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Señor

SUPERINTENDENTE DE INDUSTRIA Y COMERCIO

DIVISIÓN DE NUEVAS CREACIONES

E. _____ S. _____ D. _____

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



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Re: **PI.-GLAXOSMITHKLINE BIOLOGICALS S.A.-Solicitud de Patente de Invención en Fase Nacional PCT para "PROCESO PARA FABRICAR VACUNAS".-Clase A61K 39/116; A61P 31/04.-Expediente número 07-136.721-**

1. Me refiero al auto dictado por ese Despacho notificado por fijación en lista de 1º de Junio de 2012.

2. Con el fin de responder las objeciones expuestas por el señor Examinador en su Concepto Técnico correspondiente al Oficio No. **09226**, con respecto a la Claridad, Concisión y la presencia de materia relacionada con usos y exenta de patentabilidad dentro del capítulo reivindicatorio, así como también con la Novedad y el Nivel Inventivo de la solicitud en estudio, me permito dirigir su atención a las siguientes modificaciones y argumentos técnicos por medio de los cuales se superan completamente dichas objeciones.

3. **CLARIDAD Y CONCISIÓN DEL CAPÍTULO REIVINDICATORIO.**

(i) En relación al capítulo reivindicatorio actualmente presentado, el señor Examinador afirma que las reivindicaciones 1 a 43 no presentan las características técnicas esenciales del método para la preparación de una composición inmunogénica objeto de la presente solicitud, impidiendo así establecer el alcance de la invención que se reclama. Asimismo, señala el señor Examinador que tales reivindicaciones contienen términos y expresiones que restan claridad a la materia reclamada por la solicitud en estudio.

Tomando en consideración estos señalamientos, me permito manifestar que es deseo del solicitante modificar el capítulo reivindicatorio actualmente en estudio con el propósito de definir de manera más adecuada el objeto de la presente invención e identificar con mayor claridad sus características técnicas novedosas.

Respecto a estas modificaciones, me permito resaltar que no implican de ninguna manera una ampliación al alcance de la solicitud, tal como fue originalmente presentada, toda vez que se está restringiendo el alcance de la solicitud de conformidad con las realizaciones preferidas de la invención, como se presenta a continuación:

Reivindicación 1: Corresponde a la reivindicación 1 actualmente presentada en donde para el paso b) de las opciones I), II) y III) se ha cambiado el intervalo de tiempo

de adición de la alícuota a "de 1 minuto a 5 horas" de conformidad con lo revelado en la reivindicación 3 actualmente presentada y en, por ejemplo, desde la línea 23 de la página 7 hasta la línea 7 de la página 8 de la memoria descriptiva actualmente presentada para la solicitud.

Adicionalmente, el método reclamado ha sido restringido especificando dentro del paso b) de las opciones I), II), y III) la manera característica como se realiza la adición de la alícuota del componente final (carbodiimida, sacárido o proteína portadora), mencionando que "uno o más cuartos de la alícuota se agregan durante la primera mitad del periodo, y uno o más cuartos de la alícuota durante la segunda mitad del periodo". Sustento para esta modificación se encuentra en la reivindicación 22 actualmente presentada y en, por ejemplo, las líneas 8 a 23 de la página 8 del texto de la descripción de la solicitud.

Más aún, el método de la reivindicación 1 ha sido restringido a sus realizaciones más preferidas especificando las características técnicas de los reactivos utilizados y las condiciones particulares en las que cada etapa del proceso es llevado a cabo. De manera específica, las características técnicas adicionadas son como se leen a continuación:

"y en donde:

la alícuota de carbodiimida es de 0.01 a 3 mg de carbodiimida/mg de sacárido;

el sacárido está presente en una concentración final de 0.5-50 mg/ml en el paso b);

la proteína portadora está presente en una concentración final de 1-50 mg/ml en el paso b);

la proporción inicial de proteína portadora respecto a sacárido es de 5:1 a 1:5 (p/p);

el pH de la reacción en el paso b) es un pH de 4.5 a 6.5, o un pH de 4.5 a 7.5 si un compuesto está presente en el paso b) el cual mantiene el intermediario de la reacción estable; y

la temperatura de la reacción en el paso b) es de 4-37 °C"

Sustento para esta modificación se encuentra en las reivindicaciones 5, 26, 27, 29, 30, 31 y 32 actualmente presentadas y adicionalmente en las líneas 3 a 13 de la página 13 de la descripción de la solicitud.

De conformidad con estas modificaciones, la totalidad de la materia de las reivindicaciones 26 y 29 actualmente presentadas queda ahora incorporada en la reivindicación 1 modificada.

Adicionalmente, me permito señalar que la expresión "por ejemplo como parte de su unidad de repetición" incluida dentro de la reivindicación 1 actualmente presentada ha sido eliminada de la reivindicación 1 ahora modificada.

Así las cosas, la reivindicación 1 ahora modificada es como se lee a continuación:

"Un método para preparar una composición inmunogénica que comprende conjugar un sacárido a una proteína portadora usando química de condensación de carbodiimida, caracterizado porque el sacárido comprende, o ha sido derivado para que comprenda, grupos amino y/o grupos carboxilo, y caracterizado además porque la proteína portadora comprende, o ha sido derivada para que comprenda, grupos amino y/o grupos carboxilo, el método comprende las etapas de:

I) - si la proteína portadora comprende grupos amino y carboxilo y el sacárido comprende cualquier grupo amino o carboxilo:

a) mezclar el sacárido y la alícuota de carbodiimida requerida para realizar la conjugación, y

b) agregar la alícuota de proteína portadora requerida por un periodo de 1 minuto a 5 horas, en donde uno o más cuartos de la alícuota se agregan durante la primera mitad del periodo, y uno o más cuartos de la alícuota durante la segunda mitad del periodo;

II) - si el sacárido comprende grupos amino y carboxilo y la proteína portadora comprende cualquier grupo amino o carboxilo:

a) mezclar la proteína portadora y alícuota de carbodiimida requerida para realizar la conjugación, y

b) agregar la alícuota de sacárido requerida por un periodo de 1 minuto a 5 horas, en donde uno o más cuartos de la alícuota se agregan durante la primera mitad del periodo, y uno o más cuartos de la alícuota durante la segunda mitad del periodo;

III) - si el sacárido comprende grupos amino y carboxilo y la proteína portadora comprende grupos amino y carboxilo:

a) mezclar la proteína portadora y el sacárido, y

b) agregar la alícuota de carbodiimida requerida para realizar la conjugación por un periodo de 1 minuto a 5 horas, en donde uno o más cuartos de la alícuota se agregan durante la primera mitad del periodo, y uno o más cuartos de la alícuota durante la segunda mitad del periodo;

y en donde:

la alícuota de carbodiimida es de 0.01 a 3 mg de carbodiimida/mg de sacárido;

el sacárido está presente en una concentración final de 0.5-50 mg/ml en el paso b);

la proteína portadora está presente en una concentración final de 1-50 mg/ml en el paso b);

la proporción inicial de proteína portadora respecto a sacárido es de 5:1 a 1:5 (p/p);

el pH de la reacción en el paso b) es un pH de 4.5 a 6.5, o un pH de 4.5 a 7.5 si un compuesto está presente en el paso b) el cual mantiene el intermediario de la reacción estable; y

la temperatura de la reacción en el paso b) es de 4-37 °C." (subrayado fuera de texto)

Reivindicaciones 2 y 3: Corresponden de manera respectiva a las reivindicaciones 2 y 3 actualmente presentadas las cuales han sido restringidas de conformidad con las realizaciones preferidas ahora reclamadas en la reivindicación 1 modificada.

Reivindicación 4: Corresponde a la reivindicación 4 actualmente presentada, la cual no ha sido modificada.

Reivindicación 5: Corresponde a la reivindicación 5 actualmente presentada, la cual ha sido restringida de conformidad con las realizaciones preferidas ahora reclamadas en la reivindicación 1 modificada.

Reivindicaciones 6 a 9: Corresponden de manera respectiva a las reivindicaciones 6 a 9 actualmente presentadas las cuales no han sido modificadas.

Reivindicación 10: Corresponde a la reivindicación 10 actualmente presentada en la que se ha corregido un error involuntario contenido en su preámbulo el cual hacía referencia a una composición inmunogénica de las reivindicaciones 7 a 9, dicho error tipográfico ha sido corregido y la reivindicación 10 ahora modificada hace coherentemente referencia al método de las reivindicaciones 7 a 9.

Adicionalmente, a fin de dar mayor claridad al capítulo reivindicación se ha especificado que ADH es dihidrazida de ácido adípico. Sustento para esta modificación se encuentra en las líneas 1 a 6 de la página 2 de la memoria descriptiva actualmente presentada para la solicitud.

Reivindicaciones 11 a 14: Corresponden de manera respectiva a las reivindicaciones 11 a 14 actualmente presentadas las cuales no han sido modificadas.

Reivindicación 15: Corresponde a la reivindicación 15 actualmente presentada en la que a fin de dar aún más claridad al capítulo reivindicatorio se ha especificado que CDAP es tetrafluoroborato de 1-ciano-4-dimetilamino piridinio. Sustento para esta modificación se encuentra entre la línea 23 de la página 1 a la línea 3 de la página 2 de la descripción actualmente presentada para la solicitud.

Reivindicaciones 16 a 21: Corresponden de manera respectiva a las reivindicaciones 16 a 21 actualmente presentadas las cuales no han sido modificadas.

Reivindicación 22: Corresponde a la reivindicación 22 actualmente presentada en donde la expresión "al menos una" ha sido sustituida por la expresión "una o más" la cual establece claramente la manera en que la alícuota es adicionada.

Reivindicaciones 23 a 25: Corresponden de manera respectiva a las reivindicaciones 23 a 25 actualmente presentadas las cuales no han sido modificadas.

Reivindicaciones 26, 27 y 28: Corresponden de manera respectiva a las reivindicaciones 27, 28 y 30 actualmente presentadas, en donde las reivindicaciones 27 y 30 actuales han sido restringidas de conformidad con las modificaciones ahora realizadas en la reivindicación 1.

Adicionalmente, dentro de la nueva reivindicación 27 se ha eliminado la expresión "por ejemplo NaCl" contenida en la reivindicación 28 actualmente en estudio, esto a fin de dar mayor claridad al capítulo reivindicatorio.

Por ultimo, se han ajustado las dependencias de estas reivindicaciones de conformidad con la nueva numeración dada.

Reivindicaciones 29 a 41: Corresponden de manera respectiva a las reivindicaciones 31 a 43 actualmente presentadas, en las que se han modificado sus dependencias de conformidad con la nueva numeración ahora dada.

Adicionalmente, dentro de las nuevas reivindicaciones 36, 38 y 39 se han eliminado el término "por ejemplo" con lo cual se restringe de manera clara la materia reclamada por la solicitud a las realizaciones más preferidas de la misma.

En este sentido, me permito señalar que en la nueva reivindicación 36 el sacárido queda restringido a un *"sacárido capsular bacteriano derivado de una bacteria seleccionada de una lista que consiste en N. meningitidis serogrupo A, B, C, W135 o Y, Streptococcus pneumoniae serotipos 1, 2, 3, 4, 5, 6A, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F o 33F, Streptococcus Grupo B, grupo Ia, Ib, II, III, IV, V, VI, o VII, Staphylococcus aureus tipo 5, Staphylococcus aureus tipo 8, Salmonella typhi (sacárido Vi), Vibrio cholerae, o H. influenzae tipo b."*

De manera similar, en la nueva reivindicación 39 el sacárido queda restringido a un sacárido que *"es un lipooligosacárido o lipopolisacárido bacteriano, derivado de una bacteria seleccionada de una lista que consiste en: N. meningitidis, H. influenzae, E. coli, Salmonella o M. catarrhalis."*

Reivindicación 42: Corresponde a la reivindicación 44 actualmente presentada, en donde a fin de restringir la materia reclamada se ha incorporado también la materia contenida en las reivindicaciones 36 y 39 ahora modificadas.

Sumado a lo anterior, me permito señalar que dentro de todo el capítulo reivindicatorio ahora modificado se ha reemplazado el término "vehículo de proteína" por "proteína portadora" a fin de dar mayor claridad a la materia reclamada.

Por ultimo, me permito señalar que las reivindicaciones 45 a 47 actualmente presentadas han sido **eliminadas** del capítulo reivindicatorio ahora modificado.

De esta forma, el capítulo reivindicatorio modificado queda constituido por **42 reivindicaciones** que se refieren de manera clara y concisa a las realizaciones preferidas del método para preparar una composición inmunogénica objeto de la presente invención.

(ii) En vista de lo anterior me permito manifestar que quedan totalmente superadas las objeciones presentadas por el señor Examinador en cuanto a la necesidad de incluir dentro de la reivindicación 1 y sus reivindicaciones dependientes, los elementos específicos que caracterizan el método reclamado como los reactivos y sus proporciones y además las condiciones bajo las cuales se llevan a cabo cada uno de los pasos del procedimiento tales como la temperatura, tiempos de adición, ajuste de pH, etc., toda vez que el capítulo reivindicatorio ahora modificado menciona de manera específica dentro de la reivindicación 1 y sus reivindicaciones dependientes tales características particulares que diferencian del estado del arte al método reclamado como será evidente para la persona versada en la materia de la lectura de dicho capítulo reivindicatorio modificado como se expuso anteriormente.

(iii) Adicionalmente, ese Despacho señala en el Oficio antes mencionado que la reivindicación 1 actualmente presentada carece de claridad y concisión por cuanto:

- La reivindicación menciona que *"el método consiste en conjugar un sacárido a un vehículo de proteína, pero no especifica a que se refiere con el término "conjugar", también menciona que dicho método usa "química de condensación" pero no queda claro a que se refiere con dicha expresión"*.

En respuesta a este señalamiento, de manera respetuosa me permito manifestar que para la persona medianamente versada en la materia los términos "conjugar" y "química de condensación" son completamente claros y evidentes.

En primer lugar, me permito manifestar que tales términos deben ser puestos en el contexto de la presente solicitud, para lo cual es necesario señalar el preámbulo completo de la reivindicación 1 ahora modificada de la solicitud en trámite el cual se lee como sigue:

"Un método para preparar una composición inmunogénica que comprende conjugar un sacárido a una proteína portadora usando química de condensación de carbodiimida, caracterizado porque el sacárido comprende, o ha sido derivado para que comprenda, grupos amino y/o grupos carboxilo, y caracterizado además porque la proteína portadora comprende, o ha sido derivada para que comprenda, grupos amino y/o grupos carboxilo, el método comprende las etapas de:"

En vista de lo anterior, me permito señalar que el término "conjugar" evidentemente hace referencia a una reacción de acoplamiento químico, tal como por ejemplo se señala en las líneas 13 a 16 de la página 2 de la descripción de la solicitud en estudio. De manera particular, la persona versada en la materia comprenderá sin ambigüedad de la atenta lectura del preámbulo de la reivindicación 1 modificada que las reacciones de conjugación que se realizan en la presente solicitud se refieren a reacciones de adición-eliminación nucleofílica en un carbono carboxílico (que puede estar presente en el sacárido o en la proteína portadora) en donde el nucleófilo adicionado claramente es

el grupo amino que puede estar en el sacárido o en la proteína portadora, reacciones conocidas en el estado del arte de manera general como reacciones de adición-eliminación nucleofílica en un carbono acílico o reacciones de sustitución nucleofílica acílica. En este sentido, me permito señalar que tales reacciones de conjugación, o de acoplamiento químico, entre un grupo carboxilo y un nucleófilo (como un grupo amino para el caso de la presente solicitud) son bien conocidas en el estado del arte tal como se muestra en, por ejemplo, el documento de Graham Solomon y Craig Fryhle C. B¹ el cual comprende los conocimientos básicos de química orgánica que comúnmente posee la persona versada en la materia. Dicho documento muestra de manera general en sus páginas 822 a 824 como se llevan a cabo las reacciones de conjugación de compuestos químicos por medio de adición-eliminación nucleofílica en un carbono acílico, lo que produce el acoplamiento o "conjugación" de compuestos químicos. De manera aún más particular, dicho documento también muestra en sus páginas 838 a 840 como se llevan a cabo las reacciones de conjugación de un ácido carboxílico y una amina para dar lugar a una amida (como es el caso de la presente solicitud) a través de adición-eliminación nucleofílica en un carbono acílico, de las cuales se hablará a continuación.

De manera similar, con relación al término "química de condensación" me permito señalar que para contextualizar dicho término es necesario hacer referencia a la expresión "química de condensación de carbodiimida" contenida en el preámbulo de la reivindicación 1 ahora modificada. Ahora bien, para la persona versada en la materia será evidente de la lectura de dicho preámbulo de la reivindicación 1 que el término "química de condensación" hace referencia una vez mas a reacciones de adición-eliminación nucleofílica en un carbono carboxílico (que puede estar presente en el sacárido o en la proteína portadora) en donde el nucleófilo adicionado es el grupo amino que puede estar en el sacárido o en la proteína portadora, sin embargo, la mención de "química de condensación de carbodiimida" hace referencia de manera clara que tal reacción de adición-eliminación nucleofílica en un carbono acílico se lleva a cabo mediante la intervención de una carbodiimida que, tal como es bien conocido en el estado del arte, promueven la formación de amidas por reacción con un grupo carboxílico de un ácido y su activación para llevar a cabo la adición-eliminación nucleofílica.

Tan es así, que documentos del estado del arte que comprenden los conocimientos básicos de la persona medianamente versada en la materia como el documento de Graham Solomon y Craig Fryhle C. B² (páginas 838 a 840) y el documento de Carey F. A.³ (páginas 1079 a 1082) muestran que es bien conocido el uso de carbodiimidas para realizar **reacciones de condensación entre un ácido carboxílico y una amina**. Para tales efectos me permito mostrar que el documento de Carey muestra en su página 1081 el mecanismo de reacción por medio del cual se lleva a cabo dicho tipo de **reacción de condensación entre un ácido carboxílico y una amina** asistidas por una carbodiimida, como es el caso de la presente solicitud:

¹ Graham Solomon T. W. and Fryhle C. B., "Organic Chemistry", Seventh Edition, Editorial John Wiley and Sons, Inc., 2000, páginas 822 a 824 y 838 a 840.

² Ibid pag 8.

³ Carey F. A. "Organic Chemistry", Fourth Edition, Editorial Mc Graw Hill, 2000, páginas 1079 a 1082.

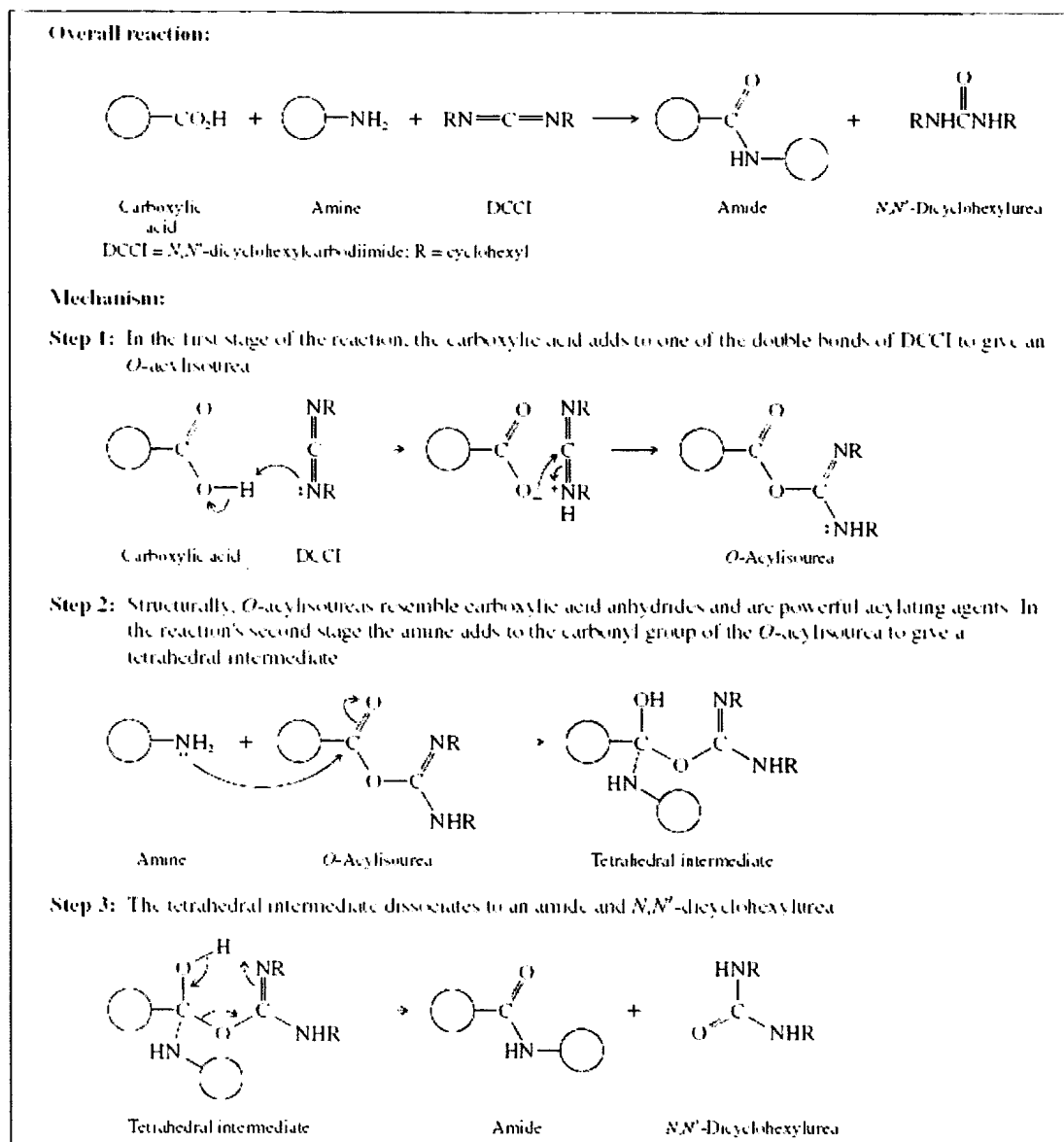


FIGURE 27 13 The mechanism of amide bond formation by *N,N'*-dicyclohexylcarbodiimide-promoted condensation of a carboxylic acid and an amine.

En vista de lo anterior, para la persona versada en la materia será evidente que los términos “conjugar” y “química de condensación” incluidos en la reivindicación 1 modificada de la presente solicitud, son términos totalmente claros que le permiten a la persona versada en la materia establecer sin ambigüedad el tipo de reacciones y procesos químicos que se llevan a cabo en el método reclamado por la presente solicitud, quedando así superadas las objeciones que en este sentido presentó el señor Examinador en su Concepto Técnico antes mencionado.

(iv) Además, el señor Examinador manifiesta que “las expresiones “por ejemplo como parte de su unidad de repetición” (Reiv 1), “por ejemplo” (Reiv. 1, 28, 38, 40 y 41), “exceso grande” (Reiv 13), “velocidad constante” (Reiv. 20), y “al menos” (Reiv 22) le restan claridad al pliego reivindicatorio ya que son términos imprecisos y no permiten limitar el alcance de la invención.”

Acatando este señalamiento, me permito manifestar que las expresiones “por ejemplo como parte de su unidad de repetición” de la reivindicación 1, “por ejemplo” y “al

menos” han sido eliminadas del capítulo reivindicatorio ahora modificado, tal como se expuso anteriormente.

Por otro lado, me permito señalar que el término “exceso grande” de la reivindicación 13 del capítulo reivindicatorio modificado que se adjunta al presente memorial, resultará completamente claro para la persona versada en la materia en vista que en la reivindicación 1 ahora modificada se han especificado las proporciones y concentraciones de sacárido y proteína portadora con lo cual la persona que desee reproducir la invención puede establecer claramente con base en esta información un exceso grande cualquiera del enlazante utilizado para realizar la derivatización mencionada en la reivindicación 13.

Asimismo, el término “velocidad constante” de la reivindicación 20 del capítulo reivindicatorio modificado que se adjunta al presente memorial, resultará completamente claro para la persona versada en la materia en vista que en la reivindicación 1 ahora modificada se han restringido los tiempos del paso b) de las opciones I), II) y III), con lo cual la persona versada en la materia que desee reproducir la presente invención podrá establecer fácilmente (en función del tiempo de adición que desee utilizar) una velocidad constante que permita la adición del sacárido, la proteína portadora o la carbodiimida en el tiempo establecido.

Así las cosas, es completamente claro que los términos “exceso grande” de la reivindicación 13 y velocidad constante” de la reivindicación 20 del capítulo reivindicatorio modificado que se adjunta al presente memorial son completamente claros para la persona medianamente versada en la materia que desee reproducir la presente invención, en función de las limitaciones y características técnicas ahora incluidas en la reivindicación 1 modificada.

(v) Con relación al señalamiento que hace el señor Examinador en cuanto a la necesidad de clarificar el por qué se menciona dentro de la reivindicación 1 que la proteína portadora comprende o ha sido derivatizada para comprender grupos amino y/o grupos carboxilo, me permito señalar que la mención a que la proteína pueda ser derivatizada para comprender grupos amino y/o grupos carboxilo hace referencia a una realización preferida de la invención en donde la proteína portadora puede ser derivatizada para comprender grupos amino y/o grupos carboxilo adicionales que están presentes en un enlazador que se une a la proteína para posteriormente realizar un acoplamiento indirecto de la proteína portadora y el sacárido por medio de dichos grupos funcionales del enlazante unido a la proteína. Tal realización preferida de la invención se muestra por ejemplo en las páginas 3 y 4 de la descripción de la solicitud, así como en las reivindicaciones 6 a 13 de la solicitud en cuestión.

