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Patente de Invención	☒	Patente de Modelo de Utilidad	☐
Vía Nacional	☐		
Vía PCT (Fase Nacional)	☒	Capítulo I	☒
		Capítulo II	☐
No. Solicitud Internacional	PCT/IB2008/003283		
Fecha de Presentación Internacional	17/Octubre/2008		

Como resultado del Examen de Fondo realizado, con fundamento en el Artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se comunica:

1. Documentos estudiados

Descripción:	Folios 8 a 75	04/Mayo/2010
Reivindicaciones:	Folios 76 a 77	04/Mayo/2010
Secuencias:	Folios 78 a 85	04/Mayo/2010
Dibujos:	Folios 86 a 87	04/Mayo/2010
Prioridad:	US 60/999,590	19/Octubre/2007

2. Análisis de la Prioridad

Se acepta la reivindicación de prioridad porque esta solicitud fue presentada dentro del plazo establecido (Art. 9 D 486) y su contenido técnico corresponde con el de la prioridad reivindicada.

3. Objeto de la invención

Título de la solicitud: "Formulaciones de Vacuna Meningococica".

El problema planteado en esta solicitud consiste en suministrar una formulación de vacuna contra el serogrupo B de *Neisseria meningitidis* ("Men-B") con buena estabilidad e inmunogenicidad.

Para resolver este problema técnico la solicitud en estudio proporciona una formulación dual de (i) un adyuvante de emulsión de aceite en agua y (ii) un componente inmunogénico de Men-B en forma liofilizada.

El objeto de la presente solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): A61K 39/95.

4. De la Solicitud de Patente

Titulo

Se sugiere al solicitante hacer el titulo más concreto según el objeto de la solicitud.

Reivindicaciones (Art 30 D 486, Art 22 PCT)

El kit de la reivindicación 1 no se encuentra caracterizado y carece de concisión, puesto que se refiere a un primer recipiente que contiene un adyuvante, el cual se pretende definir únicamente por el tipo de emulsión, sin definir inequívocamente cuales son los ingredientes que forman la emulsión de aceite en agua y sus proporciones; respecto al segundo recipiente, éste contiene una composición que comprende un inmunógeno, el cual se define de la siguiente forma "un inmunógeno para provocar una respuesta inmunitaria contra el serogrupo B de *Neisseria meningitidis*", que es un resultado a alcanzar, no define al antígeno presente en la composición ni a la composición, puesto que la reivindicación incluiría no sólo la solución propuesta por el solicitante, sino todas las alternativas presentes o futuras que lleguen a ese resultado, se recuerda al solicitante que un antígeno se define por su secuencia exacta de aminoácidos y una composición por ingredientes y sus proporciones. Se sugiere al solicitante caracterizar el kit definiendo cada uno de sus componentes adecuadamente por todas sus características esenciales.

La reivindicación 2 se refiere a un sacárido capsular conjugado que se puede encontrar en la composición antigénica liofilizada, el cual se define por los serogrupos de *N. meningitidis* de los cuales se obtiene. Se sugiere definir inequívocamente el sacárido capsular conjugado por las características técnicas del sacárido y la proteína.

La reivindicación 3 se refiere a una composición antigénica liofilizada que comprende un inmunógeno que al igual que en el kit de la reivindicación 1 se encuentra definido por un resultado a alcanzar, para lo cual, se reitera que esta forma de definir no permite caracterizar el objeto de la solicitud; la composición también comprende un sacárido capsular conjugado que presenta la misma objeción realizada para la reivindicación 2. Se sugiere al solicitante incluir todas las características técnicas esenciales de la composición.

La expresión: "y/o" en la reivindicación 3 precediendo una característica no es limitativa, porque hace opcional a la característica y no limita el alcance de la reivindicación. Se sugiere incluir las características opcionales o preferentes en una reivindicación dependiente.

Las reivindicaciones 7, 8 y 9 presentan falsa dependencia porque dicen se encuentran dependientes de cualquier reivindicación anterior, y puesto que en las reivindicaciones anteriores se encuentran 2 productos diferentes (un kit y una composición) y un método, que corresponden a objetos diferentes y de distintas categorías. Se sugiere corregir las dependencias teniendo en cuenta que las reivindicaciones 4 – 6 no son susceptibles de patentabilidad por carecer de aplicación industrial.

Las reivindicaciones 7, 8 y 9 se refieren a vesículas de membrana, proteínas recombinantes y lipolisacáridos definiéndolos solo porque son de la cepa del serogrupo B de *N. meningitidis*, esta forma de definir no es adecuada porque da lugar a un sinnúmero de opciones que no permiten definir el alcance de la solicitud. Adicionalmente, no se encuentra soporte descriptivo de la obtención o prueba de composiciones o kits como los reclamados en estas reivindicaciones, por lo tanto se sugiere al solicitante eliminar estas reivindicaciones del alcance del pliego reivindicatorio.

En la reivindicación 9 se encuentra la palabra "lipoolisacárido", que puede corresponder a un error de transcripción. Se sugiere realizar las correcciones necesarias.

Descripción (Art 28 D 486)

Se solicita definir las marcas Menjugate^{MR}, Meningitec^{MR}, Neisvac-C^{MR}, Menactra^{MR} (Pág. 17, Fl. 24), Dowfax^{MR} (Pág. 28, Fl. 35) y Prevnar^{MR} (Pág. 53, Fl. 60), Span, Tween, Tritin X-100 (Pág. 28, Fl. 35).

La figura 3 (Fl. 87) no es legible, se solicita allegar una copia legible de la misma.

Se requiere al Solicitante hacer las aclaraciones necesarias.

5. Unidad de invención (Art 25 D 486)

La Solicitud no cumple con el requisito de unidad de invención porque composiciones que comprenden un inmunógeno del serogrupo B de *Neisseria meningitidis* y un sacárido capsular conjugado de los serogrupos A, C, W135 y Y ya se encontraban divulgadas en el documento D1, por lo tanto la solicitud se refiere a 2 grupos inventivos diferentes.

Los grupos inventivos están definidos por las siguientes características **esenciales**:

- 1° Kit que comprende: (i) un primer recipiente que contiene un adyuvante que comprende una emulsión de aceite en agua; y (ii) un segundo recipiente que contiene una composición antigénica liofilizada que comprende un inmunógeno para provocar una respuesta inmunitaria contra el serogrupo B de *Neisseria meningitidis* (Documentos D1 y D2).
- 2° Composición que comprenden un inmunógeno del serogrupo B de *Neisseria meningitidis* y un sacárido capsular conjugado de los serogrupos A, C, W135 y Y (Documentos D1 y D3).

Se sugiere presentar las solicitudes divisionales que considere necesarias, ó limitar la solicitud inicial a una sola invención, eliminando de ella las otras invenciones.

6. Determinación del Estado de la Técnica

El estado de la técnica se determinó con base en la fecha de presentación de la solicitud de prioridad: 19/Octubre/2007 y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

Ítem	Documento	Título	Fecha de Publicación
D1	US 2007/0082014	Injectable Vaccines Against Multiple Meningococcal Serogroups	12/Abril/2007

Ítem	Documento	Título	Fecha de Publicación
D2	GRANOFF D. ET AL, Infection and Immunity, Vol. 65, No. 5, Págs. 1710 - 1750	MF59 Adjuvant Enhances Antibody Responses of Infant Baboons Immunized with Haemophilus influenza Type b and Neisseria meningitides Group C Oligosaccharide-CRM ₁₉₇ Conjugate Vaccine	Mayo/1997
D3	ARIGITA, C. ET AL, Vaccine, Vol. 22, Págs. 629 - 642	Stability of Mono- and Trivalent Meningococcal Outer Membrane Vesicle Vaccines	2004

D1 <http://worldwide.espacenet.com/publicationDetails/originalDocument?CC=US&NR=2007082014A1&KC=A1&FT=D&ND=3&date=20070412&DB=EPODOC&locale=en> EP divulga (Resumen, Pág. 1, párrafos 7 y 8) una composición inmunogénica inyectable que comprende sacáridos capsulares de al menos dos de los serogrupos A, C, W135 y Y de *Neisseria meningitidis*, los cuales están conjugados a una proteína y/o a oligosacáridos, la composición además comprende un antígeno del serogrupo B de *N. meningitidis*.

D2 divulga (Pág. 1710, Resumen, Fl. 151) una composición vacunas de un conjugado del grupo C de *Neisseria meningitidis* y *Haemophilus influenzae* tipo B que es reconstituida antes de su uso con un adyuvante en emulsión aceite en agua.

D3 divulga (Pág. 269, resumen, Fl. 156) una composición de vesículas de la membrana externa de *Neisseria meningitidis* grupo B estable en forma liofilizada.

7. Examen de Patentabilidad (Art 14 D486)

Nivel Inventivo (Art18 D486)

La reivindicación 1 consiste en: un kit, caracterizado porque comprende: (i) un primer recipiente que contienen un adyuvante que comprende una emulsión de aceite en agua; y (ii) un segundo recipiente que contiene una composición antigénica liofilizada que comprende un inmunógeno para provocar una respuesta inmunitaria contra el serogrupo B de *Neisseria meningitidis*.

Comparación de las características técnicas **esenciales**, con las del Estado de la Técnica:

Características esenciales	D1	D2
Un kit que comprende	Kit que comprende	Kit que comprende
Un primer recipiente que contiene un adyuvante que comprende una emulsión de aceite en agua	No se menciona	Un recipiente que contiene un adyuvante que comprende una emulsión de aceite en agua
Un segundo recipiente que contiene una composición antigénica liofilizada que comprende un inmunógeno para provocar una respuesta inmunitaria contra el serogrupo de <i>Neisseria meningitidis</i>	Un inmunógeno del serogrupo B de <i>Neisseria meningitidis</i>	No se menciona

Los documentos D1 y D2 constituyen el estado de la técnica cercano a la invención. El documento D1 revela un kit que comprende dos viales (Pág. 9, párrafo 103) y uno de estos viales contiene una composición de un antígeno del serogrupo B de *Neisseria meningitidis* (Pág. 1, párrafo 8; Pág. 17, párrafo 216) y enseña que esta composición puede estar liofilizada (Pág. 9, párrafo 101).

El documento D1 también enseña que se pueden utilizar adyuvantes de emulsión de escualeno-agua como el MF59 (Pág. 10, párrafo 113) y divulga que la composición comprende sacáridos capsulares de los serogrupos A, C, W135 y Y de *Neisseria meningitidis*, los cuales están conjugados a una proteína y/o a oligosacáridos (Pág. 1, párrafos 7 y 8; Pág. 15, párrafo 188 – 191).

El documento D2 da a conocer una composición de vacuna liofilizada de un conjugado de grupo C de *Neisseria meningitidis* y *Haemophilus influenzae* tipo B que es reconstituida antes de su uso con un adyuvante en emulsión aceite en agua (Pág. 1710, Resumen, Fl. 151).

El kit objeto de esta solicitud se diferencia del kit del documento D1, en que el segundo recipiente contiene un adyuvante que comprende una emulsión de aceite en agua.

El hecho de dejar en el segundo recipiente el adyuvante en emulsión aceite en agua permite suministrar una formulación de vacuna contra el serogrupo B de *Neisseria meningitidis* ("Men-B") con buena estabilidad e inmunogenicidad.

Por tanto, el problema técnico objetivo consiste en "cómo modificar el kit que comprende un recipiente con un antígeno contra el serogrupo B de *Neisseria meningitidis* conocido en el documento D1 para obtener una formulación con buena estabilidad e inmunogenicidad".

El documento D2 describe una manera de solucionar el problema técnico, mediante la utilización de un adyuvante en emulsión aceite en agua para reconstituir antes de su uso una composición que comprende un conjugado del grupo C de *Neisseria meningitidis* (Pág. 1710, Resumen, Fl. 151) mostrando una mejor inmunización respecto a otros adyuvantes (Pág. 1712, Figura 1, Fl. 153).

Adicionalmente, es conocido por la persona normalmente versada en la materia que la liofilización es una buena alternativa para el almacenamiento de una composición de vesículas de la membrana externa de *Neisseria meningitidis* grupo B en estado líquido y permite un ventajoso tiempo de almacenamiento de más de 1 año⁽¹⁾.

La reivindicación es obvia, porque la persona del oficio normalmente versada en la materia enfrentada al problema de obtener una formulación de antígeno contra el serogrupo B de *Neisseria meningitidis* con estabilidad e inmunogenicidad mejoradas, obtendría un kit que contiene un recipiente con un antígeno del serogrupo B de *Neisseria meningitidis* como el divulgado en D1 con un adyuvante que comprende una emulsión de aceite en agua en un segundo recipiente como se enseñan en el documento D2, aplicado medios técnicos conocidos para otro serogrupo de *Neisseria meningitidis* logrando de esta forma el kit objeto de la solicitud.

Por lo cual el kit de la Reivindicación 1 carece de Nivel Inventivo.

Puesto que las reivindicaciones dependientes 2 y 7 – 9 no mencionan características inventivas adicionales tampoco se pueden considerar inventivas.

Conclusión:

Las reivindicaciones 1, 2 Y 7 - 9 cumplen el requisito de novedad (Art. 16), pero no cumplen con el requisito de nivel inventivo (Art. 18).

