptic conditions (because the latter would be prohibitively difficult), the final product being rendered bacteriologically sterile by membrane filtration.

Chemical tests are not carried out on the final bulk but rather on aliquots of lots of purified polysaccharide. If these meet all the requirements they may be pooled to form a final bulk of any desired size. Moreover, any lot of purified polysaccharide that fails to meet any of the chemical requirements may be re-treated and if it is then able to meet the requirements it may be included in a final bulk.

Each of the following sections constitutes a recommendation. The parts of each section that are printed in large type have been written in the form of requirements so that, if a health administration so desires, these parts as they appear may be included in definitive national requirements. The parts of each section that are printed in small type are comments and recommendations for guidance. Detailed descriptions of technical methods that may be used are given in Part C of these requirements.

Should individual countries wish to adopt these requirements as the basis for their national regulations concerning meningococcal vaccine, it is recommended that a clause be included that would permit modifications of manufacturing requirements on the condition that it be demonstrated, to the satisfaction of the national control authority, that such modified requirements ensure a degree of safety and a potency of the vaccine at least equal to those provided by the requirements formulated

below. It is desirable that the World Health Organization should then be informed of the action taken.

The terms "national control authority" and "national control laboratory", as used in these requirements, always refer to the country in which the vaccine is manufactured.

PART A : MANUFACTURING REQUIREMENTS

I. DEFINITIONS

1.1 International name and proper name

The international name shall be *Vaccinum meningitidis cerebrospinalis* followed in parentheses by the serogroup specificity, thus: polysaccharide Group A and/or polysaccharide Group C. The proper name shall be the equivalent of the international name in the language of the country of origin.

The use of the international name should be limited to vaccines that satisfy the requirements formulated below.

1.2 Descriptive definition

Vaccinum meningitidis cerebrospinalis shall consist of purified Group A and/or Group C meningococcal polysaccharide(s). The polysaccharides shall be prepared from strains of *Neisseria meningitidis* that have satisfied the requirements of Part A, section 3.1.1, and purified by methods outlined in Part A, sections 3.3.3 and 3.4. The final preparation shall satisfy all the requirements formulated below.

1.3 Terminology

Parent seed lot. A quantity of living *Neisseria meningitidis* organisms of a specific strain processed together and of uniform composition. A parent seed lot may be maintained in the dried form or frozen at temperatures below -45°C .

Working seed lot. A quantity of living *Neisseria meningitidis* organisms derived from the parent seed lot by selecting a colony, growing the organisms and maintaining them in aliquots in the frozen state at -45°C or below.

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Single harvest. A sediment obtained on the same day by centrifugation of one culture irrespective of the volume of hexadecyl trimethylammonium bromide¹ that has been added to precipitate the group-specific polysaccharide.

Lot of purified polysaccharide. The material obtained after final purification. The lot may be derived from a single harvest or from several single harvests.

Final bulk. A pool of lots of purified polysaccharide that have been dissolved and filtered through membranes under aseptic conditions. The final bulk is contained in a single vessel from which the final containers are filled.

Filling lot (final lot). A collection of sealed final containers that are homogeneous with respect to the risk of contamination during filling or drying. A filling lot must, therefore, have been aseptically filtered, filled, and dried together.

2. GENERAL MANUFACTURING REQUIREMENTS

The general manufacturing requirements contained in the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories)² shall apply.

Staff assigned to the production of meningococcal vaccine should be vaccinated against the particular serogroup that is under production.

3. PRODUCTION CONTROL

3.1 Control of source of material

3.1.1 Strains of *Neisseria meningitidis*

The strains of *Neisseria meningitidis* Group A and the strains of *N. meningitidis* Group C used for preparing polysaccharide shall be approved by the national licensing authority. They shall also have been shown to be capable of producing polysaccharide known to be safe and effective in man.

The following strains have been shown to be suitable: for Group A polysaccharide—A1, M1027; for Group C polysaccharide—C11, C2241.

¹ The international nonproprietary name for this substance is cetyltrimonium bromide.

² WHO Technical Report Series, No. 323, 1966, pp. 11-22.

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The parent seed lot used shall be identified by a record of its history, including the source from which it was obtained and particulars of all tests made periodically for the verification of strain characters. The cultures shall have the following characteristics: (1) stained smears made from a culture shall be typical of *N. meningitidis*; (2) the organism shall grow at 37°C but not at 25°C; (3) the culture shall ferment glucose and maltose; (4) the colonies on agar shall be oxidase-positive; and (5) a suspension of the culture shall be agglutinated specifically with an appropriate grouping serum.

3.1.2 Seed lot system

The production of meningococcal vaccine shall be based on a seed lot system. Cultures of the working seed lot shall have the same characteristics as cultures of the strains from which the parent seed lot was derived (Part A, section 3.1.1). The preparation of seed lots shall be in compliance with the requirements of Part A, section 3.2.

A suitable menstruum for drying or preserving a parent or working seed lot in the frozen state is one containing 5% monosodium glutamate and 5% bovine plasma; in some countries skimmed milk is used. Mueller-Hinton agar medium may be used for the preparation of working seed lots.

3.2. Production precautions

The general production precautions, as formulated in the requirements of Part A, section 3, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories)¹ shall apply.

Particular efforts are necessary to maintain the culture tanks and all other equipment scrupulously clean. In particular, efforts should be made to avoid cross-contamination of the polysaccharides.

3.2.1 Culture medium for vaccine production

The fluid culture medium used for vaccine production shall be free from ingredients that will form a precipitate on addition of hexadecyl trimethylammonium bromide to a final concentration of 1 g/l. It shall be free also from substances that may give rise to sensitization in man if the ingredients appear in the final vaccine.

¹ WHO Technical Report Series, No. 323, 1966, p. 15.

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The basal medium described by Frantz¹ supplemented with 2 g/l dialysate of yeast extract may be used. The basal medium may be autoclaved but the supplement, consisting of yeast extract dialysate, magnesium sulfate, and dextrose, should be sterilized by filtration.

3.2.2 Method of production

The growth of the cultures shall take place at $35.5^{\circ}\text{C} \pm 1.5^{\circ}\text{C}$. A working seed lot shall be plated on solid medium and after 12-18 hours of incubation the culture shall be used as an inoculum for liquid precultures. As these precultures reach the stationary phase they shall be used as an inoculum for the production tanks, and the production cultures shall be harvested during the late logarithmic or early stationary phase.

The moment of harvest of the production culture should be determined keeping two considerations in mind. Late harvest tends to improve the final yield of polysaccharide but its average molecular size will be lower. Most often the optimum time for harvest has been 10-12 hours after inoculation.

3.3 Control of production culture

3.3.1 Freedom from contamination

Samples of the culture taken before killing shall be tested for bacterial contamination by microscopic examination of Gram-stained smears and by inoculation into appropriate media. Several microscopic fields shall be examined at high magnification such that at least 10 000 organisms have been inspected. If any contaminating bacteria are found the culture or any product derived from it shall be discarded.

3.3.2 Treatment of production culture

As soon as freedom from contamination is established by examination of stained smears purification shall be undertaken.

The culture may be harvested directly or after killing the organisms by heating at 56°C for a period of 10 minutes. Two methods exist; either hexadecyl trimethylammonium bromide is added to the culture to a final concentration of 1 g/l, or the culture is first clarified by centrifugation after which hexadecyl trimethylammonium bromide is added to the supernate.

¹ FRANTZ, I. D. *Journal of bacteriology*, 43: 757 (1942).