(vi) Por otro lado, con relación al término “vehículo de proteína” me permito señalar que este término al igual que el término “proteína portadora” son términos

intercambiables entre sí, que son comúnmente usados por la persona versada en la materia en el campo de producción de composiciones inmunogénicas.

En conexión con lo anterior, me permito señalar que tales términos se encuentran referidos indistintamente en inglés como "protein carrier" o "carrier protein" en diversidad de documentos del estado del arte relacionado con la invención, como por ejemplo se encuentra en los documentos de Schneerson R. et al.⁴ y de Dintzis R. Z.⁵

Sin embargo, aún cuando el término "vehículo de proteína" se encuentra en el estado del arte, a fin de dar mayor claridad al capítulo reivindicatorio modificado de la presente solicitud se ha remplazado dicho término por "proteína portadora" (traducción al español de carrier protein) el cual se encuentra ampliamente usado en el estado del arte.

Así las cosas, la objeción presentada por el señor Examinador a este respecto queda totalmente superada en vista de las modificaciones anteriormente expuestas para el capítulo reivindicatorio.

(vii) Aunado a lo anterior, el señor Examinador afirma que:

- En la reivindicación 1 no se está definiendo el sacárido, la proteína y la carbodiimida a utilizar.
- En las reivindicaciones 1 a 3 la definición del intervalo de tiempo en la etapa b) le resta concisión a la materia allí reclamada, toda vez que serían rangos inespecíficos.
- En la reivindicación 4 se sugiere especificar cuál otra carbodiimida puede usarse en el método.
- La reivindicación 5 carece de concisión debido a que los rangos de volumen presentados serían poco específicos.
- En la reivindicación 7 se sugiere especificar el enlazante hetero u homofuncional.
- Las reivindicaciones 20 a 34 actualmente presentadas mencionan diferentes rangos que en general serían muy inespecíficos como: 4-100 etapas (Reiv 23), 0.5-50 mg/ml concentración de sacárido (Reiv 26), concentración de NaCl (Reiv 28), concentración de la proteína (Reiv 29), pH y temperatura de la reacción en el paso b) (Reiv. 30-32, 34) y tiempo de reacción (Reiv. 33).

⁴ Schneerson R. et. al. "Quantitative and Qualitative Analyses of Serum Antibodies Elicited in Adults by *Haemophilus influenzae* Type b and *Pneumococcus* Type 6A Capsular Polysaccharide-Tetanus Toxoid Conjugates", *Infection and Immunity*, 1986 Vol. 52, No. 2, pp. 519-528.

⁵ Dintzis R. Z., "Rational Design of Conjugate Vaccines", *Pediatric Research* 1992, Vol. 32, No. 4, pp. 376-385.

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En respuesta a estos señalamientos, en primer lugar me permito manifestar con respecto a las reivindicaciones 1 y 4 del capítulo reivindicatorio ahora modificado, que para la persona versada en la materia será evidente que las características técnicas que hacen novedoso e inventivo el método objeto de la solicitud en trámite no son el tipo de sacárido, proteína portadora, carbodiimida o enlazante particularmente usados sino cada una de las etapas y las condiciones particulares de reacción ahora especificadas en la reivindicación 1 para las etapas del método. Por lo tanto, la persona versada en la materia reconocerá que dentro de las reivindicaciones 1 y 4 del capítulo reivindicatorio ahora modificado no se hace necesario especificar los sacáridos, proteínas portadoras ni carbodiimidas usadas en el método, toda vez que como será claro para la persona versada en la materia cualquier método para preparar una composición inmunogénica que comprende conjugar un sacárido con una proteína portadora utilizando química de carbodiimidas se encuentra dentro del alcance de la presente solicitud siempre y cuando incluya las etapas y las condiciones particulares ahora reveladas para el método en la reivindicación 1, e independientemente del sacárido, proteína portadora, carbodiimida o enlazante usado.

Sumado a lo anterior, me permito manifestar que el incluir los sacáridos, proteínas portadoras y carbodiimidas particulares usadas en el método reclamado, así como restringir aún más los intervalos de tiempo, rangos de volumen, pH, temperatura, concentración de compuestos, etc. constituiría una limitación injusta al objeto de la presente solicitud dado que un competidor podría cambiar los reactivos utilizados por otros del mismo tipo o podría variar ligeramente las condiciones de reacción del método y podría usufructuar dicho método que estaría fuera del alcance de la reclamación hecha por la solicitud (si se modificaran las reivindicaciones como lo sugiere el señor Examinador), pero que seguiría proporcionando un método, para preparar una composición inmunogénica que comprende conjugar un sacárido a una proteína portadora usando química de condensación de carbodiimida, obtenido gracias al aporte revelado por esta solicitud.

En vista de lo anterior, es a todas luces evidente que la reivindicación 1 y sus reivindicaciones dependientes 2 a 42 ahora modificadas son suficientemente claras.

(viii) Por otro lado, el señor Examinador indica que la reivindicación 10 actualmente presentada se encabeza mediante una composición inmunogénica de las reivindicaciones 7 a 9 pero estas no están referidas a tal materia sino a un método, razón por la cual sugiere aclarar esta situación y establecer una correcta dependencia.

Acatando esta sugerencia, me permito manifestar que la reivindicación 10 ha sido modificada, tal como se expuso anteriormente, corrigiendo este error involuntario contenido en su preámbulo, el cual hacía referencia a una composición inmunogénica de las reivindicaciones 7 a 9, dicho error tipográfico ha sido corregido y la reivindicación 10 ahora modificada hace referencia coherentemente al método de las reivindicaciones 7 a 9.

(ix) En las reivindicaciones 10 y 15 ahora modificadas se han especificado el nombre químico de los compuestos mencionados por las siglas ADH y CDAP, con lo cual se supera por completo la objeción que en este sentido presentó el señor Examinador en su Concepto Técnico antes mencionado.

(x) En cuanto a las reivindicaciones 7 a 9 y 11 a 19 el señor Examinador sugiere aclarar de ser el caso, si el método de derivación es un paso mas dentro del método para preparar una composición inmunogénica reivindicado en la reivindicación independiente 1.

En respuesta a este señalamiento, me permito manifestar que tal como se señala en las reivindicaciones 1, 7 a 9 y 11 a 19 ahora modificadas de la solicitud es evidente que el paso de derivación, o derivatización, corresponde a una realización alternativa del método de la reivindicación 1 de la invención, en donde el sacárido o la proteína portadora pueden ser derivatizados para comprender grupos amino y/o grupos carboxilo que están presentes en un enlazador que se une a la proteína portadora (o sacárido) para posteriormente realizar un acoplamiento indirecto de la proteína portadora (o sacárido) y el sacárido (o proteína portadora) por medio de dichos grupos funcionales del enlazante, tal como también se enseña por ejemplo en las páginas 3 y 4 de la memoria descriptiva de la presente solicitud.

(xi) El señor Examinador sostiene que los pasos adicionales revelados en las reivindicaciones 35 a 43 actualmente presentadas deben ser incluidos como parte de la reivindicación independiente.

Frente a este señalamiento, me permito manifestar que el incluir dentro de la reivindicación 1 los pasos adicionales revelados en las reivindicaciones 35 a 43 actualmente presentadas (como son por ejemplo la purificación del conjugado (paso c), filtración estéril (paso d), formulación (paso e)) constituiría una restricción inapropiada e incluso injusta de la materia objeto de la presente solicitud, toda vez que un tercero podría utilizar el método de la reivindicación 1 de la presente solicitud sin realizar alguno de los pasos adicionales mencionados por estas reivindicaciones dependientes, lo cual estaría fuera del alcance de la presente solicitud. Sin embargo, dicho método seguiría proporcionando un método para preparar una composición inmunogénica que comprendería conjugar un sacárido a una proteína portadora usando química de condensación de carbodiimida, el cual tendría las mismas características técnicas esenciales del método reclamado por la reivindicación 1 ahora modificada de la solicitud y que habría sido obtenido gracias al aporte revelado por esta solicitud.

Como consecuencia de lo anterior, el capítulo reivindicatorio ahora modificado es totalmente claro y conciso, superando totalmente las objeciones que en este sentido fueron presentadas por el Señor Examinador en su Concepto Técnico antes referenciado, y ajustándose así totalmente a lo establecido por el Artículo 30 de la Decisión Andina 486 de 2000.

4. **PRESENCIA DE MATERIA RELACIONADA CON USOS Y EXENTA DE PATENTABILIDAD DENTRO DEL CAPÍTULO REIVINDICATORIO**

A este respecto me permito señalar que las reivindicaciones 45, 46 y 47 han sido eliminadas del capítulo reivindicatorio ahora modificado, superando las objeciones que en este sentido presento el señor Examinador en su Concepto técnico frente a estas reivindicaciones.

Por lo tanto, el capítulo reivindicatorio ahora modificado solo hace referencia a materia patentable y no hace referencia a usos con lo cual se ajusta completamente a las disposiciones de la Decisión Andina 486 de 2000.

5. **NOVEDAD**

Por otra parte, el Señor Examinador considera que la solicitud en estudio sería anticipada por las enseñanzas de los documentos de Kossaczka Z., et. al., "*Synthesis and Immunological properties of Vi and Di-O-acetyl pectin protein conjugates with adipic acid dihydrazide as the linker*", *Infection and Immunity*, 1997, Vol. 65, No. 6, pp. 2088-2093 (D2) y de Coen Beuvery E., et. al. "Preparation and Immunochemical Characterization of Meningococcal Croup C Polysaccharide-Tetanus Toxoid Conjugates as a New Generation of Vaccines", *Infection and Immunity*, 1983, Vol 40, No. 1, pp. 39-45 (D4).

En relación con estos documentos el Señor Examinador indica que los elementos característicos del método reivindicado ya eran conocidos en el estado del arte y por lo tanto, dicho método no pueden considerarse como nuevo.

Sobre este particular me permito señalar que existen diferencias significativas entre las enseñanzas de los citados documentos y el objeto de la presente invención que hacen que dichos documentos no anticipen la materia reclamada, tal como se discute a continuación.

5.1. **Novedad de la solicitud frente al documento Kossaczka Z., et. al., "*Synthesis and Immunological properties of Vi and Di-O-acetyl pectin protein conjugates with adipic acid dihydrazide as the linker*", *Infection and Immunity*, 1997, Vol. 65, No. 6, pp. 2088-2093 (D2)**

El documento D2 revela un método para producir composiciones inmunogénicas que comprende conjugar un sacárido (*S. typhi* Vi) y una proteína (BSA o rEPA).

De manera particular, el documento D2 revela en la columna izquierda de la página 2089 bajo el título "Conjugates of Vi or OAcP with AH-protein" una realización en la que, como en la opción I) de la reivindicación 1 ahora modificada para la presente solicitud, el paso final del proceso de conjugación del sacárido y la proteína portadora, es la adición de la proteína portadora. De la lectura atenta de dicha realización la persona versada en

la materia reconocerá que la adición de la proteína derivatizada con hidrazida de ácido adípico (proteína-AH) fue adicionada gota a gota.

Sin embargo, para la persona versada en la materia será evidente que **el documento D2 no revela de manera específica, tal como la reivindicación 1 ahora modificada de la presente solicitud, que la adición de la proteína portadora deba realizarse en un intervalo de tiempo que va desde 1 minuto hasta 5 horas, así como tampoco revela de manera específica que dicha adición se realice de manera que uno o más cuartos de la alícuota se añaden durante la primera mitad del periodo, y uno o más cuartos de la alícuota durante la segunda mitad del periodo.** Adicionalmente, en gracia de discusión, me permito señalar que en vista de los pequeños volúmenes utilizados en el documento D2, la adición gota a gota de la proteína tomaría menos de un minuto.

En relación con lo anterior, me permito señalar que dentro del ámbito de las patentes es un hecho aceptado que para afectar la novedad de una solicitud el arte previo debe divulgar de manera explícita todas y cada una de las características técnicas de la invención, tal como por ejemplo lo señalan la Guía para el examen de solicitudes en la Oficina Europea de Patentes⁶:

*"Un documento le quita la novedad a cualquier materia reclamada **derivable de manera directa y sin ambigüedades** a partir de ese documento que incluye todos las características implícitas para una persona versada en el arte el cual está expresamente mencionado en el documento (...)*

La limitación de materia "derivable de manera directa y sin ambigüedades" a partir del documento es importante. Así, cuando se está considerando la novedad, no es correcto interpretar la enseñanzas de un documento como que comprende equivalentes bien conocido que no son revelados en los documentos; esto es un asunto de obviedad".

Adicionalmente, me permito señalar que en el Manual Andino de Patentes, -una guía práctica que no constituye un marco normativo para el estudio y análisis de patentabilidad de una solicitud en trámite, se establece lo siguiente en relación con el requisito de novedad:

*"Para determinar la novedad de la invención, se debe comprobar si existen anticipaciones del estado de la técnica que contengan explícitamente todas las características técnicas esenciales de la invención. El examen de novedad se efectúa comparando elemento por elemento de la invención tal como está definido por las reivindicaciones con los del estado de la técnica."*⁷(Resaltado fuera del texto).

⁶ European Patent Office, "Guidelines for Examination in the European Patent Office" Munich, Abril 2010. Parte C, Capítulo IV-27 y -28, numeral 9.2.

⁷ SECRETARÍA GENERAL DE LA COMUNIDAD ANDINA. Manual para el examen de solicitudes de patentes de invención en las Oficinas de propiedad Industrial de los Países de la Comunidad Andina; Lima: Perú; 2004. Páginas 65 y 66.

Más aún, en este mismo sentido considero pertinente resaltar que para que la novedad de una invención se vea afectada por un documento del estado de la técnica, dicho documento debe revelar de manera idéntica la invención reclamada, tal como es contemplado dentro del "Instructivo Examen De Solicitudes De Patentes de Invención y Modelo de Utilidad"⁸, documento recientemente preparado por la Superintendencia de Industria y Comercio de Colombia el cual pretende reunir los lineamientos básicos con los cuales se evalúan las solicitudes de patente en Colombia, en donde bajo su numeral 2.12 refiriéndose a la novedad de una solicitud señala que:

"- Verificar, según la comparación anterior, si la invención reivindicada es **idéntica** a lo revelado en el estado de la técnica, en cuyo caso se considera que no tiene novedad" (página 62) (resaltado fuera de texto).

Consecuentemente, en el contexto de las patentes es claro que para que un documento afecte la novedad de una solicitud, este debe revelar de manera **explícita y específica** todas y cada una de las características de la invención, interactuando de la misma manera que lo hacen en la solicitud.

En vista de lo anterior, aún cuando el documento D2 revela un método para producir composiciones inmunogénicas que comprende conjugar un sacárido y una proteína con algunas características técnicas comunes a las reveladas en la reivindicación 1 modificada de la solicitud en estudio, es evidente que este documento no revela "explícitamente todas las características técnicas esenciales" de la invención en cuestión, dado que **D2 no revela un método para producir composiciones inmunogénicas que comprenda conjugar un sacárido y una proteína portadora, como el de la presente solicitud, en donde la adición de la proteína portadora se realice en un intervalo de tiempo que va desde 1 minuto hasta 5 horas y en donde uno o más cuartos de la alícuota de proteína portadora se añaden durante la primera mitad del periodo, y uno o más cuartos de la alícuota durante la segunda mitad del periodo**, es evidente que el documento D2 no puede afectar la novedad de la reivindicación 1 ahora modificada de la presente solicitud, ni a ninguna de sus reivindicaciones dependientes, toda vez que lo anterior constituye una característica técnica esencial que diferencia la presente invención del estado del arte comprendido en D2.

Consecuentemente, es a todas luces evidente que la reivindicación 1 ahora modificada de la presente solicitud y sus reivindicaciones dependientes son Novedosas frente a las enseñanzas del documento D2.

5.2. Novedad de la solicitud frente al documento de Coen Beuvery E., et. al. "Preparation and Immunochemical Characterization of Meningococcal Croup C Polysaccharide-Tetanus Toxoid Conjugates as a New Generation of Vaccines", Infection and Immunity, 1983, Vol 40, No. 1, pp. 39-45 (D4).

⁸ http://www.sic.gov.co/recursos_user/documentos/propiedad_industrial/Formatos/PI02-I06.pdf

Por otro lado, el documento D4 divulga un método para la producción de vacunas que comprende el polisacárido de meningococo grupo C y toxoide tetánico.

De manera particular, el documento D4 revela en su página 40, primer párrafo de la columna izquierda, que en dicho método la carbodiimida es el reactivo que se adiciona al final, de manera análoga al paso b) de la opción III) de la reivindicación 1 de la solicitud en estudio.

Sin embargo, **la reivindicación 1 ahora modificada de la solicitud señala que para el paso b) de la opción III) el sacárido debe comprender "tanto grupos amino como grupos carboxilo".** Contrario a esto, la persona versada en la materia reconocerá que el sacárido utilizado en D4 es de N. Meningitidis grupo C (Men C) y tal como se revela en la tabla de las páginas 16 y 17 de la descripción de la presente solicitud, el sacárido Men C no posee un grupo amino (NH₂) natural.

Por lo tanto, **dado que el documento D4 no revela un método para producir composiciones inmunogénicas que comprenda conjugar un sacárido y una proteína portadora, en donde, cuando la carbodiimida es el reactivo que se adiciona al final, el sacárido comprenda tanto grupos amino como grupos carboxilo como es el caso de la opción III) del método reclamado por la presente solicitud,** es evidente que el documento D4 no puede afectar la novedad de la reivindicación 1 ahora modificada de la presente solicitud, ni a ninguna de sus reivindicaciones dependientes, toda vez que lo anterior constituye una característica técnica esencial que diferencia la presente invención del estado del arte comprendido en D4.

En consecuencia, es a todas luces evidente que la reivindicación 1 ahora modificada de la presente solicitud y sus reivindicaciones dependientes son Novedosas frente a las enseñanzas del documento D4.

6. **NIVEL INVENTIVO**

Finalmente, el Señor Examinador objeta la altura inventiva de la solicitud en estudio a la luz de las enseñanzas de los documentos **WO 9918121 A1 (D1)** y Wilkinson J., et. al. "Immune Response to a Mucosally Administered Aflatoxin B₁ Vaccine", Poultry Science, 2003, Vol. 82, pp. 1565-1572 **(D3)**.

Con respecto a estos documentos el Señor Examinador asegura que "la persona del oficio normalmente versada en la materia estaría motivada a realizar la conjugación de una proteína y un sacárido usando condensación por carbodiimidas como se divulga en D1 siguiendo el orden de adición divulgado en D3, y además realizar la adición del ultimo reactivo lentamente, por ejemplo en un lapso de tiempo de 35 segundos a 6 horas, con el fin de propender la formación intermolecular del enlace amida y evitar o minimizar la

formación de enlaces intramoleculares que generan polimerización de la molécula que contiene ambos grupos (amino y carboxilo)⁹, con la expectativa de obtener conjugados con mejores rendimientos y un mejor control en la conjugación".

Sobre este particular me permito señalar que existen diferencias significativas entre los métodos para conjugar una proteína portadora y un sacárido revelados en el arte previo y aquel que hace parte de la presente invención, diferencias que son aún más evidentes en virtud de las modificaciones ahora realizadas en el capítulo reivindicatorio, las cuales hacen que la persona versada en la materia no pudiera de ninguna manera obtener los compuestos reclamados partiendo de las enseñanzas de dichos documentos, tal como me permito exponer a continuación.

6.1 Nivel Inventivo de la solicitud frente al documento **WO 9918121 A1 (D1)**

En primer lugar me permito señalar que el documento D1 revela la preparación de conjugados de sacarido-proteína usando una carbodiimida, en donde en el método revelado para la reacción de conjugación, el componente final (EDAC) es adicionado rápidamente.

Por lo tanto, **D1 se diferencia de la presente solicitud en que en el método allí revelado no se enseña que el componente final adicionado a la reacción de conjugación sea adicionado durante un intervalo de tiempo que va entre 1 minuto hasta 5 horas, como sí se hace en la reivindicación 1 ahora modificada de la solicitud en trámite.**

En conexión con lo anterior, me permito señalar que las ventajas técnicas obtenidas mediante la adición del componente final a la reacción de conjugación durante un intervalo de tiempo que va entre 1 minuto hasta 5 horas son el incremento del rendimiento del sacárido en el conjugado, capacidad del conjugado de ser filtrado de manera estéril, mejor control de la conjugación, reproducibilidad mas fácil y/o la prevención de enlaces cruzados entre porciones (página 5 de la solicitud).

Sumado a esto, otra ventaja obtenida mediante el método objeto de la invención es el **sorprendente e inesperado aumento de la inmunogenicidad del conjugado obtenido**, como se revela en la página 31 de la descripción actualmente presentada para la solicitud en donde se enseña que:

*"También se llevaron a cabo experimentos para añadir el EDAC de una sola vez a la reacción, pero la proporción final de TT/PS (2.771) (p/p) del conjugado fue más baja que la del conjugado obtenido por medio de la reacción en donde se añadió EDAC durante 10 minutos (3.3/1); **adicionalmente, las antigenidades de aTT y aPS fueron ambas***

⁹ Cross-linking reagents Technical Handbook 2005. "En los procesos de acoplamiento, cuando hay exceso del agente reticulante es probable que ocurra la polimerización debido a que las proteínas son moléculas que contienen a la vez grupos amino y carboxilo"

menores que la medida con respecto al conjugado elaborado mediante la reacción en donde se añadió EDAC durante 10 minutos. (resaltado fuera de texto)

Así las cosas, el problema técnico objetivo a ser resuelto es incrementar la inmunogenicidad de la vacuna conjugada.

En este sentido, me permito manifestar que para la persona versada en vacunas, campo al que pertenece la presente invención, no resultaría obvio que la adición del componente final a la reacción de conjugación en un intervalo de tiempo mayor al establecido en el estado del arte produciría el aumento en la inmunogenicidad del conjugado obtenido.

En conexión con lo anterior, me permito manifestar que cuando la persona versada en la materia se ve enfrentada al problema de incrementar la inmunogenicidad de una vacuna de proteína-polisacárido, debe pensar en un gran número de parámetros a modificar a fin de ver un efecto, por ejemplo, el incremento de la dosis de la vacuna de conjugado, el incremento del número de inmunizaciones, el uso de proteínas portadoras alternativas, o la formulación de la vacuna con uno o más adyuvantes.

Por lo tanto, es claro que la persona versada en el campo de vacunas debería considerar un gran número de estrategias para mejorar la inmunogenicidad de una vacuna antes de llegar a pensar en alterar la química de carbodiimida que se usa en la conjugación.

Además, vale la pena resaltar que **para la persona versada en el campo de vacunología no resultaría obvio que el alterar la química de conjugación mediada por carbodiimida, cuyas reacciones y procedimientos experimentales se encuentran bien establecidos en el estado del arte, podría incrementar la inmunogenicidad de la vacuna.**

A este respecto, me permito señalar que en el estado del arte no hay ninguna motivación o enseñanza que discuta la adición del componente final de la reacción de conjugación mediada por carbodiimida, en un periodo de tiempo mayor. Adicionalmente, también vale la pena resaltar que dentro del estado del arte no existe ninguna sugerencia o enseñanza que haga mención a algún problema relacionado con el uso convencional de la química de carbodiimida, ni una sugerencia a modificarla en la manera reclamada en la reivindicación 1 de la solicitud ahora modificada, a fin de incrementar la inmunogenicidad de una vacuna.

Consecuentemente, me permito manifestar que cualquier sugerencia o motivación aparentemente obtenida del estado del arte hacia la modificación de la reacción de conjugación mediada por carbodiimida, solo puede ser obtenida a través de un análisis ex-post facto (o hind-sight) de nivel inventivo, **lo cual es particularmente cierto si consideramos que el estado del arte de la reacción de conjugación mediada por**

carbodiimida para la producción de vacunas ya fue bien establecida y no se percibe ningún problema en la misma.

En este sentido, me permito respetuosamente manifestar a ese Despacho que el señor Examinador a traído soluciones al problema objeto de la presente solicitud a partir de su conocimiento general sin ninguna evidencia clara cuando señala por ejemplo que *"y además realizar la adición del último reactivo lentamente, por ejemplo en un lapso de 35 segundos a 6 horas... con la expectativa de obtener conjugados con mejores rendimientos y un mejor control en la conjugación."* Por supuesto, el parámetro correcto para el análisis de nivel inventivo de una solicitud es preguntarse si teniendo en cuenta el estado del arte la invención es o no obvia para la persona versada en la materia. Para el caso que nos ocupa, tal persona versada en la materia es un científico que se desempeña en el campo de la producción de vacunas conjugadas. En este campo la existencia de la química de carbodiimidas se encuentra bien establecida y ha sido usada para hacer vacunas comerciales sin que se encuentre una sola palabra escrita sobre si esta química de conjugación necesita ser (o si podría ser) mejorada.

A este respecto, me permito dirigir la atención de ese Despacho al documento de Schneerson et al.¹⁰, el cual describe la conjugación de la primer vacuna conjugada de polisacárido-proteína ensayada y probada en ensayos clínicos, comercializada por muchos años y usada como un modelo exitoso a seguir en el campo de las vacunas conjugadas. En este artículo, la vacuna comprende un conjugado de proteína-polisacárido (polisacárido capsular PRP de *Haemophilus influenzae* B y toxoide tetánico) hecho por un **método en el cual los reactivos son mezclados inmediatamente**, tal como se evidencia de las enseñanzas de dicho documento:

"El TT fue enlazado covalentemente a los derivados de hidracida CPS por condensación mediada por carbodiimida, usando 1-etil-3-dimetilaminopropil carbodiimida. La mezcla de reacción contenía 10.0 mg de hidracida de CPS y TT por ml y 0.1 M de 1-etil-3-dimetilaminopropil carbodiimida. La mezcla de reacción fue agitada a pH 5.0, a 4 °C por 3 horas ..." (véase la columna 2 de la página 520).