(1) ARIGITA, C. ET AL, Stability of Mono- and Trivalent Meningococcal Outer Membrane Vesicle Vaccines, Vaccine, Vol. 22, 2004, Págs. 629 – 642.

La reivindicación 3 consiste en una composición antigénica liofilizada, caracterizada porque comprende (i) un inmunógeno para provocar una respuesta inmunitaria contra el serogrupo B de *Neisseria meningitidis*, y (ii) un sacárido capsular conjugado de uno o más de los serogrupos A, C, W135 y/o Y de *Neisseria meningitidis*.

Comparación de las características técnicas **esenciales**, con las del Estado de la Técnica:

Características esenciales	D1	D3
Una composición antigénica liofilizada que comprende:	Composición inmunogénica	Composición inmunogénica liofilizada
Un inmunógeno contra el serogrupo B de <i>Neisseria meningitidis</i>	Un inmunógeno del serogrupo B de <i>Neisseria meningitidis</i>	Un inmunógeno de vesículas de la membrana externa del grupo B de <i>Neisseria meningitidis</i> .
Un sacárido capsular conjugado de uno o más de los serogrupos A, C, W135 y/o Y de <i>Neisseria meningitidis</i>	Sacáridos capsulares de los serogrupos A, C, W135 y Y de <i>Neisseria meningitidis</i>	No se menciona

El documento D1 se considera el estado de la técnica cercano a la invención definida en la reivindicación 3 porque divulga una composición que comprende sacáridos capsulares de los serogrupos A, C, W135 y Y de *Neisseria meningitidis*, los cuales están conjugados a una proteína y/o a oligosacáridos y un antígeno del serogrupo B de *N. meningitidis* (Resumen, Pág. 1, párrafos 7 y 8; Pág. 15, párrafo 188 – 191; Pág. 17, párrafo 216). El documento D2 revela una composición de vesículas de la membrana externa de *Neisseria meningitidis* grupo B estable en forma liofilizada (Pág. 269, resumen, Fl. 156).

La diferencia entre la invención, definida en la reivindicación 1 y la composición del documento D1 es que los ejemplos específicos se encuentran en forma liofilizada.

El efecto que logra tener la composición en forma liofilizada es la obtención de una forma farmacéutica estable.

Por lo tanto, el problema técnico objetivo que pretende resolver esta invención se puede formular así: “cómo modificar la composición de sacáridos capsulares de los serogrupos A, C, W135 y Y de *Neisseria meningitidis* conjugados y un antígeno del serogrupo B de *N. meningitidis* conocida en el documento D1 para obtener una composición estable”.

Sin embargo, el documento D1 enseña que la composición puede estar liofilizada (Pág. 9, párrafo 101) y el documento D2 ya divulgaba que la liofilización es una buena alternativa para el almacenamiento en estado líquido y permite un ventajoso tiempo de almacenamiento de más de 1 año (Pág. 641, Fl. 168).

En consecuencia, la persona del oficio normalmente versada en la materia obtendría una composición como la divulgada en el documento D1 en forma liofilizada como se enseña en el documento D2 para llegar así al objeto de la reivindicación 3 de la solicitud. Por lo cual se considera obvia.

Puesto que la reivindicación dependientes 7 – 9 no mencionan características inventivas adicionales tampoco se pueden considerar inventivas.

Conclusión:

Las reivindicaciones 3 y 7 – 9 cumplen el requisito de novedad (Art. 16), pero no cumplen con el requisito de nivel inventivo (Art. 18).

Aplicación industrial (Art 19 D 486)

Las reivindicaciones 4 – 6 se refieren a un método de mezclar un adyuvante que comprende una emulsión de aceite en agua y una composición antigénica liofilizada, este método corresponde a un proceso de reconstitución que al ser realizado antes de la administración del producto no tiene aplicación industrial. Además, en la descripción se encuentra la siguiente frase: "los antígenos liofilizados de Men-B se pueden reconstituir en forma líquida con adyuvantes en el momento del uso listo para administración a un paciente". Se sugiere al solicitante, eliminar estas reivindicaciones del alcance del pliego reivindicatorio.

8. Conclusión

Los requisitos de Patentabilidad de la presente Solicitud están afectados así:

Ítem	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	US 2007/0082014	1 - 3, 7 - 9	Nivel Inventivo
D2	GRANOFF D. ET AL, Infection and Immunity, Vol. 65, No. 5, Págs. 1710 - 1750	1, 2, 7 - 9	Nivel Inventivo
D3	ARIGITA, C. ET AL, Vaccine, Vol. 22, Págs. 629 - 642	3, 7 - 9	Nivel Inventivo

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de sesenta (60) días contados a partir de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única No. 10 de 2001.


JOSÉ LUIS SALAZAR LÓPEZ

Director de Nuevas Creaciones (C)

El anterior requerimiento fue notificado por fijación en lista, de fecha
16 OCT. 2012

Elaborado por: Jeny Paola Villate Becerra *P. Villate*
Revisado por: Consuelo Leguizamón *CL*

MF59 Adjuvant Enhances Antibody Responses of Infant Baboons Immunized with *Haemophilus influenzae* Type b and *Neisseria meningitidis* Group C Oligosaccharide-CRM₁₉₇ Conjugate Vaccine

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The ability of the adjuvant MF59 to enhance the immunogenicity of polysaccharide-protein conjugate vaccines was investigated in infant baboons. MF59 consists of stable droplets (<250 nm) of the metabolizable oil squalene and two surfactants, polyoxyethylene sorbitan monooleate and sorbitan trioleate, in an oil-in-water emulsion. In humans, MF59 is well tolerated and enhances the immunogenicity of recombinant protein subunit or particle vaccines. Its effect on the immunogenicity of polysaccharide-protein conjugate vaccines is unknown. Baboons 1 to 4 months of age were immunized intramuscularly with *Neisseria meningitidis* group C and *Haemophilus influenzae* type b (Hib) oligosaccharide-CRM₁₉₇ conjugate vaccines. The lyophilized vaccines were reconstituted with phosphate-buffered saline (PBS), Al(OH)₃ (alum), or MF59. Groups of five animals each were given three injections of the respective formulations, with one injection every 4 weeks. Four weeks after each immunization, the MF59 group had up to 7-fold-higher geometric mean anticapsular-antibody titers than the alum group and 5- to 10-fold-higher *N. meningitidis* group C bactericidal-antibody titers. Twenty-one weeks after the third immunization, the MF59 group still showed 5- to 10-fold-higher anticapsular-antibody titers. The antibody responses of the animals given the vaccines reconstituted with PBS were low at all times measured. Both the MF59 and alum groups, but not the PBS group, showed booster antibody responses to unconjugated Hib and *N. meningitidis* group C polysaccharides, results consistent with induction of memory B cells. Thus, MF59 may be useful for accelerating and augmenting immunity to polysaccharide-protein conjugate vaccines in infants.

Once the major cause of bacterial meningitis and other serious infections in infants and children, diseases caused by *Haemophilus influenzae* type b (Hib) have been virtually eliminated by incorporation of Hib polysaccharide (PS)-protein conjugate vaccines into the routine infant and toddler immunization schedules (1, 4, 8, 25).

The success of Hib conjugate vaccines also has stimulated development of new conjugate vaccines for prevention of diseases caused by other encapsulated organisms, such as *Streptococcus pneumoniae* (36) and *Neisseria meningitidis* groups A and C (10, 19, 20, 38). However, in contrast to Hib conjugate vaccines, which contain only a single serotype-specific PS, these new vaccines require the use of multiple serotype- or serogroup-specific PS, each conjugated separately to a carrier protein. Maintaining optimal immunogenicity of each component may be difficult with conjugate vaccines containing multiple PS antigens.

One approach to increasing both the immunogenicity and the effectiveness of PS-protein conjugate vaccines is the use of an adjuvant. An effective adjuvant also may permit the use of lower dosages and fewer injections, thereby decreasing the cost of an immunization program. Currently, the only vaccine adjuvant licensed for human use by the U.S. Food and Drug Administration is alum (several salts of aluminum). While

alum has a good safety record, it is a relatively weak adjuvant. A promising, more potent adjuvant, designated MF59, has been developed at Chiron Vaccines. MF59 consists of small, uniform, stable droplets (<250 nm) of the metabolizable oil squalene with two surfactants, polyoxyethylene sorbitan monooleate (Tween 80) and sorbitan trioleate (Span 85), in an oil-in-water emulsion. When compared to alum or incomplete Freund's adjuvant (39), MF59 has been shown in mice, guinea pigs, rabbits, and nonhuman primates to augment antigen-specific humoral and T-cell responses to a variety of experimental vaccines. In humans, the MF59 adjuvant is generally well tolerated. To date (1996), MF59 has been administered to more than 6,000 humans (estimated total number of injections, >15,000) as part of clinical trials of recombinant glycoprotein subunit vaccines for herpes simplex virus type 2 (gB and gD) (6, 18), cytomegalovirus (gB) (42), human immunodeficiency virus (gp120) (16), or inactivated influenza vaccine (split virion or purified subunits) (22, 23, 27). In these studies, no abscesses or severe local reactions have been reported. Other local reactions were limited largely to transient pain at the injection site (usually mild) and minimal erythema or induration that usually resolved within 2 to 3 days (18). Fever and systemic reactions were infrequent. MF59 also has been investigated as an adjuvant in human newborns immunized with gp120 (human immunodeficiency virus vaccine) (17, 43) and toddlers immunized with gB (cytomegalovirus vaccine) (24a). In both studies, the respective vaccines were well tolerated and elicited high immune responses. Thus, MF59 is a promising candidate

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adjuvant for augmenting immune responses of vaccines intended both for adults and for infants and toddlers.

The purpose of the present study was to determine the ability of MF59 to enhance serum antibody responses of infant baboons to a combined investigational Hib and *N. meningitidis* group C oligosaccharide-protein conjugate vaccine. Infant baboons were selected as the model system because their antibody responses are likely to be more predictive of the anti-PS antibody responses of human infants than are adult primate or rodent models (3, 15, 32, 33, 37, 40, 41).

MATERIALS AND METHODS

Vaccines and adjuvants. *N. meningitidis* group C conjugate vaccine (development lot J35D18L1) and Hib conjugate vaccine (development lot L10HC24) were produced by using selective end-reducing group activation of sized oligosaccharides followed by subsequent coupling to the protein carrier CRM₁₉₇ through a hydrocarbon spacer (7). CRM₁₉₇ is a nontoxic genetic mutant of diphtheria toxin. The respective conjugates were filter sterilized and lyophilized in unit doses. On the day of vaccination, the *N. meningitidis* group C and Hib glycoconjugate vaccines were reconstituted with Dulbecco's modified phosphate-buffered saline without calcium or magnesium (PBS), with aluminum hydroxide (alum; 1 mg per dose; lot 010394; Chiron Vaccines, Siena, Italy), or with MF59 lot KJA342; Chiron Vaccines). The MF59 was prepared under high-pressure homogenization as previously described (26). After reconstitution, each 0.5-ml dose of vaccine contained approximately 10 µg of each saccharide and a total of 40 µg of CRM₁₉₇ protein.

To assess immunologic B-cell priming by the conjugate vaccination (10, 12, 19), animals were given boosters of plain (unconjugated) *N. meningitidis* group C PS vaccine (bulk lot 82) and Hib PS vaccine (bulk lot 25). Both vaccines were prepared at Chiron Vaccines (Siena, Italy). Each 0.5-ml dose contained 5 µg of the respective PS in PBS.

Animals. Infant baboons (*Papio cynocephalus anubis*), estimated on the basis of weight and color to be 1.5 to 4 months of age at study initiation, were assigned to groups of five animals each to ensure an equal distribution of ages and genders (2:3 male to female). The baboons were housed with their mothers at Southwest Foundation for Biological Research, San Antonio, Tex., a registered research facility accredited by the American Association for Accreditation of Laboratory Animal Care. Animal welfare experimentation guidelines as established by the National Institutes of Health, American Association for Laboratory Animal Science, and Food and Drug Administration were followed in the conduct of all animal research throughout the course of this study.

Immunizations and blood samples. There were four study groups. Animals in the first three groups received three immunizations with a combined *N. meningitidis* group C and Hib conjugate vaccine. The immunizations were given intramuscularly (i.m.) 1 month apart. Group 1 received vaccine administered with PBS, group 2 received vaccine with alum, and group 3 received vaccine with MF59. Animals in group 4 (controls) received three monthly 0.5-ml i.m. injections of acellular pertussis vaccine combined with diphtheria and tetanus toxoids adsorbed to aluminum hydroxide (Pertugen; Chiron Vaccines, Siena, Italy; referred to herein as DTaP) (29, 30).

Baboons were bled by venipuncture prior to each immunization and at 4 and 21 weeks after the first immunization. After the week 21 blood collection, all animals were given boosters of 5 µg of plain (unconjugated) *N. meningitidis* group C PS and 5 µg of plain (unconjugated) Hib PS. These vaccines were administered i.m. in separate limbs. Sera were obtained 7, 28, and 90 days after the booster injections.

