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|---------------------------------------------------|--------------------------------------------|
| SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO          |                                            |
| RAD: 11-144904- -16                               | FECHA: 2016-05-12 16:07:23                 |
| DEP: 2020 DIRECCION DE NUEVAS CREACIONES          | EVE: 378 FASE NACIONAL INCLUIDO CAPITULO I |
| TRA: 11 PCT-PATENTE DE INVENCION CAPITULOS I Y II | FOLIOS: 9                                  |
| ACT: 519 TRASLADOINFORME                          |                                            |

DIRECCIÓN DE NUEVAS CREACIONES

2020  
Bogotá, D.C.,

Abogado(a)/Señor(a)  
JUAN PABLO CADENA SARMIENTO

Asunto:      Radicación:      11-144904- -16  
                 Trámite:            11  
                 Evento:            378  
                 Actuación:           519  
                 Oficio:                    5557  
                 Folios:                    9

|                                     |                   |            |   |                               |  |
|-------------------------------------|-------------------|------------|---|-------------------------------|--|
| Patente de Invención                | X                 |            |   | Patente de Modelo de Utilidad |  |
| Vía Nacional                        |                   |            |   |                               |  |
| Vía PCT (Fase Nacional)             | X                 | Capítulo I | X | Capítulo II                   |  |
| No. Solicitud Internacional         | PCT/US2010/028968 |            |   |                               |  |
| Fecha de Presentación Internacional | 26/03/2010        |            |   |                               |  |

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

1. Documentos estudiados

|                   |                                  |                 |
|-------------------|----------------------------------|-----------------|
| Descripción:      | Radicado N° 11-144904-00000-0000 | 26/Octubre/2011 |
| Reivindicaciones: | Radicado N° 11-144904-00000-0000 | 26/Octubre/2011 |
|                   | Radicado N° 11-144904-00006-0000 | 08/Enero/2013   |
|                   | Radicado N° 11-144904-00009-0000 | 01/Abril/2014   |
| Dibujos:          | Radicado N° 11-144904-00000-0000 | 26/Octubre/2011 |
| Secuencias:       | Radicado N° 11-144904-00000-0000 | 26/Octubre/2011 |
| Prioridad:        | US 61/164,385                    | 27/Marzo/2009   |
|                   | US 61/164,387                    | 28/Marzo/2009   |
|                   | US 61/164,388                    | 28/Marzo/2009   |
|                   | US 61/164,389                    | 28/Marzo/2009   |
|                   | US 61/313,676                    | 12/Marzo/2010   |

## 2. Análisis de la Prioridad

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

## 3. Objeto de la invención

De acuerdo con lo establecido en el numeral 1.2.2.2 del capítulo primero del Título X de la Circular Única para efectos del examen de patentabilidad, se tendrá en cuenta la tasa pagada por la solicitante al momento de comenzar el examen para las reivindicaciones adicionales; esto es que cuando la solicitud contenga más de diez (10) reivindicaciones y no se haya pagado la tasa complementaria, sólo se estudiarán las cubiertas por la tasa pagada. En el presente caso se estudiarán las reivindicaciones 1 a 53 del capítulo reivindicatorio (Radicado N°. 11-144904-000009-0000).

Título de la solicitud: “COMPOSICIONES INMUNOGÉNICAS QUE COMPRENDEN UNA GLUCOPROTEÍNA DE SUPERFICIE DEL VIRUS DE LA GRIPE” (Modificado mediante resolución No: 56505 del 23 de septiembre de 2014).

El problema planteado en esta solicitud consiste en la necesidad de elaborar una vacuna candidata de la gripe, útil en la terapia antiviral contra el virus de la gripe.

Para resolver este problema técnico la solicitud en estudio proporciona polipéptidos víricos parcialmente glicosilados, útiles para generar composiciones inmunógenas, ampliamente reactivas, efectivas contra el virus de la gripe.

El objeto de la presente solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): A61K 39/12 // 39/145 // 39/155 // 39/21 // 39/29; A61P 31/12 // 31/14 // 31/16 // 31/18; C07K 14/11 // 14/135 // 14/155 // 14/18; C12P21/02.

## 4. Excepción a la Patentabilidad (Art 20 D 486 literal (d))

El objeto de las reivindicaciones 35 - 37 no es patentable de acuerdo con el Art citado. Si bien dichas reivindicaciones están encabezadas como vacunas, su parte característica está orientada a la administración de dicha vacuna a un sujeto y a la posterior producción de anticuerpos asociada a la misma, por lo que es evidente que se refieren a métodos de tratamiento.

## 5. De la solicitud de patente

### Reivindicaciones (Art 30 D 486, Art 22 PCT)

1. Las reivindicaciones 1 y 18 no son claras ni concisas porque no definen las características técnicas esenciales de la composición inmunógena y de la vacuna que se reclaman, que son sus ingredientes y proporciones. De esta forma, se encuentran las siguientes inexactitudes:
  - La expresión “una glucoproteína parcialmente glicosilada del virus de la gripe o un fragmento de la misma” es muy general e insuficiente para caracterizar el antígeno presente en la composición reclamada. Es importante mencionar que dicha expresión hace referencia a una gran variedad de moléculas no soportadas por la descripción. Se sugiere caracterizar dicho antígeno de acuerdo a la secuencia de aminoácidos de la glucoproteína o sus fragmentos, e incluir los residuos sobre los que se da la

glicosilación y los patrones de carbohidratos que hacen parte de la misma, tal como se menciona en la reivindicación 14.

- La expresión “*en dónde la glucoproteína antígeno se glucosila parcialmente en uno o más sitios de glucosilación, en comparación con un estado de glucosilación en el sitio de glucosilación de la glucoproteína antígeno cuando se expresa en una célula huésped*” no es clara porque no es posible comprender las características técnicas de la glucosilación mencionada, tales como el número de glucosilaciones, el sitio de glucosilación y el patrón de carbohidratos presente en las mismas. Se sugiere modificar la redacción de la reivindicación para indicar dichas características, evitando emplear términos ambiguos como “*parcialmente*” y “*uno o más*”.
- No se menciona la concentración o la proporción en la que se encuentra la glucoproteína antígeno en la composición reclamada, por lo que se sugiere que sea incluida.

2. La reivindicación 2 no es clara ni concisa porque:

- Los términos “*total o parcialmente glucosilada*” y “*fragmento de la misma*” son muy generales e impiden comprender el alcance de la materia reclamada. Se sugiere eliminar dichos términos y caracterizar el antígeno mediante la secuencia de aminoácidos, incluyendo los sitios de glucosilación y el patrón de carbohidratos presente.
  - Se menciona un grupo muy general de las posibles proteínas empleadas como antígenos, de las cuales únicamente se tiene sustento mediante pruebas técnicas para vacunas que comprenden variantes glucosiladas de hemaglutinina (HA). Se sugiere restringir la solicitud a una razonable generalización del tipo de composiciones inmunogénicas que han sido producidas y comprobadas, es decir, que se encuentran debidamente sustentadas.
3. Las reivindicaciones 3 y 24 no son claras porque el término “*sitio de O-glucosilación o N-glucosilación*” es ambiguo y no establece las características de los sitios sobre los cuales se da la glucosilación. Se sugiere a la solicitante que defina de manera concreta la secuencia de dichos sitios.
4. Las reivindicaciones 5 a 8, 17, 18, 20 y 49 no son claras porque no establecen las características técnicas del antígeno, es decir de la glucoproteína. Se sugiere definir dicho antígeno mediante su secuencia de aminoácidos y el patrón de glucosilación encontrado, es importante mencionar que la descripción únicamente presenta información sobre el desarrollo de vacunas o composiciones inmunogénicas que comprenden una hemaglutinina monoglicosilada con N-acetilglucosamida en los residuos de asparagina (Figura 5), por lo que se sugiere a la solicitante que se limite a estas modalidades.
5. Las reivindicaciones 9 y 29 no son claras porque el término “*región Sa*” no define de manera concreta las características de la región sobre la cual se da la glucosilación. Se sugiere a la solicitante que defina de manera concreta la secuencia de dicho sitio.
6. La reivindicación 10 no es clara porque el término “*parcial*” no limita de manera concreta la materia reclamada, toda vez que no es posible interpretar dicho término sin conocer la estructura del antígeno. Se sugiere eliminar dicho término e incluir las características técnicas de los patrones de glucosilación de la proteína.

7. Las reivindicaciones 11, 12, 24, 28, 31, 38 y 43 no son claras porque caracterizan la materia reclamada en términos de resultados a alcanzar, tales como: la especificidad y la afinidad, la inducción de producción de anticuerpos de unión a virus respiratorios, la capacidad de unión a un receptor siliado, la actividad antivírica significativa contra cepas del virus de la gripa o la capacidad de mantener una conformación terciaria. Dichos resultados no son características técnicas esenciales de la materia reclamada, por lo que se sugiere que sean eliminados del capítulo reivindicatorio o que sean remplazados por las características que permiten alcanzarlos.
8. La reivindicación 16 no es clara porque menciona la modificación en la región transmembranal de la proteína HA, sin embargo no menciona en que consiste dicha modificación, sino que la caracteriza únicamente en términos de los resultados obtenidos al realizar dicha modificación. Se sugiere indicar en que consiste dicha modificación de manera concreta y eliminar la referencia a los resultados a alcanzar.
9. La reivindicación 18 no es clara porque hace referencia a una vacuna que presenta las mismas características técnicas esenciales de la reivindicación 1, sin embargo es independiente de la misma. Se sugiere a la solicitante que modifique la dependencia de dicha reivindicación o que establezca de manera concreta la diferencia entre las modalidades reclamadas.
10. Las reivindicaciones 19 y 39 no son claras porque definen de manera general al coadyuvante presente en la vacuna reclamada. Se sugiere a la solicitante incluir la definición de dicho adyuvante de acuerdo a sus ingredientes y proporciones.
11. Las reivindicaciones 21 a 23 no son claras porque definen únicamente el origen de la glucoproteína presente en la composición inmunológica, sin embargo esta no es una característica esencial de la misma. Se reitera a la solicitante que el antígeno debe estar caracterizado por su secuencia de aminoácidos, los sitios de glucosilación y el patrón de carbohidratos presentes en la misma.
12. La reivindicación 40 no es clara porque no define el método reclamado ni las características del mismo, toda vez que:
  - Reclama de forma general un “*método de fabricación*” mas no define el objeto fabricado o producido.
  - Establece las etapas que lo conforman empleando términos y expresiones generales como: “*glucoproteína de superficie vírica glucosilada*”, “*fragmento inmunológicamente activo*”, “*eliminar una porción*” y mediante resultados a alcanzar tales como la conformación de la proteína lograda luego del procesamiento. Se sugiere incluir las características técnicas del antígeno de acuerdo a la secuencia de aminoácidos de la glucoproteína o de sus fragmentos, incluyendo los residuos sobre los que se da la glicosilación y los patrones de carbohidratos que hacen parte de la misma, así como las características del tratamiento mediante la cual se da la eliminación de la porción de glicanos.
13. La reivindicación 41 no es clara porque menciona de manera general “*métodos químicos o enzimáticos*” los cuales no se encuentran soportados por la descripción, en donde únicamente se encuentra sustento para el tratamiento con enzimas tales como neuramidasa y Endoglucosidasa H (Ejemplo 2). Se sugiere restringir la solicitud a una razonable generalización del tipo de procesos que ha

sido probado, es decir, que se encuentran debidamente sustentados e incluir las características técnicas de dicho método.

14. La reivindicación 42 no es clara porque no define las características del método reclamado, toda vez que establece las etapas que lo conforman empleando términos y expresiones generales como: “proveer una célula huésped eucariota”, “constructo apropiado”, “polipéptido recombinante” y “aislar el polipéptido”. Se sugiere incluir las características técnicas de la célula huésped de acuerdo a su número ATCC o certificado de depósito, del constructo de acuerdo a su secuencia de nucleótidos y del método de aislamiento.
15. Las reivindicaciones 44 y 45 no son claras porque no definen las condiciones en las que se da la digestión de los carbohidratos, tales como la temperatura, el pH o la concentración de la enzima, la naturaleza del “método químico” con el cual se logra la digestión selectiva de los carbohidratos. Se sugiere establecer dichas características, las cuales limitan el método reclamado y por lo tanto son determinantes para la comparación con el estado de la técnica. Por otra parte, la descripción únicamente menciona el uso de métodos químicos para la síntesis de glucopéptidos HA (Ej. 16), mientras que no se demuestra su uso para la eliminación de glicanos.

Si bien en el capítulo reivindicatorio se mencionan fragmentos de la proteína de superficie vírica, la descripción no proporciona información que demuestre el desarrollo de composiciones inmunogénicas o vacunas que comprendan dichos fragmentos así como tampoco pruebas técnicas del efecto antigénico de los mismos, por lo que se sugiere a la solicitante que elimine este tipo de estructuras del capítulo reivindicatorio.

El capítulo reivindicatorio no es conciso ya que presenta reivindicaciones independientes de la misma categoría que comparten las mismas características técnicas esenciales, esto es evidente al comparar las reivindicaciones 1 y 18, así como las reivindicaciones dependientes de cada una de ellas. Se sugiere eliminar las reivindicaciones redundantes y presentar una reivindicación independiente por categoría de producto o procedimiento.

Se sugiere hacer las correcciones o aclaraciones pertinentes.

### Descripción (Art 28 D 486)

La descripción no divulga la invención de manera suficientemente completa para que la persona del oficio normalmente versada en la materia pueda ejecutarla ó reproducirla, puesto que si bien se mencionan composiciones inmunogénicas que comprenden fragmentos de proteínas de superficie vírica, no proporciona información que demuestre el desarrollo de dichas composiciones así como tampoco se presentan pruebas técnicas del efecto antigénico de dichas composiciones.

Se requiere a la solicitante hacer las aclaraciones necesarias.

## 6. Determinación del Estado de la Técnica

El estado de la técnica se determinó con base en la fecha de presentación de la solicitud de prioridad: 27 de marzo de 2009, y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

| Ítem | Documento             | Título                          | Fecha de Publicación |
|------|-----------------------|---------------------------------|----------------------|
| D1   | Treanor <i>et al.</i> | “Safety and immunogenicity of a | 08/Febrero/2001      |

|    |                                                                       |                                                                                                                                       |                   |
|----|-----------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------|-------------------|
|    | Vaccine (2001),<br>Vol. 19, No. 13-14,<br>Pág.1732–1737               | recombinant hemagglutinin vaccine for H5<br>influenza in humans”                                                                      |                   |
| D2 | Wang <i>et al.</i><br>Vaccine, Vol. 24,<br>No. 12, Pág. 2176–<br>2185 | “Expression and purification of an<br>influenza hemagglutinin—one step closer<br>to a recombinant protein-based influenza<br>vaccine” | 10/Noviembre/2005 |
| D3 | WO 2007150054                                                         | “Immunogenic compositions having<br>altered glycosylations”                                                                           | 27/Diciembre/2007 |

El documento D1<sup>1</sup> divulga un estudio de la seguridad e inmunogenicidad de una vacuna de una hemagglutinina recombinante (H5 rHA) derivada de un aislado humano (Pág. 1733, Col. 1, líneas 21-22).