En vista de lo anterior, **en primer lugar me permito resaltar que entre la publicación en 1986 del documento de Schneerson et al. y la publicación del documento D1 en 2004, el método de conjugación de proteína y polisacárido por química de carbodiimida ha permanecido sin cambios en el campo de las vacunas; por tal razón será evidente que no había ninguna motivación para que la persona versada en este campo cambiara el método de conjugación.**

Adicionalmente, me permito señalar que el método revelado en dicho documento de Schneerson et al. es el modelo para las ahora exitosas vacunas ActHIB® y Hiberix® elaboradas por Sanofi-Pasteur y GlaxoSmithKline respectivamente. **Este hecho pone de manifiesto una vez mas que el estado del arte del método de conjugación de**

¹⁰ Ibid Schneerson R. et. al. pág 12.

proteína portadora y polisacárido por química de carbodiimida ha permanecido sin cambios por mas de 20 años, y habiendo sido objeto de ensayos clínicos, se consideró que la química de conjugación mediada por carbodiimida para vacunas conjugadas de polisacárido y proteína portadora fue optima y por lo tanto el estado del arte a este respecto estaba culminado para la persona versada en la materia.

Lo anterior se enfatiza a partir de las revelaciones de los documentos de Dintzis¹¹ y de Lindberg¹² los cuales se relacionan con el diseño de vacunas:

En el documento de Dintzis se listan en la página 377 los "Problemas en el Diseño de Vacunas Conjugadas", sin embargo, en dicho documento nunca se menciona que la química de acoplamiento debiera ser mejorado. Asimismo, en la página 380 el autor lista los "Parámetros moleculares que Mejoran la Inmunogenicidad", sin embargo, dicho documento tampoco menciona que la química de acoplamiento deba ser mejorada para alcanzar ese resultado.

Por otro lado, el documento de Lindberg menciona en el Resumen que cuatro vacunas conjugadas de Hib fueron introducidas en la década de los 90's (incluyendo las vacunas ActHIB® y Hiberix® antes mencionadas) y su éxito a nivel mundial en la vacunación de niños ha sido magnifico. Además, la química de EDAC existente es mencionada en el párrafo que une las páginas S29 y S30 de dicho documento, sin embargo en ningún lugar de dicho documento se revela que la metodología por la cual se realizan estas reacciones de acoplamiento mediadas por química de carbodiimida podría ser mejorada para hacer esta vacuna aún mejor.

Sumado a esto, si ahora consideramos los enfoques para las vacunas a ser todavía desarrolladas, para pneumococo (página S32, segunda columna, mitad del párrafo) vemos que los fabricantes están usando la misma estrategia integral que para los conjugados de Hib dado que se hace una referencia al documento de Schneerson mencionado anteriormente, **razón por la cual salta a la vista que no había ninguna percepción que la química existente de carbodiimida debería o podría ser mejorada para la siguiente generación de vacunas.** De manera similar, para meningococos (página S33, segunda columna, línea 6 hasta el final) vemos que los glicoconjugados están siendo desarrollados usando los mismos principios que para los glicoconjugados de Hib y de pneumococo.

En resumen, es evidente que la invención descrita en la presente solicitud logra una ventaja técnica significativa por medios aparentemente sencillos, pero la persona versada en la materia reconocerá que las enseñanzas de la invención, sus ventajas técnicas y su simplicidad no pueden ser usadas en un análisis de nivel inventivo. Esto sería un caso

¹¹ Ibid Dintzis R. Z., pág. 12.

¹² Lindberg A. A., "Glycoprotein Conjugate Vaccines", Vaccine, 1999, Vol 17, pp. S28 a S36.

clásico de un análisis ex post facto (o hind-sight) que no es permitido dentro del análisis de nivel inventivo.

En vista de lo anterior, es evidente que para solucionar el problema técnico identificado por la solicitud en estudio, una persona versada en el campo de las vacunas consideraría cambiar primero una gran variedad de parámetros diferentes (como lo sugiere por ejemplo el documento de Dintzis) pero no contemplaría cambiar la metodología de la química de carbodiimida establecida en el estado del arte de vacunas y esto debido a que ningún problema particular ha sido asociado con dicha metodología en el estado del arte.

Así las cosas, **es evidente que la reivindicación 1 del capítulo reivindicatorio ahora modificado, y sus reivindicaciones dependientes, son inventivas frente a las enseñanzas del documento D1 toda vez que el método de la presente solicitud en el que el componente final adicionado a la reacción de conjugación es adicionado en un intervalo de tiempo que va entre 1 minuto hasta 5 horas no es obvio ni evidente a partir de las enseñanzas de D1, tal como se expuso anteriormente, así como tampoco es obvio a partir de las revelaciones de D1 que la inmunogenicidad del conjugado obtenido fuera mejorada por la manera característica del método reclamado de adicionar el componente final a la reacción de conjugación.**

6.2. Nivel Inventivo de la solicitud frente a la combinación de enseñanzas de los Documentos D1 y D3

Ahora bien, a continuación me permito exponer como los documentos D1 y D3 ni aún en combinación lograrían desvirtuar la altura inventiva de la presente solicitud.

Para comenzar, me permito manifestar que el documento D3 no subsana las deficiencias del documento D1 en cuanto a sugerir la manera característica como el método de la solicitud en cuestión adiciona el componente final a la reacción de conjugación y por lo tanto no puede restarle el nivel inventivo a la solicitud en trámite.

De hecho, la persona versada en la materia reconocerá de la atenta lectura del documento D3 que las enseñanzas del mismo no son relevantes para el nivel inventivo de la solicitud en estudio, dado que D3 no menciona la conjugación de un sacárido y una proteína portadora sino que simplemente revela la adición "gota a gota" de una mezcla de alfatoxina B1-carbodiimida a una proteína portadora.

Consecuentemente, la persona versada en la materia no consideraría el documento D3 si pretendiera lograr un método para hacer una composición inmunogénica que comprende **conjugar un sacárido y una proteína portadora** usando química de condensación de carbodiimida, y esto porque sencillamente el documento D3 no hace mención a ningún tipo de conjugación entre un sacárido y una proteína portadora.

Sumado a lo anterior, y en gracia de discusión, me permito manifestar que el documento D3 solo podría contener alguna enseñanza que se dirigiera hacia la materia de las reivindicaciones de la presente solicitud si D3 explícitamente reconociera que en las circunstancias particulares de las opciones I), II), o III) de la reivindicación 1 ahora modificada, la adición "lenta" (tal como es definida en el paso b) de las opciones I), II), y III) de dicha reivindicación 1) del reactivo final fuera benéfica. Sin embargo, D3 no enseña el rango de duración de la adición que se muestra en las etapas b) de la reivindicación 1, y la revelación de adición "gota a gota" en un contexto tan diferente como la conjugación de alfatoxoide B1 no le proporcionaría a la persona versada en la materia algún indicio de las ventajas obtenidas para el conjugado de **sacárido-proteína portadora** por tal manera de realizar la adición del reactivo final.

Por otro lado, me permito señalar atentamente que contrario a lo sostenido por el señor Examinador, **la persona del oficio normalmente versada en la materia aún cuando estuviera motivada a realizar la conjugación de una proteína y un sacárido usando condensación por carbodiimidas como se divulga en D1 siguiendo el orden de adición divulgado en D3, dicha persona no encontraría ninguna motivación para realizar la adición del ultimo reactivo lentamente, por ejemplo en un lapso de tiempo de 1 minuto a 5 horas como lo reclama la reivindicación 1 ahora modificada, toda vez que a partir de ninguno de estos documentos se sugiere tal manera de adición del ultimo reactivo, y máxime cuando a partir de D1 y D3 no se sugiere de forma alguna que estas modificaciones podrían conducir a la obtención de un conjugado de sacárido-proteína portadora con propiedades mejoradas, entre ellas propiedades inmunogénicas mejoradas.**

Dentro de este marco de ideas me permito muy respetuosamente llamar la atención una vez mas a ese Despacho al hecho que el análisis presentado en el referenciado Concepto Técnico estaría enmarcado dentro de lo que en el ámbito de las patentes se conoce como "hindsight", o análisis "Ex post facto", toda vez que conociendo de antemano la invención el señor Examinador está seleccionado partes específicas de las enseñanzas contenidas dentro de los documentos D1 y D3, agrupándolas de una manera particular y suponiendo a partir de sus conocimientos del estado de la técnica una adición lo suficientemente lenta del ultimo reactivo a agregarse en la reacción de conjugación, de manera que permitan construir la materia reclamada por la presente solicitud, características que de ninguna manera podrían ser evidentes para la persona versada en la materia en el momento en el cual se desarrolló la invención.

En este sentido vale la pena resaltar que al tener conocimiento de la invención es de esperarse que dichas selección, agrupación y modificaciones particulares de las enseñanzas de D1 y D3 parezcan obvias, toda vez que ya se sabe de antemano que efectivamente estos elementos en combinación resuelven el problema planteado que es el incrementar la inmunogenicidad de la vacuna conjugada; sin embargo, esta información no era conocida por el inventor en el momento de desarrollar la invención, por lo que no

podría tener certeza de la utilidad de las condiciones particulares de reacción que caracterizan el método objeto de la solicitud, máxime cuando tal como se demostrado anteriormente, la química de conjugación mediada por carbodiimida para vacunas de polisacárido-proteína ha permanecido sin cambios en el campo de las vacunas por mas de 20 años y por lo tanto el estado del arte a este respecto estaba culminado para la persona versada en la materia.

En conexión con lo anterior, me permito referirme al señalamiento que hace el Tribunal de Justicia de la Comunidad Andina con respecto a las definiciones de "obvio" y "evidente" en la evaluación del Nivel Inventivo de una solicitud de patente¹³:

"Resulta pertinente, a los efectos del artículo 4 de la Decisión 344, definir qué se entiende por "obvio" y por "evidente" a fin de determinar qué se debe entender por nivel inventivo.

Lo obvio es aquello que salta a la vista de manera clara y directa; lo "que se encuentra o pone delante de los ojos, muy claro o que no tiene dificultad". Mientras que lo evidente consiste en la "certeza clara y manifiesta de la que no se puede dudar; cierto, claro, patente y sin la menor duda". De forma que algo que resulte obvio no es necesariamente evidente; empero lo que es evidente, es también obvio.

En este orden de ideas, se puede concluir que una invención goza de nivel inventivo cuando a los ojos de un experto medio en el asunto de que se trate, se necesita algo más que la simple aplicación de los conocimientos técnicos en la materia para llegar a ella, es decir, que de conformidad con el estado de la técnica el invento no sea consecuencia clara y directa de dicho estado."

En vista de todo lo anterior, **al no "saltar a la vista de manera clara y directa" a partir de los documentos D1 y D3 un método con específicamente las condiciones particulares de reacción del método que ahora es objeto de la presente invención, de manera particular un método en donde el componente final adicionado a la reacción de conjugación sea adicionado durante un intervalo de tiempo que va entre 1 minuto hasta 5 horas (como se hace en la reivindicación 1 ahora modificada de la solicitud en tramite) y al no tenerse "certeza clara y manifiesta" de que dichas condiciones particulares de reacción le permitirían al método obtener conjugados de sacárido y proteína portadora con propiedades inmunogénicas mejoradas, es claro que los documentos D1 y D3 no pueden de ninguna manera afectar el Nivel Inventivo del método reclamado por la solicitud en estudio.**

7. **CONCLUSIÓN.**

Para finalizar me permito resaltar que es evidente que el capítulo reivindicatorio modificado presentado junto con este memorial es totalmente claro y conciso al reclamar el método objeto de la invención, de acuerdo con las realizaciones preferidas reveladas a lo largo de la descripción de la solicitud, capítulo del que también se ha eliminado aquella

¹³ Tribunal de Justicia de la Comunidad Andina, PROCESO 192-IP-2005

materia exenta de patentabilidad y la relacionada a usos, cumpliendo así a cabalidad las disposiciones legales de la Decisión Andina 486 de 2000.

Por otro lado, en virtud de las modificaciones realizadas al capítulo reivindicatorio y de los argumentos discutidos con respecto a los documentos D2 y D4 se estableció que la materia objeto de la presente solicitud no es anticipada por dichos documentos y por lo tanto es nueva.

Finalmente, en virtud de las modificaciones realizadas al capítulo reivindicatorio y de los argumentos discutidos con respecto a los documentos citados D1 y D3, se estableció de manera incontrovertible que la persona versada en la materia no podría obtener de manera obvia o evidente a partir de las enseñanzas de estos documentos, el método que es objeto de la presente invención y que tampoco podría haber esperado de manera obvia o evidente que las condiciones particulares que caracterizan el método reclamado condujeran a obtener un conjugado de sacárido y proteína portadora con propiedades de inmunogenicidad mejoradas, y por lo tanto es irrefutable el Nivel Inventivo que conlleva la solicitud en estudio.

8. Anexo me permito presentar:

(i) Copia de las páginas **57 a 62** que corresponden al capítulo reivindicatorio modificado, tal como se discutió en el presente memorial, constituido por 42 reivindicaciones;

(ii) Copia del documento Graham Solomon T. W. and Fryhle C. B., "Organic Chemistry", Seventh Edition, Editorial John Wiley and Sons, Inc., 2000, páginas 822 a 824 y 838 a 840.

(iii) Copia del documento Carey F. A. "Organic Chemistry", Fourth Edition, Editorial Mc Graw Hill, 2000, páginas 1079 a 1082.

(iv) Copia del documento Schneerson R. et. al. "*Quantitative and Qualitative Analyses of Serum Antibodies Elicited in Adults bt Haemophilus influenzae Type b and Pneumococcus Type 6A Capsular Polysaccharide-Tetanus Toxoid Conjugates*", Infection and Immunity, 1986 Vol. 52, No. 2, pp. 519-528.

(v) Copia del documento Dintzis R. Z., "*Rational Design of Conjugate Vaccines*", Pediatric Research 1992, Vol. 32, No. 4, pp. 376-385.

(vi) Copia del document Lindberg A. A., "*Glycoprotein Conjugate Vaccines*", Vaccine, 1999, Vol 17, pp. S28 a S36.

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9. En vista de lo anterior, ruego a usted proceder a estudiar nuevamente la solicitud a la luz de las modificaciones y los argumentos presentados y conceder el privilegio de patente solicitado.

Señor Superintendente,



ALICIA LLORED RICAURTE

T. P. 53.215

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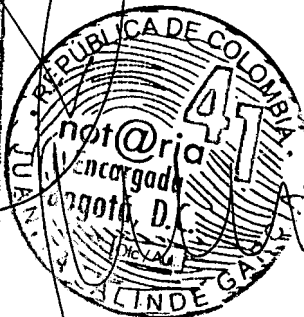
NOTARIA 41 DEL CÍRCULO DE BOGOTÁ D.C.
DEPARTAMENTO DE CUNDINAMARCA
REPÚBLICA DE COLOMBIA

El Notario 41 del círculo de Bogotá da fe de que compareció personalmente Alicia Lloreda Ricaurte identificada (o) con: C.C. 39.690.713 y tarjeta profesional de abogado No. 53215 del Consejo Superior de la Judicatura, quien autentica la firma puesta por ella (el) mismo en este escrito.

(Art. 84 C., P. C. Decreto. 2282/89 mod. 36)

22 AGO 2012
Bogotá ALICIA LLORED RICAURTE
C.C. No. 39.690.713 de Usaquén

FIRMA DE LA (EL) COMPARECIENTE



REIVINDICACIONES

1. Un método para preparar una composición inmunogénica que comprende conjugar un sacárido a una proteína portadora usando química de condensación de carbodiimida, caracterizado porque el sacárido comprende, o ha sido derivado para que comprenda, grupos amino y/o grupos carboxilo, y caracterizado además porque la proteína portadora comprende, o ha sido derivada para que comprenda, grupos amino y/o grupos carboxilo, el método comprende las etapas de:

I) - si la proteína portadora comprende grupos amino y carboxilo y el sacárido comprende cualquier grupo amino o carboxilo:

a) mezclar el sacárido y la alícuota de carbodiimida requerida para realizar la conjugación, y

b) agregar la alícuota de proteína portadora requerida por un periodo de 1 minuto a 5 horas, en donde uno o más cuartos de la alícuota se agregan durante la primera mitad del periodo, y uno o más cuartos de la alícuota durante la segunda mitad del periodo;

II) - si el sacárido comprende grupos amino y carboxilo y la proteína portadora comprende cualquier grupo amino o carboxilo:

a) mezclar la proteína portadora y alícuota de carbodiimida requerida para realizar la conjugación, y

b) agregar la alícuota de sacárido requerida por un periodo de 1 minuto a 5 horas, en donde uno o más cuartos de la alícuota se agregan durante la primera mitad del periodo, y uno o más cuartos de la alícuota durante la segunda mitad del periodo;

III) - si el sacárido comprende grupos amino y carboxilo y la proteína portadora comprende grupos amino y carboxilo:

a) mezclar la proteína portadora y el sacárido, y

b) agregar la alícuota de carbodiimida requerida para realizar la conjugación por un periodo de 1 minuto a 5 horas, en donde uno o más cuartos de la alícuota se agregan durante la primera mitad del periodo, y uno o más cuartos de la alícuota durante la segunda mitad del periodo;

y en donde:

la alícuota de carbodiimida es de 0.01 a 3 mg de carbodiimida/mg de sacárido;

el sacárido está presente en una concentración final de 0.5-50 mg/ml en el paso b);

la proteína portadora está presente en una concentración final de 1-50 mg/ml en el paso b);

la proporción inicial de proteína portadora respecto a sacárido es de 5:1 a 1:5 (p/p);

el pH de la reacción en el paso b) es un pH de 4.5 a 6.5, o un pH de 4.5 a 7.5 si un compuesto está presente en el paso b) el cual mantiene el intermediario de la reacción estable; y

la temperatura de la reacción en el paso b) es de 4-37 °C.

2. El método de la reivindicación 1, caracterizado además porque en el paso b) el periodo es de 1 minuto a 4 horas, de 2 minutos a 3 horas, de 3 minutos a 2 horas, de 4 a 60 minutos, de 5 a 50 minutos, de 6 a 40 minutos, de 7 a 30 minutos o de 8 a 20 minutos.

3. El método de la reivindicación 1, caracterizado además porque en el paso b) el periodo es de 10 minutos a 4 horas, de 20 minutos a 3 horas, de 30 minutos a 2 horas, de 40 a 90 minutos, o de 50 a 70 minutos.

4. El método de las reivindicaciones 1-3, caracterizado además porque la carbodiimida es EDAC (1-etil-3-(3-dimetil-aminopropil)carbodiimida) o una carbodiimida distinta de EDAC.

5. El método de las reivindicaciones 1-4, caracterizado además porque la alícuota de carbodiimida requerida para realizar la conjugación es de 0.05 a 2 o de 0.09 a 1 mg/mg de sacárido.

6. El método de las reivindicaciones 1-5, caracterizado además porque el sacárido y/o proteína portadora ha sido derivado para que contenga grupos amino o carboxilo.

7. El método de la reivindicación 6, caracterizado además porque la derivación se lleva a cabo mediante la adición de un enlazante hetero- u homo-bifuncional.

8. El método de la reivindicación 7, caracterizado además porque el enlazante tiene entre 4 y 12 átomos de carbono.

9. El método de la reivindicación 7 u 8, caracterizado además porque el enlazante tiene dos grupos amino reactivos.

10. El método de las reivindicaciones 7-9 caracterizada además porque el enlazante es dihidrazida de ácido adípico (ADH).

11. El método de la reivindicación 7 u 8, caracterizado además porque el enlazante tiene dos grupos reactivos ácido carboxílico.

12. El método de la reivindicación 7 u 8, caracterizado además porque el enlazante tiene un grupo amino reactivo en un extremo y un grupo reactivo ácido carboxílico en el otro extremo.

13. El método de las reivindicaciones 7-12, caracterizado además porque la derivación tiene lugar haciendo reaccionar un exceso grande de enlazante con el sacárido y/o proteína portadora que va a ser derivada.

14. El método de las reivindicaciones 7-13, caracterizado además porque el sacárido comprende un grupo hidroxilo reactivo como parte de su unidad de repetición, el cual está derivado parcialmente por medio de un grupo amino en el enlazante.

15. El método de la reivindicación 14, caracterizado además porque el sacárido es derivado parcialmente con química de tetrafluoroborato de 1-ciano-4-dimetilamino piridinio CDAP.

16. El método de las reivindicaciones 7-13, caracterizado además porque el sacárido comprende un grupo amino reactivo como parte de su unidad de repetición, el cual está derivado parcialmente por medio de un grupo carboxilo en el enlazante.

17. El método de la reivindicación 16, caracterizado además porque el sacárido es derivado parcialmente con química de condensación por carbodiimida.

18. El método de las reivindicaciones 7-13, caracterizado además porque el sacárido comprende un grupo carboxilo reactivo como parte de su unidad de repetición, el cual está derivado parcialmente por medio de un grupo amino en el enlazante.

19. El método de la reivindicación 18, caracterizado además porque el sacárido es derivado parcialmente con química con carbodiimida.

20. El método de las reivindicaciones 1-19, caracterizado además porque en el paso b) la alícuota de carbodiimida, sacárido o proteína portadora se añade a una velocidad constante usando una bomba.

21. El método de las reivindicaciones 1-19, caracterizado además porque en el paso b) la alícuota de carbodiimida, sacárido o proteína portadora se añade por etapas durante el periodo.

22. El método de la reivindicación 21, caracterizado además porque uno o más cuartos de la alícuota se añaden durante la primera mitad del periodo, y uno o más cuartos de la alícuota durante la segunda mitad del periodo.

23. El método de la reivindicación 21 o 22, caracterizado además porque la alícuota "a" se añade en 4-100 etapas "s".

24. El método de la reivindicación 23, caracterizado además porque a/s de la alícuota se añade en cada etapa.

25. El método de la reivindicación 23 o 24, caracterizado además porque si una etapa tiene lugar en el momento cero del periodo "p", cada etapa subsiguiente tiene lugar en un momento que es $p/(s-1)$.

26. El método de las reivindicaciones 1-25, caracterizado además porque la proporción inicial de proteína portadora respecto a sacárido es desde 4:1 hasta 1:1, o desde 3:1 hasta 2:1 (p/p).

27. El método de las reivindicaciones 1-26, caracterizado además porque la concentración de sal presente en el paso b) es de 0-2, 0.1-1 o 0.2-0.5 M.

28. El método de las reivindicaciones 1-27, caracterizado además porque el pH de la reacción en el paso b) se mantiene en un pH de 4.7-6.0, o 5-5.5.

29. El método de las reivindicaciones 1-27, caracterizado además porque N-hidroxisuccinimida también está presente en la reacción en el paso b), y el pH de la reacción en el paso b) se mantiene en 4.5-7.5.

30. El método de las reivindicaciones 1-29, caracterizado además porque la temperatura de la reacción en el paso b) se mantiene a 10-32, 17-30, o 22-27 °C.

31. El método de las reivindicaciones 1-30, caracterizado además porque después de que la alícuota ha sido añadida toda en el paso b) la reacción se mantiene por otros 10 minutos hasta 72 horas, 20 minutos hasta 48 horas, 30

minutos hasta 24 horas, 40 minutos hasta 12 horas, 50 minutos hasta 6 horas, o 1-3 horas.

32. El método de las reivindicaciones 1-31, caracterizado además porque una vez que está terminada la reacción el pH es ajustado a 7.5-9.

33. El método de las reivindicaciones 1-32, que además comprende un paso subsiguiente c), en donde el conjugado sacárido-proteína es purificado en una columna para cromatografía por exclusión de tamaño.

34. El método de las reivindicaciones 1-33, que además comprende un paso subsiguiente d), caracterizado además porque el conjugado sacárido-proteína es filtrado de manera estéril.

35. El método de las reivindicaciones 1-34, que comprende un paso subsiguiente e), caracterizado además porque una dosis efectiva del conjugado sacárido-proteína se formula con un excipiente aceptable farmacéuticamente para fabricar una composición inmunogénica o vacuna.

36. El método de las reivindicaciones 1-35, caracterizado además porque el sacárido es un sacárido capsular bacteriano, derivado de una bacteria seleccionada de una lista que consiste en: *N. meningitidis* serogrupo A, B, C, W135 o Y, *Streptococcus pneumoniae* serotipos 1, 2, 3, 4, 5, 6A, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F o 33F, *Streptococcus* Grupo B, grupo Ia, Ib, II, III, IV, V, VI, o VII, *Staphylococcus aureus* tipo 5, *Staphylococcus aureus* tipo 8, *Salmonella typhi* (sacárido Vi), *Vibrio cholerae*, o *H. influenzae* tipo b.

37. El método de las reivindicaciones 1-36, caracterizado además porque el peso molecular promedio en peso del sacárido es 1000-2000000, 5000-1000000, 10000-500000, 50000-400000, 75000-300000, o 100000-200000.

38. El método de las reivindicaciones 1-37, caracterizado además porque el sacárido es un polisacárido natural o está dimensionado por un factor de no más de $\times 10$.

39. El método de las reivindicaciones 1-35, caracterizado además porque el sacárido es un lipooligosacárido o lipopolisacárido bacteriano, derivado de una bacteria seleccionada de una lista que consiste en: *N. meningitidis*, *H. influenzae*, *E. coli*, *Salmonella* o *M. catarrhalis*.

40. El método de las reivindicaciones 1-39, caracterizado además porque la proteína portadora comprende uno o más epítomos T auxiliares.

41. El método de las reivindicaciones 1-40, caracterizado además porque la proteína portadora se selecciona del grupo que consiste en: TT, DT, CRM197, fragmento C de TT, proteína D de H. influenzae, PhtD neumocócico, y neumolisina neumocócica.