Serology. Serum anti-*N. meningitidis* group C PS antibody titers were measured by an enzyme-linked immunosorbent assay (ELISA), using a procedure adapted from that previously described for measuring human serum antibody to group B PS (13). In the present study, the solid-phase test antigen consisted of an adipic dihydrazide-derivatized form of the *N. meningitidis* group C PS (ADH-group C PS) instead of biotinylated ADH-group B PS. The ADH-group C PS (1 µg/ml in PBS) was used directly to coat 96-well U-bottom microtiter plates (Immulon II; Dynatech, Chantilly, Va.) by incubating them for 1 h at 37°C. After washing, plates were blocked with 1% bovine serum albumin (radioimmunoassay grade; fraction V; Sigma Chemical Co., St. Louis, Mo.) in PBS, pH 7.4, for 1 h at room temperature. Serial dilutions of sera were incubated overnight at 4°C. After washing, bound antibody was detected with alkaline phosphatase-labeled, affinity-isolated rabbit anti-monkey immunoglobulin G (IgG) (Sigma). Titers were expressed as the serum dilution giving an optical density at 405 nm of 0.5 after 30 min of incubation with substrate.

Complement-mediated bactericidal activity against *Neisseria meningitidis* group C was assayed as previously described for *N. meningitidis* group B (21) except that Gey's buffer was used instead of barbital buffer. The group C test strain used, 60F, was obtained from Wendell Zollinger (Walter Reed Army Institute of Research, Rockville, Md.). All test serum samples were heat treated (56°C for 30 min) to inactivate endogenous complement activity. For the bactericidal reaction, 20% serum from a healthy human adult whose serum alone showed no killing of the test organism was used as an exogenous complement

source. Bactericidal titers were defined as the reciprocal dilution of baboon serum giving a 50% decrease in CFU after 60 min of incubation at 37°C with bacteria and complement compared to CFU present at time 0. (In the presence of test serum alone or complement alone, CFU counts during the 60 min normally increased by $\geq 50\%$.)

Concentrations of serum antibody to Hib PS were measured by a radioantigen binding assay using ¹²⁵I-labeled Hib PS and a procedure adapted from that described by Robbins et al. (31). Immune complexes containing radiolabeled antigen were precipitated with an equal volume of saturated ammonium sulfate. After washing, counts per minute were measured in the precipitate as described elsewhere (2). Anti-Hib antibody concentrations in the baboon sera were assigned from a standard curve generated by using serial twofold dilutions of the U.S. Center of Biologic Evaluation and Research human serum reference pool, lot 1983 (U.S. Food and Drug Administration, Bethesda, Md.).

Statistics. Antibody concentrations were transformed ($\log_{10}$). For these calculations, baboons anti-*N. meningitidis* group C ELISA titers of less than 1:50 were assigned a value of 1:25, and anti-group C bactericidal-antibody titers of less than 1:25 were assigned a value of 1:12.5. Anti-Hib PS anticapsular-antibody concentrations of less than 0.14 µg/ml were assigned a value of 0.07. Geometric means and 95% confidence intervals were computed by using the log transformed means and standard errors computed from a one-way analysis of variance (ANOVA) model. Differences between each pair of groups with respect to geometric means were tested by using the *P* values from the ANOVA model. To evaluate the fold change in titers between different days within each group, the logarithm of the ratio was computed for each animal and then analyzed by using a one-way ANOVA model.

RESULTS

Safety of MF59 when administered with conjugate vaccines to infant baboons. All animals appeared to tolerate the vaccinations without difficulty. On close inspection of the injection sites at the time of reimmunization or obtaining blood samples, there was no evidence of granulomas or abscesses. One animal assigned to the alum group died 3 days after the first immunization from apparent starvation. The animal caretakers attributed this death to insufficient milk production from its mother.

MF59 enhances anti-*N. meningitidis* group C and anti-Hib PS antibody responses of infant baboons. Figure 1 summarizes the anti-PS antibody responses of the infant baboons immunized with Hib and *N. meningitidis* group C conjugate vaccines (Fig. 1B and A, respectively) administered with PBS, alum, or MF59. Although not shown, the preimmunization sera had very low or undetectable antibody levels. Also, with the exception of two animals, none of the control animals immunized with DTaP alone showed more than twofold increases in levels of antibody to *N. meningitidis* group C or Hib PS in serum. The two exceptions had 0.24 and <0.14 µg of anti-Hib PS antibody per ml detected in serum from the first bleed, and these levels increased to 0.57 and 1.46 µg/ml, respectively, in sera obtained 1 month after the third immunization. The reasons for these increases in serum anti-Hib PS antibody concentrations are not known. These two animals showed no detectable increases in titer of serum antibody to *N. meningitidis* group C PS.

The baboons given the conjugate vaccines with PBS showed minimal anti-Hib PS antibody responses after one, two, or three immunizations (geometric mean antibody concentrations of <0.14, <0.14, and 0.15 µg/ml, respectively). The corresponding values in the alum group were 0.32, 5.0, and 5.2 µg/ml (*P* $\leq$ 0.03 compared to the respective values of the PBS group). After doses 1 and 2, the MF59 group showed geometric mean antibody concentrations of 0.75 and 15.1 µg/ml, respectively. After dose 3, the geometric mean serum antibody concentration was 37 µg/ml, which is sevenfold higher than that in the alum group (*P* < 0.03).

The immunogenicity of the MenC conjugate showed a pattern similar to that of the respective anti-Hib PS antibody responses. When the conjugate vaccine was administered without adjuvant (i.e., with PBS), the geometric mean titer (GMT) of IgG anti-group C PS as measured by ELISA was below detectable levels (<1:50) after one and two immunizations and

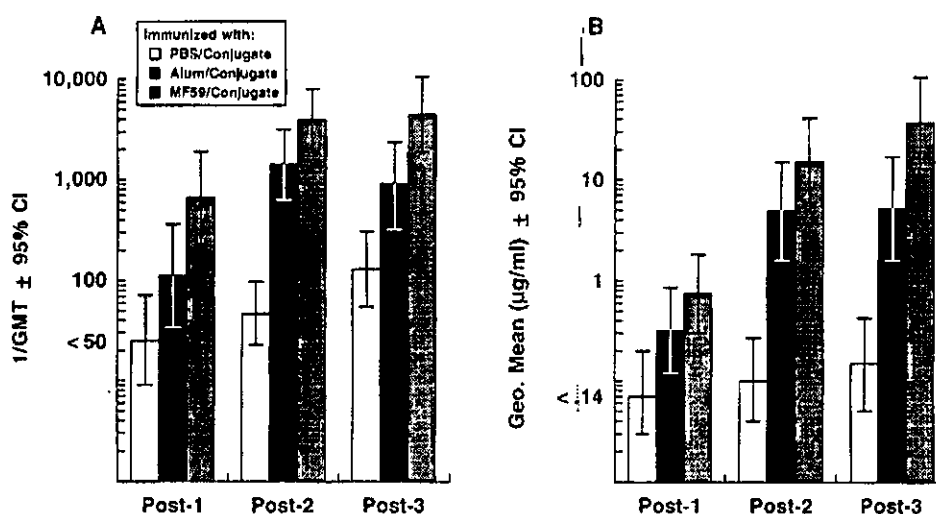


FIG. 1. Effect of MF59 adjuvant on serum anticapsular antibody responses of infant baboons immunized at 1-month intervals with *N. meningitidis* group C and Hib oligosaccharide-CRM₁₉₇ conjugate vaccines. The lyophilized vaccines were administered with PBS, alum, or MF59. (A) IgG anti-group C PS antibody responses. Animals administered vaccine with alum had higher GMTs than those given vaccine with PBS ($P \leq 0.06$ for each injection). Animals administered vaccine with MF59 had higher GMTs than those given vaccine with alum ($P < 0.03$ for each injection). (B) Anti-Hib PS antibody responses. Animals administered vaccine with alum had higher geometric mean total antibody concentrations than those given vaccine with PBS ($P < 0.03$ for each injection). Animals administered vaccine with MF59 had higher geometric mean concentrations than those given vaccine with alum ($P > 0.10$ after the first and second injections and $P < 0.03$ for the 4 weeks post-third injection). CI, confidence interval.

rose to a GMT of 1:131 4 weeks following the third immunization. With alum, the antibody responses peaked after dose 2. The corresponding GMTs after doses 1, 2, and 3 were 1:111, 1:1,432, and 1:924 ($P < 0.06$, 0.001, and 0.01 compared to the respective titers in the PBS group). The highest IgG anti-group C PS antibody responses were observed in the group of animals receiving the conjugate vaccines with MF59: GMTs of 1:669, 1:3,957, and 1:4,548 after one, two, and three injections, respectively ($P < 0.03$ for each injection compared to the respective GMTs of the alum group).

MF59 augments serum antibody functional activity against *N. meningitidis* group C. Table 1 summarizes the serum anti-group C bactericidal antibody responses of the baboons immunized with the conjugate vaccines administered with PBS, alum, or MF59. There were no detectable bactericidal responses in the group administered the vaccines with PBS (GMTs of $<1:25$ after all three doses). The group administered the conjugate vaccines with alum showed no detectable bactericidal antibody response after dose 1 but had a >18 -fold increase in GMT after dose 2 ($P < 0.001$) and no significant further change after dose 3 ($P > 0.3$). The highest antibody responses were observed in the group given vaccine with MF59 (4-fold higher than that with alum after the first immunization [$P > 0.10$], approximately 6-fold higher after the second immunization [$P < 0.01$], and 11-fold higher after the third immunization [$P < 0.01$]). Thus, MF59 not only augments IgG anticapsular-antibody responses measured by an ELISA (Fig. 1A) but also augments *N. meningitidis* group C bactericidal titers, a measure of functional antibody that correlates in humans with protection against disease caused by *N. meningitidis* (5, 9, 24).

Serum antibody response to plain Hib and *N. meningitidis* group C PS booster. Twenty-one weeks after the third immunization, concentrations of antibody to both Hib PS and MenC PS in serum had declined, on average, approximately 10- to 20-fold in the MF59 and alum groups compared to the respective peak GMTs present 1 month after the third injection (compare respective GMTs at time zero of the booster injection [Fig. 2B and D] to GMTs after the third conjugate vaccine injection [Fig. 1A and B]). However, 21 weeks after the third conjugate vaccination, the group administered the conjugate vaccines with MF59 still had 5- and 12-fold-higher GMTs to group C and Hib than the respective titers of the group administered the vaccines with alum ($P < 0.001$ for each comparison).

To assess whether the conjugate vaccination had primed the animals for the ability to respond to unconjugated PS (i.e., induction of B-cell memory), all animals were boosted at the 21-week follow-up visit with 5 µg of plain *N. meningitidis* group C PS vaccine and 5 µg of Hib PS vaccine, both given i.m. in separate hind limbs. At the time of this booster, the animals ranged from 8 to 10 months of age. The groups previously primed with the conjugate vaccines administered with alum or MF59 showed increases in the respective geometric mean anti-Hib PS antibody concentrations, which peaked at 7 days and returned to prebooster levels by 28 days (Fig. 2B). These booster responses appeared to parallel those of the unprimed control group that had previously received only DTaP (Fig. 2A). The lowest responses were observed in the group previ-

ously primed with the conjugate vaccines administered with alum or MF59 showed increases in the respective geometric mean anti-Hib PS antibody concentrations, which peaked at 7 days and returned to prebooster levels by 28 days (Fig. 2B). These booster responses appeared to parallel those of the unprimed control group that had previously received only DTaP (Fig. 2A). The lowest responses were observed in the group previ-

TABLE 1. Serum anti-group C bactericidal antibody responses of infant baboons^a

Adjuvant	1/GMT (95% confidence interval)		
	1 mo p.i. 1	1 mo p.i. 2	1 mo p.i. 3
PBS	$<25^b$ (9-23)	$<25^c$ (9-28)	41^b (17-100)
Alum	$<25^c$ (7-75)	427^d (100-1,816)	277^d (73-1,052)
MF59	102^d (9-1,190)	$2,390^e$ (833-6,860)	$3,091^f$ (490-19,479)

^a Infant baboons (five per group) were immunized monthly for 3 months with *N. meningitidis* group C and Hib vaccines reconstituted with PBS, alum, or MF59 at the time of use. Bactericidal titers were measured in sera collected prior to each immunization and 4 weeks after the third immunization. For calculation of GMTs, titers of $<1:25$ were assigned a value of 1:12.5 p.i. 1, post-injection 1.

^b versus ^c and ^c versus ^d, not significant ($P > 0.05$); ^e versus ^f and ^f versus ^d, $P < 0.01$; ^b versus ^c, and ^c versus ^d, $P < 0.01$.