El documento D2<sup>2</sup> divulga un proceso rápido capaz de purificar una proteína hemagglutinina recombinante rHA a partir de un fermentado (Pág. 2178, Col. 1, líneas 53-55).

El documento D3<sup>3</sup> divulga métodos de producción e identificación de composiciones para ser usadas como vacunas, donde éstas comprenden moléculas inmunogénicas parcial o completamente desglicosilado (Resumen).

**7. Examen de Patentabilidad (Art 14 D486)**

**Novedad (Art 16 D486)**

La reivindicación 40 consiste en: *“Un método de fabricación que comprende:”* (Radicado N°. 11-144904-00009-00000).

Comparación de las características técnicas esenciales, con las del estado de la técnica:

| Características esenciales                                                                                                                        | D2                                                                                                                                                         |
|---------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Método de fabricación:                                                                                                                            | Deglicosilación (Pág. 2178, Col. 2 <i>Deglycosylation</i> )                                                                                                |
| Proveer una glucoproteína de superficie vírica glucosilada del virus de la gripe o un fragmento inmunológicamente activo de la misma, y           | Proteína recombinante purificada rHA (hemagglutinina del virus de la gripe)                                                                                |
| Eliminar una porción de los glicanos ligados a uno o más sitios de glucosilación en la proteína para formar una proteína parcialmente glucosilada | Tratamiento con tripsina y posteriormente con PNGasa F o Endo H, que logra la deglicosilación limitada de la proteína hemagglutinina del virus de la gripe |

De acuerdo con la tabla anterior, las características esenciales del método de la reivindicación 40 habían sido divulgadas previamente en el documento D2, por lo tanto, la materia reivindicada no es nueva.

Adicionalmente, el documento D2 enseña que el método comprende la clonación y la expresión en células de insecto (Pág. 2177, Col. 2, *Cloning and expression of influenza HA*), y el tratamiento enzimático de dicha proteína (Pág. 2178, Col. 2, *Deglycosylation*).

1 Safety and immunogenicity of a recombinant hemagglutinin vaccine for H5 influenza in humans. Treanor *et al.* Vaccine (2001), Vol. 19, No. 13-14, Pág.1732–1737. Disponible en: <http://www.sciencedirect.com/science/article/pii/S0264410X00003959>.  
2 Expression and purification of an influenza hemagglutinin—one step closer to a recombinant protein-based influenza vaccine Wang *et al.* Vaccine, Vol. 24, No. 12, Pág. 2176–2185. Disponible en <http://www.sciencedirect.com/science/article/pii/S0264410X05011308>.  
3 Disponible en: [http://worldwide.espacenet.com/publicationDetails/originalDocument?CC=WO&NR=2007150054A2&KC=A2&FT=D&ND=&date=20071227&DB=&&locale=en\\_EP](http://worldwide.espacenet.com/publicationDetails/originalDocument?CC=WO&NR=2007150054A2&KC=A2&FT=D&ND=&date=20071227&DB=&&locale=en_EP).

En consecuencia, la reivindicación 40 no es nueva o carece de novedad, según lo divulgado en el documento D2.

Las reivindicaciones dependientes 41 – 44, 46 y 49, no mencionan alguna característica que haga nuevo al método de la reivindicación 40, por lo cual se considera que no son nuevas.

### Nivel inventivo (Art 18 D486)

La reivindicación 1 consiste en: *“Una composición inmunogénica que comprende: una glucoproteína parcialmente glucosilada del virus de la gripe o un fragmento de la misma, en donde la glucoproteína incluye uno o más sitios de glucosilación y en donde la glucoproteína antígeno se glucosila parcialmente en uno o más sitios de glucosilación, en comparación con un estado de glucosilación en el sitio de la glucoproteína antígeno cuando se expresa en una célula huésped”* (Radicado N°. 11-144904-00009-00000).

Comparación de las características técnicas esenciales, con las del estado de la técnica:

| Características esenciales                                                           | D1                                                                     | D2                                                                                      |
|--------------------------------------------------------------------------------------|------------------------------------------------------------------------|-----------------------------------------------------------------------------------------|
| Composición inmunogénica                                                             | Vacuna que comprende (Pág. 1733, Col. 1, <i>Vaccines and viruses</i> ) | Vacuna contra la influenza (Pág.2176, Resumen)                                          |
| Glucoproteína parcialmente glucosilada del virus de la gripe o fragmento de la misma | Una proteína recombinante H5 HA expresada en baculovirus               | Proteína hemaglutinina recombinante rHA                                                 |
| Comprende una mono-glucosilación, una di-glucosilación o una tri-glucosilación       | No se menciona                                                         | Que esta parcial o totalmente deglicosilada (Pág. 2178, Col. 2 <i>Deglycosylation</i> ) |

El documento D1 se considera el estado de la técnica más cercano a la invención definida en la reivindicación 1 porque divulga una vacuna que comprende una proteína hemaglutinina recombinante H5 HA derivada de un aislado humano H5N1 (Pág. 1733, líneas 21-22).

La diferencia entre la invención, definida en la reivindicación 1 y la vacuna del documento D1, se encuentra en la estructura del componente inmunogénico, puesto que en la solicitud este se encuentra glucosilado parcialmente, mientras que en la vacuna del documento D1 no se menciona el estado de glucosilación de la proteína hemaglutinina.

El efecto técnico asociado a la eliminación parcial de los glicosidos en la proteína hemaglutinina de la composición reclamada es un aumento en la respuesta inmune, es decir una inmunogenicidad mejorada (Pág. 45-46, Párr. [0108]; Fig. 5).

Por lo tanto, el problema técnico objetivo que pretende resolver esta invención se puede formular como la necesidad de modificar la vacuna divulgada en el documento D1 para mejorar respuesta inmune.

Sin embargo, la modificación por deglucosilación de la proteína hemaglutinina de la influenza, se había divulgado en el documento D2, que enseña un sistema de monitoreo de la expresión de la hemaglutinina recombinante rHA y un proceso de purificación rápido, (Pág. 2176, Resumen). Este documento enseña la deglucosilación total y parcial lograda por el tratamiento con PNGasa o Endo H (Págs. 2178, Col. 2,

*Deglycosylation*).

En consecuencia, la persona del oficio normalmente versada en la materia, modificaría la glucosilación de la proteína hemaglutinina de acuerdo a lo enseñado en el documento D2, en la vacuna divulgada por el documento D1, para llegar así al objeto de la reivindicación 1 de la solicitud, más aun cuando la persona del oficio normalmente versada en la materia conoce que la eliminación de los residuos de azúcar posee un efecto en la inmunogenicidad de la hemaglutinina<sup>4</sup>. Por lo cual se considera obvia.

Puesto que las reivindicaciones dependientes 2 – 5, así como las reivindicaciones 18-26, 38, y 39 no mencionan características inventivas adicionales tampoco se pueden considerar inventivas.

Las reivindicaciones 1 – 5, 18-26, 38 y 39 cumplen el requisito de novedad (Art. 16), pero no cumplen con el requisito de nivel inventivo (Art. 18).

La reivindicación 45 consiste en: *“El método de la reivindicación 40, en donde la porción de los glicanos se elimina utilizando un método químico que digiere selectivamente los carbohidratos en un sitio de glucosilación N- o O- enlazado”* (Radicado N°. 11-144904-00009-00000).

Comparación de las características técnicas esenciales, con las del estado de la técnica:

| Características esenciales                                                                                                                                                                                                                                                                                                                                                       | D2                                                                                                                                                        | D3                                                                                                                                                                                                                       |
|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Método de fabricación:                                                                                                                                                                                                                                                                                                                                                           | Deglucosilación (Pág. 2178, Col. 2 <i>Deglycosylation</i> )                                                                                               | Un método para la identificación de una composición inmunogénica que comprende (Pág. 17, Reivindicación 1):                                                                                                              |
| Proveer una glucoproteína de superficie vírica glucosilada del virus de la gripe o un fragmento inmunológicamente activo de la misma, y                                                                                                                                                                                                                                          | Proteína recombinante purificada rHA (hemaglutinina del virus de la gripe)                                                                                | Material de partida de un patógeno<br><br>Donde el dicho material es un material de envoltura vírico, y el patógeno se selecciona de un grupo que incluye el virus de la influenza A (Pág. 17, reivindicaciones 10 y 12) |
| Eliminar una porción de los glicanos ligados a uno o más sitios de glucosilación en la proteína para formar una proteína parcialmente glucosilada, en donde uno, dos o tres glicanos permanecen ligados al sitio parcialmente glucosilado en la proteína utilizando un método químico que digiere selectivamente los carbohidratos en un sitio de glucosilación N- o O- enlazado | Tratamiento con tripsina y posteriormente con PNGasa F o Endo H, que logra la deglucosilación limitada de la proteína hemaglutinina del virus de la gripe | Serie de deglucosilaciones.<br><br>Donde la deglucosilación se realiza químicamente (Pág. 17, Reivindicación 5)                                                                                                          |

El documento D2 se considera el estado de la técnica más cercano a la invención definida en la reivindicación 45 porque divulga un método para la deglucosilación de una proteína hemaglutinina del virus de la gripe que comprende la clonación y la expresión en células de insecto (Pág. 2177, Col. 2, *Cloning and expression of influenza HA*), y la eliminación de los glicanos presente en dicha proteína (Pág. 2178, Col. 2,

<sup>4</sup>Munk *et al.* Carbohydrate masking of an antigenic epitope of influenza virus haemagglutinin independent of oligosaccharide size. *Glycobiology* (1992) Vol. 2 No. (3), Pág. 233-240.

Deglycosylation).

La diferencia entre la invención, definida en la reivindicación 45 y el método del documento D2, se encuentra en el medio empleado para hacer la eliminación de los glicanos, puesto que en la reivindicación 45 se establece que esto se logra por medio de tratamiento “químico”, mientras que en el método del documento D2 este se logra por un tratamiento enzimático.

No existe evidencia de un efecto técnico asociado a la modificación del agente que elimina o degrada los glicanos presentes en la glucoproteína de superficie.

Por lo tanto, el problema técnico objetivo que pretende resolver esta invención se puede formular como la necesidad de proporcionar métodos alternativos al divulgado en el documento D2 para eliminar los glicanos de una glucoproteína del virus de la gripe.

Sin embargo, la utilización de métodos químicos para deglucosilación parcial de un componente glucoproteico en un patógeno, se había divulgado en el documento D3, que se refiere a divulga métodos de producción e identificación de composiciones para ser usadas como vacunas, donde éstas comprenden moléculas inmunogénicas parcial o completamente deglucosiladas (Resumen).

En consecuencia, la persona del oficio normalmente versada en la materia, incluiría el tratamiento con agentes químicos de acuerdo a lo enseñado en el documento D3, en el proceso de deglucosilación de la hemaglutinina del virus de la gripe enseñado en el documento D2 para llegar así al objeto de la reivindicación 45 de la solicitud. Por lo cual se considera obvia.

Puesto que las reivindicaciones dependientes 47 y 48 no mencionan características inventivas adicionales tampoco se pueden considerar inventivas.

Las reivindicaciones 45, 47 y 48 cumplen el requisito de novedad (Art. 16), pero no cumplen con el requisito de nivel inventivo (Art. 18).

## 8. Conclusión

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

| Ítem | Documento                                                               | Reivindicaciones afectadas        | Requisito que afecta |
|------|-------------------------------------------------------------------------|-----------------------------------|----------------------|
| D1   | Treanor <i>et al.</i> Vaccine (2001), Vol. 19, No. 13-14, Pág.1732–1737 | 1 – 5, 18-26, 38 y 39             | Nivel Inventivo      |
| D2   | Wang <i>et al.</i> Vaccine, Vol. 24, No. 12, Pág. 2176–2185             | 41 – 44, 46 y 49                  | Novedad              |
|      |                                                                         | 1 – 5, 18-26, 38, 39, 45, 47 y 48 | Nivel Inventivo      |
| D3   | WO 2007150054                                                           | 45, 47 y 48                       | Nivel inventivo      |

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

Si como respuesta a este requerimiento el solicitante llegara a modificar: i) el capítulo reivindicatorio, ii) la descripción de la invención a proteger, iii) o presente un nuevo capítulo reivindicatorio, y desee que se realice un nuevo examen de patentabilidad a esta modificación, no deberá pagar la tasa de modificación voluntaria pero sí la correspondiente a la tasa de examen de patentabilidad. En todo caso, el número de requerimientos por no patentabilidad estará supeditado a que el término del estudio de patentabilidad no sea superior a 18 meses, contados desde la publicación de la solicitud de patente de invención. Adicionalmente, se le recuerda al solicitante que si como respuesta a este requerimiento llegara a modificar el capítulo reivindicatorio y éste contenga más de diez (10) reivindicaciones deberá pagar la tasa por reivindicación adicional.