42. Una composición inmunogénica o vacuna que se puede obtener mediante el método de las reivindicaciones 1-41, caracterizado porque el sacárido es un sacárido capsular bacteriano, derivado de una bacteria seleccionada de una lista que consiste en: N. meningitidis serogrupo A, B, C, W135 o Y, Streptococcus pneumoniae serotipos 1, 2, 3, 4, 5, 6A, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F o 33F, Streptococcus Grupo B, grupo Ia, Ib, II, III, IV, V, VI, o VII, Staphylococcus aureus tipo 5, Staphylococcus aureus tipo 8, Salmonella typhi (sacárido Vi) o Vibrio cholerae, o en donde el sacárido es un lipooligosacárido o lipopolisacárido bacteriano, derivado de una bacteria seleccionada de una lista que consiste en: N. meningitidis, H. influenzae, E. coli, Salmonella o M. catarrhalis, y en donde una dosis efectiva del conjugado sacárido-proteína es formulado con un excipiente farmacéuticamente aceptable.

ORGANIC CHEMISTRY

seventh edition

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Craig B. Fryhle

Pacific Lutheran University



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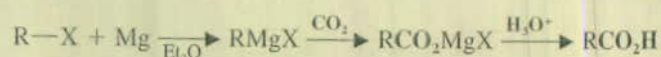
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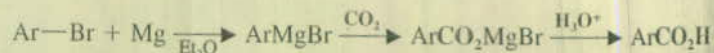
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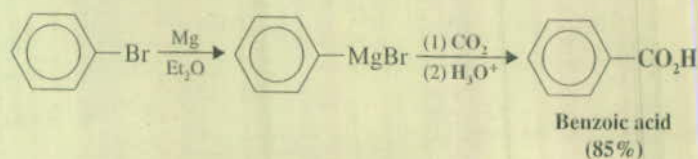
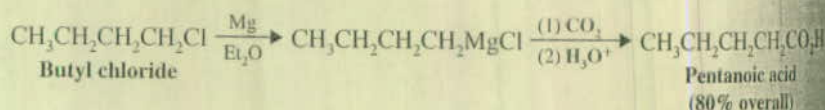
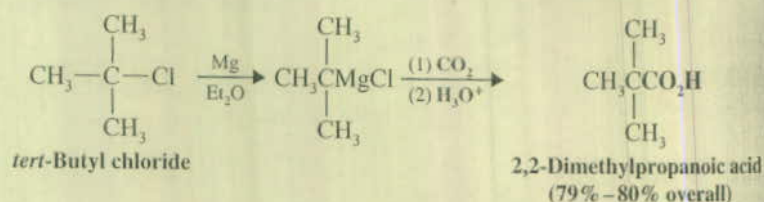
6. **By carbonation of Grignard reagents.** Grignard reagents react with carbon dioxide to yield magnesium carboxylates. Acidification produces carboxylic acids:



or



This synthesis of carboxylic acids is applicable to primary, secondary, tertiary, allyl, benzyl, and aryl halides, provided they have no groups incompatible with a Grignard reaction (see Section 12.8B).



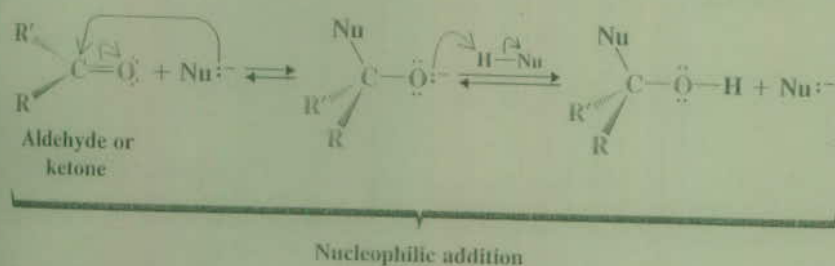
- Problem 18.6** ➤ Show how each of the following compounds could be converted to benzoic acid.
- | | | |
|------------------|----------------------------|--------------------|
| (a) Ethylbenzene | (c) Acetophenone | (e) Benzyl alcohol |
| (b) Bromobenzene | (d) Phenylethene (styrene) | (f) Benzaldehyde |

- Problem 18.7** ➤ Show how you would prepare each of the following carboxylic acids through a Grignard synthesis.
- | | | |
|--------------------------------|--------------------------|-------------------|
| (a) Phenylacetic acid | (c) 3-Butenoic acid | (e) Hexanoic acid |
| (b) 2,2-Dimethylpentanoic acid | (d) 4-Methylbenzoic acid | |

- Problem 18.8** ➤ (a) Which of the carboxylic acids in Problem 18.7 could be prepared by a nitrile synthesis as well? (b) Which synthesis, Grignard or nitrile, would you choose to prepare $HOCH_2CH_2CH_2CH_2CO_2H$ from $HOCH_2CH_2CH_2CH_2Br$? Why?

18.4 NUCLEOPHILIC ADDITION-ELIMINATION AT THE ACYL CARBON

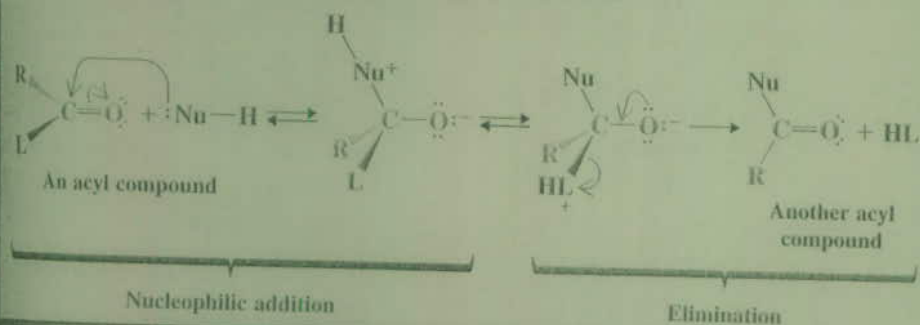
In our study of carbonyl compounds in Chapter 17, we saw that a characteristic reaction of aldehydes and ketones is one of *nucleophilic addition* to the carbon-oxygen double bond.



As we study carboxylic acids and their derivatives in this chapter we shall find that their reactions are characterized by a **nucleophilic addition–elimination** mechanism that takes place at their acyl (carbonyl) carbon atoms. We shall encounter many reactions of the following general type.

A Mechanism for the Reaction

Acyl Transfer by Nucleophilic Addition–Elimination



Many reactions of this type occur in living organisms, and biochemists call them **acyl transfer reactions**. Acetyl-coenzyme A, discussed in Special Topic D, often serves as a biochemical acyl transfer agent.

Although the final results obtained from the reactions of acyl compounds with nucleophiles (substitutions) differ from those obtained from aldehydes and ketones (additions), the two reactions have one characteristic in common. *The initial step in both reactions involves nucleophilic addition at the carbonyl carbon atom.* With both groups of compounds, this initial attack is facilitated by the same factors: the relative steric openness of the carbonyl carbon atom and the ability of the carbonyl oxygen atom to accommodate an electron pair of the carbon–oxygen double bond.

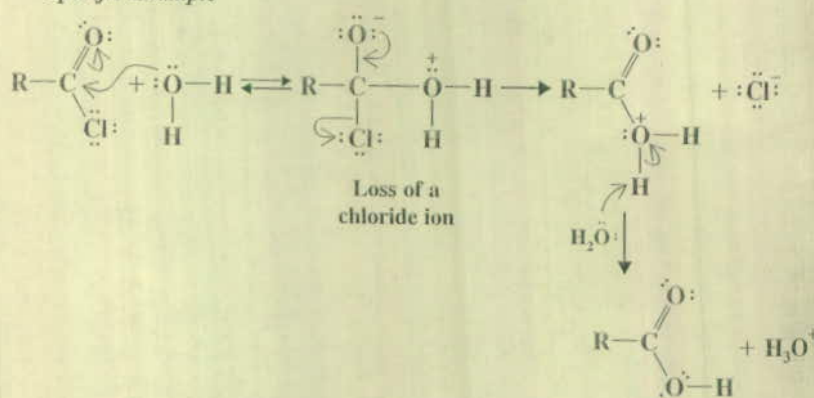
It is after the initial nucleophilic attack has taken place that the two reactions differ. The tetrahedral intermediate formed from an aldehyde or ketone usually accepts a proton to form a stable addition product. In contrast, the intermediate formed from an acyl compound usually **eliminates** a leaving group; this **elimination** leads to regeneration of the carbon–oxygen double bond and to a **substitution product**. The overall process in the case of acyl **substitution** occurs, therefore, by a **nucleophilic addition–elimination** mechanism.

Acyl compounds react as they do because they all have good, or reasonably good, leaving groups (or they can be protonated to have good leaving groups) attached to the carbonyl carbon atom. An acyl chloride, for example, generally reacts by losing a *chloride* ion—a very weak base and thus a very good leaving group. The reaction of an acyl chloride with water is an example.



Although there are many reactions in this chapter, if you bear in mind the general mechanism for acyl transfer you will be able to see the common mechanistic aspects among them.

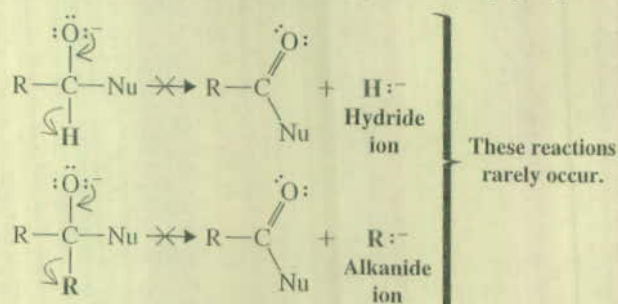
Specific Example



An acid anhydride generally reacts by losing a *carboxylate anion* or a molecule of a *carboxylic acid*—both are weak bases and good leaving groups. We consider the mechanism of acid anhydride hydrolysis in Section 18.6B.

As we shall see later, esters generally undergo nucleophilic addition–elimination by losing a molecule of an *alcohol* (Section 18.7B), acids react by losing a molecule of *water* (Section 18.7A), and amides react by losing a molecule of *ammonia* or of an *amine* (Section 18.8F). All of the molecules lost in these reactions are weak bases and are reasonably good leaving groups.

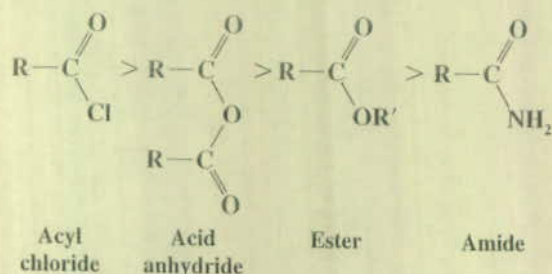
For an aldehyde or ketone to react by nucleophilic addition–elimination, the tetrahedral intermediate would have to eject a hydride ion (H^-) or an alkoxide ion (R^-). Both are *very powerful bases*, and both are therefore *very poor leaving groups*.



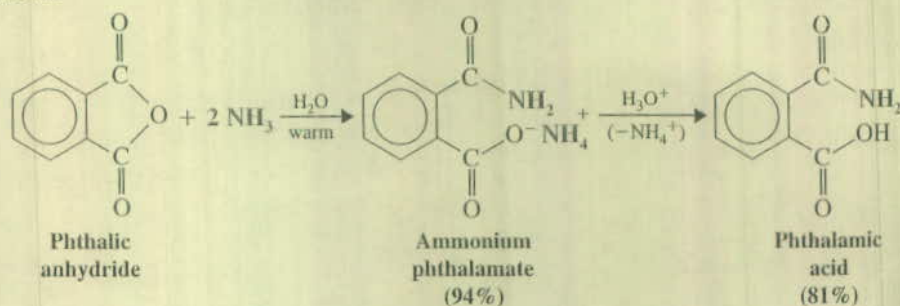
[The haloform reaction (Section 17.3C) is one of the rare instances in which an alkoxide anion can act as a leaving group, but then only because the leaving group is a weakly basic trihalomethyl anion.]

18.4A Relative Reactivity of Acyl Compounds

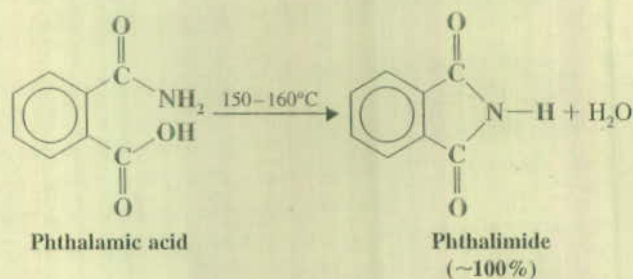
Of the acid derivatives that we study in this chapter, acyl chlorides are the most reactive toward nucleophilic addition–elimination, and amides are the least reactive. In general, the overall order of reactivity is



monium salt. Acidifying the ammonium salt gives a compound that is both an amide and an acid:

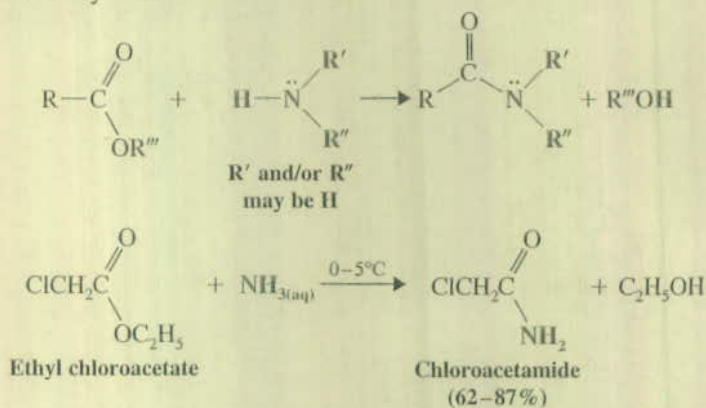


Heating the amide acid causes dehydration to occur and gives an *imide*. Imides contain the linkage $-\text{C}(=\text{O})-\text{NH}-\text{C}(=\text{O})-$.



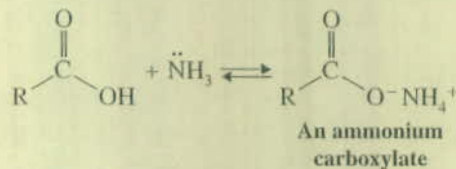
18.8D Amides from Esters

Esters undergo nucleophilic addition-elimination at their acyl carbon atoms when they are treated with ammonia (called *ammonolysis*) or with primary and secondary amines. These reactions take place more slowly than those of acyl chlorides and anhydrides, but they are synthetically useful.

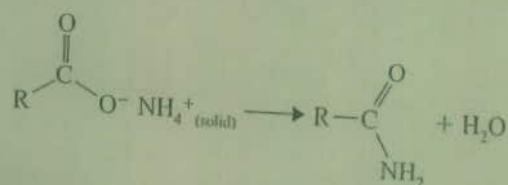


18.8E Amides from Carboxylic Acids and Ammonium Carboxylates

Carboxylic acids react with aqueous ammonia to form ammonium salts.



Because of the low reactivity of the carboxylate ion toward nucleophilic addition-elimination, further reaction does not usually take place in aqueous solution. However, if we evaporate the water and subsequently heat the dry salt, dehydration produces an amide.

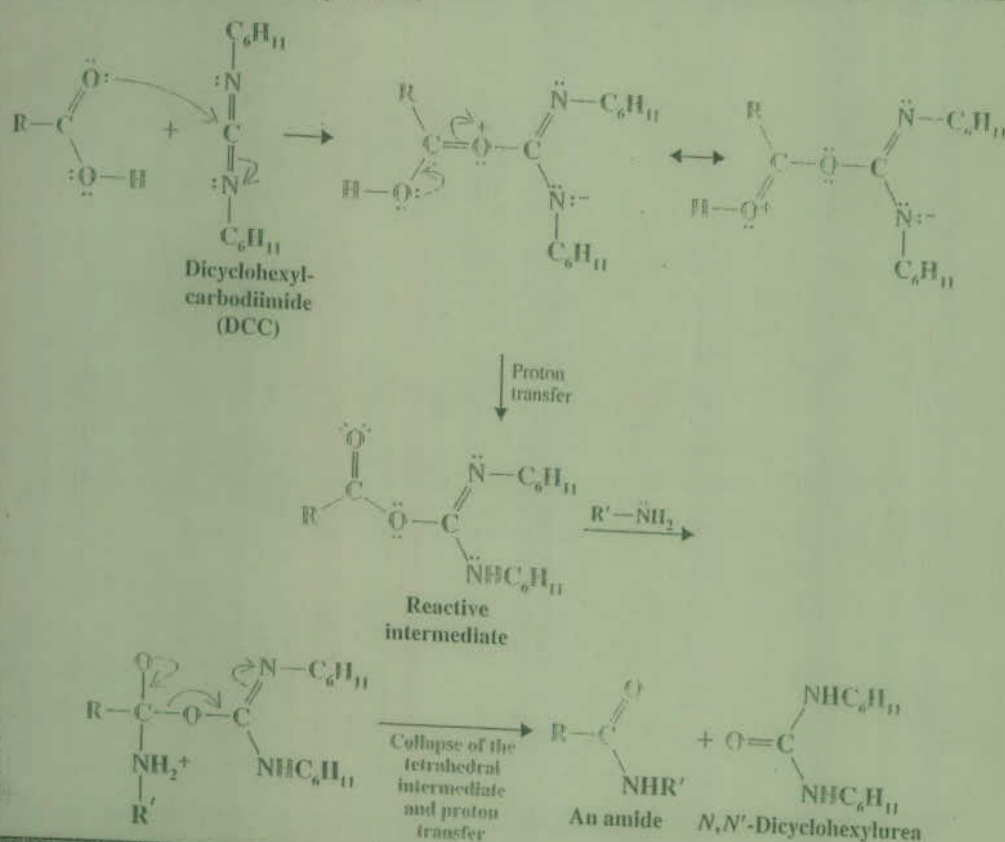


This is generally a poor method for preparing amides. A much better method is to convert the acid to an acyl chloride and then treat the acyl chloride with ammonia or an amine (Section 18.8B).

Amides are of great importance in biochemistry. The linkages that join individual amino acids together to form proteins are primarily amide linkages. As a consequence, much research has been done to find new and mild ways for amide synthesis. One especially useful reagent is the compound dicyclohexylcarbodiimide (DCC), $\text{C}_6\text{H}_{11}-\text{N}=\text{C}=\text{N}-\text{C}_6\text{H}_{11}$. Dicyclohexylcarbodiimide promotes amide formation by reacting with the carboxyl group of an acid and activating it toward nucleophilic addition-elimination.

A Mechanism for the Reaction

DCC-Promoted Amide Synthesis

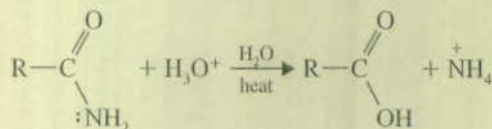


The intermediate in this synthesis does not need to be isolated, and both steps take place at room temperature. Amides are produced in very high yield. In Chapter 24 we shall see how dicyclohexylcarbodiimide can be used in an automated synthesis of peptides.

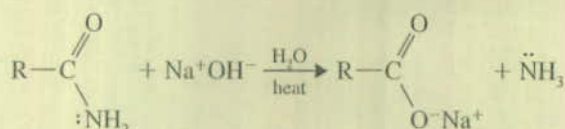
18.8F Hydrolysis of Amides

Amides undergo hydrolysis when they are heated with aqueous acid or aqueous base.

Acidic Hydrolysis



Basic Hydrolysis

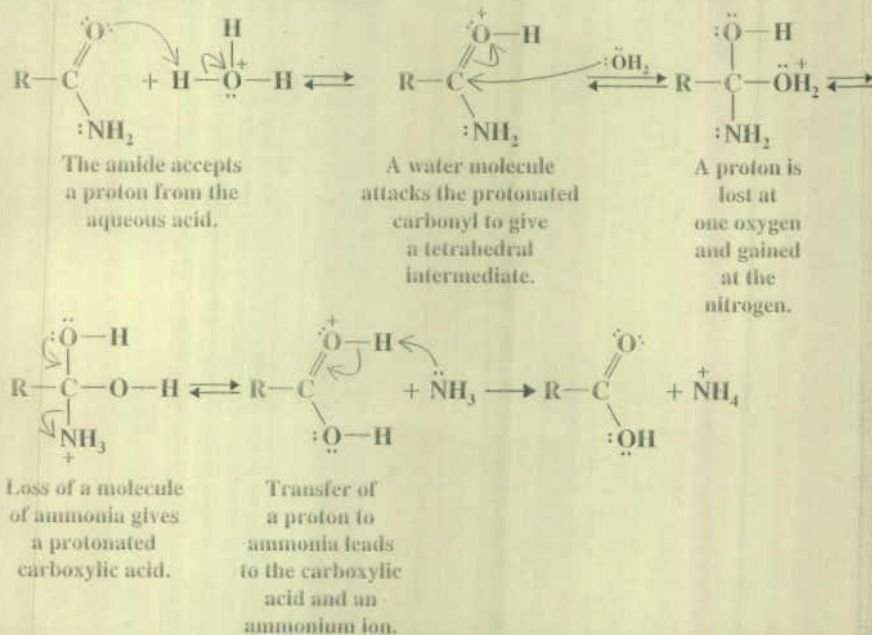


N-Substituted amides and *N,N*-disubstituted amides also undergo hydrolysis in aqueous acid or base. Amide hydrolysis by either method takes place more slowly than the corresponding hydrolysis of an ester. Thus, amide hydrolyses generally require more forcing conditions.

The mechanism for acid hydrolysis of an amide is similar to that given in Section 18.7A for the acid hydrolysis of an ester. Water acts as a nucleophile and attacks the protonated amide. The leaving group in the acidic hydrolysis of an amide is ammonia (or an amine).

A Mechanism for the Reaction

Acidic Hydrolysis of an Amide



GlaxoSmithKline Biologicals SA

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fourth edition

ORGANIC CHEMISTRY

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University of Virginia



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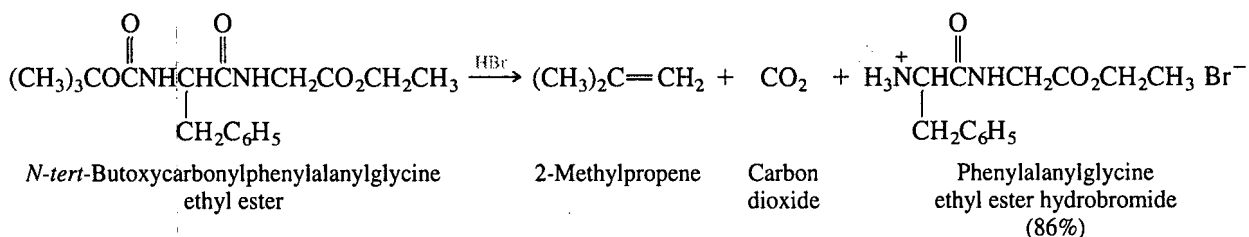
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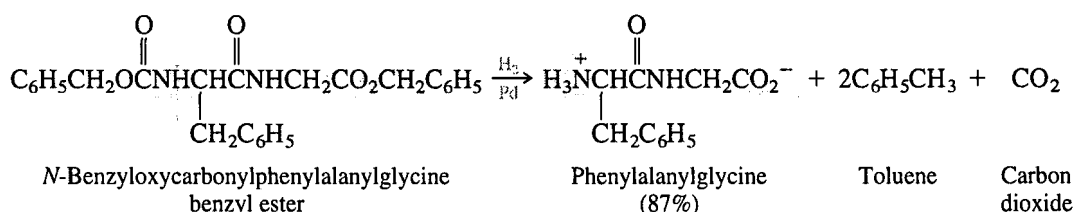


The *tert*-butyl group is cleaved as the corresponding carbocation. Loss of a proton from *tert*-butyl cation converts it to 2-methylpropene. Because of the ease with which a *tert*-butyl group is cleaved as a carbocation, other acidic reagents, such as trifluoroacetic acid, may also be used.

An experiment using Boc protection in the synthesis of a dipeptide can be found in the November 1989 issue of the *Journal of Chemical Education*, pp. 965–967.

27.16 CARBOXYL GROUP PROTECTION

Carboxyl groups of amino acids and peptides are normally protected as esters. Methyl and ethyl esters are prepared by Fischer esterification. Deprotection of methyl and ethyl esters is accomplished by hydrolysis in base. Benzyl esters are a popular choice because they can be removed by hydrogenolysis. Thus a synthetic peptide, protected at both its N terminus with a Z group and at its C terminus as a benzyl ester, can be completely deprotected in a single operation.

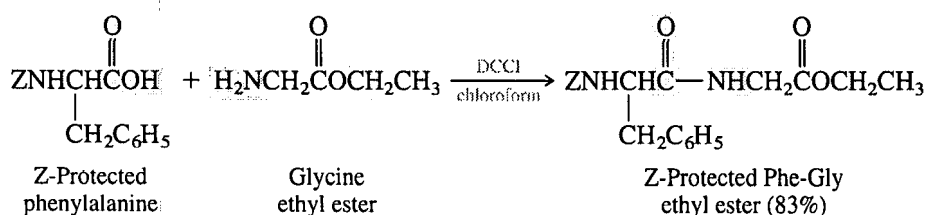


Several of the amino acids listed in Table 27.1 bear side-chain functional groups, which must also be protected during peptide synthesis. In most cases, protecting groups are available that can be removed by hydrogenolysis.

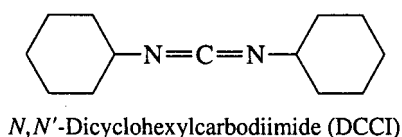
27.17 PEPTIDE BOND FORMATION

To form a peptide bond between two suitably protected amino acids, the free carboxyl group of one of them must be *activated* so that it is a reactive acylating agent. The most familiar acylating agents are acyl chlorides, and they were once extensively used to couple amino acids. Certain drawbacks to this approach, however, led chemists to seek alternative methods.