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3.3.3 Purification of polysaccharide

All steps in the purification procedure shall be carried out in clean glass or plastic ware. Only plastic ware compatible with the solvents in use during the particular step of the procedure shall be used. Reagent grade chemicals or equivalent shall be employed, and aqueous solutions of calcium chloride, sodium acetate, or other salts shall be filtered to remove particulate matter present in these materials. All purification steps shall be carried out in a cold room at a temperature of $8^{\circ}\text{C} \pm 5^{\circ}\text{C}$ with chilled reagents. The precipitated detergent-polysaccharide complex shall be collected as rapidly as possible by centrifugation and, unless preliminary purification is carried out immediately, the sediments shall be stored frozen at -20°C or lower.

3.3.3.1 Preliminary purification of meningococcal polysaccharide. The precipitate of hexadecyl trimethylammonium bromide and polysaccharide shall be partially purified, the material resulting from this preliminary purification being referred to as intermediate product. This product shall be stored at -20°C or below. The method of purification shall be approved by the national control authority.

Suitable methods for preliminary purification are shown in the Appendix to these Requirements.

3.3.3.2 Final purification of meningococcal polysaccharide. The intermediate product, which contains approximately 50% by weight of the desired polysaccharide (the remainder being principally protein and lipopolysaccharide with endotoxic activity), shall be subjected to further purification. The method of further purification shall be approved by the national control authority.

Suitable methods for further purification are shown in the Appendix to these Requirements.

After final purification the material is referred to as the lot of purified polysaccharide. The final dry product shall be handled with sterile precautions and stored at -20°C or lower.

If tests carried out on the lot of purified meningococcal polysaccharide indicate that nucleic acid, protein, or endotoxic contamination is present, the purification procedure applicable to the particular contaminant may be repeated, followed by the procedure for drying the polysaccharide.

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3.4 Chemical requirements for lots of purified polysaccharide

All chemical analyses of all lots shall be based on the dry weight of polysaccharide in the salt form. The moisture content of the purified polysaccharide shall be determined by thermogravimetric analysis at 50°C. In laboratories lacking facilities for thermogravimetric analysis, the moisture content may be determined by the Karl Fischer method or by drying *in vacuo* over P_2O_5 at 37°C until a constant weight is reached. These data shall be used for the calculation of the dry weight.

3.4.1 Protein content

Each lot of purified polysaccharide shall contain less than 10 mg of protein per gram of polysaccharide as determined by the method of Lowry et al.¹ using bovine plasma albumin as a reference.

3.4.2 Nucleic acid content

Each lot of purified polysaccharide shall contain less than 10 mg of nucleic acid per gram of polysaccharide as determined by spectroscopy, assuming that the internal transmission density of a 10 g/l solution contained in a cell 1 cm wide at 260 nm is 200.

3.4.3 O-acetyl content

The O-acetyl content of each lot of purified polysaccharide shall be equal to or greater than 2 mmol/g of polysaccharide for Group A and 1.5 mmol/g of polysaccharide for Group C. The O-acetyl content shall be determined by the method of Hestrin.²

3.4.4 Phosphorus content

Each lot of Group A polysaccharide shall contain at least 80 mg/g of phosphorus.

3.4.5 Sialic acid content

The sialic acid content of the purified polysaccharide, calculated as free N-acetylneuraminic acid (molecular weight 309), shall be not less than 800 mg/g of the dry weight of the isolated product as determined

¹ Lowry, O. H. et al. *Journal of biological chemistry*, 193 : 265 (1951).

² HESTRIN, S. *Journal of biological chemistry*, 160 : 249 (1949).

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by the method of Svennerholm,¹ using the reference sialic acid, *N*-acetylneuraminic acid, as the reference preparation.

3.4.6 Molecular size

The molecular size of each lot of purified polysaccharide shall be estimated by gel filtration using Sepharose 4B. Chromatography shall be carried out in a solvent having a concentration of 0.2 ionic strength. The molecular weight shall be determined by measuring the distribution constant K_D of the polysaccharide at the main peak of the elution curve. The K_D value must be no greater than 0.40.²

There are clinical data indicating that vaccines with a K_D value as high as 0.45 are immunogenic for man. As there is a general relationship between immunogenicity and molecular weight, however, material with the lower K_D value of 0.40 is considered preferable. Attempts should be made to produce material with still lower K_D values, and when such vaccines are being produced regularly these regulations may be amended to require a reduction in K_D value below the currently specified upper limit of 0.40.

3.5 Preparation of final bulk

The final bulk shall be prepared either from a single lot of purified polysaccharide or from several pooled lots. The polysaccharide shall be dissolved under aseptic conditions in a sterile solution containing mannitol or other suitable materials as the menstruum for freeze-drying and shall be sterilized by membrane filtration. The solvent used shall be free from pyrogenic substances.

Membranes with a pore size of 0.22 μm have been found satisfactory.

3.5.1 Sterility test on the final bulk

Each final bulk shall be tested for bacterial sterility according to the requirements given in Part A, section 5, of the revised Requirements for Biological Substances No. 6 (General Requirements for the Sterility of Biological Substances).³

¹ SVENNERHOLM, L. *Biochimica et biophysica acta*, 24: 604 (1957).

² The technology of meningococcal polysaccharide production is such that it is not easy to obtain agreement on an absolute figure for molecular size. In some countries it has been suggested that the K_D value should be less than or equal to 0.40 and that at least 65% of the material should elute with a K_D value less than or equal to 0.30.

³ WHO Technical Report Series, No. 530, 1973, pp. 48-52.

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3.5.2 Test for serological specificity

The final bulk of meningococcal Group A polysaccharide and the final bulk of meningococcal Group C polysaccharide used for the combined vaccine shall be tested for serological specificity and identity by the haemagglutination inhibition assay procedure. The meningococcal Group A final bulk shall specifically inhibit haemagglutination of erythrocytes sensitized with Group A meningococcal antigen in the presence of Group A antibody. There shall be no inhibition of haemagglutination by the vaccine at a minimum final concentration of 100 mg (dry weight) per litre in the presence of meningococcal Group C antiserum and erythrocytes sensitized with Group C meningococcal antigen. The meningococcal Group C final bulk shall specifically inhibit haemagglutination of erythrocytes sensitized with Group C meningococcal antigen in the presence of Group C antibody. There shall be no inhibition of haemagglutination by the vaccine at a minimum final concentration of 100 mg (dry weight) per litre in the presence of meningococcal Group A antiserum and erythrocytes sensitized with Group A meningococcal antigen.

4. FILLING AND DRYING

The requirements concerning filling and containers given in Part A, section 4, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories)¹ shall apply. The meningococcal vaccine shall be freeze-dried. The Group A and the combined Group A and Group C vaccines shall be stored at a temperature of -20°C or lower whereas the Group C vaccine shall be stored at a temperature of 5°C or lower.

5. CONTROL TESTS ON FINAL PRODUCT

5.1 Identity test

An identity test shall be performed on at least one labelled container from each filling lot. The presence of the group-specific antigen(s) shall be confirmed by tests described in Part A, section 3.5.2.

5.2 Test of contamination by heterologous polysaccharide

The monovalent vaccine shall be tested for the presence of heterologous polysaccharide by the test described in Part A, section 3.5.2.

¹ WHO Technical Report Series, No. 323, 1966, pp. 16-17.