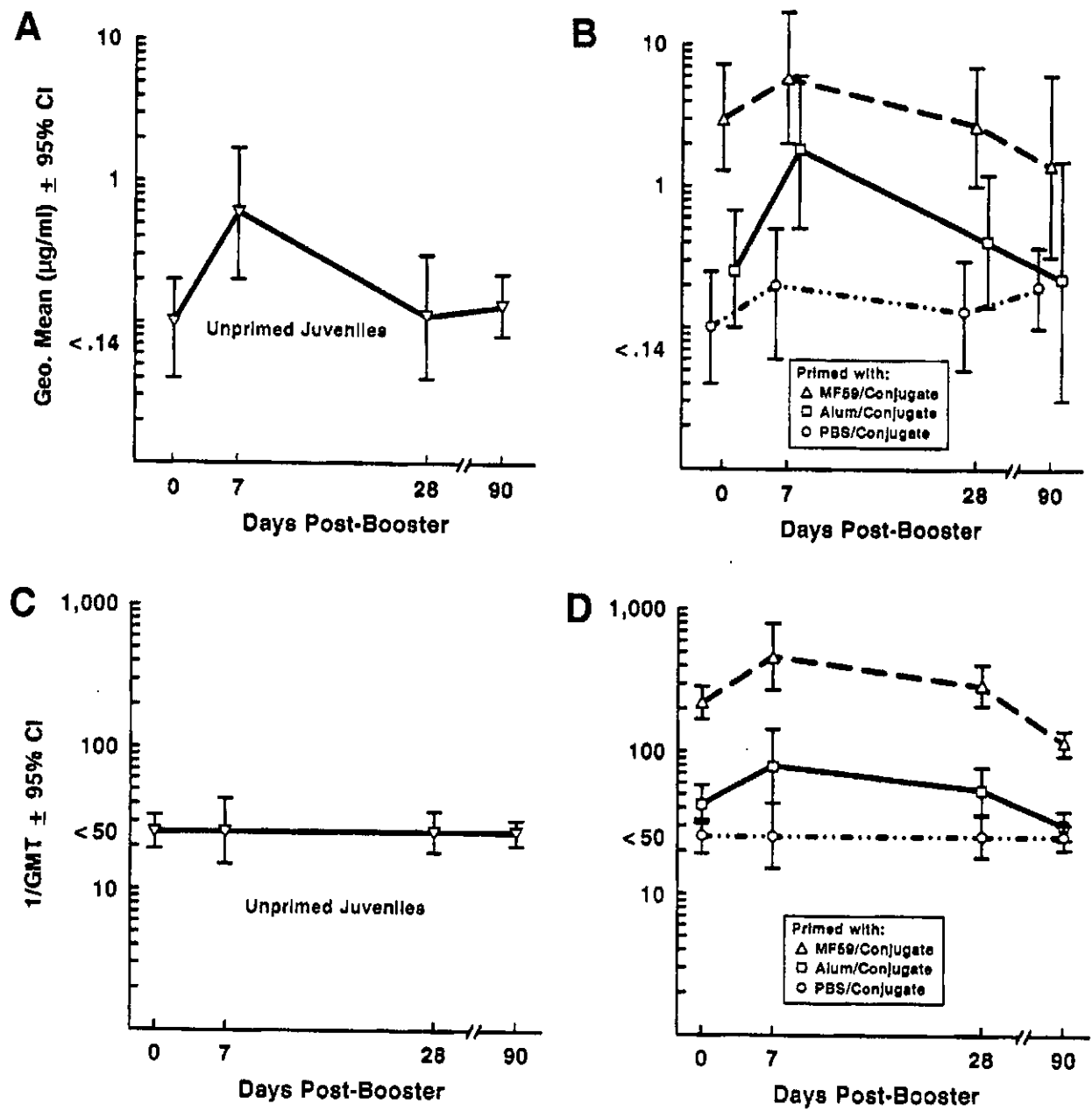


FIG. 2. Serum anticapsular antibody responses of juvenile baboons administered booster injections of unconjugated Hib PS vaccine and *N. meningitidis* group C PS vaccine administered as separate injections 21 weeks (time zero) after the three-dose primary conjugate vaccination schedule had been completed. (A) Anti-Hib PS antibody responses of unprimed control baboons previously vaccinated with DTaP only. (B) Anti-Hib PS antibody responses of baboons previously primed with the conjugate vaccines administered with PBS, alum, or MF59. (C and D) Respective IgG antibody responses to *N. meningitidis* group C PS for the same groups as shown in panels A and B.

ously vaccinated with the conjugate vaccines given with PBS (less than a twofold increase in anti-Hib PS antibody concentration at day 7 versus day 0).

With one exception, the IgG anti-group C PS antibody responses of the respective groups to the plain PS vaccine booster immunization (Fig. 2C and D) paralleled the anti-Hib PS antibody responses. The exception was that the unprimed juvenile baboons (i.e., the DTaP control group) showed no detectable serum antibody responses to group C PS vaccine (Fig. 2C), whereas this group had responded to the plain Hib PS vaccination (Fig. 2A).

Ninety days after the plain group C PS and Hib PS booster

injections, serum anti-group C and anti-Hib PS antibody concentrations still remained higher in the animals previously administered conjugate vaccine with MF59 compared to the respective titers in the group given the conjugate vaccines with alum ($P < 0.01$ for anti-Hib and $P < 0.001$ for anti-group C) or the conjugate vaccines with PBS ($P < 0.01$ for both comparisons) (Fig. 2B and D, respectively).

DISCUSSION

The serum anticapsular antibody responses of the infant baboons observed in this study paralleled those observed in

human infants previously administered an *N. meningitidis* group C oligosaccharide-CRM₁₉₇ conjugate vaccine with alum at 2, 3, and 4 months of age (35). Specifically, both the immunized human infants and the immunized infant baboons showed modest serum anti-group C PS antibody responses after the first injection, very high booster antibody responses to the second injection, and minimal or no further increases in serum antibody titer after a third injection. The baboons also showed evidence of induction of immunologic priming by the *N. meningitidis* group C conjugate vaccination, as measured by booster IgG antibody responses to plain *N. meningitidis* group C PS vaccination administered 21 weeks after a series of three doses of conjugate had been completed. In contrast, unprimed (control) juvenile baboons vaccinated with plain group C PS for the first time showed no detectable antibody response (Fig. 2C). In human infants, priming to *N. meningitidis* group C PS also has been observed with an earlier, prototype version of this conjugate vaccine (a combined group A/group C conjugate vaccine administered with alum [19]). Thus, the pattern of the antibody responses of the infant baboons to group C conjugate administered with alum appears to be predictive of the primary and booster responses of human infants and toddlers to this glycoconjugate vaccine. The only notable difference is the very short duration of the antibody responses to plain group C PS booster injection in the baboons (<28 days).

The most important finding of this study is that MF59, an adjuvant that has been widely evaluated in humans with a variety of glycoprotein subunit vaccines and inactivated influenza vaccines, both accelerates and augments serum anti-PS antibody responses of infant baboons immunized with Hib and *N. meningitidis* group C PS-protein conjugate vaccines. In addition, the animals vaccinated with the MF59-adjuvanted vaccines showed higher titers during the extended follow-up period. These animals also were capable of mounting memory serum antibody responses to unconjugated *N. meningitidis* group C and Hib PS booster immunizations. The immunologic priming appeared to be similar in the animals primed with the conjugate vaccine administered either with alum or MF59 (Fig. 2C).

Because only small numbers of infant baboons could be investigated, many important questions such as the optimal conjugate vaccine dose when given with MF59 or the optimal immunization schedule could not be addressed. Also, we did not examine the influence, if any, of simultaneous separate injections of other vaccines, such as DTaP or diphtheria-tetanus-pertussis, that contain diphtheria toxoid, which might influence serum anticapsular antibody responses to conjugate vaccines containing CRM₁₉₇, a nontoxic mutant of diphtheria toxin. This question will be important to investigate in the future, since in previous studies, immunity to the carrier protein was shown to affect (positively or negatively) the ability to mount anti-PS antibody responses to conjugate vaccines (11, 14, 28, 34).

The present study provides only limited information on the safety of administration of PS-protein conjugate vaccines with MF59. First, only a small number of infant baboons were used (five per group). Second, the observations on vaccine tolerability were limited, because any handling of the infant animals by the staff required separation of the infants from their mothers and the use of a general anesthesia on the mothers. Nevertheless, on general daily inspection, the animals appeared to tolerate the vaccinations without difficulty, as judged by the presence of normal activity and feeding patterns. Also, there was no gross evidence of severe local reactions such as granulomas or abscesses at the injection site; however, close inspection of the injection sites was performed only at the time of the

blood draws and booster immunizations, usually 30 days after each immunization.

In summary, the excellent immunogenicity of *N. meningitidis* group C and Hib conjugate vaccines when administered with MF59 to infant baboons and the lack of apparent toxicity are consistent with the excellent immunogenicity and safety record of MF59 used with other vaccines in clinical trials in humans (summarized in the introduction). These data, together with the potential of an adjuvant either to permit the use of lower conjugate vaccine dosages and/or fewer injections or to enhance the immunogenicity of multicomponent PS-protein conjugate vaccines given alone or in combination with other vaccines, support the initiation of phase I safety and immunogenicity trials of MF59 and glycoconjugate vaccines in humans.

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Stability of mono- and trivalent meningococcal outer membrane vesicle vaccines

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Abstract

The stability during storage of outer membrane vesicles (OMVs) of *Neisseria meningitidis* group B was studied. Three types of OMVs were compared for their stability, containing either one (monovalent) or three different PorA subtypes (trivalent), the latter with and without class 4 outer membrane protein (OMO, RmpM).

Aqueous formulations were stored freeze-dried (4 °C), frozen (−70 °C) and in liquid form at 4, 37 and 56 °C. Physico-chemical properties and immunogenicity of the OMVs as well as PorA conformation and antigenicity (P1.7-2.4, the subtype present in all formulations) were monitored during 1 year. At −70 or 4 °C, the structure and immunogenicity of OMVs was preserved. Storage of OMVs at high temperatures (37 or 56 °C) induced destruction of the OMV structure and denaturation of PorA, followed by chemical degradation. Immunogenicity decreased or was lost completely. Changes observed in the fluorescence spectra of degraded OMVs were also seen in tryptophan (Trp) and tyrosine (Tyr) derivatives incubated at 56 °C, indicating the occurrence of chemical degradation of tryptophan and tyrosine residues in PorA. Trivalent OMVs were slightly more stable at 37 °C than monovalent OMVs as assessed by *in vitro* methods, but these differences did not result in differences in the immunogenicity. The stability of trivalent OMVs was not affected by the presence of RmpM. Both trivalent and monovalent OMVs could be freeze-dried with preservation of their immunogenicity.

In conclusion, OMVs are sensitive to elevated temperatures, but are stable in the frozen or freeze-dried state or when stored at 4 °C in the liquid state.

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Keywords: *Neisseria meningitidis*; PorA; Vesicle; Vaccine; Stability; Freeze-drying

1. Introduction

Neisseria meningitidis is a human pathogen and one of the major causes of bacterial meningitis and sepsis [1]. Vaccines against meningococci of serogroups A, C, Y and W-135 are based on capsular polysaccharides. However, the polysaccharide of group B meningococci is poorly im-

munogenic and cross-reactive with human tissues [2]. Vaccines containing outer membrane proteins (OMP) have been developed and have shown to induce protective immune responses [3,4]. The vaccines developed at the Netherlands Vaccine Institute (NVI, formerly a division of the National Institute of Public Health and the Environment, RIVM) consist of outer membrane vesicles (OMVs) purified from genetically modified meningococcal strains expressing either one (monovalent) or three (trivalent) subtypes of class 1 OMP (PorA). Two different trivalent OMVs are mixed to form a hexavalent vaccine that represents the majority of circulating subtypes in Western Europe. This vaccine has been proven to be safe and immunogenic [5]. The monovalent OMV vaccine contains PorA P1.7-2.4, the most prevalent subtype in The Netherlands and the subtype causing an epidemic in New Zealand [1,9]. This vaccine is also safe and immunogenic in toddlers [6]. The trivalent strains contain class 4 outer membrane protein (RmpM). RmpM is

Abbreviations: BSA, bovine serum albumin; DLS, dynamic light scattering; ELISA, enzyme-linked immunosorbent assay; Gnd-HCl, guanidinium hydrochloride; LPS, lipopolysaccharide; MAh, monoclonal antibody; NATrA, *N*-acetyl tryptophan amide; NATyA, *N*-acetyl tyrosine amide; OMP, outer membrane protein; OMVs, outer membrane vesicles; PD, polydispersity index; RmpM, reduction modifiable protein M; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Trp, tryptophan; Tyr, tyrosine

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firmly associated to PorA in the bacterial outer membrane, which is probably essential for the localization and the stability of the PorA trimers in the outer membrane [15].

In previous studies, we have shown that both the conformation and the presentation form determine the quality of the immune response against PorA [7]. Thus, both characteristics have to be maintained upon storage in order to preserve the vaccine functionality.

The composition of OMVs is complex. The main constituents are: PorA, phospholipids, lipopolysaccharide (LPS), residual DNA, RmpM, and residual detergent [12]. The interaction of these compounds with each other through various non-covalent forces and determine the physico-chemical properties and stability of OMVs. Diverse degradation processes may occur simultaneously during storage of OMV vaccines. Not only can the protein undergo chemical and conformational changes, but also the OMV structure may change or even be destroyed. However, it is not clear if changes in OMVs affect the potency and efficacy of meningococcal vaccines. The bactericidal activity of sera obtained from mice or humans after vaccination with OMVs is accepted as correlate for protection [8].

To our knowledge, no studies on long-term stability of protein-containing vesicular systems such as PorA-OMVs have been reported. In this study, trivalent OMVs containing PorA subtypes P1.7-2.4, P1.5-2.10 and P1.12.13 were compared to monovalent OMVs containing PorA subtype P1.7-2.4. The possible stabilizing effect of RmpM on trivalent OMVs was also investigated. OMVs containing subtype P1.7-2.4 were chosen, since 43% of all strains causing bacterial meningitis in The Netherlands belongs to this subtype [9]. At the same time, P1.7-2.4 is one of the weakest immunogens in multivalent OMV vaccines [5].