In one method, treatment of a solution containing the N-protected and the C-protected amino acids with *N,N'*-dicyclohexylcarbodiimide (DCCI) leads directly to peptide bond formation:



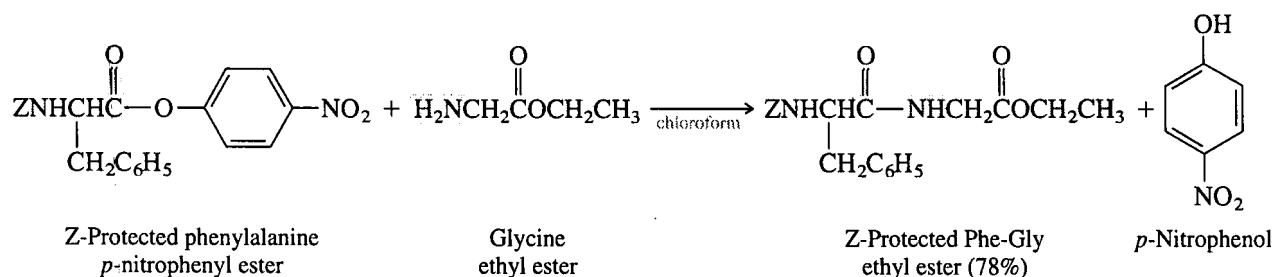
N,N'-Dicyclohexylcarbodiimide has the structure shown:



The mechanism by which DCCI promotes the condensation of an amine and a carboxylic acid to give an amide is outlined in Figure 27.13.

PROBLEM 27.17 Show the steps involved in the synthesis of Ala-Leu from alanine and leucine using benzyloxycarbonyl and benzyl ester protecting groups and DCCI-promoted peptide bond formation.

In the second major method of peptide synthesis the carboxyl group is activated by converting it to an *active ester*, usually a *p*-nitrophenyl ester. Recall from Section 20.11 that esters react with ammonia and amines to give amides. *p*-Nitrophenyl esters are much more reactive than methyl and ethyl esters in these reactions because *p*-nitrophenoxide is a better (less basic) leaving group than methoxide and ethoxide. Simply allowing the active ester and a C-protected amino acid to stand in a suitable solvent is sufficient to bring about peptide bond formation by nucleophilic acyl substitution.

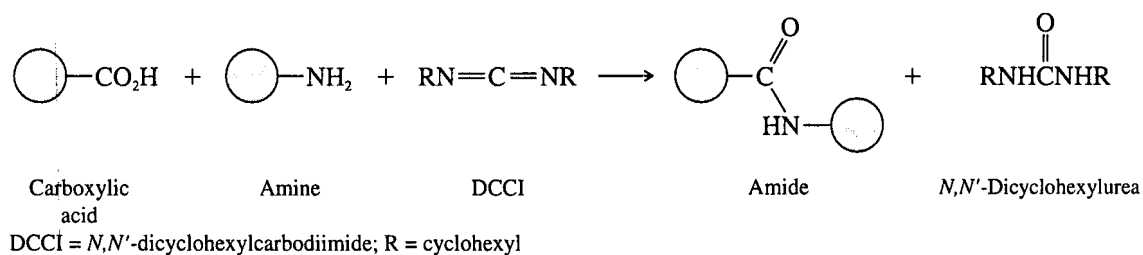


The *p*-nitrophenol formed as a byproduct in this reaction is easily removed by extraction with dilute aqueous base. Unlike free amino acids and peptides, protected peptides are not zwitterionic and are more soluble in organic solvents than in water.

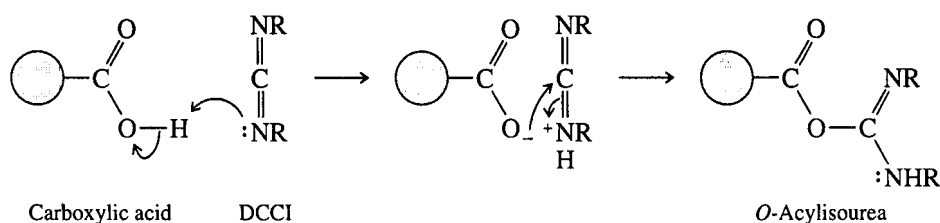
PROBLEM 27.18 *p*-Nitrophenyl esters are made from Z-protected amino acids by reaction with *p*-nitrophenol in the presence of *N,N'*-dicyclohexylcarbodiimide. Suggest a reasonable mechanism for this reaction.

PROBLEM 27.19 Show how you could convert the ethyl ester of Z-Phe-Gly to Leu-Phe-Gly (as its ethyl ester) by the active ester method.

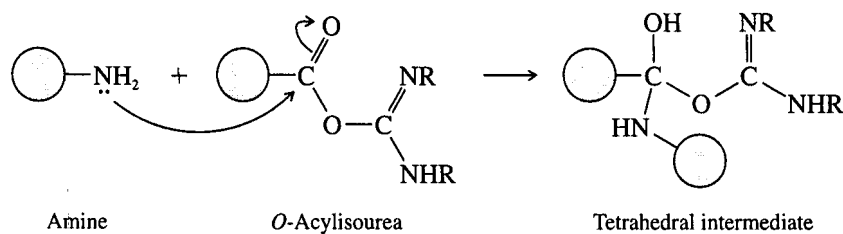
Higher peptides are prepared either by stepwise extension of peptide chains, one amino acid at a time, or by coupling of fragments containing several residues (the **fragment condensation** approach). Human pituitary adrenocorticotrophic hormone (ACTH), for example, has 39 amino acids and was synthesized by coupling of smaller peptides containing residues 1–10, 11–16, 17–24, and 25–39. An attractive feature of this approach is that the various protected peptide fragments may be individually purified, which simplifies the purification of the final product. Among the substances that have been synthesized by fragment condensation are insulin (51 amino acids) and the protein ribonuclease A (124 amino acids). In the stepwise extension approach, the starting

Overall reaction:**Mechanism:**

Step 1: In the first stage of the reaction, the carboxylic acid adds to one of the double bonds of DCCI to give an *O*-acylisourea.



Step 2: Structurally, *O*-acylisoureas resemble carboxylic acid anhydrides and are powerful acylating agents. In the reaction's second stage the amine adds to the carbonyl group of the *O*-acylisourea to give a tetrahedral intermediate.



Step 3: The tetrahedral intermediate dissociates to an amide and *N,N'*-dicyclohexylurea.

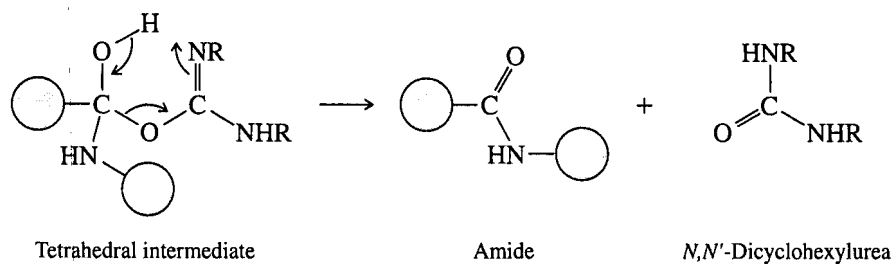


FIGURE 27.13 The mechanism of amide bond formation by *N,N'*-dicyclohexylcarbodiimide-promoted condensation of a carboxylic acid and an amine.

peptide in a particular step differs from the coupling product by only one amino acid residue and the properties of the two peptides may be so similar as to make purification by conventional techniques all but impossible. The following section describes a method by which many of the difficulties involved in the purification of intermediates have been overcome.

27.18 SOLID-PHASE PEPTIDE SYNTHESIS: THE MERRIFIELD METHOD

Merrifield was awarded the 1984 Nobel Prize in chemistry for developing the solid-phase method of peptide synthesis.

In 1962, R. Bruce Merrifield of Rockefeller University reported the synthesis of the nonapeptide bradykinin (see Section 27.14) by a novel method. In Merrifield's method, peptide coupling and deprotection are carried out not in homogeneous solution but at the surface of an insoluble polymer, or *solid support*. Beads of a copolymer prepared from styrene containing about 2% divinylbenzene are treated with chloromethyl methyl ether and tin(IV) chloride to give a resin in which about 10% of the aromatic rings bear $-\text{CH}_2\text{Cl}$ groups (Figure 27.14). The growing peptide is anchored to this polymer, and excess reagents, impurities, and byproducts are removed by thorough washing after each operation. This greatly simplifies the purification of intermediates.

The actual process of solid-phase peptide synthesis, outlined in Figure 27.15, begins with the attachment of the C-terminal amino acid to the chloromethylated polymer in step 1. Nucleophilic substitution by the carboxylate anion of an *N*-Boc-protected C-terminal amino acid displaces chloride from the chloromethyl group of the polymer to form an ester, protecting the C terminus while anchoring it to a solid support. Next, the Boc group is removed by treatment with acid (step 2), and the polymer containing the unmasked N terminus is washed with a series of organic solvents. Byproducts are removed, and only the polymer and its attached C-terminal amino acid residue remain. Next (step 3), a peptide bond to an *N*-Boc-protected amino acid is formed by condensation in the presence of *N,N'*-dicyclohexylcarbodiimide. Again, the polymer is washed thoroughly. The Boc-protecting group is then removed by acid treatment (step 4), and after washing, the polymer is now ready for the addition of another amino acid residue by a repetition of the cycle. When all the amino acids have been added, the synthetic peptide is removed from the polymeric support by treatment with hydrogen bromide in trifluoroacetic acid.

By successively adding amino acid residues to the C-terminal amino acid, it took Merrifield only 8 days to synthesize bradykinin in 68% yield. The biological activity of synthetic bradykinin was identical with that of natural material.

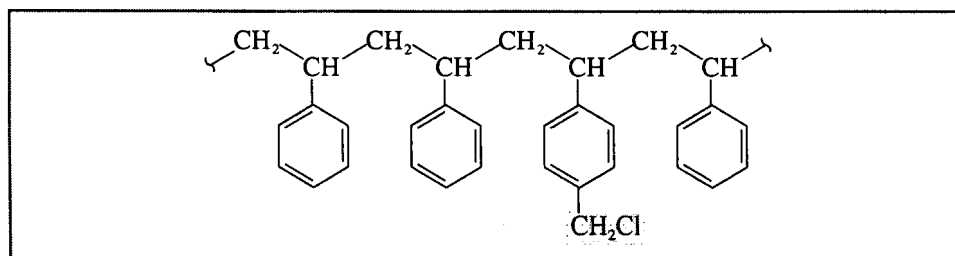


FIGURE 27.14 A section of polystyrene showing one of the benzene rings modified by chloromethylation. Individual polystyrene chains in the resin used in solid-phase peptide synthesis are connected to one another at various points (cross-linked) by adding a small amount of *p*-divinylbenzene to the styrene monomer. The chloromethylation step is carried out under conditions such that only about 10% of the benzene rings bear $-\text{CH}_2\text{Cl}$ groups.

Quantitative and Qualitative Analyses of Serum Antibodies Elicited in Adults by *Haemophilus influenzae* Type b and Pneumococcus Type 6A Capsular Polysaccharide-Tetanus Toxoid Conjugates

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Covalent binding to immunogenic proteins increases the immunogenicity of the capsular polysaccharides of *Haemophilus influenzae* type b (Hib) and pneumococcus type 6A (Pn6A). Conjugates composed of Hib, Pn6A, or the cross-reacting *Escherichia coli* K100 covalently bound to tetanus toxoid (TT) were injected into young adult volunteers. Local reactions were common and were probably due to Arthus reactivity mediated by the preexisting antibodies reacting with the TT component of the conjugates. Fever occurred in about 10% of the volunteers after the first injection; no volunteers had fever after the second injection. Similar levels of Hib or Pn6A antibodies were elicited by either 50- or 100- μ g doses or by concurrent injection of two different conjugates (Hib-TT and Pn6A-TT or Hib-TT and K100-TT). The Hib-TT elicited about a 180-fold increase in Hib antibodies, and the Pn6A-TT conjugate elicited about an 8-fold increase in Pn6A antibodies after one injection. Booster reactions were not elicited in adults; similar levels of antibodies in the five experimental groups suggested that the responses elicited by the conjugates were maximal. A one-way cross-reaction was noted as Pn6A conjugates elicited rises of Hib antibodies in 13 of 20 volunteers; only 4 of 59 volunteers immunized with Hib-TT had increases in Pn6A antibodies. The preimmunization Hib antibodies were composed of immunoglobulin M (IgM), IgA, and IgG. The postimmunization sera showed an increase in all three isotypes; the elevation of the IgG was the highest of the three isotypes. Conjugate-induced antibodies to both the polysaccharide and TT exerted biological activities that have been correlated with immunity. Adsorption of the Hib-TT onto aluminium hydroxide resulted in higher levels and an earlier Hib antibody response in infant rhesus. These results encourage the evaluation of Hib and Pn6A conjugates in human children and infants.

Serum capsular polysaccharide (CPS) antibodies, whether "natural" or induced by convalescence from disease or by vaccination, confer immunity against invasive diseases caused by encapsulated bacteria (6, 16-18, 32, 40, 41, 43-46). There is abundant evidence that CPS are immunogenic and protective in older children and adults. However, CPS vaccines have limitations in infants and children, the age group with the highest attack rate of invasive diseases due to *Haemophilus influenzae* type b (Hib) and pneumococcus type 6 (6, 25, 30, 32, 37, 41, 43-46, 52). These limitations are (i) age-related immunogenicity and (ii) T-cell-independent properties. An injection of either Hib or pneumococcus type 6A (Pn6A) CPS failed to elicit protective levels of antibodies in most infants and children up to about 24 months of age; reinjection did not induce a booster response (4, 6, 32, 43-46, 52).

To overcome these limitations, the approach of covalent binding of the CPS to proteins, as suggested by Avery and Goebel (7), was used. We reported a synthetic scheme for

preparing Hib and Pn6A CPS-protein conjugates which showed both a greater immunogenicity than CPS alone and had T-cell-dependent properties in mice, rabbits, and primates (12, 48, 50). Concurrent injection of two different conjugates did not interfere with the antibody responses to the polysaccharide or the protein components of either conjugate (12). Injection of the carrier protein with the conjugate both accelerated the formation and increased the level of Hib antibodies in juvenile and infant rhesus (50). Hib conjugates prepared by this scheme, with adipic acid dihydrazide-derivatized diphtheria toxoid as the carrier protein (48), were more immunogenic than the Hib CPS and elicited booster responses in children but not in adults (4, 20, 34).

In this study, three conjugates composed of Hib, Pn6A, and *Escherichia coli* K100 CPS and tetanus toxoid (TT) as the carrier were injected into adult volunteers. The cross-reacting *E. coli* K100 CPS was chosen to study its potential for augmenting the Hib antibody response in infants (26, 49). Pn6A was chosen because this type is a frequent cause of invasive disease and of otitis media in infants, and because it

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TABLE 1. Characteristics of Hib, Pn6A, and *E. coli* K100 CPS used for investigational conjugate vaccines

CPS	K_d^a	% (wt/wt)		<i>Limulus</i> lysate ^b (EU/ml)
		Protein	Nucleic acid	
<i>E. coli</i> K100 ^c	0.54	0.96	0.07	0.05
	0.53	0.91	0.05	0.05
Hib	0.44	0.62	0.17	<0.005
Pn6A	0.30	0.10	0.19	Not done

^a Partition coefficient through CL-4B Sepharose (15, 55).^b *Limulus* lysate, 1 EU = 0.1 ng of U.S. Food and Drug Administration standard *E. coli* lipopolysaccharide (23), with amebocyte as the endpoint (34).^c Two preparations of *E. coli* K100 CPS were used.

is the least immunogenic type in the polyvalent pneumococcal vaccine (6, 43). The three polysaccharides share a ribitol 5-phosphate moiety, and their cross-reactivity has been reported (12).

MATERIALS AND METHODS

Polysaccharides. The Hib CPS was prepared from strain Madigan, and the *E. coli* K100 CPS was prepared from strain Easter (12). The Pn6A CPS was purified by cold phenol extraction and ultracentrifugation from a lot manufactured by Eli Lilly & Co., Indianapolis, Ind., under contract to the National Institute of Allergy and Infectious Diseases (lot no. 812483) (12). All polysaccharides were dissolved in pyrogen-free water and passed through a 0.45- μ m sterile filter (Nalge, Rochester, N.Y.) before derivatization. The CPS were analyzed for protein, nucleic acids, pyrogen, and molecular size by methods outlined for pneumococcal and meningococcal vaccines (15, 55) (Table 1).

TT. A lot of TT (500 ml containing 3,125 Lf/ml and 3.61 mg of total nitrogen per ml), manufactured by the Shanghai Institute of Biological Products, Shanghai, People's Republic of China, and kindly donated by Chiayung Chu, was used. The TT was centrifuged at $12,000 \times g$ at 4°C for 30 min, and the pellet was discarded. Sucrose was then added to a final concentration of 5%, and samples of 50 ml were passed upward through a 5- by 95-cm column of S-300 Sephacryl (Pharmacia Fine Chemicals, Piscataway, N.J.) equilibrated in 0.2 M NaCl. The elution profile was monitored by absorbance at 280 nm. The second peak, corresponding to a molecular weight of about 150,000, was concentrated by vacuum dialysis, dialyzed against 0.15 M NaCl for 24 h at 3 to 8°C, centrifuged at $16,000 \times g$ at 4°C for 2 h, and passed through a 0.45- μ m sterile filter. The final material contained 155.5 mg of protein per ml and was stored at 3 to 8°C.

Hydrazide derivatives of the CPS. Each CPS was activated at pH 10.5 at 4°C for 6 min with 1.0 mg of cyanogen bromide (Eastman Chemical Products, Inc., Rochester, N.Y.) per mg of CPS (12, 29, 50). Adipic acid dihydrazide (Sigma Chemical Co., St. Louis, Mo.) recrystallized from ethanol was dissolved in 0.5 M NaHCO₃ and added to the cyanogen bromide-activated CPS to a final concentration of 0.3 M, the pH was adjusted to 8.5 with 0.2 N HCl, and the reaction mixture was allowed to tumble overnight at 3 to 8°C. The resultant solution was centrifuged at $16,000 \times g$ at 4°C for 1 h. The supernatant was dialyzed against 0.2 M NaCl for 72 h at 3 to 8°C and then passed through a 5- by 95-cm column of CL-4B Sepharose (Pharmacia) equilibrated in the same

solution. The first peak of the eluate, monitored for its polysaccharide content by the Bial reaction, was pooled, dialyzed against distilled water for 24 h with changes at 3 to 8°C, and freeze-dried (30). The K_d values of the CPS after derivatization with adipic acid dihydrazide were unchanged. The percent of hydrazide on the derivatized CPS was measured by the 1,3,5-trinitrobenzenesulfonic acid assay (Eastman) with adipic acid dihydrazide as a standard (25).

Chemical binding of the CPS hydrazide derivatives to TT. The TT was covalently bound to the CPS hydrazide derivatives by carbodiimide-mediated condensation using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (Bio-Rad Laboratories, Richmond, Calif.). The reaction mixture contained 10.0 mg each of CPS hydrazide and TT per ml and 0.1 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide. The reaction mixture was stirred at pH 5.0 at 4°C for 3 h and dialyzed against 0.2 M NaCl overnight at 3 to 8°C. The resultant material was centrifuged at $10,000 \times g$ at 4°C for 1 h, the supernatant was passed through a 5- by 95-cm CL-4B Sepharose column equilibrated in 0.2 M NaCl, and the void volume was collected (12). The Hib-TT and Pn6A-TT conjugates did not pass through 0.45- μ m sterile filters. All lots were made 0.01% in thimerosal (Eli Lilly) and stored at 3 to 8°C. The K100 conjugates were passed through 0.45- μ m sterile filters, made 0.01% in thimerosal, and stored at 3 to 8°C.

All glassware was autoclaved and kept overnight at 250°F (121.1°C) in dry heat. All fluids were autoclaved at 254°F (123.3°C) for 2 h.

Characterization of the conjugates. The polysaccharide and protein contents of the conjugates are listed in Table 2. The conjugates were diluted with phosphate-buffered saline (pH 7.4) to a final concentration of 100 μ g of CPS/ml; thimerosal was added to a final concentration of 0.01% and dispensed in 2.7-ml samples into capped vials by the Pharmacy Branch, Clinical Center, National Institutes of Health. The conjugates in the final containers were tested for sterility, safety, toxin reversion, and pyrogenicity by Donald Hochstein, Office of Biologics Research and Review, Food and Drug Administration, and found to be satisfactory.

Adsorption of Hib-TT on aluminium hydroxide. Hib-TT was mixed with preformed aluminium hydroxide (Alhydrogel, lot E1929; Superfos, Denmark) and diluted to a final concentration of 20 μ g of CPS and 1.0 mg of Al(OH)₃ per ml. The supernatant of the adsorbed Hib-TT contained less than 1% of the total Hib CPS.

Two groups of four 2-month-old rhesus monkeys were injected subcutaneously with 10 μ g of either the Hib-TT or the adsorbed Hib-TT in 0.15 M NaCl three times at 3-week intervals. A blood sample was taken before each injection and 3 weeks after the last injection.

Serology. Hib and Pn6A antibodies were measured by radioimmunoassay (44, 47). Tetanus toxin antibodies (ATT) were measured by enzyme-linked immunosorbent assay

TABLE 2. Composition of conjugates used in clinical study

Conjugate	Polysaccharide (μ g/ml)	Protein (μ g/ml)	Protein/polysaccharide	<i>Limulus</i> lysate ^a (EU/ml)	Pyrogen ^b
Hib-TT	100.4	252.4	2.52	125	Neat
K100-TT	100.3	238.0	2.38	125	1:10
Pn6A-TT	101.1	161.0	1.61	125	1:10

^a See footnote b of Table 1.^b Milliliters of vaccine per kilogram rabbit body weight (dilution not yielding fever).

(ELISA) (13, 50). Representative sera from each group of volunteers were also assayed for ATT by toxin neutralization (8). Bactericidal activity was measured using precolostral calf serum as a source of complement and Hib strain Rab (49). The mouse protective activity of three representative sera from the volunteers was assayed by intraperitoneal challenge of 15- to 17-g NIH mice with Pn6A strain BoB23 (50). The 50% lethal dose of this strain was 5.2 organisms. Each concentration of pneumococci was tested in a group of 10 mice.

The immunoglobulin M (IgM), IgA, and IgG isotype composition of the Hib antibodies in the sera of 16 volunteers, representative of the five groups, were measured by direct (sensitivity, 50 ng of antibody per ml) and indirect (sensitivity 50 to 80 pg of antibody per ml) ELISA (9, 10, see Table 8). Hib antibody references (OBRR; Food and Drug Administration) were purified from a pool of sera from adults injected with Hib vaccine (51). The Hib antibody content of the pooled sera and the immunoglobulin fractions were assigned the following values: serum, 80 µg/ml; IgM, 1.3 µg/ml; IgA, 4.0 µg/ml; and IgG, 170 µg/ml. Human immunoglobulins used to check specificity of the antiimmunoglobulins and immunoadsorbents were as follows: IgM myeloma, mu/kappa (kindly donated by Richard Wistar, Naval Medical Research Institute, Bethesda, Md.), IgG purified from sera by DEAE-anion-exchange chromatography, and IgA purified from colostrum by two cycles of DEAE anion-exchange chromatography and gel filtration through G-200 Sephadex. Affinity-purified goat anti human IgM (lot 085-6), IgA (lot 097-1), and IgG (lot 066-5) (Atlantic Antibodies, Scarborough, Maine), were passed through the appropriate immunoglobulin-4B Sepharose immunosorbents. Murine monoclonal anti-human gamma chain (lot 090382) and mu chain (lot 021484) were kindly donated by C. B. Reimer, Centers for Disease Control, Atlanta, Ga. The indirect ELISA used rabbit anti-goat IgG (heavy and light chain specific) purchased from Cappel Laboratories, West Chester, Pa., and absorbed with human immunoglobulin-4B Sepharose columns. Antiimmunoglobulin reagents were coupled to alkaline phosphatase (Sigma) as described previously (54). Each enzyme-antibody conjugate was assayed for its antigenic specificity by direct binding and by inhibition of binding to the human immunoglobulins and for enzyme activity. Microtiter plates (Dynatech Laboratories, Alexandria, Va.) were coated with avidin at 2 µg/ml (Boehringer Mannheim Corp., Indianapolis, Ind.) overnight at 24°C followed by 4.0 µg of biotinylated Hib CPS per ml for 2 h at

TABLE 3. Clinical protocol for evaluation of conjugates in young adults

Group	Immunization (µg)		No. of samples ^a				
	First	Second	Pre	1st	Second immunization	2nd	3rd
1	Hib-TT (50)	Hib-TT (50)	20	20	18	20	15
2	Pn6A-TT (50)	Pn6A-TT (50)	20	19	19	20	14
3	Hib-TT (50) and Pn6A (50)	Hib-TT (50)	14	14	12	14	12
4	Hib-TT (50) and K100-TT (50)	Hib-TT (50)	19	18	18	19	14
5	Hib-TT (100)	Hib-TT (50)	20	19	18	20	19

^a Serum samples were as follows: Pre, preimmune sera; 1st, sera taken 3 weeks after the first immunization; 2nd, sera taken 2 weeks after the second immunization; 3rd, sera taken 6 months after the first immunization.