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5.3 Concentration of polysaccharide

At least one final container shall be checked to determine that it contains the stated amount of polysaccharide. It shall be shown that Group A vaccine contains 75 mg of phosphorus per gram of polysaccharide and that Group C vaccine contains 750 mg of *N*-acetylneuraminic acid per gram of polysaccharide.¹

5.4 Sterility tests

Each filling lot shall be tested for sterility according to the requirements given in Part A, section 5, of the revised Requirements for Biological Substances No. 6 (General Requirements for the Sterility of Biological Substances).²

5.5 Innocuity tests

5.5.1 Pyrogenicity test

Each filling lot shall be tested for pyrogenicity by the intravenous injection of rabbits. Three or more healthy rabbits that have not been injected previously shall be used. The vaccine, reconstituted in the form in which it is to be used, shall be diluted further in pyrogen-free physiological saline so that each rabbit shall receive, by injection into the ear vein, the following doses of dry weight polysaccharide per kilogram of rabbit weight:

Group A vaccine, 0.0025 µg
Group C vaccine, 0.0025 µg
combined Groups A and C vaccine, 0.0050 µg

In each instance the specified dosage level of polysaccharide for each rabbit shall be suspended in 1 ml of physiological saline per kilogram of rabbit weight. The criteria for passing the test shall be those specified in the International Pharmacopoeia.³

¹ The content of phosphorus and *N*-acetylneuraminic acid in the final containers is inevitably somewhat lower than the content of these substances in the vaccine lots, as given in sections 3.4.4 and 3.4.5 of this annex.

² WHO Technical Report Series, No. 530, 1973, pp. 48-52.

³ A detailed description of the test for pyrogens is given in: *Specifications for the quality control of pharmaceutical preparations — second edition of the International Pharmacopoeia*. Geneva, World Health Organization, 1967, Appendix 43, p. 747.

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5.5.2 Tests for abnormal toxicity

5.5.2.1 *Guineapig toxicity.* No fewer than five guineapigs weighing approximately 350 g each shall be injected intraperitoneally with 500 µg of the polysaccharide(s). The animals shall be observed for 7 days and the injection shall cause neither significant symptoms nor death during this period.

5.5.2.2 *Mouse toxicity.* No fewer than five mice weighing approximately 18 g each shall be injected intraperitoneally with 100 µg of the polysaccharide(s). The animals shall be observed for 7 days and the injection shall cause neither significant symptoms nor death during this period.

5.6 Estimation of molecular size

The molecular size of the polysaccharide in at least one final container from each filling lot shall be determined by Sepharose 4B gel filtration as outlined in Part A, section 3.4.6. The polysaccharide shall elute with a distribution constant, K_D , of 0.40 or less.¹

5.7 Test for residual moisture

A test shall be performed on at least one container from each filling lot to determine the amount of moisture in the product. The filling lot shall pass the test if the moisture content is not more than 3%. The method used for the determination of the moisture content shall be approved by the national control authority.

5.8 Storage

The Group A vaccine shall be stored at a temperature of -20°C or lower. The Group C vaccine shall be stored at a temperature of 5°C or lower.

6. RECORDS

The requirements given in Part A, section 6, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories)² shall apply.

¹ See footnote on p. 59.

² WHO Technical Report Series, No. 323, 1966, p. 17.

7. SAMPLES

The requirements given in Part A, section 7, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories)¹ shall apply.

8. LABELLING

The requirements given in Part A, section 8, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories)¹ shall apply, with the addition of the following.

The label on the container shall show :

- the number of micrograms of polysaccharide(s) in the human dose
- the volume and nature of the reconstituting fluid.

Furthermore, the label on the container or the label on the carton enclosing several containers, or the leaflet accompanying the container, shall contain the following additional information :

- a statement that after the dried vaccine has been reconstituted it should be used within 8 hours ;
- a statement that the Group A vaccine shall be stored at -20°C or lower ;
- a statement that the Group C vaccine shall be stored at 5°C or lower ;
- a statement that a combined Group A and Group C vaccine shall be stored at -20°C or lower.

9. DISTRIBUTION AND SHIPPING

The requirements given in Part A, section 9, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories)¹ shall apply.

¹ WHO Technical Report Series, No. 323, 1966, p. 18.

10. STORAGE AND EXPIRY DATE

10.1 Storage conditions

The manufacturer shall recommend such conditions of storage and shipping as will ensure that the vaccine conforms to these requirements until the expiry date as stated on the label. The Group A vaccine shall be stored at -20°C or lower; the Group C vaccine shall be stored at 5°C or lower.

10.2 Expiry date

The expiry date for dried vaccines shall be not more than 5 years from the date of harvest or not more than 18 months from the date of issue. The manufacturer shall provide data to confirm the stability of the product.

PART B :

NATIONAL CONTROL REQUIREMENTS

1. GENERAL

The general requirements for control laboratories contained in Part B of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories)¹ shall apply.

In addition, the national control authority shall give directions to manufacturers concerning the most suitable strains from which polysaccharide vaccine shall be produced.

2. RELEASE AND CERTIFICATION

A vaccine lot shall be released only if it fulfils all requirements set forth in Part A of the present document.

A statement signed by the appropriate official of the national control laboratory shall be provided at the request of the manufacturing establishment and shall certify whether the lot of vaccine in question meets all national requirements as well as Part A of the present requirements.

¹ WHO Technical Report Series, No. 323, 1966, pp. 19-22.

The certificate shall also state the date of the last satisfactory test for molecular weight, the lot number, the number under which the lot was released, and the number appearing on the labels of the containers. In addition, a copy of the official national release document shall be attached.

The purpose of the certificate is to facilitate the exchange of meningococcal polysaccharide vaccine between countries.

3. REACTIVITY AND IMMUNOGENICITY OF VACCINE IN MAN

The national control authorities shall satisfy themselves that adequate control of meningococcal vaccine has been achieved by arranging for studies in man¹ to be made on the first series of production lots and thereafter at regular intervals on some of the vaccine lots. Such studies shall be repeated if any changes in production methods are made.

The national control authorities shall ensure that the studies are done in an adequate number of subjects to obtain statistically valid data on reactivity and immunogenicity.

The following methods of demonstrating safety and efficacy may be used.

Healthy adult human subjects should be immunized in the manner prescribed for the product and their temperatures measured between 4 and 6 hours, and again between 24 and 48 hours, after injection. Such temperatures, as well as all adverse reactions during the first 48-hour period should be reported.

Samples of sera from each subject taken immediately prior to injection and again at 2-4 weeks after immunization should be assayed for bactericidal antibodies. The bactericidal assay should be performed with paired sera from each subject in serial twofold dilutions against the A1 strain for Group A and/or the C-11 strain for Group C or equivalent strains of *Neisseria meningitidis*. The antibody titre should be expressed as the reciprocal of the highest dilution that effects 50% or greater killing of the test organisms. The antibody titres of the sera from at least 90% of the subjects should show a fourfold or greater rise after immunization. If the sera from less than 90% but greater than 80% of the subjects show such a rise, one re-test of the product may be allowed, but in such a case the sera from at least 90% of all subjects in the two tests combined should show a fourfold or greater antibody increase.

¹ The persons should be volunteers.

Appendix

DETAILS OF SOME METHODS AND TESTS USED IN THE MANUFACTURE AND CONTROL OF MENINGOCOCCAL POLYSACCHARIDE VACCINES

These methods and tests are given as a guide to control laboratories.