The stability of liquid (stored 4, 37 and 56 °C) and frozen (–70 °C) PorA-OMVs has been assessed during 12 months of storage. The possibility to freeze-dry OMVs preserving its physico-chemical and immunogenic characteristics was also studied. The stability of the presentation form was assessed with electron microscopy and dynamic light scattering (DLS) measurements. The physico-chemical stability of PorA was monitored by fluorescence spectroscopy, SDS-PAGE and ELISA. The functional immunogenicity (i.e. bactericidal response) of the vaccines was evaluated *in vivo*. The optimal storage conditions for functionally stable OMVs were determined.

2. Materials and methods

2.1. Bacterial strains and growth conditions

N. meningitidis strain F91 (P1.7-2.4, PorB[–], RmpM[–], low expression of Opa/Opc) was used for the production of monovalent PorA-OMVs. Strains JB10124 (P1.5-2.10, P1.12.13, P1.7-2.4, PorB[–], RmpM[–], low expression Opa/Opc) and HP10124 (P1.5-2.10, P1.12.13, P1.7-2.4,

PorB[–], RmpM⁺, low expression Opa/Opc) were used for the production of trivalent PorA-OMVs. Bacteria were grown in a defined medium, with as main components L-glutamic acid, glucose, L-cysteine and ammonium chloride, at 35 °C for 15 h in a 40 l stirred fermentor under pH and *pO*₂ control.

2.2. Preparation of OMV formulations

PdrA-OMVs were prepared as previously described [7]. Briefly, PorA-OMVs were extracted from bacteria with EDTA. DNA was partially removed by DNase treatment. Lipopolysaccharide was partly depleted by gel permeation chromatography (GPC) in the presence of deoxycholate (DOC). In order to remove DOC, a second GPC step was performed, which also served to formulate the OMVs in 10 mM Tris-HCl, 3% sucrose pH 7.4.

OMVs derived from the three strains were stored at –70, 4, 37 and 56 °C in 2 ml plastic tubes with screw caps (Greiner, Alphen a/d Rijn, The Netherlands). For storage at 37 and 56 °C, 0.01% Na azide was added. For fast freezing of OMVs, 2 ml samples were submerged in an ethanol/dry ice mixture and immediately stored at –70 °C.

Freshly prepared OMVs were freeze-dried in aliquots of 2 ml in 5 ml glass vials. The rubber stoppers were dried at 85 °C for 2 days prior to use. The vials containing the OMV formulations were placed directly on the shelf of a Leybold GT 4/6 freeze-dryer with a temperature of –35 °C. In the first step of the freeze-drying process, the temperature of the shelf was maintained at –35 °C and the chamber pressure set at 20 Pa. After 1–3 h, the shelf temperature was raised to –25 °C and the pressure set to 5–6 Pa. These conditions were maintained for 65 h, followed by additional secondary drying steps at plate temperatures of –20, –5, 10, 25 and 35 °C, each for at least 5 h at a pressure of 4–5 Pa. At the end of the freeze-drying process the vials were closed under vacuum with the dried rubber stoppers and capped with aluminium seals.

Freeze-dried OMVs were stored at 4 °C until reconstitution. Analysis of frozen and liquid formulations was performed before storage (*t* = 0) and after 3, 6 and 12 months. Freeze-dried OMVs were reconstituted and analyzed after 3 months storage.

2.3. Composition of OMVs

The protein content of the OMVs was measured according to Peterson [10] with BSA as standard. The PorA content (relative to total protein content) was determined by SDS-PAGE (see below). The presence of LPS was analyzed by gas chromatography (GC). In short, LPS was hydrolyzed in 3.45 M NaOH in 50% (v/v) methanol. Then, the fatty acids were methylated in 5.5 M HCl in 27% (v/v) methanol and extracted with a hexane/methyl *tert*-butyl ether mixture (1/1, v/v). Methylated fatty acids were separated and quantified by GC on an HP Ultra column (25 m × 0.2 mm,

Agilent). The different fatty acids were identified based on their retention time using a calibration mix of known fatty acids (Calmix, Midi, Newark, DE). The amount of C14:3OH fatty acids was calculated with a calibration curve of *Serratia marcescens* LPS. As internal standard, C14:2OH was used. DNA content was determined by an ethidium bromide assay [11] with salmon sperm DNA (ICN, Rotterdam, The Netherlands) as standard.

The water content of freeze-dried OMVs was measured with the Karl–Fisher method using a Mitsubishi Moisture analyzer. Differential scanning calorimetry was performed to determine the glass-transition temperature of the freeze-dried OMVs directly after drying and after 12 months of storage at 4 °C.

2.4. Physical characteristics of OMVs

To check the OMV structure after freeze-drying and storage, electron microscopy was performed as described by Claassen et al. [12]. The particle size of monovalent and trivalent OMVs was measured by dynamic light scattering at 25 °C with a Malvern 4700 system equipped with a 75 mW Argon ion laser (488 nm, Uniphase, San José, CA, USA), a remote interface controller and PCS software, version 1.35 (Malvern Ltd., Malvern, UK). For refractive index and viscosity the values of water were used. The particle size distribution was reflected in the polydispersity index (PD), which ranges from 0.0 for a monodisperse to 1.0 for a heterodisperse dispersion. The zeta potential of the OMVs was measured with a Malvern Zetasizer 2000 with an aqueous dip-in cell and a computer with PCS software (version 1.35, Malvern).

2.5. Fluorescence measurements and guanidine denaturation assay

The local environment of tryptophan (Trp) in PorA was monitored with fluorescence spectroscopy. Steady-state fluorescence measurements were performed with an LS-50B Luminescence spectrophotometer (Perkin-Elmer, Norwalk, CT, USA).

Fluorescence emission spectra after excitation at 295 nm were recorded from 300 to 450 nm at 150 nm/min, with excitation and emission band widths of 2.5 and 5 nm, respectively. Samples contained either 25 µg/ml protein (F91, JB10124) or 20 µg/ml (HP10124). Sets of five spectra were averaged and corrected for buffer background.

Upon incubation with increasing concentrations of guanidinium hydrochloride (Gnd-HCl), PorA in OMVs denatures, causing increased exposure of fluorescent groups (Trp), which results in a shift of the fluorescence maximum towards higher wavelengths [13]. Samples were diluted with Gnd-HCl to final Gnd-HCl concentrations ranging from 1 to 5 M, and incubated for 2 h at room temperature before measurement. Fluorescence spectra of these samples were measured as described above. Sets of five spectra were

averaged and corrected for buffer/Gnd-HCl background. The emission maximum was plotted against the Gnd-HCl concentration. The curves obtained were fitted, assuming a two-state equilibrium between the native and the denatured protein [14]. The concentration of Gnd-HCl necessary to obtain 50% denaturation of PorA was determined based on the midpoint of the denaturation curve.

2.6. Analysis of the denaturation of model compounds

N-Acetyl tryptophan amide (NATrA), *N*-acetyl tyrosine amide (NATyA) were dissolved in 10 mM Tris-HCl, 3% sucrose pH 7.4 plus 0.01% Na azide and incubated at 56 °C. Spectral properties of the samples were monitored during 6 months. UV spectra (200–400 nm) of samples were taken with a Perkin-Elmer spectrophotometer and normalized ($Abs_{280} = 1$). Samples used for fluorescence measurements had an absorbance maximum ≤ 0.1 . Fluorescence emission spectra after excitation at 295 (for tryptophan) and 280 nm (tyrosine) were recorded as described above.

2.7. SDS-PAGE and Western blotting

The trimeric folding of PorA was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 'native' gels as described by Jansen et al. [15]. Prior to electrophoresis, samples were incubated for 10 min at room temperature or 100 °C in sample buffer [16] containing 0.05% SDS. Polyacrylamide gels were prepared as described [16], except that SDS was omitted from the stacking and running gels. The acrylamide concentration of the running gel was 12%. Gels were run at 20 mA in a temperature-controlled room (4 °C) to prevent denaturation of the formulations containing folded PorA. Gels were stained with Coomassie Brilliant Blue.

Relative PorA content was measured by SDS-PAGE under denaturing conditions [16]. Gels were scanned after running and staining in a Agfa Fotolook 3.5 scanner and analyzed with Phoretix 1D Quantifier software (version 5.10, Biozym, Landgraaf, The Netherlands) for determination of the relative PorA content.

Western blotting was performed as described [17] after SDS-PAGE under denaturing conditions. The monoclonal antibodies (mAb) used to detect the different PorA subtypes were MN20B9.34 (anti-P1.4); MN20F4.17 (anti-P1.10); MN20A7.10 (anti-P1.12) and MN3B9F (anti-RmpM).

2.8. PorA inhibition ELISA

Antigenicity of the P1.4 epitope of PorA P1.7-2.4 in OMVs was tested by an inhibition ELISA as previously described using the mAb MN20B9.34 [7]. Briefly, a dilution series of the sample was incubated with a fixed concentration of mAb. Free mAb was quantified with an OMV-coated ELISA plate. The coating OMV batch contained at least 90% PorA, as determined by SDS-PAGE. The results are

shown as the ratio between the PorA concentration determined by ELISA and the protein concentration determined by Peterson (total protein) corrected for protein impurities. As a reference, the antigenicity of freshly produced monovalent OMVs was arbitrarily set at 1.

2.9. Immunization experiments

BALB/c mice, 6–8 weeks old (10 animals in each group) were immunized subcutaneously on days 0, 14 and 28. PorA (1.5 µg/subtype) in OMVs were administered adjuvated with 1.5 mg AlPO₄ (NVI) (0.25 ml per mouse) (see Table 3). The mice were bled on day 42, and sera were collected and stored at 4 °C.

2.10. Anti-PorA whole-cell ELISA

The antibody titer (total IgG) of each individual mouse serum was determined by whole-cell ELISA as described [18]. *N. meningitidis* isogenic H44/76-based strains (B:15P1.7,16:L3,7,9) expressing P1.7-2,4 (H44/76 7-2,4), P1.12,13 (H44/76 12,13) or P1.5-2,10 (H44/76 5-2,10), and the H44/76-derived mutant strain H15 (lacking PorA) were used. Titers were determined with goat anti-mouse (GAM) total IgG conjugates, labeled with horseradish peroxidase (HRP) (Southern Technology Associates; dilution 1:5000). The titer is defined as the dilution of the serum where 50% of the OD_{max} in the assay is reached.

2.11. Serum bactericidal assay

The serum bactericidal activity was measured as previously described [19] against the four *N. meningitidis* strains: H44/76 7-2,4; H44/76 12,13; H44/76 5-2,10 and H15 (PorA[−]). From each group of 10 mice, two pools were made (five mice/pool) and every pool was tested at least twice. Pooled sera were heat inactivated for 30 min at 56 °C prior to use. Serum samples and bacteria were incubated for 10–15 min at room temperature before the addition of complement. A final concentration of 80% (v/v) rabbit

serum was used as complement source. As positive controls, the bactericidal anti-P1.4 (MN20B9.34) and anti-LPS (MN15A17F12) monoclonal antibodies were used. Also, test sera were incubated without complement as a negative control. The serum bactericidal titer was measured as the reciprocal serum dilution showing more than 90% killing of the number of bacteria used.

2.12. Statistical methods

Before statistical analysis, antibody and bactericidal titers were log₁₀ converted. IgG titers are expressed as the mean log₁₀ titer of 10 independent observations. Bactericidal titers are expressed as the mean log₁₀ titer of at least four independent observations. Analysis of variance was used for statistical evaluation of the data. The significance of the differences between the mean values of the antibody and bactericidal titers was determined by the least-significant-difference (LSD) test at a confidence level of 95%.

3. Results

3.1. Characterization of OMVs

The composition of the OMVs derived from the three strains (F91, JB10124 and HP10124) is shown in Table 1. The protein present in all OMVs was mainly PorA. Most impurities were found in OMVs from strain JB10124. OMVs had a negative surface charge (zeta potential) ranging between −40 and −50 mV.

The average particle size of freshly prepared OMVs ranged between 150 and 350 nm, with a rather high polydispersity around 0.4, typical of OMVs (Table 1). Both particle size and polydispersity were smaller for OMVs derived from the trivalent strain JB10124, indicating that these OMVs were less aggregated than the other ones. The presence of partly aggregated vesicles with a diameter around 80 nm for all OMVs was shown by electron microscopy (see Fig. 2A for a representative picture). Both freezing

Table 1
Physico-chemical characteristics of the OMVs used in this study

Strain	PorA ^{a,b} (%)	PorA subtypes	Class 4 protein ^c	LPS content (%) ^b	DNA content (%) ^b	Particle size (nm)	Polydispersity ^d	Zeta potential (mV)
F91 (monovalent)	94	P1.7-2,4	−	9.4	7.7	362	0.42	−49
JB10124 (trivalent)	76	P1.7-2,4 P1.12,13 P1.5-2,10	−	10.6	19.2	138	0.22	−42
HP10124 (trivalent)	87	P1.7-2,4 P1.12,13 P1.5-2,10	+	3.7	8.2	304	0.45	−48

^aDetermined by scanning the SDS-PAGE profile

^bRelative to the total amount of protein present in the formulation.

^cThe presence of class 4 protein was demonstrated by Western blotting with monoclonal antibody MN3B9F.

^dIndication of the size distribution of the OMVs; ranges from 0.0 for a monodisperse to 1.0 for an entirely heterodisperse dispersion.