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



**JOSÉ LUIS SALAZAR LÓPEZ**  
Director de Nuevas Creaciones (c)

El anterior requerimiento fue notificado por fijación en lista, de fecha 2016-05-17

Elaboró: Ruth Andrea Rodriguez  
Revisó: Diego Armando Galvis Sanchez

|                                                                                                       |                                                         |                                                                                                                                                                                                                                 |                 |                                   |                                                                  |         |
|-------------------------------------------------------------------------------------------------------|---------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------|-----------------------------------|------------------------------------------------------------------|---------|
| INFORME DE BÚSQUEDA DE PATENTES PARA EL No de Radicación: 11-144904-00009                             |                                                         |                                                                                                                                                                                                                                 |                 |                                   |                                                                  |         |
| CRITERIOS PARA LA BÚSQUEDA                                                                            |                                                         |                                                                                                                                                                                                                                 |                 |                                   |                                                                  |         |
| Palabras clave utilizadas                                                                             |                                                         | (hemagglutinin or HA) AND (vaccine OR immunological composition) glycosilation                                                                                                                                                  |                 |                                   |                                                                  |         |
| CLASIFICACIONES INTERNACIONALES UTILIZADAS PARA LA BÚSQUEDA                                           |                                                         | A61K 39/12                                                                                                                                                                                                                      |                 |                                   |                                                                  |         |
| BASES DE DATOS CONSULTADAS.                                                                           |                                                         |                                                                                                                                                                                                                                 |                 |                                   |                                                                  |         |
| Google Patents                                                                                        |                                                         | Espacenet                                                                                                                                                                                                                       | NCBI-Blast      |                                   | The Lens                                                         | Patbase |
| INFORMES DE EXÁMENES REALIZADOS EN OTRAS OFICINAS (documentos patentes, literatura no patente etc...) |                                                         |                                                                                                                                                                                                                                 |                 |                                   |                                                                  |         |
| Oficina                                                                                               | Fecha del examen o de reporte de búsqueda de documentos | Anterioridades encontradas para el examen o reportados en el examen de búsqueda(relacionarlos todos)                                                                                                                            | Reiv. afectadas | RELEVANCIA (requisito que afecta) | FUERON TENIDOS EN CUENTA PARA EL EXAMEN EN LA OFICINA COLOMBIANA |         |
|                                                                                                       |                                                         |                                                                                                                                                                                                                                 |                 |                                   | SI                                                               | NO      |
| Europea                                                                                               |                                                         | WO2007015005                                                                                                                                                                                                                    | 40-49           | NI                                | X                                                                |         |
|                                                                                                       |                                                         | US6908617                                                                                                                                                                                                                       |                 |                                   |                                                                  | X       |
|                                                                                                       |                                                         | WO8603224                                                                                                                                                                                                                       |                 |                                   |                                                                  | X       |
|                                                                                                       |                                                         | US6103238                                                                                                                                                                                                                       |                 |                                   |                                                                  | X       |
|                                                                                                       |                                                         | US2009017052                                                                                                                                                                                                                    |                 |                                   |                                                                  | X       |
|                                                                                                       |                                                         | US2008241918                                                                                                                                                                                                                    |                 |                                   |                                                                  | X       |
|                                                                                                       |                                                         | US2004047877                                                                                                                                                                                                                    |                 |                                   |                                                                  | X       |
|                                                                                                       |                                                         | US2005075292                                                                                                                                                                                                                    |                 |                                   |                                                                  | X       |
|                                                                                                       |                                                         | US2007224205                                                                                                                                                                                                                    |                 |                                   |                                                                  | X       |
|                                                                                                       |                                                         | US2008050402                                                                                                                                                                                                                    |                 |                                   |                                                                  | X       |
|                                                                                                       |                                                         | US2002045594                                                                                                                                                                                                                    |                 |                                   |                                                                  | X       |
|                                                                                                       |                                                         | US2008118529                                                                                                                                                                                                                    |                 |                                   |                                                                  | X       |
|                                                                                                       |                                                         | WO2008112017                                                                                                                                                                                                                    |                 |                                   |                                                                  | X       |
|                                                                                                       |                                                         | Arora, <i>et al.</i> , Genomic Study of Hemagglutinins of Swine Influenza (H1 N1) Viruses Associated with Acute and Chronic Respiratory Diseases in Pigs. <i>Archives of Virology</i> . (1997), vol. 142, no. 2, Pág. 401 - 412 |                 |                                   |                                                                  | X       |
|                                                                                                       |                                                         | Bolmstedt A. <i>et al.</i> Enhanced immunogenicity of a human immunodeficiency virus type 1 env DNA vaccine by manipulating N-glycosylation signals. <i>Vaccine</i> . (2001) Vol:                                               |                 |                                   |                                                                  | X       |

|                                                                                                                                                                                                  |                                                                          |                                    |              |      |                 |   |
|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------|------------------------------------|--------------|------|-----------------|---|
|                                                                                                                                                                                                  |                                                                          | 20, Nr: 3-4, Page(s):<br>397 – 405 |              |      |                 |   |
| USPTO                                                                                                                                                                                            |                                                                          | US6803225                          |              |      |                 | X |
|                                                                                                                                                                                                  |                                                                          | US6103238                          |              |      |                 | X |
|                                                                                                                                                                                                  |                                                                          | US2002045594                       |              |      |                 | X |
|                                                                                                                                                                                                  |                                                                          | US2004047877                       |              |      |                 | X |
|                                                                                                                                                                                                  |                                                                          | US2005075292                       |              |      |                 | X |
|                                                                                                                                                                                                  |                                                                          | US6908617                          |              |      |                 | X |
|                                                                                                                                                                                                  |                                                                          | US2007224205                       |              |      |                 | X |
|                                                                                                                                                                                                  |                                                                          | US2008050402                       |              |      |                 | X |
|                                                                                                                                                                                                  |                                                                          | US2008118529                       |              |      |                 | X |
|                                                                                                                                                                                                  |                                                                          | US2008241918                       |              |      |                 | X |
|                                                                                                                                                                                                  |                                                                          | US2009017052                       |              |      |                 | X |
|                                                                                                                                                                                                  |                                                                          | US6803225                          |              |      |                 | X |
| DOCUMENTOS ADICIONALES ENCONTRADOS POR EL EXAMINADOR DE ACUERDO A LOS CRITERIOS DE BÚSQUEDA.                                                                                                     |                                                                          |                                    |              |      |                 |   |
| Wang <i>et al.</i> Expression and purification of an influenza hemagglutinin—one step closer to a recombinant protein-based influenza vaccine. <i>Vaccine</i> , Vol. 24, No. 12, Pág. 2176–2185. |                                                                          |                                    |              |      |                 |   |
| Treanor <i>et al.</i> Safety and immunogenicity of a recombinant hemagglutinin vaccine for H5 influenza in humans. <i>Vaccine</i> (2001), Vol. 19, No. 13-14, Pág.1732–1737.                     |                                                                          |                                    |              |      |                 |   |
|                                                                                                                                                                                                  |                                                                          |                                    |              |      |                 |   |
| BÚSQUEDA DE SECUENCIAS                                                                                                                                                                           |                                                                          |                                    |              |      |                 |   |
| Número de depósito / patente y N° de secuencia<br>% de identidad                                                                                                                                 |                                                                          |                                    |              |      |                 |   |
| SEQ ID NO<br>(de la solicitud)                                                                                                                                                                   | NCBI                                                                     | The Lens                           | EMBL-<br>EBI | DDJB | Genome<br>Quest |   |
| 4                                                                                                                                                                                                | 98% de identidad y 92% de<br>cobertura<br>SEQ ID No. 11 de US<br>7504109 |                                    |              |      |                 |   |
|                                                                                                                                                                                                  | ACP82359.1 GI:228220380                                                  |                                    |              |      |                 |   |
| 6                                                                                                                                                                                                | 80% de identidad y 90% de<br>cobertura<br>SEQ ID No. 28 de US<br>7736642 |                                    |              |      |                 |   |
|                                                                                                                                                                                                  | ADR83840.1 GI:314122541                                                  |                                    |              |      |                 |   |
| 8                                                                                                                                                                                                | 98% de identidad y 92% de<br>cobertura<br>SEQ ID No. 67 de US<br>7459162 |                                    |              |      |                 |   |
|                                                                                                                                                                                                  | ACK31212.1 GI:217307512                                                  |                                    |              |      |                 |   |
| 10                                                                                                                                                                                               | 95% de identidad y 92% de<br>cobertura<br>SEQ ID No. 65 de US<br>7459162 |                                    |              |      |                 |   |
|                                                                                                                                                                                                  | ACK31210.1 GI:217307510                                                  |                                    |              |      |                 |   |

#### Búsqueda de secuencias

SEQ ID No. 4

[Download](#) [GenPept](#) [Graphics](#)

Sequence 11 from patent US 7504109

Sequence ID: [gb|ACP82359.1|](#) Length: 564 Number of Matches: 1

[▶ See 7 more title\(s\)](#)

Range 1: 1 to 523 [GenPept](#) [Graphics](#)

[▼](#) Next Match [▲](#) Previous Match

| Score           | Expect                                                         | Method                       | Identities   | Positives    | Gaps      |
|-----------------|----------------------------------------------------------------|------------------------------|--------------|--------------|-----------|
| 1071 bits(2770) | 0.0                                                            | Compositional matrix adjust. | 513/523(98%) | 516/523(98%) | 1/523(0%) |
| Query 1         | MEKIVLLFAIVSLVKSDQICIGSHANNSTEQVDTIMEKNVTVTTHAQDILEKTHNGKLCDL  |                              |              | 60           |           |
| Sbjct 1         | MEKIVLLFAIVSLVKSDQICIG HANNSTEQVDTIMEKNVTVTTHAQDILEK HNGKLCDL  |                              |              | 60           |           |
| Query 61        | DGVKPLILRDCSVAGNLLGNPMCMDEFINVPEWSYIVEKANPANDLCYPGDFNDYEEELKHL |                              |              | 120          |           |
| Sbjct 61        | DGVKPLILRDCSVAGNLLGNPMCMDEFINVPEWSYIVEKANP NDLCYPGDFNDYEEELKHL |                              |              | 120          |           |
| Query 121       | LSRINHFEKIQIIPKSSWSSEASGVSSACPYQKGSSFFRNWVWLKKNSTYPTIKRSY      |                              |              | 180          |           |
| Sbjct 121       | LSRINHFEKIQIIPKSSWSSEAS GVSSACPYQKGSSFFRNWVWLKKNSTYPTIKRSY     |                              |              | 180          |           |
| Query 181       | NNTNQEDLLVLWGIHPNDAAEQTKLYQNPTTYSVGTSTLNQRLVPKIATRSKVNQSG      |                              |              | 240          |           |
| Sbjct 181       | NNTNQEDLLVLWGIHPNDAAEQTKLYQNPTTYSVGTSTLNQRLVP+IATRSKVNQSG      |                              |              | 240          |           |
| Query 241       | RMEFFWTILKPNDAINFESNGNFIAPYAYKIVKKGDSTIMKSELEYGNCNTKCQTPMGA    |                              |              | 300          |           |
| Sbjct 241       | RMEFFWTILKPNDAINFESNGNFIAPYAYKIVKKGDSTIMKSELEYGNCNTKCQTPMGA    |                              |              | 300          |           |
| Query 301       | INSSMPFHNIHPLTIGECPKYVKSRLVLATGLRNSPQRE-RGLFGAIAGFIEGGWQGMV    |                              |              | 359          |           |
| Sbjct 301       | INSSMPFHNIHPLTIGECPKYVKSRLVLATGLRNSPQRE RGLFGAIAGFIEGGWQGMV    |                              |              | 360          |           |
| Query 360       | DGWYGYHHSNEQSGSYAADKESTQKAIDGVTNKVNSIIDKMNTQFEAVGREFNNLERRIE   |                              |              | 419          |           |
| Sbjct 361       | DGWYGYHHSNEQSGSYAADKESTQKAIDGVTNKVNSIIDKMNTQFEAVGREFNNLERRIE   |                              |              | 420          |           |
| Query 420       | NLNKKMEDGFLDWWTYNAELLVLMENERTLDFHDSNVKNLYDKVRLQLRDNALGNGCF     |                              |              | 479          |           |
| Sbjct 421       | NLNKKMEDGFLDWWTYNAELLVLMENERTLDFHDSNVKNLYDKVRLQLRDNALGNGCF     |                              |              | 480          |           |
| Query 480       | EFYHKCDNECMESVRNGTYDYPQYSEEARLKREEISGVDIRSL                    |                              | 522          |              |           |
| Sbjct 481       | EFYHKCDNECMESVRNGTYDYPQYSEEARLKREEISGV + S+                    |                              | 523          |              |           |

SEQ ID No. 6

[Download](#) [GenPept](#) [Graphics](#)

Sequence 28 from patent US 7736642

Sequence ID: [gb|ADR83840.1|](#) Length: 617 Number of Matches: 1

Range 1: 18 to 528 [GenPept](#) [Graphics](#)

[Next Match](#) [Previous Match](#)

| Score          | Expect                                                         | Method                       | Identities   | Positives    | Gaps      |
|----------------|----------------------------------------------------------------|------------------------------|--------------|--------------|-----------|
| 887 bits(2293) | 0.0                                                            | Compositional matrix adjust. | 410/512(80%) | 459/512(89%) | 1/512(0%) |
| Query 14       | TANADTLCIGYHANNSTDTVDTVLEKNMTVTHSVNLLLEDKHNGKLCCKLRGVAPLHLGKCN |                              |              |              | 73        |
| Sbjct 18       | +A+ DT+CIGYHANNSTDTVDTVLEKNMTVTHSVNLLLED HNGKLC+L+G+APL LGKCN  |                              |              |              | 77        |
| Query 74       | IAGWILGNPECESLSTASSWSYIVETPSSDNGTCYPGDFIDYEELREQLSSVSSFERFEI   |                              |              |              | 133       |
| Sbjct 78       | IAGW+LGNPEC+ L SWSYIVETP+S+NG CYPGDFIDYEELREQLSSVSSFERFEI      |                              |              |              | 137       |
| Query 134      | FPKTSSWPNDHNSNKGVTAACPHAGAKSFYKNLIWLKKGNSYPKLSKSYINDKGKEVLVL   |                              |              |              | 193       |
| Sbjct 138      | FPK SSWPNH++N GVTAAC H G SFY+NL+WL +K SYPKL SY+N KGKEVLVL      |                              |              |              | 196       |
| Query 194      | WGIHHPSTADQOSLYQNADAYVFGSSRYSKKFKPEIAIRPKVRDQEGRMNYWTLVPEP     |                              |              |              | 253       |
| Sbjct 197      | WGIHHP S +QO+LYQN +AYV V +S Y+++F PEIA RPKVRDQ GRMNYWTL++P     |                              |              |              | 256       |
| Query 254      | GDKITFEATGNLVPRYAFAMERNAGSGIIISDTPVHDCNTTCQTPKGAINSLPQNIH      |                              |              |              | 313       |
| Sbjct 257      | GD I FEA GNL+ P YAF+ R GSGII S+ ++CNT CQTP GAIN+SLP+QNIH       |                              |              |              | 316       |
| Query 314      | PITIGKCPKYVKSTKLRLATGLRNIPSIQSRGLFGAIAAGFIEGGWTGMVDGWYGYHHQNE  |                              |              |              | 373       |
| Sbjct 317      | P+TIG+ PKYV+S KLR+ TGLRNIPSIQSRGLFGAIAAGFIEGGWTGM+DGYGYHHQNE   |                              |              |              | 376       |
| Query 374      | QSGGYAADLKSTQNAIDEITNKVNSVIEKMNTQFTAVGKEFNHLEKRIENLNKKVDDGFL   |                              |              |              | 433       |
| Sbjct 377      | QSGGYAAD KSTQNAI+ ITNKVN+VIEKMN QFTAVGKEFN LEKR+ENLNKKVDDGFL   |                              |              |              | 436       |
| Query 434      | DIWTYNAELLVLENERLTDYHDSNVKNLYEKVRSQKLNNAKEIGNGCFEFYHKCDNTCM    |                              |              |              | 493       |
| Sbjct 437      | DIWTYNAELLVLENERLTDHDSN+KNLYEKV+SQKLNNAKEIGNGCFEFYHKCDN CM     |                              |              |              | 496       |
| Query 494      | ESVKNGTYDYPKYSEEAKLNREEIDGVDIRSL                               |                              | 525          |              |           |
| Sbjct 497      | ESV+NGTYDYPKYSEE+KLNRE++DGV + S+                               |                              |              |              |           |
|                | ESVRNGTYDYPKYSEESKLNREKVDGVKLESM                               |                              | 528          |              |           |