1. Suitable methods for preliminary purification

A satisfactory method of preliminary purification is as follows. The quantities suggested assume that the sediment from 100 litres of culture is available. The sediment, which may come from a single harvest or may be a pool of several single harvests, is smoothly suspended in 1 litre of distilled water. One litre of 2 mol/l calcium chloride is added to dissociate the detergent-polysaccharide complex and the suspension is stirred for one hour. In order to precipitate the nucleic acids and the bulk of soluble protein, absolute ethanol or 95% ethanol is added to a final ethanol concentration of 250 ml/l. After one hour of standing the precipitate is sedimented by centrifugation at approximately 20 000 g for 20 minutes and the perfectly clear supernatant is retained. Unless this solution is free from particulate matter it is probable that the final product will contain an unacceptable level of nucleic acid contamination. Ethanol is added to this solution to a final concentration of 750 ml/l for the Group C polysaccharide and 800 ml/l for the Group A polysaccharide. After agglomeration of the precipitate is complete (usually within one hour), the precipitated polysaccharide is collected by centrifugation at approximately 3000 g for 10 minutes. The sediment is washed at least twice with about 1 litre of absolute ethanol (each time) to remove hexadecyl trimethylammonium bromide and calcium chloride. It is essential to use absolute ethanol because unacceptable losses of polysaccharide will occur if 95% ethanol is employed. The sediment is washed twice with approximately 1 litre of acetone and dried in vacuum.

2. Suitable methods for further purification

Two methods for further purification have been used. The first is based on the method of Sevag¹ and consists of homogenizing in a blender an aqueous solution of the intermediate product (about 20 g/l) and a mixture of chloroform and *n*-butanol (in the ratio of 3:1). After centrifugation for 10 minutes at approximately 10 000 g, the clear aqueous supernatant is decanted and the homogenization of this layer repeated until a negligible interfacial precipitate forms on centrifugation.

The other method depends on cold-phenol extraction. The intermediate product is dissolved in 0.1 saturated neutral sodium acetate to give a concentration of intermediate product of 20 g/l. It is then extracted three times with twice its volume of cold phenol (100 g crystalline phenol dissolved in 40 ml of 0.1 saturated neutral sodium acetate). The extraction consists of vigorous shaking by hand for approximately 30 seconds. To minimize losses the three phenol layers are sequentially extracted with twice their volume of distilled water and added to the first aqueous supernatant. Phase separation is achieved by centrifugation for 15 minutes at 35 000 g. The polysaccharide solution is dialysed for 24 hours against cold 0.1 mol/l calcium chloride.

¹ SEVAG, M. G. *Biochemische Zeitschrift*, 273: 419 (1934).

The solution is centrifuged for 1 hour at 100 000 g to sediment endotoxic lipopolysaccharide. Ethanol is added to the supernatant to a final concentration of 750 ml/l. The precipitated polysaccharide is collected by centrifugation, washed twice with absolute ethanol and twice with acetone. It is then dried in vacuum.

For the production of the Group A polysaccharide the cold-phenol procedure is to be preferred because the yield and molecular size are much higher. This procedure can also be used for the production of Group C polysaccharide, but frequently endotoxic activity is not readily removed by ultra-centrifugation unless one or more cycles of homogenization with a mixture of chloroform and butanol have been carried out.

3. Molecular sizing of capsular polysaccharide antigens by Sepharose 4B column chromatography

Reagents¹

Sepharose 4B gel
0.2 mol/l ammonium acetate, pH 7.0, as eluant
¹⁴C-labelled sodium acetate
Blue dextran 2000
Scintillation liquid
Reagents for chemical assay of polysaccharides

Equipment

Column (1.5 x 90 cm)
Automatic fraction collector and test-tubes
Gel and eluant reservoir
Liquid scintillation counter and counting vials
Refractometer or other appropriate monitoring device and recorder (optional)

Procedures

(1) Washing of the Sepharose 4B gel:

- To approximately 200 ml of packed Sepharose 4B gel add 400 ml of 0.2 mol/l ammonium acetate. Stir the gel thoroughly and let it sediment for about 1 hour. Remove the supernatant fluid together with the fine particles suspended in it.
- Repeat the above procedure until there are no fine particles in the supernatant.
- Decant all the supernatant and add 200 ml of fresh 0.2 mol/l ammonium acetate to the gel. De-aerate the gel suspension in a vacuum.

(2) Packing of the column:

- Mount the column vertically and connect it to the gel and eluant reservoir. Fill the column with 0.2 mol/l ammonium acetate to a depth of about 30 cm.
- Pour the washed Sepharose 4B slurry into the reservoir slowly to avoid trapping air bubbles and adjust the height of the surface of the slurry above

¹ Advice on the availability of suitable products may be obtained from Biologics, World Health Organization, 1211 Geneva 27, Switzerland.

the column outlet to about 70–75 cm. Open the column outlet and let the gel sediment at that operating pressure for about 16 hours; the flow rate of the column is approximately 15–20 ml/h.

(c) When the column is packed to a height of about 87 cm, equilibrate the gel bed with two to three column volumes of eluant. The operating pressure should always be equal to or less than that at which the column was packed. Mark the gel bed level.

(3) Calibration of the column:

(a) Void volume (V_0):

(i) The void volume is determined with blue dextran dissolved in 0.2 mol/l ammonium acetate to a concentration of 2 g/l.

(ii) Drain the excess eluant from the column until the eluant level is equal to the gel bed level; close the column outlet.

(iii) Carefully layer 1 ml of blue dextran on top of the gel bed and allow the sample to drain into the gel bed by opening the column outlet.

(iv) When the sample level is equal to the gel bed level, fill the column with eluant and connect it to the eluant reservoir. Start collecting the fractions (approximately 2 ml per fraction) with an automatic fractionator. (Simultaneously, the eluant may be monitored for blue dextran with a monitoring device and a recorder.)

(v) The internal transmission density (optical density) of each fraction is read at 260 nm and plotted against the number of fractions. V_0 is determined as the volume of eluant collected up to the position of the maximum of the first blue dextran peak in the elution diagram. Determination of the position of the apex of the elution peak may be done by extending upwards the two straight lines on the slopes. The interception of the two slopes is taken as the apex of the elution peak.

(b) Total bed volume (V_T):

(i) The total bed volume is determined with ^{14}C -labelled sodium acetate. The column is loaded with 1 ml of the sodium acetate solution containing 0.4 μCi of radioactivity.

(ii) Fractions of about 2 ml each are collected and a 0.5 ml portion from each fraction is transferred into separate scintillation vials containing 10 ml of scintillation liquid. The vials are counted in a liquid scintillation counter.

(iii) The radioactivity, expressed as counts per minute, is plotted against the respective fraction numbers. The total bed volume is the volume of eluant collected up to the position of the maximum of the ^{14}C sodium acetate peak in the elution diagram. Symmetrical peaks in the elution profiles are indicative of the acceptability of the column.

(4) Vaccine measurement:

(a) Preparations of meningococcal polysaccharide vaccine Group A or Group C or Groups A and C combined in the final containers are reconstituted with water to a concentration of 2.5 g/l of antigen for Group A or Group C or

5.0 g/l of antigens for Groups A and C combined. The vaccine pool should contain at least 2.2 ml. No more than 10 mg of polysaccharide should be used.

(b) One millilitre of the vaccine is used to charge the column, the column is eluted with a volume of 0.2 mol/l ammonium acetate using not less than 1.5 times the total bed volume of the column, and the eluate is collected.

(c) The quantity of phosphorus in fractions of Group A vaccine and the quantity of sialic acid in fractions of Group C vaccine are determined. The readings are plotted against the number of fractions. Alternatively, the eluate may be monitored with a refractometer or an absorptiometer and a recorder. However, the profile thus obtained by the monitoring device should be compared with a profile of the same type of vaccine as determined by chemical assay of fractions eluted under identical chromatographic conditions. Immunochemical analyses of the polysaccharides are not sufficiently quantitative to be reliable.