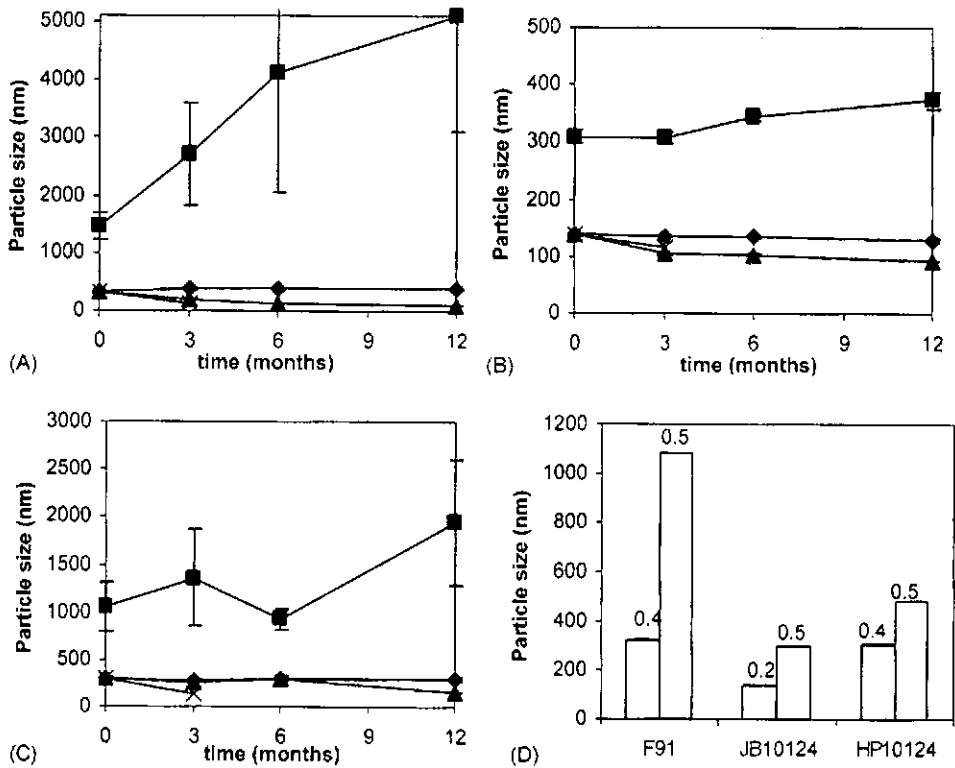


Fig. 1. Effect of storage on the particle size of OMVs of strains F91 (A), JB10124 (B) and HP10124 (C). Frozen samples were stored at -70°C (squares); liquid samples at 4°C (diamonds), 37°C (triangles) or 56°C (cross). Bars indicate S.D. ($n = 3$). (D) Effect of freeze drying on the particle size of OMVs. The bars indicate the particle size $\pm$ S.D. ($n = 3$) of OMVs before (grey) and after (white) freeze-drying and reconstitution after 3 months storage at 4°C . The numbers above the bars indicates the polydispersity of the samples.

and freeze-drying of OMVs induced the formation of large aggregates, accompanied by an increase of the polydispersity index (see Fig. 1A–D). The size of the aggregates was smaller in freeze-dried OMVs than in freeze-thawed OMVs. A representative picture of the aggregates present after freeze-thawing can be observed in Fig. 2B.

The water content of freeze-dried OMVs was below 3% for all formulations. Freeze-dried OMVs showed a glass-transition temperature of ca. 50°C . These characteristics were maintained over 1 year of storage at 4°C .

The fluorescence emission spectrum of PorA was similar in OMVs derived from the three different strains studied. The emission maximum was around 338 nm, indicating a relatively hydrophobic environment of the tryptophan residues [13]. Upon titration with Gnd-HCl, a shift in the fluorescence maximum was observed from ca. 338 nm to ca. 350 nm. This shift was in magnitude similar for OMVs from the three strains. The denaturation midpoint was at 3.8 M Gnd-HCl for monovalent OMVs and slightly higher (4.1 M) for both trivalent OMVs.



Fig. 2. Electron micrographs of OMVs stored at 4°C (strain JB10124 (A)), -70°C (strain F91 (B)) and 56°C (strain HP10124 (C)) during 6 months. The bar indicates $0.2\text{ }\mu\text{m}$. Micrographs are representative for all three OMVs stored at the indicated temperature.

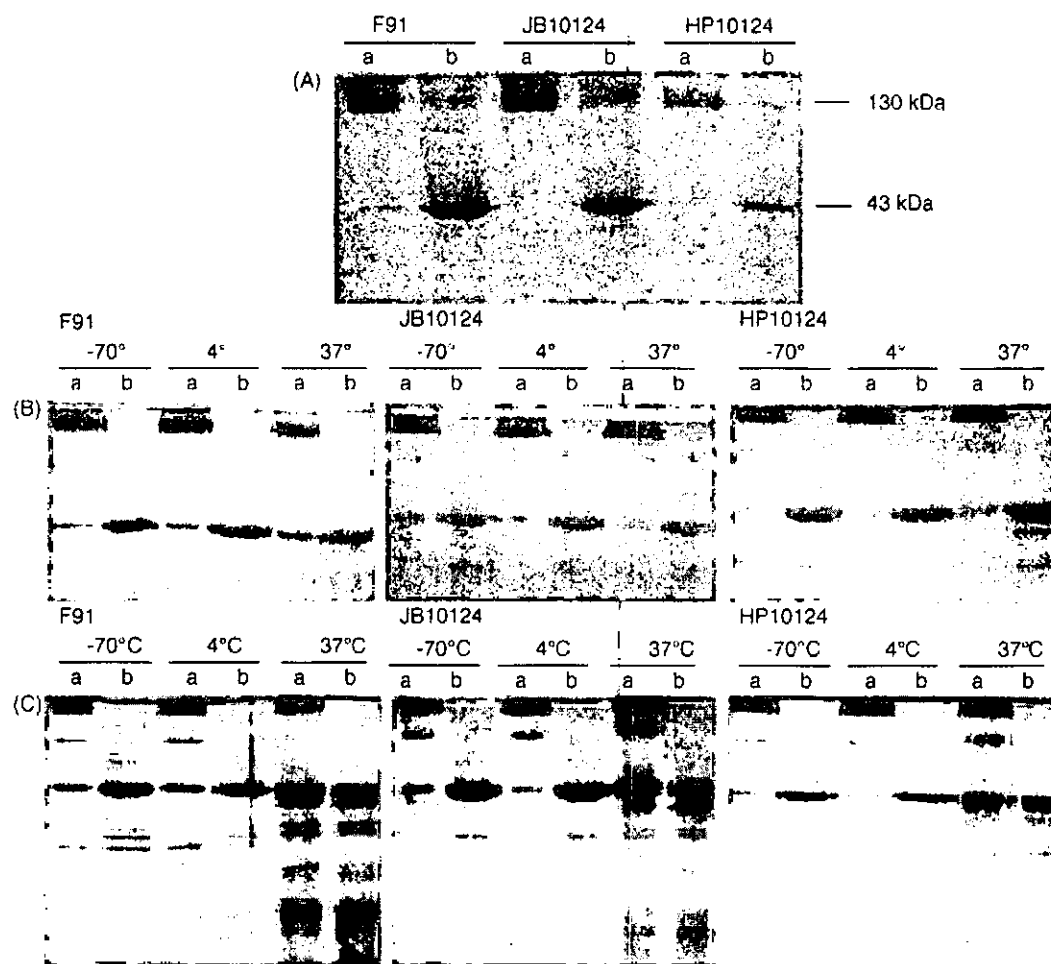


Fig. 3. SDS-PAGE under mild conditions (see Section 2.7) of OMVs after production (A), after 6 months storage (B) or after 12 months storage (C). In (A) is indicated the molecular weight of PorA monomers (43 kDa) and trimers (~130 kDa). The OMV strains and the storage temperature are indicated above the gels. Each sample was mixed with sample buffer containing 0.05% SDS and incubated either at room temperature (lanes a) or at 100 °C (lanes b).

The trimeric structure typical of native PorA was monitored by 'native' SDS-PAGE (Fig. 3) [15]. Freshly prepared PorA-containing OMVs showed the presence of a high molecular weight band (~120 kDa) corresponding to PorA trimers (Fig. 3A, lanes a). No monomers were visible. In samples incubated at 100 °C prior to electrophoresis the trimers were converted to monomers (41 kDa) (Fig. 3A, lanes b). Freeze-drying of OMVs did not induce changes in the electrophoretic characteristics of PorA in the three OMVs studied (not shown).

The interaction of PorA P1.7-2,4 with the bactericidal mAb MN10B9.34 (directed against loop 4 of this PorA subtype) was studied by ELISA (Fig. 4). When assuming that the ratio of the three PorA subtypes in trivalent OMVs is 1:1:1, the antigenicity of PorA P1.7-2,4 in trivalent OMVs is expected to be one third of that of monovalent OMVs. However, the results show that the antigenicity of PorA P1.7-2,4 in trivalent OMVs was only 1/8 and 1/4 for strains JB10124 and HP10124, respectively, as compared with monovalent OMVs. Freeze-drying and reconstitution of OMVs did not

significantly reduce the antigenicity of PorA P1.7-2,4 in any of the OMVs (Fig. 4D).

3.2. Effect of storage on the physico-chemical characteristics of OMVs

During 12 months, the pH of the preparations increased from 7.4 to 8.1 for almost all the formulations studied, except for trivalent OMVs incubated at 56 °C, where the solution acidified, reaching pH values around 6. The zeta potential of OMVs did not change upon storage under different conditions (not shown).

OMVs stored at -70 °C and thawed (at 37 °C, 10 min) showed aggregation, which tended to increase with storage time (Fig. 1), parallel to an increase in the polydispersity index. The size of OMVs in liquid formulations did not change during storage at 4 °C. At higher temperatures (37 and 56 °C), the average particle size of OMVs derived from the three strains gradually decreased (Fig. 1A–C). After 6 months, the particle size and zeta potential of samples

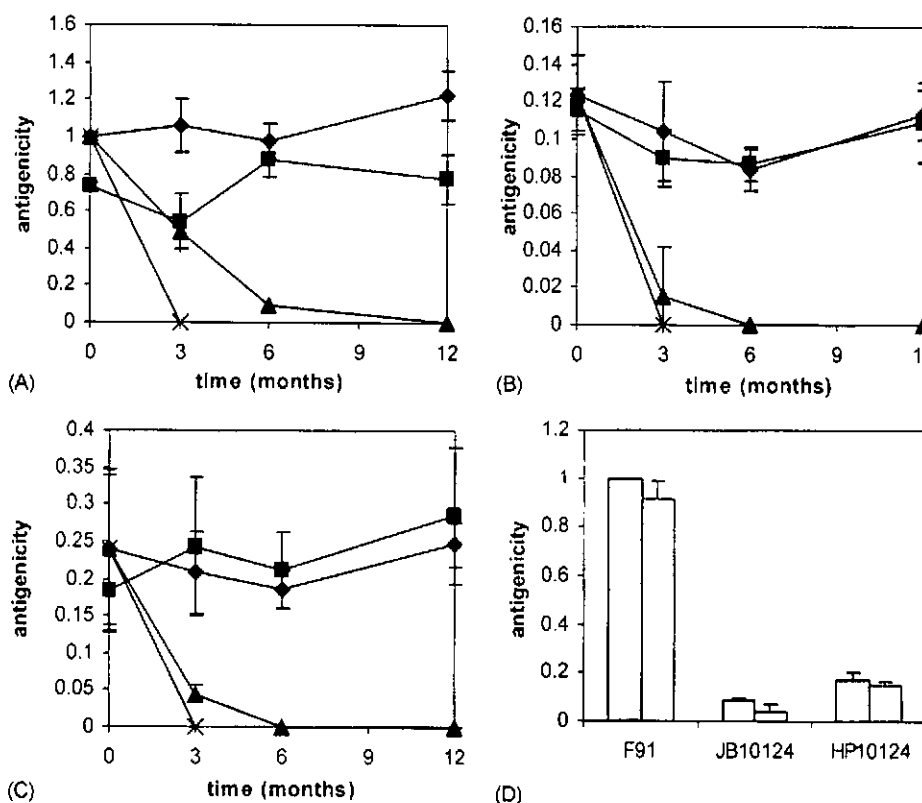


Fig. 4. Effect of storage on the antigenicity of PorA P1.7-2.4 in OMVs of the strains F91 (A), JB10124 (B) and HP10124 (C). Samples were stored at -70°C (squares), 4°C (diamonds), 37°C (triangles) or 56°C (cross). (D) Effect of freeze drying and storage on the antigenicity of PorA P1.7-2.4 in OMVs. The bars indicate the antigenicity of PorA P1.7-2.4 in OMVs before (grey) and after (white) freeze-drying in samples stored for 3 months at 4°C . The results are shown as mean values ($\pm$ S.D.) of at least three measurements.

incubated at 56°C could not be measured anymore, as the scatter intensity had become too low. This indicates total destruction of the OMVs, as confirmed by the presence of amorphous granular material in electron micrographs (Fig. 2C).