TABLE 4. Local, systemic, and combined local and systemic reactions elicited by conjugates after the first injection^a

Group	n	No. of volunteers with symptoms												
		None	Local only			Systemic only			Local and systemic					
			Mild	Mod	Sev	Mild	Mod	Sev	Local			Systemic		
									Mild	Mod	Sev	Mild	Mod	Sev
1	20	7	3	4	1	1	1	0	2	2	1	2	1	2 ^b
2	20	1	7	7	2	0	0	0	1	2	0	3	0	0
3	14	0	3	5	0	0	0	0	4	4	2	7	2	1 ^b
4	19	1	1	4	3	0	0	0	3	6	1	8	2	0
5	20	3	5	4	0	0	0	0	6	2	0	7	1	0

^a Local reactions were erythema or induration or both, classified as follows: mild (Mld), <3 cm; moderate (Mod), 4 to 8 cm; severe (Sev), >8 cm. Systemic reactions included malaise, aching, chills, and fever, classified as follows: (i) mild reaction, no fever but malaise and/or aching; (ii) moderate reaction, malaise and/or aching, temperature 99.3 to 101°F (ca. 37.4 to 38.3°C); (iii) severe reaction, malaise, aching, and chills, temperature greater than 101°F.

^b Volunteers had temperatures of 102°F (ca. 38.9°C) for 24 h after the first injection.

24°C and overnight at 3 to 8°C (52a). The plates were washed with phosphate-buffered saline containing 0.1% Brij-35 (Sigma) and blocked with 1% bovine serum albumin (Sigma). Reference and test sera were diluted in phosphate-buffered saline-0.1% Brij-0.1% bovine serum albumin, applied to the plates, incubated overnight at 24°C, and washed five times. The plates were then overlaid with alkaline phosphatase-goat anti-human immunoglobulin conjugate for the direct ELISA or with the murine anti-human immunoglobulin and then the rabbit anti-mouse immunoglobulin-alkaline phosphatase conjugate for the indirect ELISA. *p*-Nitrophenyl phosphate (Kirkgaard, Gaithersburg, Md.) was applied following incubation of the conjugate. All samples were assayed in triplicate. Pre- and postimmunization sera were assayed simultaneously in 10 to 12 serial twofold dilutions and compared with a standard curve developed with the OBRR Hib serum reference. Absorbance was measured at 405 nm at 10-min intervals and calculated to 30 or 60 min. Values greater than 1.0 were extrapolated linearly to the time at which the optical density was approximately 1.0 and reanalyzed accordingly. Test sera were retested at different dilutions to achieve a slope that fitted the reference. The results are expressed as the geometric mean of at least five determinations.

Volunteers and clinical protocol. Ninety-three college students were assigned to five groups on a random basis. Blood samples were obtained on day zero, and the volunteers were injected with conjugates (Table 3). Their temperatures were recorded at 6 and 24 h after injection and daily thereafter if they were elevated. The volunteers were contacted by J. C. Parke, Jr., and W. T. Williams from Davidson College 24 h after each injection and were asked to report any malaise, local pain, or other symptoms. Three weeks later another serum sample was taken, and the volunteers were injected again with the conjugates. Blood samples were obtained 2 weeks and about 5 months later. The number of volunteers in each group and the number of samples obtained during the course of the study are shown in Table 3.

Statistical methods. Standard methods were used to evaluate the antibody determinations (5). The average antibody responses for the pre- and postimmunization sera were expressed by the geometric mean levels; variation among the subjects in each group was characterized by the 25th and 75th percentiles of antibody measurements at each serum

TABLE 5. Hib serum antibody responses of young adult volunteers immunized with two doses of conjugates given 3 weeks apart^a

Group	Immunization (μg)		Hib serum antibody response (μg/ml) ^b			
	First	Second	Pre	1st	2nd	3rd
1	Hib-TT (50)	Hib-TT (50)	1.12 (0.55–2.53)	202.2 (95.8–422)	162.0 (71.5–350)	84.2 (48.6–166)
2	Pn6A-TT (50)	Pn6A-TT (50)	1.61 (0.59–3.38)	4.3 (1.81–10.2)	5.90 (2.14–11.4)	5.04 (1.57–11.3)
3	Hib-TT (50) and Pn6A (50)	Hib-TT (50)	0.98 (0.32–1.66)	147.6 (96.5–241)	152.3 (97.0–253)	89.4 (56.0–140)
4	Hib-TT (50) and K100-TT (50)	Hib-TT (50)	1.37 (0.70–2.60)	203.8 (156–276)	179.5 (116–264)	109.2 (56.9–187)
5	Hib-TT (100)	Hib-TT (50)	1.18 (0.33–4.52)	235.7 (149–410)	206.8 (131–430)	83.6 (49.6–166)

^a Volunteers, randomized into five groups (see Table 3 for immunization regimens), were injected subcutaneously with either one or two of the three conjugates on day 0 and again 3 weeks later as outlined in Materials and Methods. Blood samples were taken before the two injections, 3 weeks after the second injection, and 6 months after the first injection. Hib antibodies were assayed by radioimmunoassay (44).

^b Results are given as geometric means, with the ranges from the 25th to 75th percentiles given within parentheses. Serum samples are as indicated in footnote *a* of Table 3.

sampling. The average change in antibody response from the baseline values was analyzed by paired *t* tests in log₁₀ units, and comparisons among groups at sampling were made by analysis of variance in log₁₀ units.

RESULTS

Reactions. The adverse reactions after the first injection are listed in Table 4. The reactions were classified as local, systemic, or local and systemic combined. The highest rates of systemic reactions were observed in groups 3 and 4, which received two different conjugates concurrently. Only 1 out of 33 volunteers in these two groups had no local or systemic reaction. About one-third of the volunteers in these two groups had moderate systemic reactions. Three systemic reactions classified as severe were observed, two volunteers in group 1 and one volunteer in group 3. Neither of the three volunteers had signs or symptoms 48 h after receiving the vaccines. Most volunteers in group 2 (16 of 20) had local reactions; 3 experienced mild systemic reactions. Eight volunteers declined to receive the second injection, including one of the two with severe systemic reactions.

On the basis of our review of the side reactions, groups 1 and 2 were reinjected with the same dose of conjugate as used for the first injection. Groups 3, 4, and 5 were injected with 50 μg of the Hib-TT conjugate for their second injection. No moderate or severe systemic reactions were observed in any of the volunteers after the second injection. One volunteer in group 3, one volunteer in group 4, and two volunteers in group 5 experienced several hours of malaise after the second injection. There were 41 mild, 11 moderate, and 3 severe local reactions; none of these reactions was debilitating or lasted more than 2 days.

Hib antibodies. Table 5 shows the geometric means of the Hib antibody levels and the levels between the 25th and 75th percentiles. The increase over the preinjection levels was 150- to 200-fold in the vaccinees that received the Hib-TT (groups 1, 3, 4, and 5). The postimmune levels of Hib antibodies after the first injection and 2 weeks after the

second injection in these groups were statistically indistinguishable. There were no nonresponders; all volunteers in these four groups had a fourfold or greater rise in antibodies over their preinjection levels in the three postinjection samples. Group 2, which received the Pn6A-TT, had about a threefold increase in Hib antibodies. The rise in Hib antibodies elicited by the Pn6A conjugate is consistent with reported structural similarities and cross-reactions between these two CPS (10).

There was a slight, but not significant, decline in the Hib antibodies in groups 1, 4, and 5 after the second injection. Seven of the 73 volunteers in groups 1, 3, 4, and 5 did not receive the second injection. Nevertheless, they too maintained fourfold or greater levels of Hib antibodies over their preimmunization levels in the samples taken 5 weeks after their immunization with Hib-TT.

Hib antibodies declined at the 6-month bleeding to about 50% of the levels after the two immunizations. The geometric mean levels of Hib antibodies were statistically indistinguishable among the vaccinees that received the Hib-TT (groups 1, 3, 4, 5). The levels of Hib antibodies observed in the volunteers injected with Pn6A-TT (group 2) declined only slightly in the 6-month samples.

Pn6A antibodies. The Pn6A antibodies elicited by the conjugates are shown in Table 6. The geometric mean increase was approximately eightfold in groups 2 and 3. The postimmunization Pn6A antibody levels of groups 2 and 3 were statistically indistinguishable. Seroconversion, as defined by a twofold or greater increase, occurred in all but one of the volunteers injected with Pn6A-TT alone or with Hib-TT. The geometric mean levels of Pn6A antibodies in the groups that did not receive Pn6A-TT (groups 1, 4, and 5) remained the same throughout the study period. The decline in Pn6A-TT antibodies in the samples taken at 6 months was similar to that observed with Hib antibodies; the reduction was about 45% in volunteers that received Pn6A-TT (group 2) and 32% in those that were injected with Pn6A-TT and Hib-TT.

TABLE 6. Pn6A serum antibody responses of young adult volunteers immunized with two doses of conjugates given 3 weeks apart^a

Group	Immunization (μg)		Pn6A serum antibody response (ng/ml)			
	First	Second	Pre	1st	2nd	3rd
1	Hib-TT (50)	Hib-TT (50)	196.7 (131–231)	219.6 (156–268)	229.8 (147–295)	259.3 (173–352)
2	Pn6A-TT (50)	Pn6A-TT (50)	166.2 (95–300)	1,385.2 (1,145–2,508)	1,609.2 (1,095–2,216)	773.2 (606–1,234)
3	Hib-TT (50) and Pn6A (50)	Hib-TT (50)	230.0 (126–367)	1,408.1 (899–2,577)	1,183.1 (876–1,626)	959.3 (530–1,718)
4	Hib-TT (50) and K100-TT (50)	Hib-TT (50)	198.8 (157–261)	245.2 (170–340)	251.6 (184–354)	263.4 (209–328)
5	Hib-TT (100)	Hib-TT (50)	190.1 (141–316)	229.5 (161–360)	201.4 (115–396)	264.4 (172–466)

^a See footnotes *a*, *b*, and *c* of Table 5. Pn6A antibodies were assayed by radioimmunoassay (47).

TABLE 7. ATT serum antibody responses of young adult volunteers injected twice 3 weeks apart with conjugate vaccines^a

Group	Immunization (μg)		ATT serum antibody response (U/ml)			
	First	Second	Pre	1st	2nd	3rd
1	Hib-TT (50)	Hib-TT (50)	20.5 (11.9–31)	185.3 (118–326)	201.6 (140–359)	73.9 (43–133)
2	Pn6A-TT (50)	Pn6A-TT (50)	14.6 (5.8–34)	133.9 (83–216)	134.1 (83–229)	39.9 (26–71)
3	Hib-TT (50) and Pn6A (50)	Hib-TT (50)	17.1 (7.8–30)	166.9 (70–302)	189.8 (86–438)	78.3 (33–185)
4	Hib-TT (50) and K100-TT (50)	Hib-TT (50)	16.3 (7.8–29)	265.0 (166–483)	275.0 (176–395)	131.3 (72–236)
5	Hib-TT (100)	Hib-TT (50)	14.6 (8.3–28)	193.6 (107–369)	202.2 (100–360)	69.2 (41–91)

^a See footnotes a, b, and c of Table 5. ATT were assayed by ELISA (13, 50).

Cross-reactivity between Hib-TT and Pn6A conjugates. There was an increase in the geometric mean level of Hib antibodies in group 2, which received only Pn6A-TT, from 1.61 μg/ml preimmunization to 4.30 and 5.9 μg/ml after the first and second immunizations, respectively ($P < 0.001$). This level of Hib antibodies declined slightly to 5.04 μg/ml in the sample taken 6 months later. About half of the volunteers in group 2 had an increase in Hib antibodies; 13 of 20 had a 2-fold or greater increase, and 7 of these 13 vaccinees responded with a 5- to 10-fold increase in Hib antibodies. This cross-reaction was predominately one way, since Hib-TT did not elicit an increase in the geometric mean level of Pn6A antibodies in groups 1, 4, and 5, which did not receive Pn6A-TT; only 4 of 59 volunteers in these groups (1 volunteer in groups 1 and 5 and 2 volunteers in group 4) responded with a twofold or greater increase.

ATT antibodies. Table 7 shows the ATT responses elicited

by the conjugates. The composition and dosage of the conjugates differed, so that each group received different amounts of TT. Group 1 received 126.2 μg of TT two times, group 2 received 80.5 μg of TT two times, group 3 received 206.5 μg of TT for the first injection and 126.2 μg of TT for the second injection, group 4 received 245.2 μg of TT for the first injection and 126.2 μg of TT for the second injection, and group 5 received 252.4 μg of TT for the first injection and 126.2 μg of TT for the second injection. All volunteers had greater than 1.0 U of ATT per ml, as measured by ELISA, in their preimmunization sera (estimated protective level is 0.01 U/ml). The geometric mean increase of ATT ranged from 10-fold in group 1 to about 20-fold in group 4. The ATT responses of all groups were statistically indistinguishable, but there were some differences that should be mentioned. The lowest ATT levels throughout the study were in group 2, which received the lowest dose of TT in the conjugate. The

TABLE 8. Total and isotype composition of Hib antibodies of young adult volunteers injected with polysaccharide or conjugate vaccines^a

Group/ volunteer no.	Hib antibody (μg/ml)															
	Total				IgM				IgA				IgG			
	Pre	1st	2nd	3rd	Pre	1st	2nd	3rd	Pre	1st	2nd	3rd	Pre	1st	2nd	3rd
1/29	5.5	221.0	253.3	145.1	1.4	43.8	48.9	32.8	0.9	17.3	21.9	26.6	2.2	205.3	230.8	132.5
1/70	4.2	185.7	195.9	101.9	1.7	23.8	27.8	38.4	0.7	15.5	16.8	21.1	1.7	120.7	134.1	90.4
1/98	9.4	253.6	198.2	152.8	1.1	19.9	13.3	15.6	0.3	4.4	7.6	6.6	7.7	246.0	190.6	111.1
1/159	3.1	124.7	106.7	98.9	1.1	19.1	21.7	22.5	1.0	9.3	8.9	10.0	1.4	95.9	82.7	81.5
2/40	3.0	29.2	25.8	19.3	1.5	3.3	2.6	2.5	1.1	1.8	3.2	2.8	1.2	20.4	15.9	9.4
2/148	4.0	46.2	31.4	21.9	1.1	5.7	6.3	6.0	1.0	4.8	4.6	4.1	1.8	31.6	22.0	21.8
2/151	7.4	86.6	71.2	62.0	1.9	7.7	8.6	5.5	0.7	6.1	6.3	5.3	2.1	63.0	53.3	45.0
3/6	1.4	216.9	137.7	126.8	1.0	18.2	16.8	20.2	0.5	8.3	9.0	9.3	1.2	150.5	100.1	104.5
3/59	0.9	129.5	164.6	92.9	0.7	5.6	7.3	7.5	1.5	16.1	16.9	19.3	0.9	95.9	99.1	61.5
3/157	5.5	186.5	196.7	132.9	1.9	34.3	36.2	40.3	1.3	23.8	25.6	22.9	1.5	144.9	156.5	97.9
4/81	1.5	131.0	181.3	85.9	1.4	5.1	5.2	8.2	0.8	7.7	14.2	17.8	1.7	82.0	77.4	67.5
4/69	15.0	468.9	399.5	341.1	1.5	81.8	45.3	50.8	1.5	49.3	69.8	74.3	2.0	376.9	344.2	219.9
4/149	4.0	228.5	176.5	114.4	0.8	33.4	34.0	36.0	0.8	10.5	10.0	13.4	1.3	158.7	141.4	140.0
5/31	3.6	422.0	246.1	139.7	1.5	62.6	46.6	34.5	1.0	20.7	20.1	19.1	1.8	246.1	160.2	90.4
5/67	16.2	262.1	289.1	195.7	2.4	63.5	43.5	29.8	1.3	32.7	46.3	33.0	2.3	143.9	148.2	115.4
5/153	11.8	345.0	312.7	139.5	1.6	32.6	39.1	25.3	1.3	30.8	30.4	37.7	1.8	315.7	275.5	98.9
Hib-AS ^b	4.0	17.7			0.4	2.0			1.	1.8			2.2	21.7		
Hib-JBR ^b		15.1	15.1	17.3		2.7	2.7	3.3		1.6	2.1	2.6		10.8	9.9	12.8
Hib-DLR ^b						6.3				1.0				14.4		
Hib-RS ^b		7.7	10.3			1.7	2.4			1.5	2.1			7.4	9.2	

^a Total and isotype-specific Hib antibodies were measured by ELISA (9, 10). Hib antibodies were measured by ELISA in representative sera from volunteers in the five groups and in sera from four volunteers immunized with Hib polysaccharide. Each value represents the geometric mean of 5 to 10 assays of duplicates of 10 to 12 serial dilutions of each serum. Standards included a reference human total, IgM, IgA, and IgG Hib antibodies and purified myeloma proteins. Serum samples are as indicated in footnote a of Table 3.

^b Sera from volunteers injected with the purified Hib polysaccharide in another study (44).

TABLE 9. Hib antibodies and bactericidal activity of sera from volunteers immunized with conjugates^a

Group/ volunteer no.	Serum	μg of antibody per ml	Titer
1/29	Preimmunization	1.18	1:4
1/29	After first immunization	602.0	>1:4,096
1/29	5 wk after first immunization ^b	960.0	>1:4,096
1/56	Preimmunization	0.69	1:16
1/56	After first immunization	336.0	1:1,024
1/56	After second immunization	120.0	1:512
2/40	Preimmunization	2.80	1:4
2/40	After second immunization	44.0	1:16
2/105	Preimmunization	0.46	1:2
2/105	After first immunization	5.8	1:8
2/105	After second immunization	6.1	1:16
3/59	Preimmunization	0.74	1:2
3/59	After first immunization	208.0	1:2,048
3/59	After second immunization	336.0	1:1,024
4/69	After second immunization	1,728.0	>1:4,096
4/107	Preimmunization	0.20	<1:2
4/107	5 wk after first immunization ^b	512.0	>1:4,096

^a Bactericidal activity and Hib antibodies, assayed by radioimmunoassay, are compared. Sera were taken from volunteers immunized with Hib-TT, Pn6A-TT, Hib-TT and Pn6A-TT, and Hib-TT and K100-TT.

^b Immunized only once. Bleeding at 5 weeks after first injection.

highest ATT levels throughout the study were observed in group 4, which was one of the two groups that received the highest dose of TT. No booster response was noted after the second immunization. The ATT levels at 6 months after the first injection ranged from about 30 to 50% of their postimmunization levels.

Isotype composition of Hib antibodies. The pre- and postimmunization sera of 16 representative volunteers from the five groups were analyzed for their Hib antibodies by ELISA with antiimmunoglobulin (total Hib antibodies) and isotype-specific antibody reagents (Table 8). Sera from four adult volunteers injected with Hib CPS alone as part of another study were also examined by this technique (51). The preimmunization levels of Hib antibodies ranged from 0.75 to 10.0 μg/ml. Hib antibodies of all three isotypes were detected in all the sera. An increase in the three isotypes was observed in the postimmunization sera; predictably, the highest levels were observed in the 13 volunteers injected with the Hib-TT, alone or in combination. The highest levels and greatest fold increases after the first and second immunizations were observed for the IgG isotype. The Hib IgG antibodies of 7 of the 16 volunteers remained at about the same levels in the 6-month samples; IgG Hib antibodies in the remaining 10 samples declined to about one-half of their maximal levels. IgM Hib antibodies increased about 10-fold after the first immunization. The IgM Hib antibodies were maintained in most of the volunteers in the 6-month bleedings. A similar pattern of reactivity was observed for IgA Hib antibodies. The IgG and total Hib antibody levels were most related, showing their highest rise after the first injection and a decline to about one-third to two-thirds of these maximal values in the 6-month bleedings. The sera from four volunteers immunized with the Hib CPS alone showed this same pattern of Hib antibodies distributed among all isotypes, with the highest levels of the IgG isotype.

Bactericidal activity of Hib antibodies elicited by conjugates. Hib antibodies, as measured by radioimmunoassay,

and the bactericidal titers of pre- and postimmunization sera from representative volunteers in groups 1 through 4 are shown in Table 9. Volunteers immunized with Hib-TT alone (nos. 29 and 56) or in combination with Pn6A-TT (no. 59) or K100-TT (no. 69 and 107) showed an increase of both Hib antibodies and bactericidal titers. There was a good correlation between the levels of Hib antibodies and the bactericidal titers. The cross-reacting antibodies elicited by the Pn6A-TT conjugate in volunteers no. 40 and 105 also had bactericidal activity which was related to their level of Hib antibodies as measured by radioimmunoassay.

Mouse protective activity of Pn6A antibodies elicited by conjugates. Table 10 shows the mouse protective activity and the Pn6A antibodies (radioimmunoassay) in the pre- and postimmunization sera of two volunteers in group 2 injected with Pn6A-TT. The preinjection serum of volunteer no. 3 showed low levels of both Pn6A antibodies and mouse protective activity. The two postinjection sera, in contrast, showed both high Pn6A antibodies and high mouse protective activity.

Tetanus toxin-neutralizing activity of ATT elicited by conjugates. Representative sera from volunteers in groups 1, 2, 4, and 5 were analyzed for their tetanus toxin-neutralizing activity. Table 11 shows their TT antibodies, measured by ELISA, and by toxin neutralization. The values obtained by these two methods show a fair correlation and comparable fold increases. These data indicate that both conjugates elicited TT antibodies with neutralization activity.

Comparison of Hib and Pn6A antibodies elicited by conjugates or polysaccharides in adult volunteers. Table 12 shows that the fold increase and postimmunization geometric mean for both Hib and Pn6A antibodies elicited by the conjugates are considerably higher than those achieved in young adult volunteers injected with the Hib CPS alone or with pneumococcal vaccines containing the type 6A or type 6B CPS, thereby confirming the increased immunogenicity of the conjugates compared with the polysaccharide alone.

Effect of adsorption of Hib-TT onto aluminium hydroxide. The antibody response to protein antigens, but not Hib-polysaccharide, is enhanced by their adsorption onto aluminium salts (21, 36). Accordingly, the effect of adsorbing Hib-TT onto aluminium salts upon antibody synthesis was studied in infant rhesus. One injection of 10 μg of Hib-TT in saline did not elicit Hib antibodies in four infant rhesus (Table 13). In contrast, protective levels of Hib antibodies were elicited with one injection of the same dose when Hib-TT was adsorbed onto aluminium hydroxide. The adsorbed Hib-TT elicited about 20-fold higher levels of Hib

TABLE 10. Pn6A antibodies and mouse protective activity of sera from volunteers immunized with conjugates^a

Group/ volunteer no.	Serum	ng of anti- body nitrogen per ml	LD ₅₀ ^b
2/3	Preimmunization	122.5	85
2/3	After second immunization (1:2)	1,514.9	>7 × 10 ⁴
2/148	After first immunization	2,544.9	1 × 10 ⁵

^a Representative sera, taken before and after one or two injections of Pn6A-TT were assayed for their mouse protective activity by intraperitoneal challenge with live pneumococci. Pn6A antibodies were determined by radioimmunoassay. The serum of volunteer no. 3 was diluted 1:2 with saline before it was titrated.

^b The 50% lethal dose (LD₅₀) of Pn6A in saline was 5.2 organisms.

antibodies than did the unabsorbed conjugate after the second injection; similar levels were observed in both groups after the third injection.

DISCUSSION

Local reactions elicited by the conjugates were typical of Arthus-type reactivity and are a common complication of immunization with TT in recently immunized adults (36, 38, 53). TT vaccines contain about 25 to 60 µg of protein. The conjugates used in this study contained between 80 µg (group 2) and 252 µg (group 5) of TT protein. None of the local reactions was debilitating or prolonged. Such local reactions are not expected in children with their lower levels of ATT.

We do not yet have an explanation for the systemic reactions elicited by the investigational vaccines. The timing and nature of the reactions are consistent with a small amount of lipopolysaccharide in the vaccines, yet the pyrogen test in rabbits and the *Limulus polyphemus* lysate gelation assays show levels of lipopolysaccharides in the vaccines that would be predicted to elicit little or no systemic reactions in adult volunteers (23, 33, 40). One explanation, therefore, is that the specific activity of two bioassays is lower for lipopolysaccharide in the presence of the conjugates than for the lipopolysaccharide alone or with CPS. No fever or moderate or severe systemic reactions were observed in the volunteers that received only Pn6A-TT (group 2); 16 of the 20 volunteers in this group experienced local reactions. The absence of systemic reactions after the second injections could be explained by induction of lipopolysaccharide-neutralizing antibodies by the first injection (22). The lack of a dose effect between the 50-µg (group 1) and 100-µg (group 5) doses of Hib-TT argues against lipopolysaccharide as a cause of these systemic reactions.

Numerous studies in laboratory animals and in humans

TABLE 11. Toxin neutralization and ELISA values of sera from adult volunteers immunized with conjugates^a

Group/ volunteer no.	Serum	ELISA	Neutralization titer
1/21	Preimmunization	26.0	>1-<10
1/21	After first immunization	197.9	>10-<100
1/21	After second immunization	39.6	>10-<100
1/64	Preimmunization	24.8	>1-<10
1/64	After first immunization	504.3	>100-<1,000
1/104	Preimmunization	8.32	>1-<10
1/104	After first immunization	62.3	>10-<100
2/40	Preimmunization	15.6	>1-<10
2/40	After first immunization	82.7	>1-<10
2/103	Preimmunization	5.7	>0.1-<1
2/103	Postimmunization	198.1	>10-<100
2/142	Preimmunization	5.2	>1-<10
2/142	After first immunization	467.9	>100-<1,000
2/142	After second immunization	461.7	>100-<1,000
4/69	Preimmunization	70.7	>1-<10
4/69	After first immunization	585.8	>10-<100
5/67	Preimmunization	24.8	>1-<10
5/67	After first immunization	274.3	>10-<100
5/67	After second immunization	509.8	>100-<1,000

^a ATT were measured by ELISA and toxin neutralization (8). Toxin neutralization used 10-fold dilutions of sera, and the two values depict (A) the highest dilution that conferred protection and (B) the lowest dilution that did not protect mice against a lethal dose of tetanus toxin.