(d) The elution volume V_e is measured at the main peak of the polysaccharide elution curve, and the distribution constant is calculated from the equation:

$$K_D = \frac{V_e - V_0}{V_i - V_0}$$

using the values of V_0 and V_i determined for the column. The K_D value of the polysaccharide must be no greater than 0.40.¹

4. Determination of the O-acetyl group in meningococcal polysaccharides Group A and Group C

The methods described in this section are based on the work of Hestrin² and Kabat & Mayer.³

Instrumentation

Spectrophotometer for the ultraviolet and visible regions of the spectrum.

Mechanism

O-acetyl groups react with hydroxylamine in alkali to form hydroxamic acid. The hydroxamic acid formed is measured by the formation of a coloured complex with Fe^{2+} in acid solution.

Reagents

- (1) 2 mol/l hydroxylamine hydrochloride. Store in the cold.
- (2) 3.5 mol/l NaOH.
- (3) Concentrated HCl (specific gravity 1.18), diluted with twice its volume of water.
- (4) 0.37 mol/l solution of $FeCl_3 \cdot 6H_2O$ in 0.1 mol/l HCl.
- (5) 0.001 mol/l sodium acetate, pH 4.3.
- (6) Reference solution of acetylcholine chloride (molecular weight 181.7).

¹ See footnote on p. 59.

² HESTRIN, S. *Journal of biological chemistry*, 180: 249 (1949).

³ KABAT, E. & MAYER, M. *Experimental immunochemistry*. Springfield, Charles C. Thomas, 1971, pp. 493-494.

Method of preparing reference solution

The reference solution should contain 150 mg acetylcholine chloride per vial. To each vial 10 ml of 0.001 mol/l sodium acetate (pH 4.5) are added with a volumetric pipette. One millilitre of this solution is transferred to a test tube and mixed with 4 ml of 0.001 mol/l sodium acetate (pH 4.5), using a 5 ml volumetric flask if available. From this solution 0.1, 0.2, 0.3, 0.4, and 0.5 ml quantities are transferred accurately in duplicate to 10 test tubes, and 0.9, 0.8, 0.7, 0.6 and 0.5 ml of water are added to make the volume in each tube 1 ml. These reference tubes contain respectively 1.66, 3.32, 5.0, 6.64 and 8.30 micromoles of *O*-acetyl per millilitre. The duplicate at each concentration is used as a reference blank. The approximate internal transmission densities of these solutions at 540 nm in the visible range of the spectrophotometer are 0.2, 0.5, 0.8, 1.0 and 1.3. The readings are not taken at the maximum of absorption but rather on the shoulder of the peak. Further details are given by Hestrin.² The reaction is carried out at room temperature.

A reference blank is run for each standard prepared as indicated above. Acid is added to the blanks before the NaOH-hydroxylamine solution is added.

Procedure

(1) Prepare the sample for analysis by making up a 0.1% solution of the polysaccharide (no salt) in water. For each test use 1 ml of this solution, which contains 1 mg of polysaccharide.

(2) To 1 ml of the sample and reference preparations add 2 ml of a freshly prepared (within 3 hours) mixture of equal parts of reagents (1) and (2) (2 mol/l hydroxylamine, and 3.5 mol/l sodium hydroxide) and mix.

(3) After 4 minutes at room temperature, add 1 ml of reagent (3), HCl, to bring the pH to about 1.2 $\pm$ 0.2, and mix.

(4) Add 1 ml of reagent (4), ferric chloride, and mix.

(5) Swirl the contents of the test tubes rapidly during the addition of each of the above reagents.

(6) Both a reference blank (one blank for each concentration of the reference) and a sample blank (1 ml of 0.1% polysaccharide solution) must be included. In Hestrin's method the blank determination is called the test for non-specific colour. Acid is added to the blanks before the NaOH-hydroxylamine solution is added.

(7) Read the internal transmission density of the purple-brown colour at 540 nm promptly.

(8) Subtract the sample blank readings from those of the samples and subtract the reference blank readings from those of the reference preparations.

(9) Plot a curve of internal transmission density versus molarity of *O*-acetyl. The values of internal transmission density of the samples on the reference curve correspond to micromoles of *O*-acetyl per milligram of polysaccharide (since the test was conducted on 1 ml of the polysaccharide solution, which contains 1 mg of polysaccharide).

The weight of polysaccharide used in the 1 g/l polysaccharide solution is corrected for its moisture content³ and the final result is expressed as "micromoles of *O*-acetyl per gram of dry polysaccharide".

² Hestrin, S. *Journal of biological chemistry*, 180 : 249 (1949).

³ See section 3.4 of Annex 2.

Example: Moisture content is 10%. Thus 1 mg of "wet" polysaccharide comprises 0.1 mg of water and 0.9 mg of "dry" polysaccharide. If, for every milligram of "wet" polysaccharide there are 1.95 μ mol of O-acetyl, this figure is equivalent to:

$$\begin{aligned} & \frac{1.95 \mu\text{mol O-acetyl}}{1 \text{ mg "wet" polysaccharide}} \\ &= \frac{1.95 \mu\text{mol O-acetyl}}{0.9 \text{ mg "dry" polysaccharide}} \\ &= 2.16 \mu\text{mol O-acetyl per milligram of "dry" polysaccharide.} \end{aligned}$$

5. Procedures for haemagglutination-inhibition assay for meningococcal polysaccharide vaccine

Sensitization of erythrocytes

Sensitized erythrocytes are prepared according to the method of Arstenstein et al.¹ with the following modifications:

- (1) Dulbecco's phosphate-buffered saline, pH 7.4, with calcium and magnesium salts is used not only as a diluent but for washing cells as well.
- (2) Sheep erythrocytes (washed three times) are used.
- (3) Antisera are heated at 56°C for 30 minutes and adsorbed on to packed normal sheep erythrocytes before use.
- (4) Sensitizing antigens (Group A or Group C polysaccharides) are used at a concentration of 20 mg/l.

Titration of antisera

Sera are titrated in triplicate using microtitre U-plates.

- (1) 0.025 ml of buffered saline is added to each well.
- (2) 0.025 ml of antiserum is added to the first well of each row and dilutions are made with 0.025 ml loops down the rows of the wells.
- (3) 0.025 ml of buffer is added to each well after the antiserum has been diluted.
- (4) 0.05 ml of sensitized cells is added to each well. The plates are incubated for 1-2 hours at room temperature for Group C antigen-coated cells and at 4°C for Group A antigen-coated cells. The cell control consists of 0.05 ml of sensitized cells and 0.05 ml of normal serum or saline.

The highest serum dilution giving a definitive haemagglutination pattern is considered as having one unit of activity. The preceding well is considered to have two units of activity and the next preceding well four units, etc.

A titration of antiserum over an appropriate range of dilutions is included with each haemagglutination inhibition test.

Haemagglutination inhibition

- (1) The test vaccine to be used as inhibitor is reconstituted with diluent so that its final concentration in the first well will be at least 100 mg/l. It should be noted that, since the dilution factor for the test vaccine in the first well is 1:8, the initial vaccine concentration should be at least 800 mg/l.

¹ ARNSTEIN, M. S. et al. *Journal of infectious diseases*, 124: 277-288 (1971).

- (2) 0.025 ml diluent is added to each well.
- (3) 0.025 ml test vaccine is added to the first well of each row. Using 0.025 ml loops, make serial twofold dilutions.
- (4) The antiserum titrated as described above (see section on titration of antisera) is diluted in tubes to cover a range of at least 1, 2, 4, and 8 units. A 0.025 ml quantity of each antiserum dilution is added to each well of the appropriate rows of vaccine dilutions.
- The microtitre plate is gently swirled to ensure the mixing of the material in each well and is incubated at 37°C for 30 minutes.
- (5) 0.05 ml of the appropriate sensitized cells is added to each well.