The concentration of Gnd-HCl necessary to induce a shift in the fluorescence emission maximum decreased in liquid samples of all three strains were incubated at 37°C , as compared to non-treated OMVs. This indicates a decreased stability of these samples. The red shift of samples stored at 56°C could not be measured, as in the absence of Gnd-HCl they showed a broadened emission spectrum with a maximum above 350 nm after 3 months. After 6 and 12 months totally different spectra were observed: the tryptophan emission peak disappeared and a new spectrum was formed instead with an emission maximum of ca. 405 nm (Fig. 5). In these degraded samples, the fluorescence spectra were similar when measured at an excitation wavelength of 280 or 295 (not shown). The newly formed spectra, which cannot originate from natural aromatic amino acids, indicate the formation of (fluorescent) chemical degradation products due to storage at high temperatures. To study these observations in more detail, the fluorescent amino acids *N*-acetyl tryptophan (NATrA) and *N*-acetyl tyrosamine (NATyA) were used as a model compounds. Solutions of these amino

acids in the same buffer as OMVs were stored for 6 months at 56°C , and their fluorescence (λ_{exc} 295 and 280 nm , respectively) and UV spectra were monitored (Fig. 6). The intensity of the maximum fluorescence signal decreased in all model compounds and a new fluorescence emission peak was formed at higher wavelengths (ca. 405 nm for NATyA, 375 nm for NATrA). As expected (because NATyA does not absorb at 295 nm), when freshly prepared NATyA was

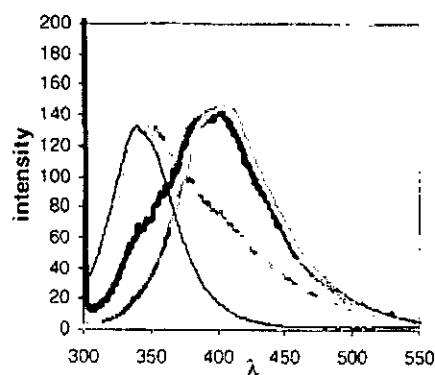


Fig. 5. Fluorescence emission spectra of monovalent OMVs stored at 56°C . Thin black line: before storage; thin gray line: after 3 months storage; thick black line: after 6 months storage; thick gray line: after 12 months storage.

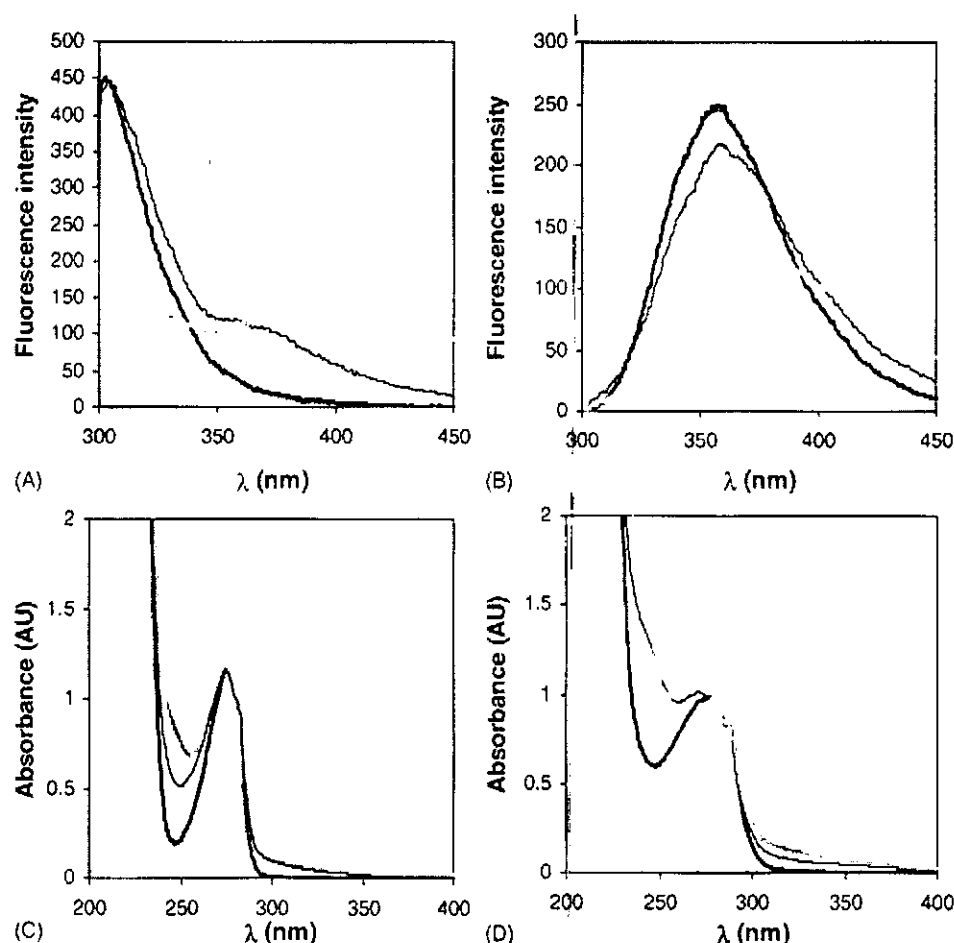


Fig. 6. Fluorescence spectra of NATyA ((A) λ_{exc} 280 nm) and NATrA ((B) λ_{exc} 295 nm) freshly prepared (thick black line) and after 3 (thin black line) and 6 months (thick grey line) incubation at 56 °C. See Section 2 for details. Normalized UV spectra of the NATyA (C) and NATrA (D).

excited at 295 nm, no emission spectra could be observed. After 3 and 6 months of incubation at 56 °C, however, the NATyA sample did absorb at 295 nm (Fig. 6A) and showed an emission spectrum with a maximum around 405 nm upon excitation at 295 nm (not shown), indicating the formation of an innovative fluorescent degradation product.

The trimeric structure of PorA was still present in frozen and liquid OMVs stored at 4 and 37 °C after 3 months (not shown). At this time point no protein bands were observed for samples incubated at 56 °C. Samples incubated at 56 °C were not studied further by SDS-PAGE. After 6 months, part of the trimeric PorA was converted to monomers for all OMVs stored at –70, 4 and 37 °C (Fig. 3B). In trivalent OMVs (JB10124 and HP10124) incubated at 37 °C, extra bands with lower molecular weights appeared below the monomer band (43 kDa) and this band was separated in two bands (Fig. 3B, lanes b). The structural changes of PorA were clearer after 12 months of incubation at all temperatures (Fig. 3C). In native samples (lanes a), trimers and monomers were still observed, and an extra band appeared at ~80 kDa, indicating the presence of PorA dimers. Degradation bands at lower molecular weight were also observed.

This degradation pattern was most apparent for samples incubated at 37 °C, especially for monovalent OMVs, where the intensity of the trimer band had strongly decreased (Fig. 3C, F91). For all samples incubated at 37 °C, a smear could be observed. The presence of P1.4, P1.5-2 and the P1.12 epitopes in degradation bands was checked by Western blotting. No degradation bands were observed for PorA subtypes P1.7-2, 4 and P1.5-2,10. Only in the case of the P1.12,13 subtype one minor degradation band in addition to the original band was observed containing the P1.12 epitope (not shown).

3.3. Effect of storage on the immunochemical characteristics of OMVs

The antigenicity of liquid samples stored at 4 °C was preserved in time. Frozen monovalent OMVs showed a slight decrease in the antigenicity, which was not observed for trivalent samples stored at –70 °C. When OMVs were incubated at 37 °C the antigenicity decreased to (almost) zero within 6 months of incubation. When samples were incubated at 56 °C the antigenicity disappeared within 3 months (Fig. 4A–C).

3.4. Effect of storage on the immunogenicity of OMVs

The ability of monovalent and trivalent OMVs stored at –70, 4, and 37 °C to induce high IgG titers against the different PorA subtypes did not decrease with time (Table 2). A pronounced decrease in the ability to elicit an IgG response was only observed with OMVs stored at 56 °C. Antibodies induced by monovalent OMVs and trivalent OMVs from strain HP10124 (containing RmpM) were mainly PorA-specific, as demonstrated by the low titers against the PorA-lacking strain HI.5. OMVs from strain JB10124 induced the formation of antibodies against other structures than PorA, probably LPS or other impurities (cf. Table 1). The antibody titers against other structures than PorA were fairly constant for samples stored during 12 months at –70 and 4 °C, but the titers were lower in sera of mice immunized with OMVs stored at 37 and 56 °C.

Sera of animals were also tested for the presence of bactericidal antibodies (Table 3). Only antibodies directed against PorA were bactericidal, as determined by the lack of bactericidal serum activity against meningococci of strain HI.5 (not shown). The capability of monovalent OMVs to induce a bactericidal response against the PorA P1.7-2.4 was unchanged in samples stored at –70 or 4 °C, and significantly reduced in OMVs stored at 37 °C for 12 months. OMVs of strain F91 stored for 3 months at 56 °C did not induce a bactericidal response at all. The P1.7-2.4-specific bactericidal activity induced by OMVs from strain JB10124

was weak or absent. OMVs from strain HP10124 induced anti-P1.7-2.4 bactericidal antibodies only in samples stored at –70 °C. OMVs from this strain stored at 4 °C did not induce bactericidal anti-P1.7-2.4 antibodies after 3 or 6 months storage. Surprisingly, after 12 months storage at 4 °C this formulation induced again significant bactericidal antibodies. This is probably due to the variability in the test and the fact the titers against this subtype are low [5]. Although samples incubated at 37 °C induced detectable bactericidal titers after 6 and 12 months, these titers were not significant above background (LSD of 0.4 and 0.6, respectively). The PorA P1.5-2.10 induced the highest bactericidal titers of all three subtypes in both trivalent strains. Also, the anti-P1.5-2.10 bactericidal titers were maintained for OMVs of both strains (JB10124 and HP10124) upon storage at all temperatures except 56 °C, where bactericidal activity of these formulations dropped to undetectable levels after 3 months storage. A similar trend was observed for the P1.12.13-specific bactericidal response. The anti-P1.12.13 bactericidal titers induced by trivalent OMVs after 12 months were relatively high (e.g. ≥2.7); the OMVs from strain HP10124 induced a bactericidal response that was even higher than the one induced by the same OMVs freshly prepared.

Freeze-drying and reconstitution did not affect the immunogenicity of monovalent OMVs and trivalent OMVs of strain JB10124 (without RmpM) (Table 4). Surprisingly, in trivalent OMVs containing RmpM, only PorA subtype P1.5-2.10 preserved the capacity to induce a bactericidal response after freeze-drying.

Table 3
Bactericidal titers induced by OMVs incubated at different temperatures

OMV strain	Storage (°C)	Bactericidal titers ^{a,b}											
		7b,4 ^c				5c,10 ^c				12,13 ^c			
		t = 0	t = 3	t = 6	t = 12	t = 0	t = 3	t = 6	t = 12	t = 0	t = 3	t = 6	t = 12
F91	–70	1.8	2.6	2.1	3.1	– ^d	–	–	–	–	–	–	–
	4	2.5	2.8	2.7	3.1	–	–	–	–	–	–	–	–
	37	2.5	2.9	2.5	1.2	–	–	–	–	–	–	–	–
	56	2.5	<1	–	<1	–	–	–	–	–	–	–	–
JB10124	–70	1.2	<1	1.0	1.6	2.5	2.9	3.3	3.3	2.4	2.5	2.2	3.1
	4	<1	1.2	<1	1.5	3.1	2.5	3.3	3.3	2.2	2.1	1.9	3.1
	37	<1	<1	<1	<1	3.1	3.1	3.3	3.4	2.2	1.5	2.8	2.7
	56	<1	<1	–	<1	3.1	2.2	–	<1	2.2	<1	–	<1
HP10124	–70	2.1	2.1	1.5	2.1	3.0	3.1	3.2	3.4	2.1	1.2	2.8	3.1
	4	2.0	<1	<1	2.7	2.8	3.1	3.0	3.3	1.8	1.6	2.5	2.7
	37	2.0	<1	1.2	1.6	2.8	3.1	3.4	3.3	1.8	1.4	1.6	2.9
	56	2.0	<1	–	<1	2.8	1.8	–	<1	1.8	1.1	–	<1

^a The bactericidal titer was determined as the reciprocal value of the serum dilution that effectuates >90% killing of H44/76-based strains expressing the indicated PorA subtype (see Section 2) and is expressed as the mean log₁₀ titer. Serum of each groups was pooled in two samples. Each pool was tested at least four times. The means were compared by the LSD test with a confidence level of 95%. LSD_{0.05} (7-2, 4_{t=0}) = 0.5; LSD_{0.05} (7-2, 4_{t=3}) = 0.4; LSD_{0.05} (7-2, 4_{t=6}) = 0.4; LSD_{0.05} (7-2, 4_{t=12}) = 0.3; LSD_{0.05} (5-2, 10_{t=0}) = 0.5; LSD_{0.05} (5-2, 10_{t=3}) = 0.4; LSD_{0.05} (5-2, 10_{t=6}) = 0.2; LSD_{0.05} (5-2, 10_{t=12}) = 0.1; LSD_{0.05} (12, 13_{t=0}) = 0.6; LSD_{0.05} (12, 13_{t=3}) = 0.6; LSD_{0.05} (12, 13_{t=6}) = 0.4; LSD_{0.05} (12, 13_{t=12}) = 0.2.

^b Sera were tested for bactericidal activity against the PorB-containing *N. meningitidis* strain HI.5. None of the pools tested induced killing of cells.