TABLE 12. Comparison of serum Hib and Pn6A antibodies elicited by polysaccharides or conjugates in adult volunteers

Vaccine	Geometric mean levels ^a of serum antibodies		Fold increase	Reference
	Preimmunization	Postimmunization		
Hib	0.56	15.9	28.4	44
Hib	1.99	29.5	14.8	20
Hib	1.20	35.0	29.1	51
Hib-DT	3.30	248.0	75.3	20
Hib-TT	1.12	202.2	180.5	This study
Pn6A	220	686	3.1	51
Pn6A	117	617	5.3	43
Pn6A-TT	161.2	1,385	8.6	This study

^a Hib antibody results are in micrograms of antibody per milliliter of serum. Pn6A results are in nanograms of antibody nitrogen per milliliter of serum.

have confirmed the increased immunogenicity of Hib, meningococcal, and pneumococcal CPS, including Pn6A, by their covalent attachment to immunogenic proteins (3, 4, 11, 12, 14, 20, 27, 28, 34, 42, 49, 50). Compared by the fold increase of the postimmunization antibody levels, the immunogenicity of the Hib was increased more than that of the Pn6A by our conjugation procedure. This difference may be related to the greater immunogenicity of the Hib CPS compared with the Pn6A CPS alone. Another possibility is that the molecular size and ratio to protein in the conjugate may affect the immunogenicity of the CPS component. It has been shown that there is a minimum size of Hib CPS component of a conjugate required for its immunogenicity (3). Makela et al., using conjugates composed of dextran and heterologous albumins, suggested that there is an optimal size of the polysaccharide component of the conjugate to achieve its maximum immunogenicity; a decrease in the level of dextran antibodies was observed with conjugates prepared with the highest- and the lowest-molecular-weight dextrans (35). The molecular weight of the Pn6A used to prepare the Pn6A-TT was higher than that of the Hib. We have used ultrasonic irradiation to prepare, in a nondestructive fashion, polysaccharides of a defined and comparatively homogeneous molecular weight to further study this possibility (S. C. Szu, G. Zon, R. Schneerson, and J. B. Robbins, Carbohydr. Res., in press).

The second injection of the conjugates did not elicit a booster response for either the CPS or TT components in any of the five groups. This was unexpected, since booster responses were elicited by these conjugates in mice and juvenile and infant rhesus (2, 50). One explanation for this failure to elicit booster responses may be related to the hyperimmune status of the volunteers to TT. All of the volunteers had been injected with TT adsorbed within 1 year. Reinjection of TT in adults does not elicit booster responses after two or more injections within a period of 1 year (39, 53). It is probable that all of the volunteers had received the United States recommended injection schedule, which includes at least five injections of bacterial vaccines containing TT during infancy and childhood (36). Considering the high levels of antibodies after the first injection and the similar response elicited by 50 and 100 µg of Hib-TT (groups 1 and 5, respectively), it is likely that the 50-µg dose elicited a maximal response and no booster effect after the second injection or that enhancement by the cross-reacting K100 or Pn6A conjugates could be induced. Although the

TABLE 13. Hib antibodies of infant rhesus injected with 10 µg of Hib-TT in saline or adsorbed onto aluminium hydroxide^a

Hib-TT prepn	Infant no.	Hib antibody (µg/ml of serum)			
		Pre	1st	2nd	3rd
Saline	B351	<0.04	<0.04	1.19	7.8
	B561	<0.04	<0.04	0.64	32.0
	A713	<0.04	<0.08	0.72	10.05
	B72	<0.04	<0.04	0.14	2.60
With Al(OH) ₃	B585	<0.04	4.50	21.0	19.0
	B557	<0.04	12.05	16.4	80.0
	B311	<0.04	0.30	3.35	7.89
	B57	<0.04	1.10	8.70	18.75

^a Hib-TT was adsorbed onto preformed aluminium hydroxide (10 µg of conjugate per 0.5 mg of Al³⁺). Hib-TT was diluted in saline to 20 µg/ml; 0.5-ml samples of the two preparations were injected subcutaneously in four 1- to 2-month-old rhesus 3 weeks apart for three doses. The monkeys were bled before each injection (Pre) and 3 weeks past the third immunization, and their serum Hib antibodies were measured by radioimmunoassay (44).

mechanism(s) has not been elucidated, a certain level of antibodies, achieved by immunization, serves as a feedback inhibition for additional immunizations (2). It is likely that this mechanism was operative in the volunteers. The two injections were spaced 3 weeks apart; this short interval may also be a factor in the lack of a booster response (36). Similar lack of booster responses in adults injected with conjugates of similar composition has been reported (20, 34).

Concurrent injection of two conjugates containing cross-reacting CPS had no effect upon the Hib or Pn6A antibodies (groups 3 and 4). We reported that concurrent injection of Hib and Pn6A conjugates, with the same or different carrier proteins, accelerated Hib antibody formation in mice (12). Our data in adult volunteers suggest that immunization with of two conjugates will not suppress antibody responses to either conjugate; whether there may be an enhanced response will be studied in children whose responses after one injection are not expected to be maximal.

The current United States infant vaccine formulation of DTP (diphtheria toxoid-tetanus toxoid-pertussis) vaccine is compounded with aluminium salts (adsorbed). The adsorption onto aluminium salts results in an adjuvant action upon the antibody responses to the diphtheria toxoid and TT components in at least two aspects; (i) the initial injections of vaccine elicits higher levels of antibodies; and (ii) the antibody levels remain elevated longer (21, 36). The adsorbed Hib-TT elicited both earlier and higher titers of Hib antibodies in the infant rhesus than did the same dose of the unadsorbed conjugate (Table 13). We did not have the opportunity to obtain additional blood samples from these monkeys. We have shown that adsorption of Hib-polysaccharide alone onto aluminium had no effect upon Hib antibody responsiveness in human infants (44). The apparent discrepancy between these two experimental observations may be due to differences in the preparation of the adsorbed products; the Hib-TT was adsorbed onto preformed aluminium hydroxide, and the insoluble aluminium salt was formed in situ. Another possibility is that the adjuvant effect exerted by the aluminium salts requires a T cell-dependent antigen. Aluminium salts have an acceptable safety record as adjuvants for DTP vaccine and other vaccines used for humans. The aluminium content of the adsorbed conjugate was below the maximum allowable level for a human dose (36). A similar adjuvant effect of aluminum hydroxide adsorption of Pn6A-diphtheria CRM 197 protein conjugates

has been recently reported (42). Accordingly, we plan to study the immunogenicity of our Hib-TT and Pn6A-TT conjugates adsorbed onto aluminium as either single components or combined with an experimental detoxified pertussis toxin (Sekura, Trollfors, and Zhang, unpublished data) (12, 48).

The conjugate-induced antibodies to both the polysaccharide and protein components exerted secondary biological activities that have been correlated with immunity. Hib antibodies, whether induced by the homologous antigen (Hib) or by cross-reacting antigen (Pn6A), had bactericidal activity. Passive immunization with Pn6A antibodies resulted in about a 100,000-fold increase in the 50% lethal dose (LD₅₀) of Pn6A organisms in mice. Similarly, TT antibodies, as measured by ELISA, paralleled increases in neutralizing antibodies (ATT). These and similar data suggest that appropriate levels of antibodies induced by these semisynthetic vaccines will confer immunity to diseases caused by encapsulated as well as toxin-producing bacteria.

Hib antibodies of IgM, IgA, and IgG isotypes were detected in all preimmunization sera of 16 volunteers in this study. Similar results were obtained for other bacterial CPS antibodies (1, 4, 6, 18, 19, 25, 31). Immunization with either the conjugates or with Hib-polysaccharide in four persons in another study (44) induced an increase in Hib antibodies of all three isotypes. Both types of Hib vaccines induced the highest rise in IgG; this isotype also had the steepest decline at the 6-month bleeding, although the IgG levels after this interval were considerably higher than the preimmunization levels. The proportion of Hib antibodies of the three isotypes elicited by either the homologous antigen (Hib) or the cross-reacting conjugates (Pn6A), by combinations of conjugates (Hib-TT and Pn6A or Hib-TT and K100-TT), or by the CPS alone were similar.

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Rational Design of Conjugate Vaccines

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

Abbreviations

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

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

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

ENHANCED IMMUNOLOGIC EFFECTIVENESS OF TD ANTIGENS

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

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

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

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a number of previously poorly immunogenic—mainly T1—bacterial antigens (1–20). The glycoconjugates thus formed not only are TD antigens, but they also usually generate antibody responses to both the carbohydrate and its protein carrier, although the levels of antibody raised against each moiety often differ.

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

PROBLEMS IN CONJUGATE VACCINE DESIGN

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

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

Protein-carrier selection. In the formulation of conjugate vaccines, should the protein carriers be natural or synthetic? What should be the nature of the linkage and coupling chemistry? The number of proteins that are acceptable for use in humans as potential carrier proteins for polysaccharide or peptide epitopes is restricted. Clinically useful proteins such as diphtheria (1, 7, 17, 20, 22) and tetanus (3–6, 11) toxoids have been used as carriers. In addition, a nontoxic native mutant form of diphtheria toxin, CRM₁₉₇, has also been successfully used in conjugate vaccines (8, 9). Because most infants will have received their first injection of a diphtheria-tetanus-pertussis (DPT) vaccine by the age of 3 mo, the use of diphtheria and tetanus toxoids as carriers for future conjugate-vaccine administrations avoids the introduction of new components into the immunization protocol for infants. In addition, because periodic booster immunizations are indicated for many immunization procedures, the presence of these toxoids in conjugate vaccines could serve to boost antibody titers to diphtheria and tetanus. However, there is some concern that excessive exposure to these proteins, both as carriers and as part of the DPT regimen, might have the adverse effect of causing tolerance rather than boosting the response. A number of con-

fusing and contradictory reports as to the effect of carrier priming and dose as well as carrier choice have appeared, indicating that additional studies are needed to clarify the issues.

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

In contrast to the above findings, questions have arisen as to whether previous exposure to the carrier protein suppresses the response to the epitope attached to it (30–32). Recent studies in mice on the effect of carrier priming on the immunogenicity of saccharide-protein conjugates indicate that both the nature of the carrier and the dose used for priming can affect the response to the saccharide (33). Low-dose priming with tetanus toxoid enhanced the response, whereas high-dose carrier priming suppressed the response. Priming with CRM₁₉₇ neither enhanced nor suppressed the subsequent response to the saccharide epitope.

To complicate matters further, a recent study on the immunogenicity of an Hib conjugate vaccine with CRM₁₉₇ as the carrier found that infant monkeys had no response to the Hib unless they were concurrently primed with diphtheria toxoid (34). Thus, in certain cases, previous exposure to a form of the carrier might prove to be not only beneficial, but essential to produce an adequate response to the epitope conjugated to that carrier.

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

PEPTIDE-PROTEIN VACCINE CONJUGATES

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

PROBLEMS IN ADJUVANT USE

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

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

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

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

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

CELLULAR MECHANISMS INVOLVED IN ANTIBODY PRODUCTION

Although the conjugation of a TD antigen to bacterial carbohydrate moieties acts to elicit a TD type of immune response against the carbohydrate, the cellular mechanisms involved are still not completely understood. The active involvement of T cells and their secretory products in an immune response seems

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

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

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

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

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

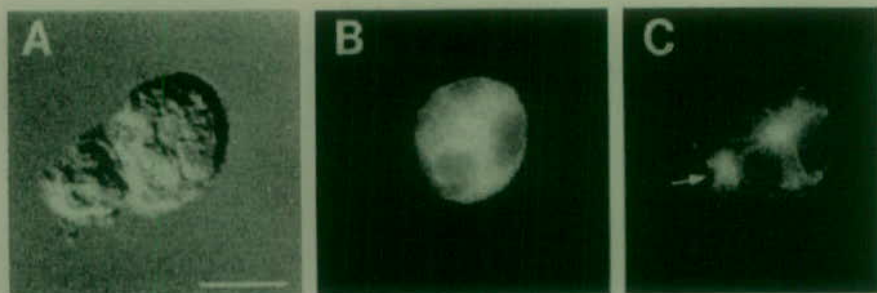


Fig. 1. A cell couple formed between a cloned helper T cell and a hybridoma B cell that had been pulsed with the specific antigen conalbumin. The couple is seen in Nomarski optics in A. The same couple was double-labeled immunofluorescently for a cell surface marker to label the B cell (B) and for microtubules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

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

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

B-cell and T-cell epitopes of the circumsporozoite protein of the malaria parasite *P. berghei* were identified and incorporated into a MAP system (50). Vaccines produced in this way were found to be strong immunogens in a number of inbred mouse strains when administered first in complete Freund's adjuvant and boosted two subsequent times in incomplete Freund's adjuvant. The effect of the stoichiometry, orientation, and arrangement of the B and T epitopes on the immunogenicity of the MAP was studied. Ten MAP models were prepared containing various arrangements of the T and B epitopes of the circumsporozoite protein of *P. berghei*. The B epitope was represented by

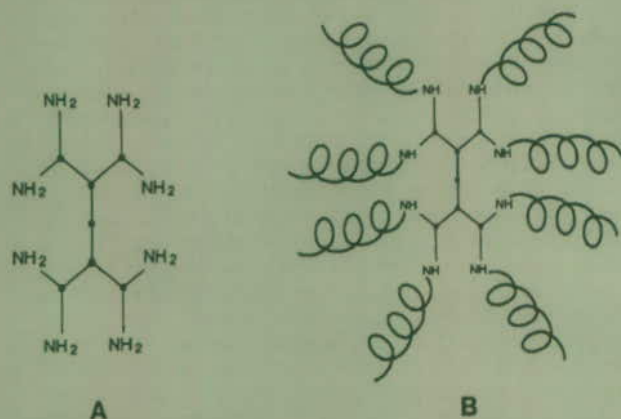


Fig. 2. Schematic representation of the core matrix of the MAP. The lysyl core of the octabranching MAP (A) and MAP with eight peptide antigens attached (B). (From Tam JP: Multiple antigen peptide system: a novel design for synthetic peptide vaccine and immunoassay. In: *Synthetic Peptides: Approaches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

the 16-residue peptide (PPPPNPND)₂, and the T epitope was represented by a 12-residue peptide, KQIRDSITEEWS. The latter peptide was identified as a T-cell epitope because it was recognized by several strains of mice and induced helper activity *in vivo* (51). Examples of the types of structures formulated as MAP models are shown in Figure 3. The immunogenicity and protective value of the MAP when these structures were tested in mice are presented in Table 1. The best arrangement of B-

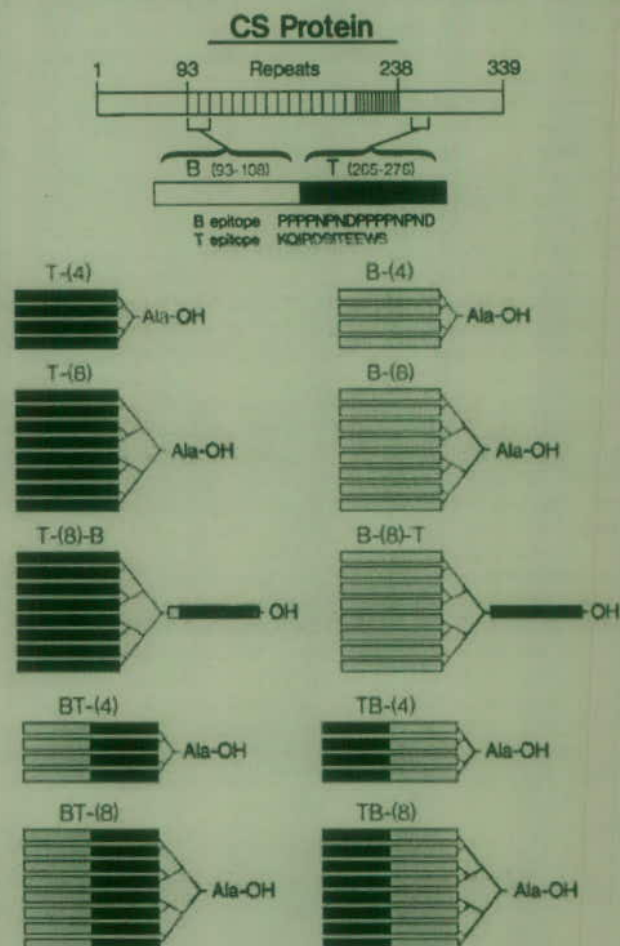


Fig. 3. Schematic representation of the structure of the CS protein of *P. berghei* of the monomeric form of a peptide containing tandem B- and T-cell epitopes and of 10 MAP models. (Reproduced from *J Exp Med* 171:299-306, 1990, by copyright permission of the Rockefeller University Press.)

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

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

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† See Figure 4 for pictorial representation of immunogen.

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

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

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

MOLECULAR PARAMETERS ENHANCING IMMUNOGENICITY

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

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

Immunogenicity of multimeric proteins. The aforementioned

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

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

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

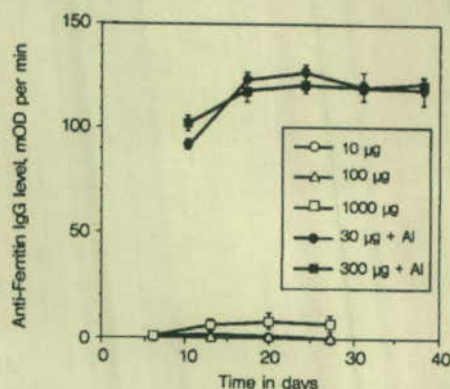


Fig. 4. The serum levels of antiferritin antibodies of the IgG class at various times after intraperitoneal injection of antigen. The open symbols show the responses to three doses of monomeric ferritin injected in saline. The solid symbols show the responses to two doses of monomeric ferritin adsorbed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

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

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

Trials of conjugate vaccines using oligosaccharides coupled to diphtheria toxoid or to CRM₁₉₇, a nontoxic mutant protein of diphtheria toxoid, demonstrated that epitope valence (number of epitopes per unit of carrier) is also a critical parameter in immunogenicity (8, 9). A higher multiplicity of saccharides per protein carrier was found to give greater immunogenicity.

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

VACCINATION AGAINST SOME COMMON INFECTIONS

Development of conjugate vaccine against P. aeruginosa. In the last 30 years, there has been a dramatic increase in the

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

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

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

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

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

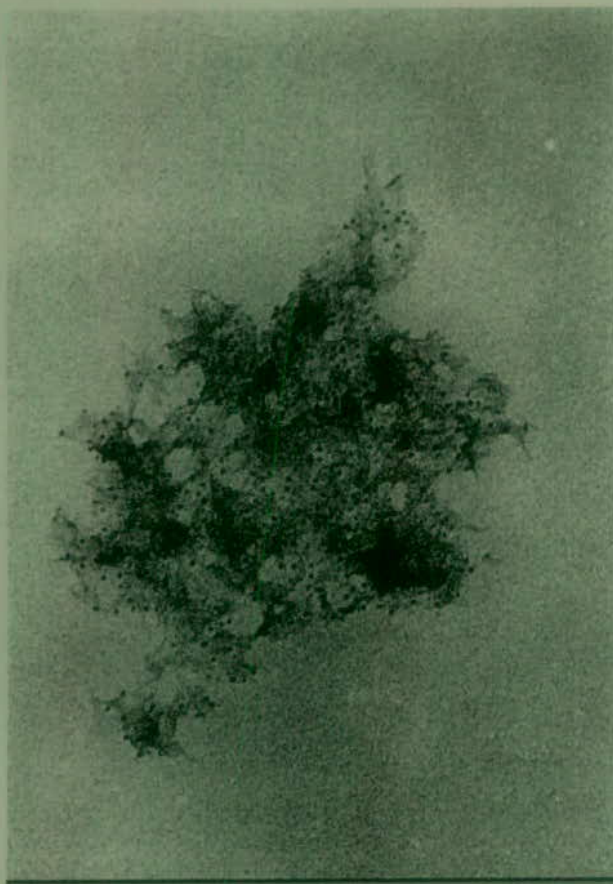


Fig. 5. A transmission electron micrograph of ferritin molecules adsorbed on the surface of an aluminum hydroxide particle. The ferritin was adsorbed at a ratio of 300 μ g of ferritin per mg of aluminum hydroxide. The sample was deposited on a collodion membrane, enlarged by electron microscope 50 000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

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

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

Vaccines for bacterial meningitis. Hib is the leading cause of bacterial meningitis in children under 4 y of age. At birth, maternal antibodies seem to confer protection, but as the maternal antibody level in the infant decreases, infant susceptibility to infection increases. Unfortunately, young infants fail to respond to the Hib capsular polysaccharide, which is a TI type of antigen. However, inasmuch as they are capable of responding to TD antigens, several groups have attempted to produce an efficacious vaccine against Hib by covalently linking the polysaccharide (or an oligosaccharide derived from the larger molecule) to a protein carrier, thereby creating a TD antigen. The carrier proteins used have been derived from different bacteria: tetanus toxoid, diphtheria toxoid, CRM₁₉₇, which is a nontoxic form of diphtheria toxin, and the OMP of group B *N. meningitidis*. Conjugate vaccines of all four types are now being studied clinically, and side-by-side comparisons of efficacy are being conducted in infants and in older children (reviewed in 63, 64).

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

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

the NPr-GBMP-tetanus toxoid as a good candidate for a conjugate vaccine against meningitis caused by either group B *N. meningitidis* or *E. coli* K1.

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

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

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

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

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

Challenge strain	Bactericidal titer‡	Anti-NPr-GBMP serum§		Normal serum§		% Reduction in viable bacterial counts	
		3 h	5 h	3 h	5 h	3 h	5 h
<i>N. meningitidis</i> 80165 (B:2b:P1.H)	Exp. 1 512	24	16	1064	2144	98	99
	Exp. 2 512	440	0	1850	2700	76	100
<i>N. meningitidis</i> 86370 (B:15:P1.16)	256	760	200	2360	350	68	84
<i>N. meningitidis</i> 87147 (C:2b:P1.2)	4	2000	2920	2180	2736	0	-6
<i>E. coli</i> 871743 (O18:K1:H7)		80	24	4658	368	98	93

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

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

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

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

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

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

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

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Glycoprotein conjugate vaccines

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Abstract

The polysaccharide capsule which surrounds bacterial species like *Haemophilus influenzae*, *Streptococcus pneumoniae*, *Neisseria meningitidis*, *Salmonella typhi*, is a potent virulence factor. It protects the bacterium from phagocytosis, but capsule specific antibodies plus complement binding to the capsule opsonise the organism for phagocytosis and elimination. Purified capsules elicit T-independent antibody responses without a memory function, and are often poorly immunogenic in infants where much of invasive *H. influenzae* type b (Hib) and pneumococcal infection is seen. Covalent linkage of the polysaccharide, or fractions thereof, to immunogenic carrier proteins creates glycoconjugates which are T-dependent antigens and which prime for boosting either with the glycoconjugate or the capsular polysaccharide; During the 1990s, four Hib glycoconjugate vaccines have been introduced and in countries that have vaccinated the majority of children, the success has been stunning. In countries with very high immunization coverage the disease has been virtually eliminated and, to a decline of over 95% in countries with slightly lower vaccine rates. Worldwide use of Hib glycoconjugate vaccines offers the possibility of elimination of invasive Hib disease. Pneumococcal (11 serotypes with coverage of approximately 85% of invasive disease) and meningococcal (A, C, W 135, Y but not B) glycoconjugates are in pre-registration phases and offer the prospect of being as successful as the Hib glycoconjugates. © 1999 Published by Elsevier Science Ltd. All rights reserved.

Keywords: Polysaccharide; Glycoconjugate; Memory

1. Introduction

Several bacterial species are surrounded by a polysaccharide capsule made up of either a polymerized homopolymer, like $\alpha 2, 8$ or $\alpha 2, 9$ linked sialic acid residues in *Neisseria meningitidis* groups B and C, respectively, or polymerized repeating units made up of di- to pentasaccharides as in most *Streptococcus pneumoniae*. The capsular structure protects the bacterium against phagocytosis and is a potent virulence factor. Once inside polymorphonuclear leucocytes the capsulated bacterium is rapidly killed. The host responds to the infection with production of specific antibodies which bind to the capsule thereby opsonising them for phagocytosis, a process mediated via both Fc and complement receptors. The antibody-dependent complement-mediated killing is thought to occur via a final insertion of the C8–C9 complex in the bacterial membrane.

The medical impact of infections caused by encapsu-

lated bacteria and in particular by pneumococci, was evident in the beginning of the century. The immunological properties of bacterial capsular polysaccharides became the target of several investigators in the 1920s and 1930s. In the mid-1940s it was evident that

1. Capsular polysaccharides, on killed cells or 'purified', elicited type-specific protective immune responses [1–4]
2. Infants and young children did not respond with type-specific antibodies neither after disease nor after vaccination [5–8]
3. Type-specific antibodies were the effector molecules which conferred protection [7,9]
4. Vaccination with polysaccharides reduced the carrier rate of bacteria of the same types as in the vaccine [4]
5. Glycoconjugates using haptenic saccharides covalently linked to a carrier protein, could in rabbits induce antibody responses which were high titered,

boostable and protected against challenge infection [10,11]

However, the successful introduction of antimicrobial agents put an effective stop to the further development of both capsular polysaccharide and glycoconjugate vaccines for a few decades. A few things rekindled the interest in vaccination in the 1970s. First it became increasingly evident that antimicrobials, although largely successful, was not the ultimate solution to handle infections. Treatment failures and the emergence of antimicrobial resistant strains forced a rethinking and the role of prophylactic immunization became one alternative. Advances in immunology with delineation of B and T cell responses, and the role of T cells for the immunological memory functions, as well as the structural elucidation of the capsular polysaccharide opened the way for development of defined glycoconjugates.