The cell control consists of two drops (0.05 ml total) of diluent in place of the test vaccine and antiserum. A known inhibitor should be included in each experiment as antigen control. Incubation is continued for 1-2 hours at room temperature for Group C antigen-coated cells and at 4°C for Group A antigen-coated cells.

A positive test for inhibition is defined as a reduction of the titre of the titrated antiserum by two twofold dilution steps or more by any vaccine dilution with a final concentration in the well of 100 mg/l or less.

6. Bactericidal antibody assay

Materials¹

Tissue culture trays, 20 x 30 cm, 96 cups, 2 ml per cup, with sterile covers
 Sterile automatic syringes, 1 and 2 ml
 Disposable serological pipettes, 1 ml, sterile glass plugged
 Nephelometer flasks with side-arm, 300 ml capacity
 Nephelometer
 Sterile pipettes and test-tubes
 Liquid medium: Mueller-Hinton broth or soybean-casein digest broth
 Solid medium: soybean-casein digest broth with 1.2% agarose
 Normal horse serum, heat-inactivated
 Difco's phosphate buffered saline (DPBS) with calcium and magnesium
 0.85% saline, sterile
 Complement from baby rabbits (3-4 weeks old), or freshly collected baby rabbit (approximately 3 weeks old) serum stored at -80°C. Complement should be pre-screened for the absence of anti-meningococcal activity.

Procedure

(1) Serum samples:

- (a) All test sera are diluted 1:2 in DPBS and inactivated at 56°C for 30 minutes. A reference is included as positive control.
- (b) Serial twofold dilutions of antisera are made by placing 0.2 ml DPBS in each cup of the tray, using a sterile 1-ml automatic syringe, and adding

¹ Advice on the availability of suitable products may be obtained from Biologicals, World Health Organization, 1211 Geneva 27, Switzerland.

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0.2 ml of inactivated test serum to the first well, using a sterile disposable 1-ml pipette aspirated by a 1-ml automatic syringe connected to it by a piece of rubber tubing (taking care not to fill the syringe). The serum is mixed, and 0.2 ml of the mixed serum is transferred to the next well using the same pipette. This dilution process is continued successively through the rest of the wells, the 0.2 ml taken from the last well being discarded.

(2) Bacteria :

(a) A lyophilized meningococcal culture or a recently transferred culture is inoculated on to the solid medium and incubated overnight at 37°C in air containing 5% CO₂. A 25-ml quantity of broth is added aseptically to a nephelometer flask and a 5-ml quantity to a sterile culture tube. Approximately $\frac{1}{2}$ loopful of the overnight culture is transferred to the 5 ml of broth in the culture tube. After mixing thoroughly, the culture is poured into the flask and incubated at 37°C on a shaker for approximately 5 hours. Turbidity is read at 530 nm and the number of cells (colony forming units) per millilitre is determined from a standard curve. The culture is diluted in saline to a concentration of 500 cells/ml.

(b) 0.2 ml of the cell suspension is added to each well, using a sterile 1-ml automatic syringe.

(3) Complement :

(a) 0.2 ml of pre-screened complement is added to each well with a 1-ml automatic syringe.

(b) The following controls are included.

(i) Cell control: 0.2 ml DPBS, 0.1 ml bacteria, and 0.1 ml heat-inactivated complement.

(ii) Complement control: 0.2 ml DPBS, 0.1 ml bacteria, and 0.1 ml of active complement.

(iii) Serum control: 0.2 ml serum, 0.1 ml bacteria, 0.1 ml DPBS.

(4) Incubation :

(a) The plates are covered and incubated at 37°C for 30 minutes in 5% CO₂.

(b) Sterile solid medium is prepared afresh each time in the following manner. Agar gel is heated to 120°C and sterilized in an autoclave. When it has cooled to approximately 45°C, normal horse serum (heat-inactivated) is added to a concentration of 10 ml/l. One millilitre of the medium is added to each well, using a warm 2-ml automatic syringe. The trays are then gently swirled and the medium is allowed to set at room temperature. The trays are covered and incubated at 37°C for 18-24 hours in 5% CO₂.

(5) Reading of results :

The growth in the complement control is used as growth control, which should be the same or only slightly less than that of the cell control. The end-point of each serum titration is the last well that gives 50 % or greater killing as compared with the cell control. Some sera, especially high-titred samples, may have a prozone. Sera showing a killing effect in the serum control should be titrated without complement to determine the extent of this killing effect.

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ACKNOWLEDGEMENTS

The first draft of this appendix was written at a meeting of interested parties held in Lyons, France, from 30 to 31 January 1975. The document was amended at a meeting of a WHO Study Group on Cerebrospinal Meningitis Control¹ held in Geneva from 27 to 31 October 1975 for presentation to the WHO Expert Committee on Biological Standardization, which amended it further. The names of all persons participating in the formulation of the requirements are as follows:

- Dr J.-P. Albert, WHO Collaborating Centre for Reference and Research on Meningococci, Microbiology Research Laboratory, Institute of Tropical Medicine of the Army Health Service, Marseilles, France
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- Dr G. Biron, Institut Mérieux, Lyons, France
- Dr B. Bytchenko, Bacterial Diseases, WHO, Geneva, Switzerland
- Dr C. Charbonnier, Institut Mérieux, Lyons, France
- Dr W. Chas. Cockburn, Director, Division of Communicable Diseases, WHO, Geneva, Switzerland
- Dr B. Cvjetanovic, Chief Medical Officer, Bacterial Diseases, WHO, Geneva, Switzerland
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- Dr I. Jód, Scientific Leader, "Human" Institute for Serobacteriological Production and Research, Budapest, Hungary
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¹ WHO Technical Report Series, No. 588, 1976.

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1. Occupational lung disease: asbestosis, byssinosis, silicosis, coal workers' pneumoconiosis, lung cancer, and occupational asthma.

2. Musculoskeletal injuries: disorders of the back, trunk, upper extremity, neck, and lower extremity; and traumatically induced Raynaud's phenomenon.

3. Occupational cancers (other than lung): leukemia; mesothelioma; and cancers of the bladder, nose, and liver.

4. Amputations, fractures: eye loss, lacerations, and traumatic deaths.

5. Cardiovascular diseases: hypertension, coronary artery disease, and acute myocardial infarction.

6. Disorders of reproduction: infertility, spontaneous abortion, and teratogenesis.

7. Neurotoxic disorders: peripheral neuropathy, toxic encephalitis, psychoses and extreme personality changes (exposure-related).

8. Noise-induced loss of hearing.

9. Dermatologic conditions: dermatoses, burns (scalding), chemical burns, and contusions (abrasions).

10. Psychologic disorders: neuroses, personality disorders, alcoholism, drug dependency.

11. Control technology research: application of scientific principles to control strategies, preconstruction review, technology forcing/new source performance concepts, technology transfer, substitution, unit operations approaches.

12. Respirator research: new and innovative respiratory protective devices; techniques to predict performance; effectiveness of respirator programs; physiologic and ergonomic factors; medical surveillance strategies; psychological and motivational aspects; and physical properties.

FOR FURTHER INFORMATION CONTACT:

For technical information: Roy M. Fleming, Sc.D., Associate Director for Grants, NIOSH, Building 1, Room 3053, Centers for Disease Control, Atlanta, GA 30333, Telephone: 404/329-3343 or FTS 236-3343

For business information: Leo A. Sanders, Chief, Grants Management Branch, Procurement and Grants Office, Centers for Disease Control, 255 E. Paces Ferry Road, NE, Room 321, Atlanta, Georgia 30305, Telephone: (404) 262-8575 or FTS 236-6572.