^c PorA subtype against which bactericidal titers are specific.

^d n.d.: not determined.

Table 4
Effect of freeze-drying on the immune response induced by OMVs

OMV strain	Storage conditions	IgG titers ^a				Bactericidal titers ^b		
		7-2,4 ^c	5-2,10 ^c	12,13 ^c	HI.5 ^d	7-2,4 ^c	5-2,10 ^c	12,13 ^c
F91	Liquid, 4 °C	3.9	– ^e	–	0.6	2.3	–	–
	F-D ^f , 4 °C	4.2	–	–	0.6	2.2	–	–
JB10124	Liquid, 4 °C	3.8	4.4	4.0	3.6	0.0	2.9	2.8
	F-D, 4 °C	3.9	4.2	4.1	3.3	0.0	2.6	2.8
HP10124	Liquid, 4 °C	3.7	4.4	4.1	1.7	2.0	3.1	2.4
	F-D, 4 °C	3.6	4.0	3.8	1.4	0.9	2.9	0.8

Freeze-dried OMVs were reconstituted after 3 months storage at 4 °C and measured in parallel to liquid OMVs stored under the same conditions.

^aThe total IgG titer of each mouse serum was determined by whole-cell ELISA and is expressed as the mean log₁₀ titer. Plates were coated with *N. meningitidis* strains: H44/76 7-2, H44/76 5-2,10, H44/76 12,13 and HI.5 (see Section 2). The means were compared by the LSD test with a confidence level of 95%: LSD_{0.05} (7-2, 4) = 0.3; LSD_{0.05} (5-2, 10) = 0.4; LSD_{0.05} (12, 13) = 0.3; LSD_{0.05} (HI.5, 4 °C) = 0.9.

^bThe bactericidal titer was determined as the reciprocal value of the serum dilution that effectuates >90% killing of H44/76-based strains expressing the indicated PorA subtype (see Section 2) and is expressed as the mean log₁₀ titer. Serum of each groups was divided in two pools. Each pool was tested at least four times. The means of these tests were compared by the LSD test with a confidence level of 95%: LSD_{0.05} (7-2, 4) = 0.5; LSD_{0.05} (5-2, 10) = 0.4; LSD_{0.05} (12, 13) = 0.8.

^cPorA subtype against which IgG/bactericidal titers are specific.

^dIgG titers specific for other structures than PorA. Sera were tested for bactericidal activity against the strain HI.5. None of the pools tested induced killing of cells.

^en.d.: not determined.

^fFreeze-dried.

4. Discussion

The stability of three types of outer membrane vesicle vaccines from *N. meningitidis* was studied, with a detailed in vitro and in vivo characterization of these OMVs. The preservation of the native PorA conformation as well as the structure of the presentation form were monitored, as both are crucial for the quality of the immune response [7]. PorA is a porin, belonging to a class of membrane proteins that appear to be exceptionally stable [20]. The physical stability of purified recombinant PorA has been previously studied, showing its ability to maintain its trimeric membrane arrangement in the presence of low SDS concentrations [15].

As expected, the physico-chemical characteristics of PorA OMVs were best preserved by storage at 4 °C. However, partial unfolding of PorA was observed in all three OMV formulations stored at this temperature after 12 months (Fig. 3). Although this unfolding did not measurably affect the antigenicity or the protective immunogenicity generated by OMVs, it may be a first step in the degradation leading to decreased immunogenicity of PorA during storage.

Only the incubation of OMVs at high temperatures (56 °C) resulted in the total destruction of both the OMV structure and the protein conformation (DLS, EM, SDS-PAGE, ELISA), accompanied by change of the pH. The modification of the PorA structure was accompanied by a dramatic change in the fluorescence maximum towards higher wavelengths (from 338 to ca. 405 nm), reflecting chemical modifications of PorA, with formation of new fluorescent degradation product(s). This was confirmed using NATrA and NATyA as model compounds. Similar changes in the fluorescence spectrum have been reported for tetanus

toxoid incubated at pH 2.5 and 37 °C after 14–28 days [21] and tetanus and diphtheria toxoids stored at 60 °C for 21 days [22]. The modifications of Trp and Tyr described in literature that lead to fluorescence shift are oxidation of Trp or dimerization of Tyr [23]. Other oxidation reactions (e.g. methionine, histidine) as well as other chemical degradation reactions (e.g. deamidation, chain scission) may also have occurred [24,25], but this was not investigated in this study.

Unfolding of PorA and degradation of the OMV structures was also observed at 37 °C but obviously, much more slowly than at 56 °C. At this temperature, the change in the conformation of PorA could also be observed by SDS-PAGE, with monomerization of PorA (Fig. 3). This was also translated in a drastic drop in antigenicity (Fig. 4). However, a decrease in functional immunogenicity could not be detected, indicating that not only the epitope investigated in our ELISA system is important for the development of a protective immune response. The unfolding of PorA was accompanied by a reduced stability of the OMV structure, as their particle size decreased (Fig. 1).

Freezing or freeze-drying of OMVs resulted in aggregation (Fig. 1). Probably, the freezing step is responsible for this aggregation. During freezing, crystal formation may partially rupture the vesicle structure, resulting in formation of larger vesicles and/or aggregates after thawing or reconstitution. The presence of 3% sucrose that could act as cryoprotectant or lyoprotectant was not enough to prevent this aggregation. However, the conformation of PorA did not change upon freeze-thawing and freeze-drying/reconstitution, which may explain the fact that the immunogenicity of aggregated OMVs was not decreased. Although the functional immunogenicity of monovalent OMVs and

trivalent OMVs from strain JB10124 was maintained when freeze-dried, lyophilized OMVs from strain HP10124 induced a decreased P1.7-2,4- and P1.12.13-specific bactericidal response upon reconstitution. This is unexpected since the amount of RmpM was low compared to the PorA amount. A negative effect of RmpM on the immunogenicity of two of the three subtypes is unlikely. It is assumed that other factors, related to batch or strain differences cause the decreased immunogenicity. This would require production and testing of several batches. No attempts were made to improve the freeze-drying process by adding other lyoprotectants than sucrose.

The trimeric PorA association was more stable in trivalent OMVs than in monovalent OMVs. This is shown by the relatively higher amounts of trimeric PorA in trivalent OMVs incubated at 37 °C (Fig. 3B and C) and the higher concentration of Gnd-HCl necessary to unfold PorA in trivalent OMVs (Fig. 7). As the composition of both trivalent and monovalent OMVs is similar, i.e. most of the protein is PorA, this increased physico-chemical stability of trivalent OMVs could be due to differences in the stability among PorA serosubtypes or differences among strains in the quality of the membrane association of PorA. With regard to this it would be interesting to know the composition of individual trimers in trivalent preparations; are they heterogeneous or are the mixtures of homogeneous trimers? We tried to establish this by 'native' SDS-PAGE but the results were inconclusive (results not shown). Other approaches require separation of intact trimers. This is hard to establish. Detergent solubilization is needed to prepare trimers but there is no way to check for complete separation of trimers. Vesicle fragments or mixed micelles containing several trimers may still exist. Furthermore, subsequent separation of trimers will be difficult. It would require affinity separation with subtype-specific monoclonal antibodies. In the near future attempts will be made to address this problem with mass spectrometry.

It is difficult to correlate physico-chemical characteristics of trivalent and monovalent OMVs with immunogenicity, as

the PorA subtype present in monovalent OMVs (P1.7-2,4) is the least immunogenic subtype in trivalent OMVs [6]. Antigenicity could not help to predict the bactericidal response induced by OMVs stored at high temperatures. Whereas the antigenicity of the P1.4 epitope in all OMVs was decreased or completely absent in OMVs stored at 37 °C, these OMVs were still able to induce polyclonal bactericidal antibodies in mice. Possibly, the immune response was directed against other epitopes of PorA subtype P1.7-2,4 than the epitope recognized by the monoclonal antibody used for the antigenicity test. Also, as previously shown in man [27], the presence of IgG in mice serum was not always accompanied by a bactericidal activity.

The ELISA data (relative to epitope P1.4 of PorA P1.7-2,4) show that the antigenicity of this epitope was lower than expected in trivalent OMVs than in monovalent OMVs. This may be due to a lower P1.7-2,4 expression in trivalent OMVs, or to a decreased accessibility of the P1.4 epitope in these OMVs for the monoclonal antibodies used in the assay. Moreover, the P1.7-2,4-mediated bactericidal serum activity induced by trivalent OMVs was reduced as compared to the bactericidal activity induced by monovalent OMVs. It was previously shown that bactericidal activity induced by the P1.7-2,4 subtype in trivalent OMVs and mixtures of three monovalent OMVs was decreased in both cases [26]. This indicates that the exposure of this PorA subtype is similar in trivalent and monovalent OMVs, but that P1.7-2,4—when simultaneously administered with other subtypes—is less efficiently processed and/or that P1.7-2,4 epitopes generate antibodies with low affinity. From the other PorA subtypes in trivalent OMVs, P1.5-2,10 induced the highest bactericidal serum activity. This subtype was also previously found to induce the highest bactericidal response in toddlers and school children [27]. Even after 3 months incubation at 56 °C, still bactericidal activity was detected against this subtype. This is very surprising, as no PorA could be seen by SDS-PAGE and samples incubated at 56 °C showed no antigenicity. This bactericidal activity could be due to the

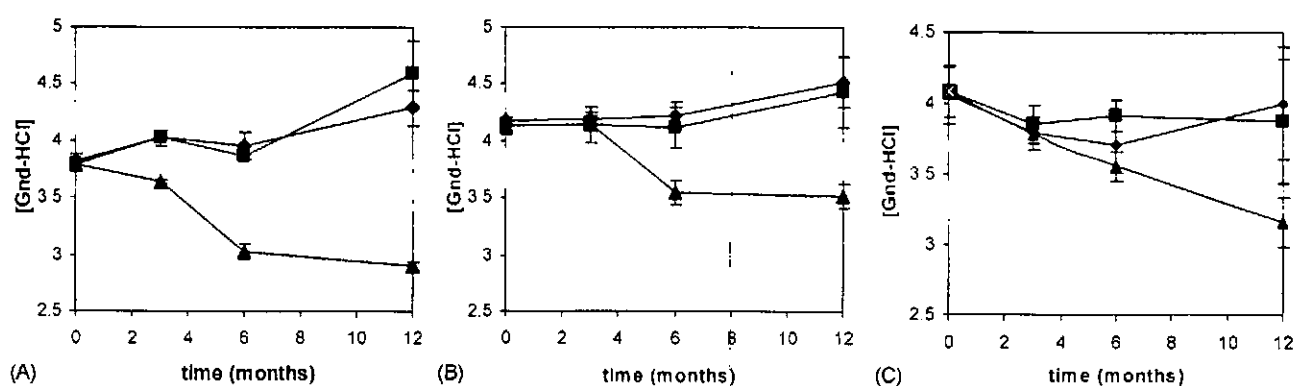


Fig. 7. Effect of time and storage conditions on the concentration of Gnd-HCl needed for 50% red shift of λ_{max} of PorA in OMVs from strain F91 (A), JB10124 (B) and HP10124 (C). Samples were stored at -70 °C (squares), 4 °C (diamonds) or 37 °C (triangles). In samples stored at 56 °C, a shift at 0 M Gnd-HCl comparable to the one obtained in samples at $t = 0$ with 4 M Gnd-HCl was already observed after 3 months storage. The results are shown as mean values ($\pm$ S.D.) of three measurements.

presence of a non-detectable fraction of intact, denatured or fragmented PorA present in the formulations.

The presence of low molecular weight bands in Western blotting, indicating the presence of degradation products containing epitopes located in the variable regions 1 and 2 (VR1 and VR2, respectively) [28], was observed for PorA subtype P1.12,13 in trivalent OMVs stored for 12 months at -70 , 4 or 37°C . The fragmentation of PorA did not affect the immunogenicity of this PorA subtype in trivalent OMVs, probably because the fragmentation was incomplete.

The presence of RmpM in the OMVs from strain HP10124 did not improve their long-term stability. On the contrary, the immunogenicity induced by these OMVs was decreased upon freeze-drying (Table 4). It is unexpected that these OMVs are (even) less stable (after freeze-drying) than trivalent OMVs that do not contain RmpM, as this protein has an important role in the localization and stability of PorA trimers in the outer membrane [15]. The yield (by means of the amount of total protein) of OMVs purified from HP10124 strain (RmpM⁺), was also three-fold lower than the yield in RmpM⁻ strains (not shown), and the amount of RmpM present in OMVs was very low, only detectable by Western blotting. A reason for these relatively low yields may be the strong effect of RmpM on the structure of the bacterial outer membrane. RmpM⁺ bacteria form a well fitted, tight outer membrane, whereas the outer membrane of RmpM⁻ strains has an 'over-sized' appearance [29]. The RmpM seems to influence the attachment of the outer membrane to the underlying peptidoglycan layer. The result is that the OMV yield of the RmpM⁻ strains is higher, at least in the case of detergent-free extraction.

In conclusion, trivalent OMVs appeared slightly more stable than monovalent OMVs, although these differences are not reflected in the in vivo activity. All OMVs are stable for at least 1 year if stored at 4°C or frozen. Freeze-drying provides a good alternative to storage in the liquid state and may be advantageous for long-term (>1 year) storage.

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