SEQ ID No. 8

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Sequence 67 from patent US 7459162

Sequence ID: [gb|ACK31212.1](#) Length: 565 Number of Matches: 1

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Range 1: 1 to 524 [GenPept](#) [Graphics](#)

[Next Match](#) [Previous Match](#)

| Score           | Expect                                                       | Method                       | Identities   | Positives    | Gaps      |
|-----------------|--------------------------------------------------------------|------------------------------|--------------|--------------|-----------|
| 1066 bits(2758) | 0.0                                                          | Compositional matrix adjust. | 513/525(98%) | 517/525(98%) | 1/525(0%) |
| Query 1         | MKVLLVLLCTFTATYADTICIGYHANNSTDVDTVLEKNVTVTHSVNLLLEDHNGKLCL   |                              |              | 60           |           |
| Sbjct 1         | MK KLLVLLCTFTATYADTICIGYHANNSTDVDTVLEKNVTVTHSVNLLLEDHNGKLCL  |                              |              | 60           |           |
| Query 61        | LKGIAPLQLGNCVAGWILGNPECELLISKESWSYIVETPNPENGTCYPGYFADYEELRE  |                              |              | 120          |           |
| Sbjct 61        | LKGIAPLQLGNCVAGWILGNPECELLISKESWSYIVETPNPENGTCYPGYFADYEELRE  |                              |              | 120          |           |
| Query 121       | QLSSVSSFERFEIFPKESSWPHTVTGVSASCSHNGKSSFYRNLLWLTGKNGLYPNLSK   |                              |              | 180          |           |
| Sbjct 121       | QLSSVSSFERFEIFPKESSWP HTVT GVSASCSHNGK+SFYRNLLWLTGKNGLYPNLSK |                              |              | 179          |           |
| Query 181       | SYANNKEKEVLVLWGVHPPNIGDQRALYHTENAYVSVSSHYSRRFTPEIAKRPKVRDQ   |                              |              | 240          |           |
| Sbjct 180       | SYNNKEKEVLVLWGVHPPNIGDQRALYHTENAYVSVSSHYSRRFTPEIAKRPKVRDQ    |                              |              | 239          |           |
| Query 241       | EGRINYWTLLLEPGDTIIFEANGNLIAPRYAFALSRGFGSGIITSNAPMDECDACQTPQ  |                              |              | 300          |           |
| Sbjct 240       | EGRINYWTLLLEPGDTIIFEANGNLIAP YAFALSRGFGSGIITSNAPMDECDACQTPQ  |                              |              | 299          |           |
| Query 301       | GAINSSLFPQNVHPVTIGECPKYVRSTKLRLMTGLRNIPSIQSRGLFGAIAGFIEGGWTG |                              |              | 360          |           |
| Sbjct 300       | GAINSSLFPQNVHPVTIGECPKYVRS AKLRMTGLRNIPSIQSRGLFGAIAGFIEGGWTG |                              |              | 359          |           |
| Query 361       | MVDGWYGYHHQNEQGSYAADQKSTQNAINGITNKVNSVIEKMNTQFTAVGKEFNKLERR  |                              |              | 420          |           |
| Sbjct 360       | MVDGWYGYHHQNEQGSYAADQKSTQNAINGITNKVNSVIEKMNTQFTAVGKEFNKLERR  |                              |              | 419          |           |
| Query 421       | MENLNKKVDDGFLDIWTYNAELLVLLLENERTLDFHDSNVKNLYEKVKSQKNNAKEIGNG |                              |              | 480          |           |
| Sbjct 420       | MENLNKKVDDGFLDIWTYNAELLVLLLENERTLDFHDSNVKNLYEKVKSQKNNAKEIGNG |                              |              | 479          |           |
| Query 481       | CFEFYHKCNDECMESVKNGTYDYPKYSEESKLNREKIDGVDIRSL                |                              | 525          |              |           |
| Sbjct 480       | CFEFYHKCN+ECMESVKNGTYDYPKYSEESKLNREKIDGV + S+                |                              | 524          |              |           |

SEQ ID No. 10

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Sequence 65 from patent US 7459162

Sequence ID: [gb|ACK31210.1](#) Length: 565 Number of Matches: 1

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Range 1: 1 to 524 [GenPept](#) [Graphics](#)

▼ Next Match ▲ Previous Match

| Score           | Expect                                                         | Method                       | Identities   | Positives    | Gaps      |
|-----------------|----------------------------------------------------------------|------------------------------|--------------|--------------|-----------|
| 1040 bits(2690) | 0.0                                                            | Compositional matrix adjust. | 499/525(95%) | 511/525(97%) | 1/525(0%) |
| Query 1         | MKVKLLVLLCTFTATYADTICIGYHANNSTDTVDTVLEKNVTVTHSVNLLLED SHNGKLCL |                              |              |              | 60        |
| Sbjct 1         | MK KLLVLLCTFTATYADTICIGYHANNSTDTVDTVLEKNVTVTHSVNLLLED SHNGKLCL |                              |              |              | 60        |
| Query 61        | LKGIAPLQLGNCSVAGWILGNPECELLISKESWSYIVETPNPENGTCYPGHFADYEELRE   |                              |              |              | 120       |
| Sbjct 61        | LKGIAPLQLGNCSVAGWILGNPECE LISKESWSYIVETPNPENGTCYPG+FADYEELRE   |                              |              |              | 120       |
| Query 121       | QLSSVSSFERFEIFPKESSWPNDVTGVSASCSHNGESSFYRNLLWLTGKNGLYPNLSK     |                              |              |              | 180       |
| Sbjct 121       | QLSSVSSFERFEIFPKESSWP H TVTGV+ASCSHNG+SSFYRNLLWLT KNGLYPNLS    |                              |              |              | 179       |
| Query 181       | SYANNKEKEVLVLWG VHPNIGDQKALYHTENAYVSVSSHYSRKFTPEIAKRPKVRDQ     |                              |              |              | 240       |
| Sbjct 180       | SY NNKEKEVLVLWG VHPNIGDQ+A+YHTENAYVSVSSHYSR+FTPEIAKRPKVR Q     |                              |              |              | 239       |
| Query 241       | EGRINYYWTLLEPGDTIIFEANGNLIAPRYAFALSRGFGSGIINSNAPMDKCDACKQTPQ   |                              |              |              | 300       |
| Sbjct 240       | EGRINYYWTLLEPGDTIIFEANGNLIAP YAFALSRGFGSGII SNAPM++CDACKQTPQ   |                              |              |              | 299       |
| Query 301       | GAINSSLPFQNVHPVTIGECPKYVRS AKLRMTGLRNIPSIQSRGLFGAIAAGFIEGGWTG  |                              |              |              | 360       |
| Sbjct 300       | GAINSSLPFQNVHPVTIGECPKYVRS KLRMTGLRNIPSIQSRGLFGAIAAGFIEGGWTG   |                              |              |              | 359       |
| Query 361       | MVDGWYGYHHQNEQSGGYAADQKSTQNAINGITNKVNSVIEKMNTQFTAVGKEFNKLERR   |                              |              |              | 420       |
| Sbjct 360       | MMDGWYGYHHQNEQSGGYAADQKSTQNAINGITNKVNSVIEKMNTQFTAVGKEFNKLERR   |                              |              |              | 419       |
| Query 421       | MENLNKKVDDGFLDIWNTYNAELLVLENERLDFHDSNVKNLYEKVKSQLKNNAKEIGNG    |                              |              |              | 480       |
| Sbjct 420       | MENLNKKVDDGFLDIWNTYNAELLVLENERLDFHDSNVKNLYEKVKSQLKNNAKEIGNG    |                              |              |              | 479       |
| Query 481       | CFEFYHKCNDECMESVKNGTYDYPKYSEESKLNREKIDGVDIRSL                  |                              | 525          |              |           |
| Sbjct 480       | CFEFYHKCN+ECMESVKNGTYDYPKYSEESKLNREKIDGV + S+                  |                              | 524          |              |           |

## Safety and immunogenicity of a recombinant hemagglutinin vaccine for H5 influenza in humans<sup>☆</sup>

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### Abstract

Recent outbreaks of avian influenza in humans have demonstrated the need for vaccines for influenza viruses with pandemic potential. Recombinant hemagglutinins are an attractive option for such vaccines because they do not require handling potentially highly pathogenic influenza viruses for vaccine production. In order to evaluate the immunogenicity, optimum dosing and timing of administration of a recombinant baculovirus-expressed H5 HA (rH5) in humans, 147 healthy adults were assigned randomly to receive intramuscular rH5 as two doses of 25, 45 or 90 µg each, one dose of 90 µg followed by a dose of 10 µg, or two doses of placebo, at intervals between doses of 21, 28 or 42 days. All doses of rH5 were well tolerated. The rH5 vaccine was modestly immunogenic at high dose. Neutralizing antibody responses to a titer of 1:80 or greater were seen in 23% (14/60) of individuals after a single dose of 90 µg, and in 52% (15/29) after two doses of 90 µg. Varying intervals between doses from 21 to 42 days had no significant effect on antibody responses to vaccination. These results suggest that baculovirus-expressed H5 HA can induce functional antibody in individuals who have not had prior exposure to H5 viruses, but that further studies to improve the immunogenicity of the vaccine are needed. © 2001 Elsevier Science Ltd. All rights reserved.

**Keywords:** Avian influenza; Vaccines; Clinical trials

### 1. Introduction

During 1997, 18 human cases of infection with avian influenza A (H5N1) viruses, including six fatalities, occurred among individuals living in Hong Kong [1,2]. Phylogenetic analysis determined that the H5N1 viruses isolated from humans were closely related to H5N1 viruses that had circulated concurrently in the live poultry markets and retail stalls of Hong Kong [3–5].

Two antigenically and genetically distinct variants of H5N1 influenza viruses were isolated from individuals with severe disease, and are represented by the prototype strains HK/156 and HK/483 [4]. Exposure to live poultry was determined to be the major risk factor for human H5N1 disease [6]. Human to human transmission of H5N1 viruses was documented, but was rare [7,8].

The ability of the avian virus to infect and cause disease in humans highlighted the need for a strategy of prevention against these viruses. Should influenza viruses with novel hemagglutinin (HA) acquire the ability to spread among humans lacking immunity to the novel virus, a pandemic having significant health and economic consequences could result. However, the highly pathogenic avian H5N1 viruses pose special

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problems for vaccine development since the virulent nature of the virus for both humans and domestic poultry requires that the viruses be handled under biosafety level (BSL)-3<sup>+</sup> conditions. The generation of vaccine candidates using conventional techniques would therefore require significant changes in current manufacturing procedures that might delay vaccine production in a pandemic situation. An alternative approach to H5 vaccine development uses a recombinant HA (rHA) protein generated in insect cells by a recombinant baculovirus. This approach eliminates the need to handle infectious influenza virus and allows the safe and simplified preparation of the candidate vaccine and its subsequent large-scale manufacturing as a highly purified product. Previous studies in humans demonstrated that intramuscular doses of up to 135 µg recombinant H1 and H3 HA were well tolerated and immunogenic, and preliminary evidence of protective efficacy of the recombinant H3 vaccine was obtained [9–11].

In the current study we evaluated the safety and immunogenicity of an H5 rHA vaccine derived from the human H5N1 isolate, A/Hong Kong/156/97 (HK/156). The study evaluated a two-dose schedule of administration since subjects had no prior exposure to H5 viruses. In the event of a pandemic, there would be considerable public health need to use supplies of a vaccine as efficiently, and to induce immunity as quickly, as possible. Therefore, the main objectives of the study were to determine the minimal immunogenic dose of the vaccine, to optimize the schedule of administration of the two doses, and to determine the kinetics of the antibody response.

## 2. Materials and methods

### 2.1. Vaccines and viruses

Baculovirus-expressed recombinant H5 HAs from the A/Hong Kong/156/97 (HK/156) and A/Hong Kong/483/97 (HK/483) viruses were prepared in serum-free *Spodoptera frugiperda* insect cell culture as described previously [9]. Recombinant H5 vaccine (rH5) was purified to greater than 95% purity using physical and chromatographic methods and was formulated in PBS with 0.01% Tween-20, and administered intramuscularly in a volume of 0.5 ml without adjuvant.

### 2.2. Study design

The study population consisted of healthy adults who had not received inactivated influenza vaccine within the previous 6 months. Subjects were randomly assigned to receive two doses of vaccine or placebo at intervals of 21, 28 or 42 days. Within each interval,

subjects were randomly assigned to receive either two doses of placebo, two doses of 25, 45 or 90 µg rH5, or one dose of 90 µg followed by one dose of 10 µg of rH5, in double-blind fashion. Thus, there were 15 groups in the study.

Each subject was seen by a study nurse on the day following each vaccination, at which time the site of injection was examined for induration and erythema, and the subject's oral temperature measured. Subjects self-reported erythema and induration on a diary card through day seven, as well as systemic and local symptoms and oral temperature. A complete blood count, serum chemistries, and urinalysis were obtained for safety evaluation before and on day 14 following the first vaccination. Sera for evaluation of systemic antibody responses were collected prior to and 14 days after the first vaccination (S1 and S2), and prior to and 1, 2, 3 and 4 weeks after the second vaccination (S3–S7).