[This program is described in the Catalog of Federal Domestic Assistance Program No. 13.282, Occupational Safety and Health Research Grants]

Dated: August 13, 1985.

Elliott S. Harris,
Acting Director, National Institute for
Occupational Safety and Health.
[FR Doc. 85-19946 Filed 8-20-85; 8:45 am]
BILLING CODE 4760-10-13

Food and Drug Administration

Advisory Committee Meeting; Filing of Annual Reports

AGENCY: Food and Drug Administration.
ACTION: Notice.

SUMMARY: The Food and Drug Administration (FDA) is announcing that, as required by the Federal Advisory Committee Act, the agency has filed with the Library of Congress the annual reports of those FDA advisory committees that held closed meetings.

ADDRESS: Copies are available from the Dockets Management Branch (HFA-305), Food and Drug Administration, 5300 Fishers Lane, Rockville, MD 20857, 301-443-1751.

FOR FURTHER INFORMATION CONTACT: Richard L. Schmidt, Committee Management Office (HFA-306), Food and Drug Administration, 5300 Fishers Lane, Rockville, Md 20857, 301-443-2765.

SUPPLEMENTARY INFORMATION: Under section 13 of the Federal Advisory Committee Act (Pub. L. 92-463, 86 Stat. 770-776 (5 U.S.C. App. I)), FDA has filed with the Library of Congress the annual reports for the following FDA advisory committees that held closed meetings during the period July 1, 1984, through June 30, 1985:

Center for Drugs and Biologics:
Anesthetic and Life Support Drugs Advisory Committee.
Anti-Infective Drugs Advisory Committee.
Blood Product Advisory Committee
Vaccines and Related Biological Products Advisory Committee.
Center for Devices and Radiological Health:
Circulatory System Devices Panel,
Immunology Devices Panel
Ophthalmic Devices Panel.

Annual reports are available for public inspection at (1) the Library of Congress, Newspaper and Current Periodical Reading Room, Rm. 1026, Thomas Jefferson Bldg., 2d and Independence Ave. SE., Washington, DC, (2) the Department of Health and Human Services Library, Rm. 1436, 330 Independence Avenue SW., Washington, DC, on weekdays between 9 a.m. and 4:30 p.m., and (3) the Dockets Management Branch (HFA-305), Food

and Drug Administration, Rm. 4-62, 5600 Fishers Lane, Rockville, MD 20857, between 9 a.m. and 4:30 p.m., Monday through Friday.

Dated: August 14, 1985.
Mervin H. Shumate,
Acting Associate Commissioner for
Regulatory Affairs.
[FR Doc. 85-19937 Filed 8-20-85; 8:45 am]
BILLING CODE 4160-01-M

[Docket No. 84D-0263]

Meningococcal Polysaccharide Vaccines; Availability of Guideline

AGENCY: Food and Drug Administration.
ACTION: Notice.

SUMMARY: The Food and Drug Administration (FDA) is announcing the availability of a guideline for the manufacture and release of Meningococcal Polysaccharide Vaccine and the bulk powders of each polysaccharide used for the manufacture of the vaccines.

ADDRESS: Written requests for a copy of the guideline or comments concerning the guideline to the Dockets Management Branch (HFA-305), Food and Drug Administration, Rm. 4-62, 5600 Fishers Lane, Rockville, MD 20857.

FOR FURTHER INFORMATION CONTACT: Carl E. Frasch, Center for Drugs and Biologics (HFN-858), Food and Drug Administration, 8800 Rockville Pike, Bethesda, MD 20205, 301-496-1920.

SUPPLEMENTARY INFORMATION: Meningococcal Polysaccharide Vaccine are biological products licensed under section 351 of the Public Health Service Act (42 U.S.C. 262).

FDA issued the first license for Meningococcal Polysaccharide Vaccine in 1974. FDA regulates production of these vaccines not only through standards in the license applications but also through requirements in the regulations on establishment and product licensing in 21 CFR Parts 600 and 601, the general biological standards in 21 CFR Part 610, the labeling requirements in 21 CFR Parts 201 and 202, and the current good manufacturing practice (CGMP) regulations in 21 CFR Part 211. FDA has not issued specific regulations for Meningococcal Polysaccharide Vaccines.

The Office of Biological Research and Review in FDA's Center for Drugs and Biologics has developed a guideline concerning the manufacture and release of Meningococcal Polysaccharide Vaccines and the polysaccharide bulk powders from which the individual polysaccharides used for making the

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vaccines are derived. FDA is making the guideline available to provide guidance to the two currently licensed manufacturers and any possible new manufacturers concerning the production of safe and effective vaccines.

FDA is making the guideline available under 21 CFR 10.90(b). That regulation provides for FDA's use of guidelines to outline procedures or standards of general applicability that are acceptable to FDA for a subject matter that falls within the laws administered by FDA. Although guidelines are not legal requirements, a person may be assured that in following an agency guideline the procedures followed and standards used will be acceptable to FDA. A person may also choose to use alternative procedures or standards for which there is scientific rationale even though they are not provided for in a guideline. A person who chooses to use procedures or standards different from procedures or standards in a guideline may discuss the matter further with the agency to prevent an expenditure of resources for work that FDA may later determine to be unacceptable.

Interested persons may submit written comments on the guideline to the Dockets Management Branch (address above). Two copies of any comments are to be submitted, except that individuals may submit one copy. Comments are to be identified with the docket number found in brackets in the heading of this document. FDA will consider the comments when determining whether amendments or revisions to the guideline are warranted. The comments will assist the agency in developing additional standards for Meningococcal Polysaccharide Vaccines. Received comments may be seen in the office above between 9 a.m. and 4 p.m., Monday through Friday.

Dated: August 14, 1985.

Ervin H. Shumate,

Acting Associate Commissioner for Regulatory Affairs.

[FR Doc. 85-19936 Filed 8-20-85; 8:45 am]

BILLING CODE 4160-01-M

Public Health Service

Privacy Act of 1974; System of Records

AGENCY: Department of Health and Human Services; Public Health Service.

ACTION: Notification of a new system of records: 09-25-0156: "Records of Participants in Programs and Respondents in Surveys used to Evaluate Programs of the National Institutes of Health, HHS/NIH/OD."

SUMMARY: In accordance with the requirements of the Privacy Act, the Public Health Service (PHS) is published notice of a proposal to establish a new system of records, 09-25-0156, "Records of Participants in Programs and Respondents in Surveys Used to Evaluate Programs of the National Institutes of Health, HHS/NIH/OD." We are also proposing routine uses for this system.

The National Institutes of Health (NIH) will use the proposed system of records to support evaluation of the methods, materials, activities and services used by NIH in fulfilling its legislated mandate to conduct and support biomedical research into the causes, prevention and cure of diseases, to support training of research investigators, and to support communication of biomedical information.

PHS invites interested persons to submit comments on the proposed routine uses on or before September 20, 1985.

DATES: PHS has sent a Report of New System to the Congress and to the Office of Management and Budget (OMB) on August 12, 1985. The system of records will be effective 60 days from the date submitted to OMB unless PHS receives comments on the routine uses which would result in a contrary determination.

ADDRESS: Comments should be addressed to the NIH Privacy Act Coordinator at the address listed below. Comments received will be available for inspection weekdays between 9 a.m. and 3 p.m. in Room 3B11, Building 31, at that address.

FOR FURTHER INFORMATION CONTACT: Dr. Kenneth Thibodeau, NIH Privacy Act Coordinator, Building 31, Room 3B07, 9000 Rockville Pike, Bethesda, MD 20205, or call 301-496-2832. (This is not a toll free number.)