2. *Haemophilus influenzae* type b (Hib)

Haemophilus influenzae is a gram-negative coccobacillus which frequently colonizes the oropharynx of man. Most *H. influenzae* strains are non-encapsulated, (non-typable *H. influenzae* — NTHI), but a few are surrounded by a polysaccharide capsule. Six capsular types, a to f, have been recognized with type b being the most common and virulent. *H. influenzae* type b, Hib, causes meningitis, epiglottitis, septicemia, facial cellulitis, pneumonia, arthritis and a variety of less common forms, i.e. invasiveness and dissemination is a characteristic trait of Hib infection.

Most Hib infections in the prevaccine era occurred in children, less than 5 years of age. The rates of Hib disease varied, but invasive disease in Europe ranged from 30 to 60 cases per 100,000 children less than 5 years old [12]. In the US the corresponding figures ranged from 50 to 100 per 100,000 children. Some population groups have been reported to have much higher rates of disease; thus native Americans and Australian Aborigines have had rates up to 300 per 100,000. Invasive Hib infections are uncommon in adults unless there is predisposing underlying disease. An annual incidence of 0.22 per 100,000 has been reported [13].

The peak incidence of Hib disease in the prevaccine era was in children between the ages of 5 and 12 months, coinciding with the waning of maternal antibodies and prior to appearance of anti-capsular antibodies. A pivotal large scale double-blind study in Finland in 1974 comprising almost a 100,000 children between 3 months and 5 years of age showed that (i) children > 18 months were protected with an efficacy > 90%, (ii) children between 12 and 18 months had questionable protection, and (iii) no protection was

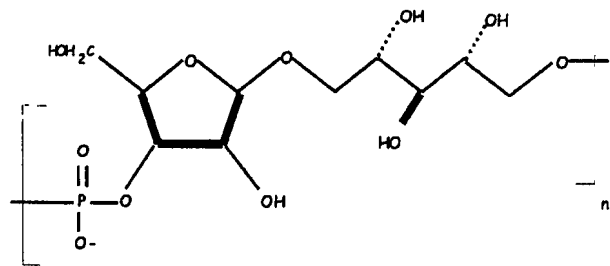


Fig. 1. *Haemophilus influenzae* type b repeating unit: structure of polyribosylribitolphosphate.

seen in the age group of 3 to 12 months [12,14]. This study certainly was a trigger point for the development of Hib glycoconjugate vaccines. After the pivotal studies of the 1920s to 1940s significant advances had been made (i) the role of T cells and B cells was being clarified, (ii) the structure of the PRP capsule (Fig. 1) was determined, and hence targets of chemical coupling strategies became evident (Fig. 2), and (iii) the immunological memory mechanisms were unraveled.

2.1. Conjugation strategies

The capsular polysaccharide of Hib does not contain chemically reactive groups such as amino or carboxyl moieties that can directly be covalently linked to a protein carrier (Fig. 1) was determined. However, the presence of both the ribitol and the phosphodiester in the repeating unit makes the polysaccharide susceptible to derivatisation. The periodate oxidation creates –CHO groups which by reductive amination are coupled to a carrier protein. This procedure was used by Anderson and coworkers at Praxis to create HbOC (Hib-Titer[™]) [15] Robbins et al. [16,17] used adipic acid dihydrazide (ADH), a bifunctional nucleophilic spacer, to facilitate binding of the capsular polysaccharide to the carrier. Cyanogen bromide is then used to introduce an active group into the ribitol moiety of

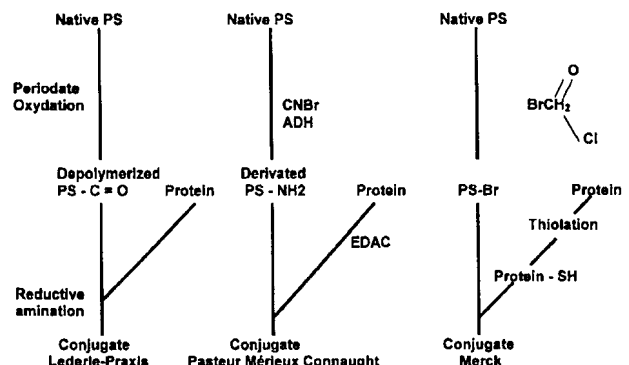


Fig. 2. Coupling schemes for the synthesis of *Haemophilus influenzae* type b glycoconjugates [15,16,17,18].

the repeating unit. The creation of a Hib adipoic hydrazide derivative subsequently allows it to be conjugated to tetanus toxoid by carbodiimide-mediated condensation. The resulting conjugate (PRP-T, Act-Hib[®]) has multipoint attachments between the polysaccharide and the carrier protein and is referred to as a lattice structure. A prior strategy first coupled ADH to the carrier protein. This methodology was used by Connaught for making the PRP-D (ProHibit[®]) conjugate which has been less immunogenic than PRP-T, and is little used today.

A different conjugate, PRP-OMP, was developed by scientists at Merck, Sharpe and Dohme [18]. They conjugated the PRP moiety to an outer membrane protein complex from a *Neisseria meningitidis* groups B strain (PedVax Hib[®]).

Thus there are three, currently used, different PRP conjugates in the market with differences in (i) carrier protein used (ii) linkage type with or without spacer, and (iii) polysaccharide size, and linked at end(s) or in the chains (Fig. 2).

The three vaccine types, not unexpectedly, differ in their immunogenicity. HbOC and PRP-T behave in the same manner each requiring two or three doses to generate substantial antibody titers. PRP-OMP elicits strong antibody responses already after a single dose, and subsequent doses add little to the immune response. Thus PRP-T and HbOC have been evaluated, and are used, in three-dose primary immunization series, whereas PRP-OMP can be used in two-dose primary series.

2.2. Correlates of protection after vaccination

The protective role of antibodies to the Hib capsular polysaccharide was known already in 1933 [7]. The inverse correlation of disease occurrence and the presence of bactericidal antibodies in serum of individuals of different ages was convincingly demonstrated. The pivotal Finnish study with purified PRP as vaccine demonstrated the same relationship for type b specific anticapsular antibodies [12].

Adults regularly have antibodies to PRP. Based on the argument that Hib disease is rare in adults as a consequence of 'protective antibodies' and that 95% of adult sera analyzed had $>0.04 \mu\text{g/ml}$ of anti PRP antibodies a limit of $>0.15 \mu\text{g/ml}$ was arbitrarily set as a level for short-term protection [14,19]. In studies of the post-immunization antibody concentrations seen in vaccines in the Finnish PRP study a concentration of $\geq 1.0 \mu\text{g/ml}$ predicted protection over the following year [20]. Thus the concentrations $>0.15 \mu\text{g/ml}$ and $>1.0 \mu\text{g/ml}$ are used as correlates for short-term and long-term protection, respectively. It is to be noted, however, that these antibody concentrations are the result of immunization with the T independent PRP

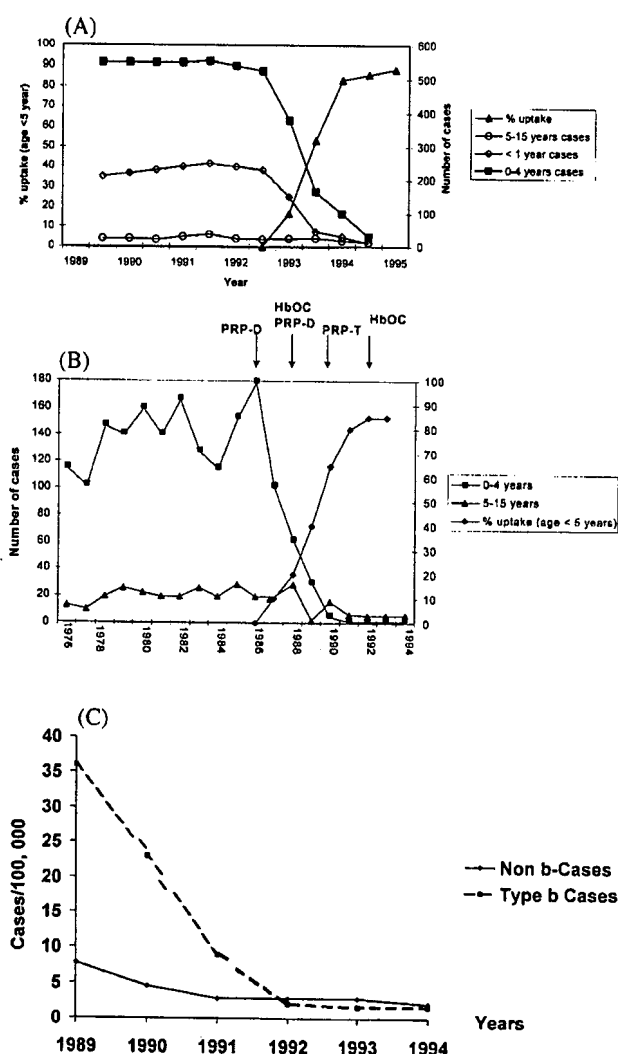


Fig. 3. (A) Number of cases of invasive *H. influenzae* type b disease in England and Wales. (B) Number of cases of invasive *H. influenzae* type b disease in children in Finland. (C) Incidence of invasive *H. influenzae* type b and non-b disease in the United States. From [80].

polysaccharide. Do these levels have any relevance for protection in an infant primed with a glycoconjugate for memory responses? It is probable that lower concentrations, with B cell clones which rapidly can expand upon stimulation, than those used as 'protective levels' following Hib conjugate vaccination are 'protective'. To determine such protective levels is, however, difficult. A better approach in the evaluation of glycoconjugate vaccine immunogenicity should be to incorporate a marker of priming. Memory responses are characterized by the production of high-avidity antibody, i.e. antibodies strongly binding to the antigen. Thus avidity can be considered as a surrogate of successful priming. In a recent study, it was shown that antibody avidity was relatively low following primary immunization, and significantly higher following boosting [21]. Most of the increase in avidity was seen

in the months after primary immunization. The concern with 'too low' anti-PRP titers after vaccination with combination vaccines containing acellular pertussis and Hib conjugate in the same formulation highlight a need for a better understanding of the relationship between primary antibody responses and long-term protection.

2.3. Hib glycoconjugate vaccine efficacy

The introduction of Hib conjugate vaccines has had a profound effect on the overall burden of Hib disease. There has been $\geq 97\%$ reduction in Finland, the UK and the USA, where different Hib conjugates have been used: in Finland PRP-D followed by HbOC [22], in the UK PRP-T [23] and in the US HbOC ($> 90\%$ up to 1994), Figure 2 in [24].

In Finland and in the US a primary series with three doses at 2, 4 and 6 months of age were used with a booster dose in the second year of life. In the UK the primary series also contained 3 doses given at 2, 3 and 4 months of age but with no booster [23]. The reported efficacy for infants 5–11 months of age was 99.1%, for 12–23 months old infants, 97.3% and for 24–35 months children it was 94.4%. Although this shows a gradual fall in the efficacy as children approach the age of 3 years, the 95% confidence limits for efficacy in each age group were found to overlap [23]. The estimated protection remained high at about 95%. The authors challenged the concept that a booster may be required in the second year of life to ensure that protection persists until school age (more than 95% of invasive Hib disease occurs before the age of 5). A basis for this proposal is a high vaccine coverage (probably $> 90\%$) with a resulting herd immunity. An added benefit of the routine use of Hib conjugate vaccines is a substantial reduction of oropharyngeal carriage of Hib [25,26]. The reduced colonization in immunized children most likely reduces transmission and hence children who lack protective antibodies have a reduced probability of being infected (see also Fig. 3).

The use of a carrier protein in the glycoconjugate vaccine may improve the immune response in yet another way. If the infant is primed by a prior tetanus toxoid vaccination the antibody response to PRP-T was found to be improved [27,28]. In the British study young infants (3–11 months of age) were found to have a high level of protection from 1 week after just one dose of vaccine [23].

2.4. Genetic basis for vaccine failure

Gm and *Km* genes code for the antigenic determinants of the heavy and light chains of immunoglobulins, respectively. *Gm* groups are localized to the

constant region of the γ heavy chain and thus are limited to IgG classes and each are exclusive to one of the four IgG subclasses. The *Gm* allotypes are located on chromosome 14. Many of the *Gm* factors are inherited together. The *Km* factors are present on χ light chains and therefore represented in all classes of immunoglobulins. The *Km* allotypes are found on chromosome 2.

There is significant variation in *Gm* and *Km* allotypes among different races in human populations [29], and important allelic associations with *Gm* and *Km* allotypes in the genetic predisposition to some infectious diseases [30,31]. The biological role of the polymorphisms of *Gm* and *Gm* is not understood, but evidence suggests that the polymorphism is protective at the population level: lack of it is associated with higher levels of infectious disease [32] and is specifically associated with lower immune responses to certain infections [33].

The responsiveness of healthy white adults and children to Hib PRP is significantly associated with the *Km* (1)-negative allotype [34,35]. When the Hib glycoconjugate was used the association between the *Km* (1)-negative allotype and the PRP disappeared [36].

It appears that the responsiveness, or lack thereof, is specific to the particular type of polysaccharide. A woman unresponsive to PRP, responded readily to pneumococcal and meningococcal capsular polysaccharides as well as *H. influenzae* outer membrane proteins [37]. Immunization with PRP-T elicited only a minimal response, and not until multiple doses given at 0, 2, 4, 8 and 15 months, did she reach a concentration of 3.1 $\mu\text{g/ml}$. Thus a selective deficiency of antibody to PRP could be overcome by repeated injections with PRP-T, i.e. a need for an individual vaccination scheme beyond what is used in routine immunizations.

Response to PRP has also been linked to the *G2m*-positive allotype [23,38]. However, there is a lack of a persistent association across racial or ethnic groups regarding *Gm* and *Km*. Hence other factors must interact with the roles of the products of the *Gm* and *Km* allotypes. Although the use of glycoconjugate vaccines may overcome some of the allotype associations with the T-cell independent responses to polysaccharide vaccines, it may not be the panacea we hoped for.

3. Streptococcus pneumoniae

Streptococcus pneumoniae continues to be a major cause of morbidity and mortality in adults and children despite the availability of effective antimicrobial therapy. As for Hib the virulence is caused by a capsular polysaccharide, and there are now 90 different capsular types described [39]. Invasiveness is dependent on the capsular structure rather than on the amount being

produced. The polysaccharide is not toxic in itself, but inhibits phagocytosis.

The immunological defence against pneumococcal infection depends on type specific anticapsular polysaccharide antibody interacting with complement to opsonise the bacteria and have them prepared for phagocytosis and clearance. Defects in any of these components of the host defence, i.e. antibody production, complement factor deficiency, splenic dysfunction including splenectomized individuals, or neutropenia are usually associated with an increased risk of serious pneumococcal infection. Of the components type specific anticapsular antibodies are by far the most important. The importance of antibody to recovery from pneumococcal infection was shown already in the 1930s. The immunogenicity and immunochemistry of pneumococcal capsular polysaccharides has been recently reviewed [40,41].

Pneumococci causes a wide variety of infections ranging from relatively mild mucosal infections like acute otitis media, to more serious bronchopneumonia and potentially life threatening meningitis. Pneumococci colonize the respiratory mucosa of both healthy and sick individuals. The carrier rates are higher in children than in adults, even higher in children attending day care centers than those staying at their homes, and still higher in those with respiratory infections compared with healthy children [42,43,44].

Clinical illness is a result of the spread of the pneumococci to tissues from the oropharynx. Although carriage may result in infection it appears as if the period just after acquisition is a period of high risk [42]. Epidemiological studies in the 1980s have found an annual incidence of pneumococcal bacteremia to vary between 9 and 18 cases per 100,000 persons of all ages [41]. In children there were 105 and 234 cases per 100,000 individuals, white and black respectively. There appear to be racial differences with Blacks and Native Americans having rates approximately 2.5 times those of Whites and Hispanics. Even higher rates have been found among the native population of Alaska. It has been estimated that in the US there is on an annual basis 3000 cases of meningitis, 50,000 of bacteremia, 500,000 of pneumonia, and 7,000,000 cases of acute otitis media [41,45,46]. Approximately 40,000 deaths caused by pneumococci occur each year in the US and it has been estimated that approximately half are potentially preventable with pneumococcal vaccines.

In developing countries pneumococcal infections cause a significant disease burden. It has been estimated that acute lower respiratory infections caused by pneumococci account for more than 4 million deaths annually, most of them in children being <5 years of age [44].

Thus it is evident that a pneumococcal vaccine elicit-

ing protective immunity in the very young as well as the elderly would have a significant public health impact.

3.1. Capsular types

Of the 90 capsular types identified only a fraction are common causes of pneumococcal disease. The seven most commonly isolated serotypes cover up to 85% of all pneumococcal strains causing invasive infections in Finnish, Israeli and US children [47].

A nanovalent vaccine for global use including serotypes 1, 5, 6B, 7F, 9V, 14, 18C, 19F and 23F would cover approximately 80% of strains causing infections in industrialized countries and approximately 70% of strains in developing countries [48,49].

There exists pneumococcal polysaccharide vaccines which are composed of the 23 most common pneumococcal serotypes. They represent 85-90% of the serotypes that cause invasive infections in adults in industrialized countries. As for Hib young children do respond poorly to most serotypes up to the age of 2 years, and the least immunogenic serotypes (6B, 14, 19F, 23F) up to the age of 5 to 10 years [49,50]. The polysaccharide as a T independent antigen also fails to induce a memory function.

The structure of each polysaccharide serotype differs from that of others serotypes and from that of PRP. Hence different coupling strategies have to be devised for each polysaccharide, either in its native or sized form (often incorrectly called oligosaccharide). The manufacturers are using the same overall strategies as for the Hib conjugates, i.e. reductive amination, ADH spacer and linkage to a *N. meningitidis* OMP complex. The carriers used are the same as for Hib conjugates, i.e. tetanus and diphtheria toxoids [17] the genetically toxoided diphtheria toxin CRM 197 [51] and the *N. meningitidis* OMP complex [18].

The resulting pneumococcal conjugate vaccine will contain from 9 to 11 different polysaccharides. However the amount of carrier protein should be restricted even if tetanus toxoid can prime for a higher antibody response for PRP-T [23], too much carrier antigen may impair the antibody response to the polysaccharides by antigen competition or carrier-mediated epitope suppression [52,53].

Several conjugates have been through the phase 1, 2 and 3 trials and several conclusions can already be drawn: (i) in adults the conjugates have been well tolerated although local reactions at the injection site appear to be more common than after vaccination with capsular polysaccharides [54], (ii) in toddlers all conjugates tested have been immunogenic, (iii) in infants all conjugates tested have been well tolerated and no immediate, serious vaccine related adverse events have been reported. Local reactions have been

similar to those seen after Hib conjugates. The reaction rates have been lower and their intensity less marked in young infants than in adults, (iv) there is priming for anamnestic IgG antibody responses to subsequent boosting with polysaccharide vaccine [55,56]. The highest dose (10 µ/ml of each polysaccharide) induced the strongest response after primary immunization (2, 4, 6 months of age), but the booster response was greatest in the group primed with the lowest dose (1 µ/ml of each polysaccharide), (v) a significant reduction in the carriage of vaccine related strains was observed after primary immunization suggesting that transmission of specific pneumococcal serotypes most often associated with disease at least partially be controlled by immunization [57], (vi) Wyeth-Lederle's heptavalent Pnc-CRM vaccine was 100% efficacious against invasive disease in a study in Northern California: 17 cases in the control group and 0 in the vaccine group (2, 4, 6 month primary series) (personal communication), and (vii) no antibody correlates for protection are yet available.

Available information suggest that we may have efficacious pneumococcal glycoconjugate vaccines on the market just after the turn of the century. Efficacy most likely will first be demonstrated for invasive infections (bacteremia and meningitis) to be followed by demonstrated efficacy against mucosal infections, i.e. acute otitis media.

3.2. Genetic regulation of immune responses

Healthy adults have a varying capacity to elicit antibodies to pneumococcal polysaccharides. A study of 61 ashkenazic family members showed that the capacity to generate anti-pneumococcal polysaccharide IgG was inherited in a mixed, codominant fashion [58]. However, no association between *G2m* allotype [23] and antibody to the polysaccharide was found. Revaccination either with the 23-valent polysaccharide vaccine (single, double dose or 20 times higher dose of a single polysaccharide) or with either Pnc-OMPC or Pnc-CRM failed to elicit antibody production to the relevant polysaccharide in a subject who had initially shown no measurable IgG after vaccination. These individuals also failed to elicit IgG antibody production, and hence the failure to respond was not caused by a failure to switch from IgM to IgG.

The data suggest that a genetically mediated defect in pneumococcal polysaccharide recognition is responsible. However, the genetics of the defect are not understood. It is tempting to speculate that occasional vaccine failures with the 23-valent may be caused at least in part by genetically determined incapacity to make IgG to the capsular polysaccharides.

4. *Neisseria meningitidis*

Meningitis caused by *Neisseria meningitidis* is still a feared disease. Although the total number of cases is small it is a disease which because of its mortality attracts much attention. Here as for Hib and pneumococci a polysaccharide capsule is an important virulence factor, and prospects for prophylactic immunization are good. *N. meningitidis* is an exclusive human pathogen, and transmission is by droplets from colonized upper respiratory mucosal membranes.

There are 12 serogroups based on the structure of the capsular polysaccharide: A, B, C, 29E, H, I, K, L, W135, X, Y, and Z. Approximately 90% of cases of meningitis are caused by strains belonging to serotypes A, B and C. All three serotypes can cause epidemics, but it is usually group A strains that are the causative agent in repeating epidemics in sub-saharan Africa [59]. In Europe and Latin America serogroup B is most prevalent, causing more than 50% of the cases, whereas serogroup C is most prevalent in North America [60,61].

Meningococcal meningitis is spread over all age groups: one-third occurs from 0 to 4 years of age, one-third among 5 to 9 years old, and the final one-third at age 20 and above [62]. Therefore there is a need to vaccinate the whole population. Already in 1913 Flexner published convincing data that serum therapy decreased the fatality rate of meningococcal meningitis [63], and 20 years later Fothergill and Wright observed that there was an inverse relationship between the incidence of meningitis at different ages and the presence of serum antibodies to the causative agent [17].

4.1. Meningococcal glycoconjugates

Purified capsular polysaccharides from serogroups A, C, W135 and Y are marketed products, and as T independent antigens elicit antibody responses with no memory function, with the possible exception of serogroup A [64]. The polysaccharide vaccine from serogroup A elicits an antibody response in infants, and even below the age of 6 months infants appear to be primed [64]. The serogroup C polysaccharide is not immunogenic in children <2 years of age, and development of antibody titers is slow see [61]. Correlates of protection have not been established. However, data suggest that for serogroups A and C serum concentrations of anti capsular antibodies should be >2.0 µg/ml [61,64]. Glycoconjugates are being developed using the same principles as for Hib and pneumococcal glycoconjugates (see above).

The A and C glycoconjugate vaccines are safe and well tolerated in infants and toddlers and using a 2,4, 6 month schedule for primary immunization all infants

had elicited specific anti-A and anti-C polysaccharide antibody titers $>2.0 \mu\text{g/ml}$ [65-67].

A functional assay is to estimate bactericidal antibodies. A titer of $>1:8$ was seen in all vaccinated, with 47% having equally high titers one year later [65].

In a study in Gambia an interesting and as yet unexplained observation was made: whereas the serogroup C conjugate, as expected, induced memory, no such observation was seen with the serogroup A conjugate [68].

So far all data indicate that meningococcal glycoconjugate vaccines will have as great a chance to be successful as the Hib and pneumococcal conjugates and licensure is expected within the next coming years. Most likely, meningococcal A+C and meningococcal A+C+W135+Y glycoconjugates will be marketed.

4.2. Meningitidis serogroup B

The meningococcal serogroup B polysaccharide, a homopolymer of $\alpha 2, 8$ linked sialic acid residues, is poorly immunogenic in man [69]. Since the same structure is formed in embryonic neural cell adhesion molecules, man is likely tolerant to the polysaccharide structure. It has been suggested that vaccine induced antibodies may interfere with the adhesion molecules causing injury [70], a theory which also has been challenged [71]. The uncertainties have definitely slowed development of a glycoconjugate vaccine, and instead focused interest on outer membrane proteins and protein-complexes. However, antibodies elicited against the serogroup B polysaccharide, although low in titer, are bactericidal [72]. Jennings has modified the group B polysaccharide by replacing the *N*-acetyl groups with *N*-propionyl groups before its conjugation to tetanus toxoid [73]. The resulting polysaccharide mimics a unique protective epitope on the surface of the native group B meningococci and elicits antibodies [74]. Although bactericidal antibody production still only has been shown in mice, the observation holds promise for the development of a human vaccine.

5. Other glycoconjugates

The spectacular success of the Hib conjugates and the likely successes of the pneumococcal and meningococcal glycoconjugates have obviously focused an interest on converting other capsular polysaccharide antigens from T-independent to T-dependent vaccines.

Group B streptococci (GBS) are the leading cause of meningitis and sepsis in neonates in the US. At least six capsular types have been associated with human disease, and type-specific antibodies are responsible for protection via opsonisation of the bacteria by antibodies and complement. Kasper and Jennings have

developed immunogenic glycoconjugates which in animal models elicit protective antibodies [75,76].

Similar attempts are underway to make immunogenic and safe vaccines against the Vi polysaccharide capsule of *Salmonella typhi* [77] and capsular polysaccharide and lipopolysaccharide of *Escherichia coli*, *Shigella sonnei*, and *S. Flexneri* [78,79].

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