### 2.3. Antibody assays

Sera were tested for neutralizing activity against the HK/156 virus by using a microneutralization assay as described previously [12], in BSL-3<sup>+</sup> laboratories at the Centers for Disease Control and Prevention (CDC; Atlanta, GA). Each serum sample was tested in at least two replicate experiments beginning at a dilution of 1:20, and the mean titer of replicate assays reported. Based on criteria established previously, a serum sample was considered positive for anti-H5 antibody if a titer of  $\geq 1:80$  was obtained in at least two independent assays and was also positive in a Western blot assay using purified HK/156 HA and serum diluted 1:100 [12]. Individuals were considered to have made a response to vaccine if one or more post-vaccination serum samples met the criteria above and demonstrated a fourfold or greater rise in neutralizing antibody with respect to the pre-vaccination titer.

Sera were also tested by enzyme-linked immunosorbent assay (ELISA) for antibody to the influenza A/Hong Kong/156/97 and A/Hong Kong/483/97 viruses. Polystyrene 96-well ELISA plates were coated with 100 ng/well of baculovirus-expressed HK/156 or HK/483 HA. For the HK/156 ELISA, sera were tested in triplicate at a single 1:1000 dilution, and the OD value reported after detection using alkaline phosphatase-conjugated goat anti-human IgG antibody (BioSource International, Camarillo, CA). For the HK/483 ELISA, sera were added to wells in serial twofold dilutions beginning at 1:400. A positive control sample consisting of a pool of four post-vaccination sera with a neutralizing titer of 1:100 was run on each plate and assigned an arbitrary value of 100 units, and the activity of each test serum was determined by comparison to the positive control sample by the reference line method [13].

Table 1

Rates of antibody responses to the A/HK/156/97 and A/HK/483/97 viruses following vaccination with recombinant H5 hemagglutinin

| Dose 1/dose 2 ( $\mu$ g) | Number with 156 neutralizing response <sup>a</sup> /number tested who were given two doses of vaccine at the following intervals |       |       |             | Number with 186 ELISA response <sup>b</sup> /number tested (%) |
|--------------------------|----------------------------------------------------------------------------------------------------------------------------------|-------|-------|-------------|----------------------------------------------------------------|
|                          | 21                                                                                                                               | 28    | 42    | Any (%)     |                                                                |
| 25/25                    | 1/10                                                                                                                             | 2/10  | 2/9   | 5/29 (17)   | 5/24 (21)                                                      |
| 45/45                    | 1/10                                                                                                                             | 4/9   | 3/10  | 8/29 (28)   | 7/24 (29)                                                      |
| 90/10                    | 4/10                                                                                                                             | 4/10  | 2/10  | 10/30 (33)  | 8/23 (35)                                                      |
| 90/90                    | 5/9                                                                                                                              | 6/10  | 4/10  | 15/29 (52)  | 8/19 (42)                                                      |
| Any rH5                  | 11/39                                                                                                                            | 16/39 | 11/39 | 38/117 (32) | 28/90 (31)                                                     |
| Placebo                  | 1/9                                                                                                                              | 0/9   | 0/8   | 1/26 (4)    | 0/20 (0)                                                       |

<sup>a</sup> Neutralizing response was defined as a fourfold increase in antibody between the first and last sera, with development of a neutralization titer of 1:80 or greater and positive Western blot assay against the 156 virus.

<sup>b</sup> ELISA response was defined as a fourfold or greater increase in units in the 483 virus ELISA between the first and last sera.

Results were reported as units of ELISA activity, and a fourfold or greater increase in units was considered evidence of a significant response to vaccination.

#### 2.4. Statistical methods

The data were analyzed using SAS version 6.12. PROC MIXED was used to test for dose and interval effects using an AR(1) covariance matrix to account for repeated measurements for the same volunteer. Sidek's method was employed to adjust for multiple comparisons. Logistic regression and Fisher's exact test were used to compare dichotomized response rates.

### 3. Results

A total of 147 subjects were enrolled in the study, of whom 146 completed vaccination. Vaccine was well tolerated, and there were no serious adverse events. No significant changes in clinical laboratory studies were seen. Complaints judged as moderate or greater in the 7 days following vaccination were rare. There were no complaints of moderate or greater arm pain or soreness in any group. One subject who received 90  $\mu$ g and one subject who received 45  $\mu$ g complained of moderate malaise following dose 1, and one subject who received 90  $\mu$ g and one subject who received placebo complained of moderate malaise following dose 2. Two individuals (one after a first dose of 90  $\mu$ g and one after a second dose of 45  $\mu$ g) experienced transient fever after vaccination. In both cases, temperatures did not exceed 10 PF and lasted for 1–2 days. There were no changes in clinical laboratory tests following vaccination.

The frequency of neutralizing antibody responses to HK/156 virus after two doses of vaccine as a function of both the dose of vaccine and the interval between doses is shown in Table 1. The frequency of responses to two doses of vaccine was dependent on the total dose of vaccine administered, with individuals who received

two doses of 90  $\mu$ g of rHA having the highest frequency of neutralizing antibody response (52%). These differences by dose were statistically significant by logistic regression analysis ( $P = 0.04$ ). In addition, only 2% (1/58) of the subjects in the combined 25  $\mu$ g plus 45  $\mu$ g groups achieved a fourfold or greater increase following a single dose of vaccine, compared with 23% (14/60) of the subjects receiving 90  $\mu$ g at dose 1 ( $P < 0.01$ , Fisher's exact test). In contrast, there was relatively little variation in the response rate depending on the interval between vaccination ( $P = 0.38$ , logistic regression analysis). The study had about 75% power to find significant differences by interval between vaccinations, assuming true response proportions of 11/39 for two of the time intervals and 22/39 for the third. Overall 32% of individuals responded to any dose of the rHA vaccine compared with a response rate of 4% in the placebo group.

The kinetics of the neutralizing antibody response to vaccination with rH5 HA are shown in Figs. 1 and 2.

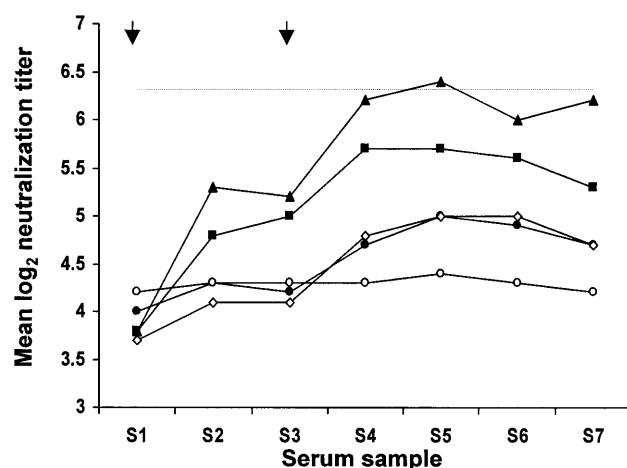


Fig. 1. Kinetics of the neutralizing antibody response to the A/HK/156 virus. Vaccines were administered at the time points indicated by the arrows. The dotted line represents a titer of 1:80. Symbols: —●—, 25  $\mu$ g; —◇—, 45  $\mu$ g; —▲—, 90  $\mu$ g; —■—, 90  $\mu$ g/10  $\mu$ g; —○—, placebo.

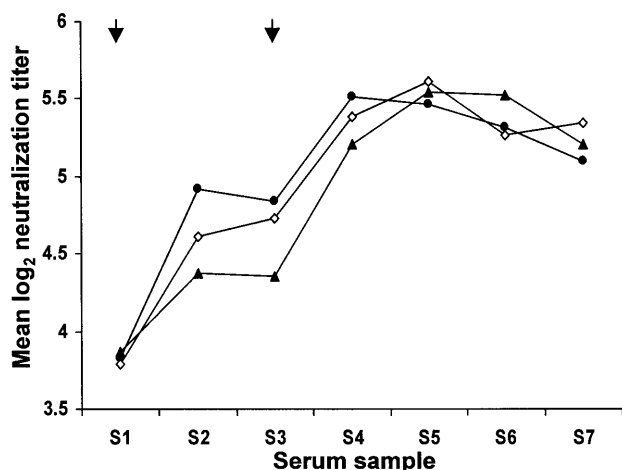


Fig. 2. Effect of varying the interval between doses on the neutralizing antibody response. Data are pooled from all vaccine dose groups except placebo. Symbols: —●—, 21 days; —□—, 28 days; —▲—, 42 days.

Fig. 1 presents neutralizing antibody responses in all individuals for a given vaccine dose, regardless of the time interval between doses. Two doses of 25 or 45  $\mu$ g rH5 HA were required to elicit a modest increase in neutralizing antibody titer. Only the group receiving 90  $\mu$ g of H5 HA demonstrated an increase in mean neutralizing antibody after the first dose of vaccine (Fig. 1). A second dose of 90  $\mu$ g substantially boosted the response with the mean neutralizing antibody titer in this group exceeding 1:80 (6.32  $\log_2$ ) 2 weeks after the second dose. Administration of 90  $\mu$ g followed by a boost with 10  $\mu$ g of rHA resulted in higher neutralizing antibody titers than did two doses of 45  $\mu$ g of vaccine, but elicited mean titers that fell short of those achieved with the 90  $\mu$ g boost.

The effect of varying the time interval (21, 28 or 42 days) between the first and second dose of vaccine is shown in Fig. 2. Although extending the interval between vaccine doses to 42 days resulted in a decrease in the mean neutralizing antibody titer prior to administration of the second dose, antibody titers achieved following the second vaccine dose were comparable in all interval groups. Modeling the entire post-vaccination data for the non-placebo recipients using a model to control for repeated observations on the same subject and controlling for multiple comparisons in pairwise testing confirmed the overall significant differences by dose ( $P < 0.001$ ) and the significant differences between the 90  $\mu$ g group and the 25  $\mu$ g ( $P < 0.001$ ) and 45  $\mu$ g ( $P = 0.01$ ) groups in the titer of neutralizing antibody achieved. This approach also confirmed the absence of a significant dosing interval effect ( $P > 0.7$ ). Similar results were obtained when sera were analyzed by ELISA against the HK/156 HA (data not shown).

To determine whether the HK/156 rHA vaccine induced antibodies that cross-reacted with HK/483 virus, we also used the HK/483 rHA protein in an ELISA assay. However, because of limited availability of this antigen, only 85% of the sera were tested. Both the frequency of response to the 483 virus by ELISA (Table 1) and the mean titer of antibody (Fig. 3) were dependent on the dose of vaccine administered, with the highest levels of H5 IgG antibody developing in individuals that received two doses of 90  $\mu$ g.

#### 4. Discussion

The avian H5N1 influenza outbreak in Hong Kong in 1997 highlighted the potential for avian influenza viruses of novel subtypes to infect and cause disease in a non-immune human population. Although H5N1 viruses have not been isolated from humans since the depopulation of poultry from Hong Kong bird markets in December 1997, H5 and other avian virus subtypes still circulate worldwide in birds. Recently, avian influenza H9N2 viruses were isolated from two children in Hong Kong. Whether for H5, H9 or yet another of the 15 influenza A subtypes, the development and evaluation of human vaccines against influenza viruses with pandemic potential remains an important task.

Recombinant hemagglutinins are an attractive approach for this objective since live influenza viruses are not used in their production and elaborate containment procedures are not necessary. The rHA H5 vaccine used in this study was ready for testing in humans less than 4 months after recognition of the outbreak in

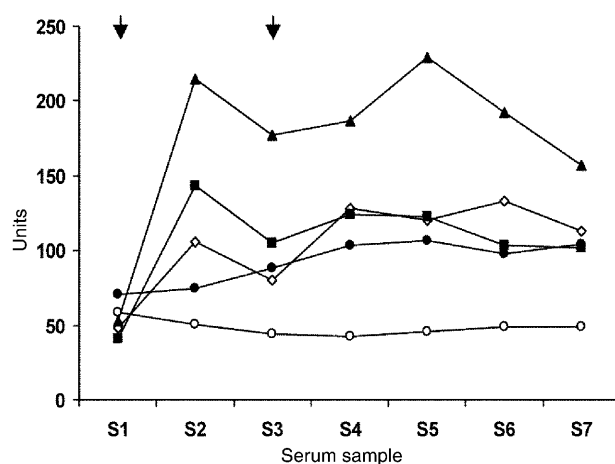


Fig. 3. Time course of the ELISA antibody response to the A/HK/483/97 virus. The antibody level of the sera were compared with a pooled reference sera composed of post vaccination sera with detectable neutralizing antibody and assigned an arbitrary number of units. Symbols are as per Fig. 1.

Hong Kong, and before any other vaccine candidates were available for human trials. As expected, the vaccine was extremely well tolerated, consistent with other trials of rHA vaccines in humans [9–11]. However, substantial neutralizing antibody responses were induced only with the highest doses of vaccine. We chose a titer of 1:80 as a marker for protective immunity based on studies showing that this titer is found in human sera after documented infection with H5N1 virus [7]. By this measure, potentially protective responses were detected in slightly over half of the subjects who received two doses of 90 µg of vaccine. A comparison of our results with those of earlier studies that evaluated antibody responses to conventional influenza vaccines in unprimed adult populations, suggests that the H5 rHA vaccine was sub-optimally immunogenic. For example, in previous studies ~60% of individuals aged 16–25 who had not previously been exposed to H1N1 viruses mounted a fourfold or greater HI response to a single dose of conventional A/USSR/77 (H1N1) vaccine containing 5–17 µg of HA, and >75% of these individuals responded to two doses [14]. The response rates seen in this study were also lower than those seen in adults receiving H1 or H3 rHA vaccines in previous studies, where between 60 and 100% of subjects manifested HAI antibody responses at doses ranging from 15 to 135 µg rHA [9–11].

There are a number of possible explanations for the relatively reduced immunogenicity of rH5 vaccine observed in this study. First, rHA vaccines may be inherently less immunogenic than conventional inactivated vaccines. In previous studies, doses of 45 µg (assessed by protein content) of the highly purified recombinant HA were required to induce the same level of immunity as that achieved with a standard dose of inactivated influenza vaccine containing 15 µg of HA antigen (as assessed by radial immunodiffusion) suggesting that on a per weight basis, rHA may be slightly less immunogenic than the standard preparations of vaccines [9–11]. Second, HA from avian viruses may be less immunogenic in mammalian species compared with HA from human viruses. Previous studies have failed to detect HI antibody responses in mammals in which avian viruses clearly replicated, although the reduced sensitivity of the HI assay may have contributed to this result [15,16]. The results of the current study are consistent with preliminary data reporting relatively infrequent and low-titred neutralizing antibody responses in healthy adult volunteers following administration of an inactivated influenza vaccine prepared from the antigenically related A/Duck/Singapore-Q/F119-3/97 (H5N3) virus at doses from 7.5 to 30 µg [17].