SUPPLEMENTARY INFORMATION: NIH was established to improve the health of the American people by conducting biomedical research, by funding such research through grants, contracts and other awards, and by promoting communication and dissemination of biomedical knowledge. NIH conducts reviews to determine how well these programs and projects achieve their goals.

The proposed system of records will cover cases where (1) NIH collects opinions, suggestions or other personal information concerning programs which do not use any system of records; or (2) program evaluation requires several categories of information which are not contained in any existing system.

Evaluation studies may take the form of surveys in which individuals who have or could participate in, contribute to, or benefit from NIH programs, provide opinions, suggestions or other information useful in evaluating the programs. For example, surveys of how much the public knows about certain methods for reducing the risk of disease may be used to evaluate dissemination of information about disease prevention. Other studies may use information from existing sources. For example, to evaluate research training programs, NIH may assemble information about publications by individuals who have received research training support, or from NIH grants files about research grants subsequently awarded to such individuals.

Evaluation studies may be conducted either by NIH staff or by other organizations under contract. Such contracts might be awarded when NIH staff is not available, when other organizations have better access to targeted populations, or when they can obtain or provide the desired information at lower cost or with less intrusion upon individuals.

The routine uses proposed for these systems are compatible with the purpose of evaluating NIH programs. The first two proposed routine uses are needed to accomplish the purpose of the system; that is, information would be disclosed under these routine uses to conduct, review, analyze or follow-up evaluation studies. The third proposed routine use for disclosure to a congressional office acting on behalf of an individual would not violate the privacy of an individual because such disclosure would be made only pursuant to a request initiated by the individual.

The proposed system will cover evaluation studies throughout NIH. Records in this system will be organized and maintained according to the particular evaluation study of which they are a part. NIH will not enter records into a general or comprehensive data base, nor create any overall index to the separate sets of records used in different evaluation projects; however, NIH is treating the separate sets of records as a single system under the Privacy Act (1) because all of the sets of records serve the same purpose, (2) in order to apply consistent policies and practices in the maintenance of such records, and (3) to make it easier for subject individuals to obtain notification of or access to their records.

When it becomes effective, the proposed system will subsume records currently covered by three existing systems: 09-25-0147, "Records of

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GUIDELINES FOR THE PRODUCTION OF
MENINGOCOCCAL POLYSACCHARIDE VACCINES

Docket No. 84D-0263

Notice of Availability Published August 21, 1985
FR 50 (162) 0.33852

Center for Drugs and Biologics
Office of Biologics Research and Review
Food and Drug Administration
5600 Fishers Lane
Rockville, Maryland 20857

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6.

Guidelines for meningococcal vaccine,
groups A, C, Y, and W135 polysaccharides combined

A. Testing procedures

1. Group A, C, Y, and W135 final bulk powders used for formulation of the final container should pass all tests described in section A.1. of the release specifications for the individual polysaccharides.

2. The following tests should be conducted using material in final containers:

a. Quantitation: The final container should contain 50.0 μg $\pm 20.0\%$ per dose of groups A, C, Y, and W135 polysaccharides as determined by the rocket immunoelectrophoretic technique of the Office of Biologics Research and Review. Other quantitative immunochemical procedures, shown to yield results equivalent to the above technique, would be acceptable after being submitted in writing and approved by the Director, Office of Biologics Research and Review. The reference standard for each meningococcal group polysaccharide to be used for this quantitative procedure should pass all tests required of the final bulk powders (see A.1. above).

b. Stabilizer content: Lactose should be present at a concentration of 2.5-5.0 mg per human dose of the vaccine.

c. Pyrogenic substances: The vaccine should meet the standards of 21 CFR 610.13(b)(3) in a test dose of 0.10 micrograms vaccine (dry weight) per milliliter per kilogram of rabbit body weight.

7.

- d. Molecular sizing: Molecular sizing of meningococcal groups A, C, Y, and W135 polysaccharides should be determined by Sepharose CL-2B (Pharmacia Fine Chemicals, Piscataway, NJ) gel filtration, using the procedure of the Office of Biologics Research and Review or a procedure shown to yield equivalent results. The individual polysaccharides should be quantitated by an immunoelectrophoretic or equivalent procedure. The recommended specifications for molecular size are maximum Kd values on CL-2B as follows: Group A, 0.70, group C, 0.70, group Y, 0.57, and group W135, 0.68.
- e. Moisture: The moisture content of the vaccine in final containers should be determined by the gravimetric procedure (21 CFR 610.13) or an equivalent procedure. The test should be performed on one vial per thousand up to a maximum of 10 vials but not less than 5 vials taken at random throughout the filling lot. The average residual moisture should not exceed 1.0%.
- f. General safety: A general safety test must be conducted as provided in 21 CFR 610.11.
- g. Sterility: Requirements with respect to sterility are set forth in 21 CFR 610.12.
- h. Identity: Requirements with respect to establishment of identity are set forth in 21 CFR 610.14.
- i. Clinical potency: A clinical potency test should normally be conducted on at least two of the first three first-filling lots of A, C, Y, W135 tetravalent vaccine.

8.

Samples of sera from each subject taken immediately prior to injection and again 3 to 4 weeks after immunization should be assayed for bactericidal antibodies.

The bactericidal assay should be performed with paired sera from each subject in serial two-fold dilutions against the following meningococcal strains: for group A strain A1, for group C strain C-11, for group Y strain S-1975, and for group W135 strain S-4383. The antibody titer should be expressed as the reciprocal of the highest dilution that effects 50% or greater killing of the test organisms. At least 90% of the subjects should show a four-fold or greater rise to all four strains after immunization. If the sera from less than 90% but greater than 80% of the subjects show such a rise, one retest of the product using not less than 25 human subjects may be performed. The sera from at least 90% of all subjects in the two tests combined should show a four-fold or greater antibody increase.

9.

At least 2 milliliters each of pre-and post-immunization sera from each of the human subjects tested should be saved by the manufacturer. The Office of Biologics Research and Review may request these samples to be submitted for testing. These sera will contain no additives, and should be stored and shipped in a manner which prevents the growth of possible microbial contaminants.

B. Manufacturing protocol. For each lot of vaccine, a release protocol which consists of a summary of the history of manufacture, filling date, date of performance and result(s) of each required test and the calculation of the actual fill weight of each of polysaccharide Group A, Group C, Group Y and Group W135 antigens should be submitted to the Director, Office of Biologics Research and Review. Protocols are to include all clinical potency results and any adverse reactions observed in the clinical test (A.2.g.), if performed. Protocols for each lot of combined A, C, Y, W135 vaccine should include the lot number of the final dry bulk for each of the polysaccharides used in formulation of the vaccine lot.

C. Samples. For each lot of vaccine, a sample of no less than 60 milligrams of the vaccine distributed in no fewer than 12 final containers, and an appropriate amount of diluent should be submitted to the Director, Office of Biologics Research and Review. Additional samples include (also described in final bulk release specifications):

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(1) 75 milligrams of powder from the Group A, Group C, Group Y, and Group W135 final dry bulks for the first lot of vaccine derived from these bulks.

(11) 10 milligrams of powder for each subsequent vaccine lot utilizing the final bulk powder.

D. Dating period

1. The in-house storage period and storage temperature of groups A, C, Y and W135 combined vaccine in filled but undated final containers should be no longer than thirty-six (36) months at -20°C or colder from date of filling into final containers.

2. The dating period and storage temperature of labeled and packaged final container vaccine should be twenty-four (24) months at $2-8^{\circ}\text{C}$ from the date of initiation by the manufacturer of the last valid potency tests (A.2. a through g), and the term of the dating period should not exceed sixty (60) months from date of filling.