Strategies to improve the immunogenicity of the rH5 vaccine could include use of higher doses of antigen and/or addition of adjuvants. In mice, addition of alum to an inactivated vaccine generated from the Duck/Sin-

gapore (H5N3) virus resulted in significantly greater immunogenicity [18], but in previous studies of the rHA vaccines in humans, addition of alum had no such effect [9]. Addition of the oil-in-water adjuvant MF-59 significantly increased the frequency with which neutralizing titers of >1:20 were achieved after vaccination of humans with the Dk/Singapore vaccine, but the GMT following two doses of adjuvanted vaccine was still quite low [17]. Other adjuvants could also be considered, including QS-21, which has recently been reported to significantly reduce the dosage of a baculovirus derived gp 160 vaccine required to induce neutralizing antibody in humans. Further studies to optimize the immunogenicity of vaccines for H5 and other avian influenza vaccines in man will be an important component of preparation for the next influenza pandemic.

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# Expression and purification of an influenza hemagglutinin—one step closer to a recombinant protein-based influenza vaccine

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## Abstract

Numerous human infections with avian influenza viruses in Asia in recent years have raised the concern that the next influenza pandemic is imminent. The most effective way to combat influenza is through the vaccination of the public. However, a minimum of 3–6 months is needed to develop an influenza vaccine using the traditional egg-based vaccine approach. The influenza hemagglutinin protein (HA), the active ingredient in the current vaccine, can be expressed in insect cells using the baculovirus expression vector system and purified rapidly. An influenza vaccine based on such a recombinant antigen allows a more timely response to a potential influenza pandemic. Here, we report an innovative monitoring assay for recombinant HA (rHA) expression and a rapid purification process. Various biochemical analyses indicate that the purified rHA is properly folded and biologically active.

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**Keywords:** Influenza; Vaccine; Hemagglutinin; Baculovirus; Recombinant protein; Purification; Chromatography

## 1. Introduction

Influenza is a highly contagious, acute viral respiratory disease, which causes significant morbidity and mortality worldwide each year [1–3]. Influenza viruses are single-stranded ribonucleic acid (RNA) viruses surrounded by a lipid containing envelope spiked with two glycoproteins: hemagglutinin (HA) and neuraminidase (NA). These glycoproteins, and HA, in particular, have been recognized as key antigens in the host response to influenza virus in both natural infection and vaccination [4,5]. The viruses are well known for their ability to mutate to circumvent immunity and re-infect the host. An antigenic shift, a major antigenic change of the virus due to the genetic re-assortment of two subtype strains that co-infected a host, can cause an influenza pandemic since the population may have no inherent immunity against the new strain [6,7]. The avian influenza A (H5N1) epizootic outbreak and numerous human infections

with H5N1 in Asia in recent years, and events such as the infection of two nurses attending to avian influenza patients in Vietnam (WHO, March 14, 2005) and a possible person-to-person transmission in a family cluster of the disease in Thailand [8], continue to raise concern that the next influenza pandemic is imminent.

A proven, effective way to combat influenza is through vaccination of the public using the trivalent vaccine produced in embryonated chicken eggs. In the current process, three influenza strains selected by WHO/CDC are propagated in chicken eggs, chemically inactivated, and semi-purified. The egg-based technology, however, is unable to respond to a pandemic crisis. Vaccine development and production take several months following the identification of potential strains and typically requires the re-assortment with a high yield strain to obtain adequate growth properties [9–12]. A minimum of 3–6 months is needed to develop an influenza vaccine using this approach. More importantly, the H5 avian influenza strains responsible for recent epizootic outbreak involving numerous human infections are lethal to chicken eggs used for vaccine production and to the chickens that

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lay the eggs. If the 2003 severe acute respiratory syndrome (SARS)-coronavirus outbreak serves as a guide, the next influenza pandemic will likely have global consequences spreading within weeks, if not within days. Thus, a system that can rapidly produce new influenza vaccine is needed to prevent or to effectively reduce the impact of pandemic influenza.

Two new approaches have shown great promise to replace the egg-based technique [9–11]. One is cell culture-based, and the other is recombinant protein (antigen)-based. The cell culture-based approach involves production of influenza viruses in cell culture followed by the current (egg-based) virus inactivation and purification for the down stream processing. The advantages are: cell cultures are easier to handle and can be scaled up in a short period of time, and the influenza vaccines produced with this approach have been tested in Phase I and Phase II clinical trials and were found to be safe and at least as effective as the vaccines produced in embryonated chicken eggs [13–15]. A limitation of the cell culture-based approach is that the process still requires the production of a high-yielding re-assorted virus. This process also may introduce cell line specific mutations in the genes that can lead to the selection of variants characterized by antigenic and structural changes in the HA protein, potentially resulting in less-efficacious vaccines [16–18]. Additional hurdles include: the production and handling of a dangerous virus requires the availability of a high containment facility; mammalian cells can harbor animal viruses that may lead to safety concerns; the residues from the expressing cells may cause some unknown side-effects since no thorough purification process has been introduced into the manufacturing process. On the other hand, the recombinant protein-based approach involves production of viral antigens such as HA and NA in cell culture with recombinant DNA technology and utilization of the purified antigens as the active ingredients in the vaccine. The rHA influenza vaccines developed using the baculovirus-insect cell expression system has been tested in several Phase I and Phase II human clinical trials involving over 1200 subjects that demonstrated safety, immunogenicity and efficacy [19–23]. In elderly adults, rHA vaccine is equally or more immunogenic than the egg-based vaccine (Treanor JJ, et al. Dose-related safety and immunogenicity of a trivalent baculovirus-expressed influenza virus HA vaccine in elderly adults, manuscript in preparation). Interestingly, two H1N1 rHA vaccines (derived from two strains of A/New Caledonia/20/99 or A/Texas/36/91) provided partial protection against the lethal challenge of a reconstructed highly lethal 1918 pandemic influenza virus (also a H1N1 strain) in mice, suggesting that cross protection against drifted strains is definitely feasible [24].

To meet the challenge of a potential influenza pandemic, however, a reliable expression system and a quick, efficient downstream purification process are needed. In this communication, we reported a rapid process capable to purify rHA (H1N1, A/New Caledonia/20/99) from the fermentation to 95% purity within 6 h with a 57% overall yield. Since all

chromatographic media used here are chemically stable and commercially available, the process can be easily scaled up in a GMP facility. Various biochemical analyses indicated that the purified rHA is properly folded and biologically active. In addition, we also developed a quick, simple analytical assay to monitor the expression of rHA in the insect cell fermentation to ensure the rHA production.

## 2. Materials and methods

### 2.1. Cloning and expression of influenza HA

The influenza vaccine strain-A/New Caledonia/20/99 (H1N1)—was obtained from the CDC. The full-length HA gene (containing the HA1 and HA2 genes) from the influenza viruses was cloned using RT-PCR and inserted into a baculovirus transfer vector developed by Protein Sciences Corporation. This specialized vector contained the promoter from the baculovirus polyhedrin gene flanked by sequences naturally surrounding the polyhedrin locus. Next, the transfer vector was co-transfected into insect cells with the linearized baculovirus genomic DNA (*Autographa Californica Nuclear Polyhedrosis Virus*) depleted of the polyhedrin gene and part of an essential gene downstream of the polyhedrin locus. The homologous recombination between the transfer plasmid and the linearized viral DNA rescued the virus, resulting in recovery efficiencies of recombinant virus of nearly 100%. Recombinant viruses were then selected by plaque assay. The plaque-derived recombinant baculovirus was then used to create a virus stock by infecting increasingly larger cultures of the proprietary insect cells (*expresSF+*<sup>®</sup>, derived from Sf9 cells) in serum-free culture medium (Protein Sciences Fortified Medium). The virus stock was then used to infect insect cells ( $2.0 \times 10^6$  cells/ml) to produce rHA in a 15-liter Applikon bioreactor. The multiplicity of infection (MOI) of the virus stock was 1 for the experiment.

To monitor the infection process and the expression of HA, 4 ml samples were taken from the bioreactor at various times. One millilitre was used for analyzing the changes in cell density, cell viability and cell size distribution. Two millilitres were centrifuged at 1600 rpm. The supernatant and pellet were stored separately at  $-80^\circ\text{C}$  to be used for SRID, gel and blot analysis. One millilitre was used for hemadsorption analysis. The rest was used for protein purification.

### 2.2. Hemadsorption assay

To 0.5 ml fermentation samples (insect cells uninfected, infected with recombinant baculovirus containing HA genes, and infected with recombinant baculovirus containing a non-HA gene) in a 1.5 ml Eppendorf tube, 0.10 ml of 5% chicken red blood cells (Charles River–Spafas, North Franklin, CT) in PBS was added and shaken gently for 10 min at room temperature. At the end of incubation, the tube was flipped gently for five times to get a homogenous suspension. Then,

10  $\mu$ l of the suspension was pipetted on a glass plate and observed under a microscope (CK2, Olympus Optical Co., Japan) in three representative view fields. On average, about 20–70 insect cells were counted in each field. To reduce the chance of false positives, only the insect cells attached by three or more RBCs were counted as the RBC-bound insect cell. The percentage of RBC-bound insect cells against the total insect cells in each time point was calculated from three fields.

### 2.3. Cell analysis

At each time point, 1.0 ml of fermentation sample was analyzed with an automated cell analyzer (Cedex AS20, Innovatis GmbH, Germany) for cell density, cell viability and cell size distribution using the procedure described by the manufacture.

### 2.4. Buffers and columns

Buffer A: 20 mM sodium phosphate, 1.0 mM EDTA, 0.01 % Tergitol-NP9, 5% glycerol, pH 5.89. Buffer B: 20 mM sodium phosphate, 0.03 % Tergitol, 5% glycerol, pH 7.02. Buffer C: 20 mM sodium phosphate, 150 mM NaCl, 0.03% Tergitol, 5% glycerol, pH 7.02. Buffer D: 40 mM sodium phosphate, 0.05% Tween-20, 5% glycerol, pH 7.20. Buffer E: 100 mM sodium phosphate, 0.05% Tween-20, 5% glycerol, pH 7.20. Buffer F: 500 mM sodium phosphate, 0.05% Tween-20, 5% glycerol, pH 7.20. Buffer G: 10 mM sodium phosphate, 150 mM NaCl, 0.01% Tween-20, pH 7.22. Sanitation buffer: 1.0 M NaCl, 0.5 M NaOH.

UNOsphere-Q (Bio-Rad, Hercules, CA) column,  $\varnothing$  1.6 cm  $\times$  10 cm, 20 ml; SP-Sepharose Fast Flow (GE/Amersham/Pharmacia, Piscataway, NJ) column,  $\varnothing$  1.6 cm  $\times$  10 cm, 20 ml; Hydroxyapatite Type I column (HX-I, Bio-Rad, Hercules, CA),  $\varnothing$  1.0 cm  $\times$  4.6 cm, 3.6 ml.

### 2.5. Purification procedure

The fermentations producing rHA were harvested by centrifugation at 56–65 h post-infection. The cell pellet (6.4 g) was extracted with 225 ml of 1% Tergitol NP-9 in buffer A by stirring on a magnetic stirrer at 4 °C for 30 min. The extract was clarified by centrifugation at 10,000  $\times$  g for 25 min. The supernatant was loaded on Q/SP columns (equilibrated with Buffer A) in tandem at 5 ml/min. After loading, the columns were washed with 140 ml of Buffer A. Then, the columns were disconnected. HA was eluted from the SP column with 140 ml of buffer B and 80 ml of buffer C, consecutively. The Q/SP columns can be regenerated by washing with 5 column volumes (CV) of sanitation buffer and 5 CV of water and equilibrated with 5 CV of buffer A.

The HA fraction in buffer B (40 ml) was loaded on a HX-I column at 2 ml/min. The column was washed with 18 ml of buffer B. The HA was eluted from the HX-I column with increasing phosphate concentration (buffers D, E and F). The

HA preparation in buffer D was further purified and concentrated by ultrafiltration with a stir cell using a 100 kDa MWCO regenerated cellulose membrane with buffer G. The HX-I column can be regenerated by washing with 10 CV of water, and equilibrated with 10 CV of buffer C.

### 2.6. Single radial immunodiffusion (SRID)

The rHA contents in all preparations were determined with SRID assay as described by Williams [25] and Manchini et al. [26]. The assay is based on the diffusion of rHA into a 1% agarose gel containing antibodies against the HA. The interaction between antigen and antibody produced a precipitation ring of which the size was directly proportional to the amount of antigen applied. The diameters of the rings in the SRID assays were determined with a measuring magnifier (Baush/Lomb, 81-34-38). The diameters of the precipitate ring were used to determine the actual concentrations based on standards provided by the Center for Biologics Evaluation and Research of FDA.

### 2.7. Deglycosylation

For complete deglycosylation, 20  $\mu$ g of purified rHA was deglycosylated with 5000 units of peptide-N-glycosidase F (PNGase F, New England BioLabs, Beverly, MA, USA) or Endoglycosidase H (Endo H, New England BioLabs) at 37 °C for 60 min as described previously [27]. For limited deglycosylation, 20  $\mu$ g of rHA was treated with 0.2  $\mu$ g of trypsin on ice for 30 min. The digestion was stopped by adding 10 $\times$  denaturing buffer and boiling for 5 min. Then the trypsin treated rHAs were deglycosylated with 2, 20 or 200 units of PNGase F or Endo H on ice, at 25 °C or at 37 °C for 2–60 min. The reactions were stopped by adding 2 $\times$  SDS sample buffer and boiling for 5 min. The protein species at various deglycosylation stages were resolved on SDS-PAGE.

### 2.8. Other biochemical analyses

Vaxigrip influenza vaccine 2004–2005 was purchased from Canada Drug Delivery (Nanaimo BC, Canada). The purity of rHA was measured on SDS-polyacrylamide gels stained with Coomassie blue using scanning laser densitometry (model 710, Bio-Rad, Hercules, CA, USA) and peak integration analysis. The total amino acid analysis was carried out with a Beckman amino acid analyzer at Keck Facility of Yale University. The N-terminal amino acid sequence analysis was executed at the Protein Core Facility of Columbia University. The molecular size of the purified rHA was analyzed on a size-exclusion columns (TSK-4000, 7.5  $\times$  300 mm, Tosa-Haas, Japan) at a constant flow rate of 0.8 ml/min, using the protein molecular weight markers as the reference (Sigma, St. Louis, USA) as previously described [28]. Elution buffer: 50 mM sodium phosphate, 50 mM NaCl and 0.001% NaN<sub>3</sub>. A trypsin resistance assay was carried out by incubating rHA

for 30 min at 0 °C without or with 50 µg/ml TPCK-treated trypsin as described by Copeland et al. [29]. For this assay, the denatured HA was produced by boiling rHA for 5 min. Hemagglutination activity assays were done essentially as described by Barrett and Inglis [30] with a 0.5% solution of fresh chicken red blood cells in a U-bottom 96-well microtiter plate.

### 3. Results

#### 3.1. Monitoring the expression of rHA in insect cell

A critical issue in production of a therapeutic protein using recombinant DNA technology is determining when to harvest the fermentation [31,32]. Too early, the yield may be suboptimal. Too late, the expressed protein may be degraded by a variety of proteases released during the lytic process of infected cells. Thus, a rapid and sensitive assay is needed to monitor protein expression and to choose the right harvest time. HA is well known for its ability to bind the sialic acid on the surfaces of red blood cells (RBCs) and agglutinate these cells [30]. This phenomenon has been success-

fully used to detect cells and tissues infected with influenza viruses. To determine whether the insect cells infected with the recombinant baculoviruses containing the HA gene can also agglutinate RBCs, both uninfected and infected insect cells were incubated with RBCs for 10 min and observed under a microscope. In the uninfected insect cell sample, the RBCs (the smaller cells) were scattered around on the slide, and no specific binding of RBCs to the insect cells (the larger cells, about 16 µm in diameter) was observed as shown in Fig. 1a. On the other hand, most of RBCs were bound to the insect cells infected with baculovirus containing the HA gene derived from influenza strain A/New Caledonia/20/99 (H1N1) (Fig. 1b). This method also works for expression of the HAs derived from other strains such as B/Jiangsu/10/2003 (Fig. 1c) and A/New York/55/2004 (H3N2) (data not shown). To verify that the observed hemadsorption is due to the HA genes and not to the other genes in the baculovirus, insect cells infected with recombinant baculovirus containing a non-HA gene were also incubated with RBCs. No binding of RBCs on insect cells was observed (Fig. 1d). These observations clearly demonstrate that RBC's binding to the surface of insect cells is HA expression dependent, not infection dependent. The data also support the conclusion that the HAs

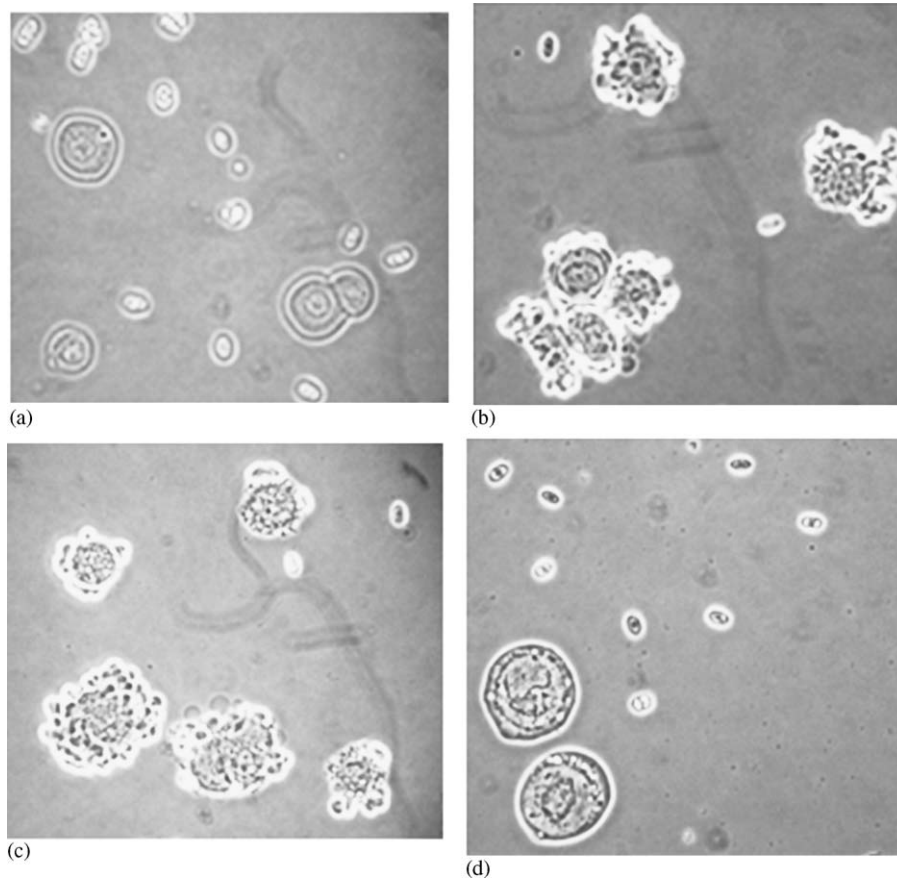


Fig. 1. Monitoring rHA expression with hemadsorption. (a) Uninfected insect cells. (b) The insect cells infected with the baculovirus containing the HA gene from the A/New Caledonia/20/99 (H1N1) influenza virus. (c) The insect cells infected with the baculovirus containing the HA gene from the B/Jiangsu/10/2003 influenza virus. (d) The insect cells infected with the baculovirus containing a non-HA gene.

Table 1  
Possible correlation among HPI, hemadsorption, cell viability and HA yield<sup>a</sup>

| HPI | %Binding | Viability | HA (mg/L) |
|-----|----------|-----------|-----------|
| 0   | 0        | 92.9      | 0         |
| 6   | 0        | 92.9      | 0         |
| 23  | 20       | 87.6      | 4         |
| 30  | 30       | 88.2      | 7         |
| 46  | 65       | 74.7      | 18        |
| 50  | 80       | 52.4      | 20        |
| 54  | 70       | 50.1      | 20        |
| 70  | 40       | 21.8      | 11        |
| 75  | 30       | 16.1      | 11        |

<sup>a</sup> HA yield was determined by SRID assay. The MOI of the virus stock was 1 for the experiment.

expressed in insect cells are properly folded and biologically active.

This hemadsorption assay was further optimized to monitor the expression of HA during fermentation. Even though the infection of insect cells can be seen by observing morphological changes as early as 6 hours post infection (HPI), the first binding of RBCs to insect cells was only observed around 23 HPI—about 20% of insect cells were bound by three to eight RBCs. Almost all insect cells were infected at 23 HPI according to cell morphology. The binding reached its peak around 45–55 HPI, when 70–80% of insect cells were bound by 8–20 RBCs (in some fermentations, almost all insect cells were bound by RBCs during this time), as shown in Table 1. At the peak of hemadsorption, clusters of 20–200 insect cells agglutinated by RBCs have also been observed. Later, the binding gradually reduces to 30–40% of insect cells, most likely caused by the breakdown of some infected cells and the loss of cell membrane, as evidenced by the rapid decrease of cell viability. To determine the rHA levels in the fermentation at each sampling time, the single radial immunodiffusion (SRID) assay has been used, since it is a simple, reproducible technique, and relatively unaffected by other proteins in the crude extract [25,26]. Consistently, the RBC binding to insect cells correlated well with the HA levels determined by SRID. Late HA expression is expected because the HA gene is regulated by the polyhedrin promoter, which is a late stage promoter in baculovirus infection. Similar results were also obtained for the expression of other HAs such as B/Jiangsu/10/2003 and A/New York/55/2004 (data not shown).

### 3.2. Designing and optimizing rHA purification process

A major challenge in the biotechnology industry is purification of biologically active recombinant proteins [33,34]. An ideal purification process should be mild, efficient and capable of achieving high purity in a short period of time. Accordingly, each purification step has been carefully designed to optimize the whole process. As demonstrated in the hemadsorption studies, rHAs are expressed, folded and transported to the cell membrane at a late stage. To extract rHA from the cell membranes, several non-ionic detergents at various

concentrations were tested for their efficiency. The best result was obtained using 1% Tergitol NP-9. To get a relatively clean extraction, a magnetic stirrer was used to avoid the disruption of cell nuclei and other organelles. The extract was clarified by centrifugation to remove cell nuclei and other debris.

The HA monomer of A/New Caledonia/20/99 influenza virus strain consists of 547 amino acids with a theoretical molecular weight of 63,156.43 and a *pI* of 6.30. Therefore, this rHA can be bound on a cation-exchange media like SP using a lower pH buffer and eluted with a higher pH buffer to achieve the primary purification and concentration. The supernatant of the extract was loaded on UNO Sphere Q/SP columns in tandem. The anion-exchange Q column acts as a scavenger by binding the negative-charged impurities that may foul the SP column. After loading and washing, the columns were detached and eluted separately. As shown in the chromatogram (Fig. 2a), about 50% of the proteins flow through Q/SP columns, a small amount of protein elutes from the SP with the pH 7 buffer and the rest bind tightly to either the SP or the Q column. As shown on the SDS-PAGE (Fig. 2b), the rHA captured on the SP at pH 6 was selectively eluted by a shift to pH 7. The pH shift resulted in a 14-fold increase in purity based on densitometry of the SDS-PAGE gel.

To further purify rHA, use of hydroxyapatite type I (HX-I) media was explored. The binding preference of HX-I media is significantly different from the ion-exchange media, and yet the binding and eluting conditions are relatively mild so as to preserve the biological activity of the target protein [35,36]. Thus, the pH 7 SP column eluate was loaded on a HX-I column. After washing, rHA was eluted from the column with increased phosphate concentration. As shown in Fig. 2c, most rHA was eluted in 40 mM phosphate, and there was a small loss in the wash and in the 100 mM phosphate elution. The purity was increased from 52% to 91% according to the densitometry of the SDS-PAGE.

However, there was still a protein contaminant of about 36 kDa in the preparation as revealed on lane 5 of Fig. 2c. Since the size of rHA trimer is around 210 kDa, the difference between rHA size and this impurity could be explored to remove this impurity. Thus, the 40 mM phosphate eluate was further purified with ultrafiltration using a stir cell (100 kDa MWCO). As demonstrated in Fig. 2d, the 36 kDa band (lane 5) was selectively removed from the retentate. The purified rHA migrated on SDS-PAGE gel as a single polypeptide (rHA) with an apparent molecular weight of approximately 70 kDa. On the blot, a small amount of rHA1 and rHA2 (the cleavage products of rHA) were also observed, with apparent molecular weights of  $\approx 50$  and  $\approx 28$  kDa, respectively (data not shown). Trace amounts of rHA dimers and trimers were also detectable, with apparent molecular weights of  $\approx 140$  and  $\approx 220$  kDa, respectively.

As summarized in Table 2, the process described here can purify rHA from the fermentation to 95% purity within 6 h with a 57% overall yield. The largest single step loss (27%) is on HX-1 column.

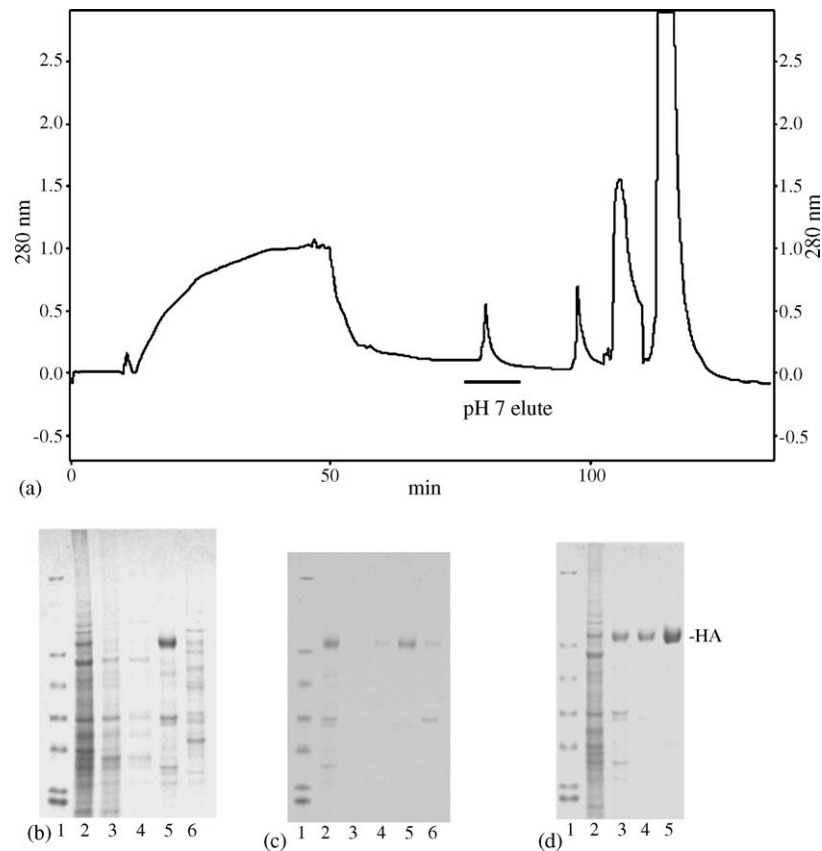


Fig. 2. The purification process of rHA. (a) The chromatogram of primary purification and concentration on Q/SP columns. (b) The SDS-PAGE of Q/SP fractions, lanes 1–6 are molecular weight markers, applied sample, flow through, wash, pH 7 (buffer B) eluate, and buffer C eluate, respectively. (c) The SDS-PAGE of secondary purification on HX-I, lanes 1–6 are molecular weight markers, applied sample, flow through, wash, 40 mM phosphate eluate, and 100 mM phosphate eluate, respectively. (d) The SDS-PAGE of step-wise purification, lanes 1–5 are molecular weight markers, Q/SP applied sample, HA after the primary purification, HA after the secondary purification, HA after the final polish using ultrafiltration.

### 3.3. Characterizing rHA protein

To confirm the authenticity of rHA, the purified protein was examined by N-terminal amino acid sequencing and total amino acid analysis (AAA). The N-terminal amino acid sequence matched the predicted one (10 cycles were used) and the signal sequence peptide (the first 17 amino acids) of the full-length HA gene was absent in rHA. The measured amino acid composition of the purified rHA was consistent with the theoretical one (data not shown). The authenticity of rHA was further verified by the Western blot using A/New Caledonia/20/99 antibody provided by the Food and Drug Administration (FDA).

The purified rHA in 0.005% Tween/PBS solution was analyzed using size-exclusion chromatography. It was eluted as a single peak at 9.1 min as shown in Fig. 3a, corresponding to a molecular weight around 800–1000 kDa, likely a complex of four to five HA trimers ((4–5) × 3 × 70 kDa). To test whether the purified rHA still retained its native structure, the purified protein was treated with trypsin on ice. As shown in the lane 3 of Fig. 3b, rHA was cleaved into only two bands, HA1-50.9 kDa and HA2-27.5 kDa. On the other hand, the heat-denatured rHA was digested into numerous small fragments. The trypsin-resistance data demonstrate that the rHA expressed in insect cells folded properly and retained its native structure after purification.

Table 2  
Stepwise mass balance of HA-NC purification by SRID assay

|                   | HA (μg/ml) | Volume (ml) | HA (mg) | Purity <sup>a</sup> (%) | Step recovery (%) | Total recovery (%) |
|-------------------|------------|-------------|---------|-------------------------|-------------------|--------------------|
| Starting material | 53         | 225         | 12      | 3.6                     |                   |                    |
| Q/SP              | 240        | 40          | 9.6     | 52                      | 80                | 80                 |
| HX-1              | 280        | 25          | 7.0     | 91                      | 73                | 58                 |
| Ultrafiltration   | 700        | 9.1         | 6.4     | 99                      | 91                | 53                 |

<sup>a</sup> Purity was determined by the densitometry of SDS-PAGE.

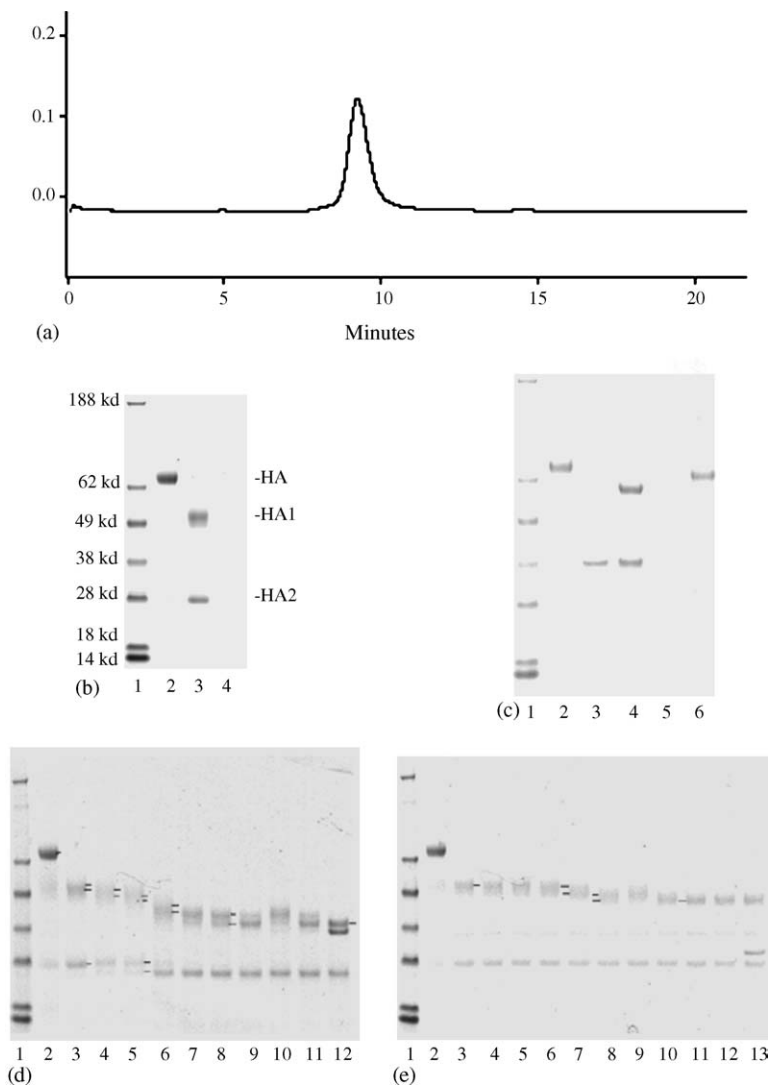


Fig. 3. Characterization of rHA. (a) The SEC profile of the purified rHA. (b) The trypsin-resistant test analyzed on SDS–PAGE, lanes 1–4 are molecular weight markers, native rHA, native rHA treated with trypsin, and heat-denatured rHA treated with trypsin, respectively. (c) Deglycosylation of rHA with PNGase F and Endo H, lanes 1–6 are molecular weight markers, rHA, PNGase F, rHA treated with PNGase F, Endo H, and rHA treated with Endo H, respectively. (d) Limited deglycosylation of rHA with PNGase F. Lane 1, molecular weight markers; lane 2, rHA; lane 3, trypsin treated rHA; lanes 4–8, the trypsin treated rHAs were deglycosylated with 2 units of PNGase F on ice, at 25 °C or at 37 °C for 2–60 min; lanes 9–11, deglycosylated with 20 units of PNGase F; lane 12, deglycosylated with 200 units of PNGase F. (e) Limited deglycosylation of rHA with Endo H. Lane 1, molecular weight markers; lane 2, rHA; lane 3, trypsin treated rHA; lanes 4–8, the trypsin treated rHAs were deglycosylated with 2 units of Endo H on ice, at 25 °C or at 37 °C for 2–60 min; lanes 9–12, deglycosylated with 20 units of Endo H; lane 13, deglycosylated with 200 units of Endo H.

Since glycosylation may play an important role in the biological function of HA [37–39], it is of interest to explore whether the rHA produced in insect cells is properly glycosylated. Thus, rHA was deglycosylated with peptide-*N*-glycosidase F (PNGase F) or endoglycosidase H (Endo H) and resolved on SDS–PAGE. As shown in Fig. 3c, the untreated HA migrated at 70.4 kDa, PNGase F deglycosylated HA at 57.9 kDa, and Endo H treated HA at 64.8 kDa, respectively. These data indicate that the rHA produced in insect cells is indeed glycosylated with N-linked oligosaccharide side chains. About 5.6 kDa of oligosaccharide chains have high mannose content susceptible to Endo H, and 6.9 kDa of oligosaccharide chains have low mannose residues

resistant to Endo H. To assess the number of N-linked oligosaccharide chains, the trypsin treated rHA was subjected to limited deglycosylation with PNGase F or Endo H under a variety of conditions. On the SDS–PAGE of PNGase F treated samples (Fig. 3d), there are seven distinguishable HA1 bands, 50.9, 49.0, 46.8, 45.2, 43.3, 42.0 and 39.8 kDa, and 2 HA2 bands, 27.1 and 25.4 kDa, respectively. On the SDS–PAGE of Endo H treated samples (Fig. 3e), there are three distinguishable HA1 bands, 51.2, 49.4 and 46.5 kDa, and one HA2 band, 27.1 kDa. The data suggest that there are six N-linked oligosaccharide chains in the HA1 region, and two of them have high mannose content. There is only one N-linked oligosaccharide chain in the HA2 region, which is

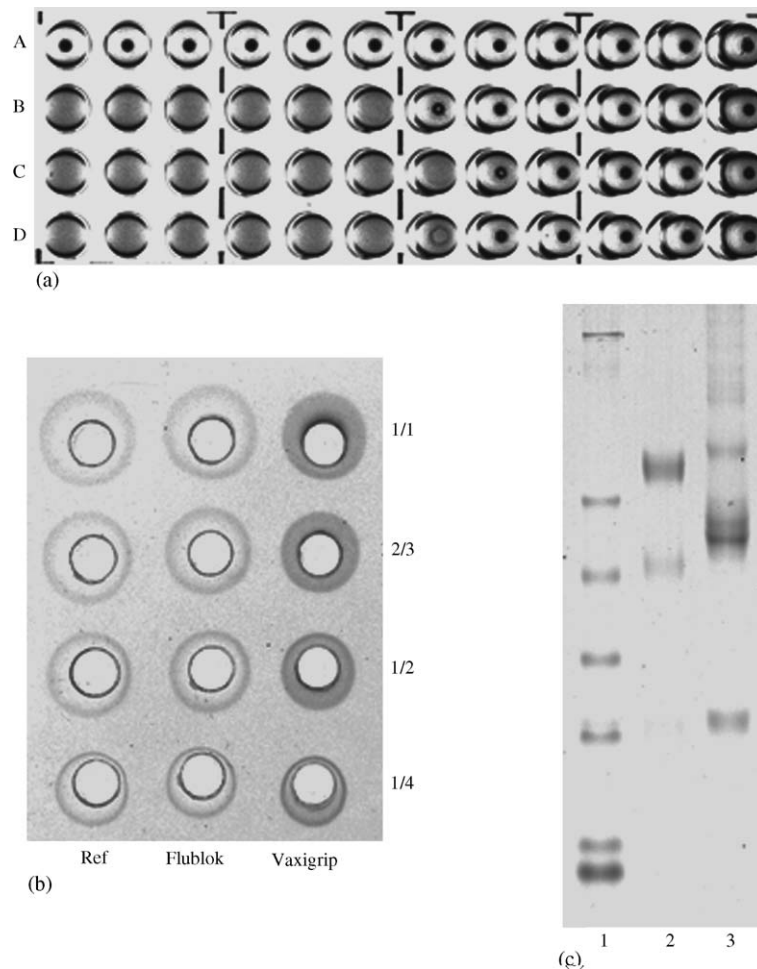


Fig. 4. The comparison of FluBIØk with Vaxigrip. (a) The hemagglutination activities of 1.0  $\mu\text{g/ml}$  rHA or equivalent amount from the vaccines. Row A, PBS as negative control; Row B, rHA; Row C, Vaxigrip; Row D, FluBIØk. (b) The evaluation of the effective antigen concentration with SRID assay. (c) The SDS-PAGE of the vaccines. Lane 1, molecular weight markers; lane 2, FluBIØk; lane 3, Vaxigrip.

likely a hybrid or a complex oligosaccharide. Consistently, there are six predicted N-linked glycosylation sites in the HA1 region and one predicted N-linked glycosylation site in the HA2 region of HA/A/New Caledonia/20/99, according to the sequence analysis (NetNGlyc) at Technical University of Denmark (<http://www.cbs.dtu.dk/services/NetNGlyc/>).

To directly compare with the A/New Caledonia antigen present in the egg-based vaccine, the purified rHA/A/New Caledonia was formulated either alone into a 15  $\mu\text{g}/0.5\text{ ml}$  solution or with 15  $\mu\text{g}$  rHA/B/Jiangsu and 45  $\mu\text{g}$  rHA/A/Wyoming in a 0.5 ml dosage (FluBIØk<sup>TM</sup>, the expected trade name of Protein Sciences' rHA vaccine). As judged by the hemagglutination assay, FluBIØk is as active as Vaxigrip (a licensed egg-based vaccine manufactured by Sanofi-Pasteur-Aventis) in agglutinating RBCs and preventing them from forming a tight pellet as shown in Fig. 4a. Antigen specific SRID assay is widely used to determine the concentration of active ingredients in a vaccine. On the SRID gel prepared with A/New Caledonia/20/99 antibody (Fig. 4b), the diffusion ring of FluBIØk is slightly larger than that of Vaxigrip, suggesting that FluBIØk is equivalent to Vaxigrip

for the active ingredient of A/New Caledonia/20/99 strain. On the SDS-PAGE (Fig. 4c), the FluBIØk lane is clean, and only three broad bands are visible, representing the HA, HA1 and HA2 of the three strains, respectively. The Vaxigrip lane is complicated with the major HA band around 59 kDa along with many minor bands. Similarly, numerous impurities have also been found in other egg-based vaccines [40].

All these data demonstrate that the purified rHA expressed in insect cells is correctly translated, properly glycosylated and folded and biologically active.

#### 4. Discussion

The traditional egg-based vaccines have been successfully used for more than 50 years to prevent influenza. They are reliable, effective (if there is a good match), and affordable. However, the production cycle of the egg-based vaccines is lengthy and heavily dependent on egg supply and unable to be developed quickly in response to the urgent need in an influenza pandemic [9–12]. To replace or supplement the

egg-based vaccines, the new vaccine has to be equally effective, reliable, economical, and capable of being developed and delivered in a short period of time. The work reported here is some important progress toward an alternative influenza vaccine—the recombinant protein-based vaccine.

A new analytical method based on hemadsorption has been developed to closely monitor the expression of HA in insect cells. This method plays a critical role in ensuring optimal HA production and in determining the right harvest time, in addition to other harvest parameters such as HPI, cell's morphology and viability. It has also been successfully used at Protein Sciences Corporation to accelerate the screening process for the recombinant baculoviruses used for HA manufacturing.

A purification process has been developed to quickly purify the recombinant HA from the bulk harvested from the bioreactor while retaining its biological activity. Previously, HA purifications were heavily dependent on affinity chromatography using specific monoclonal antibodies or various lectins [41,42]. Such methods are highly selective, but difficult to scale up to commercial levels due to a number of limiting factors: (1) some of the ligands will leach off the column during the purification and they must be removed from the final product; (2) it is difficult to regenerate an affinity column after use, and thus the performance declines after each use; (3) the batch to batch variations in the quality of affinity media make it almost impossible to have a robust purification process from time to time; (4) most affinity media are too expensive to be used at large scale. On the other hand, all chromatographic media used in the present process are chemically stable (can be regenerated repeatedly), commercially available and relatively inexpensive; thus more suitable to scale up in a GMP facility. If the reagents, columns and ancillary equipment are well prepared in advance, the whole purification can be completed in one full working day, avoiding the overnight storage of intermediate rHA preparation and possible inactivation of rHA. Several factors have made this rapid process possible. First, there is no sample manipulation between purification steps, which makes a quick, continuous purification process possible. Second, Q/SP is connected in tandem, combining two chromatographic processes into one. Third, a chemically different chromatographic media—HX-I is used to differentiate rHA from the remaining impurities. Unlike ion-exchange media, the adsorption of proteins to HX-I involves both anionic and cationic interaction. The calcium group can interact with carboxylate residues, whereas the phosphate group can bind the basic residues on the surface of the protein. The bound proteins can be eluted by an increasing phosphate gradient or a gradient of calcium, magnesium ions. It is worth to point out that the purification process described here needs to be further optimized for large-scale production, for example, a tangential flow filter should be used to replace a stir cell at the polish step. Nonetheless, we believe the strategies described here can also be used to develop a rapid purification process for other recombinant proteins.

Biologically, FluB1Øk is as active as the egg-based vaccine—Vaxigrip as determined by the hemagglutination assay. Based on the SRID assay, FluB1Øk is equivalent to Vaxigrip for the active ingredient of A/New Caledonia/20/99 strain of influenza. In challenge studies, chickens were effectively protected against the H5N1 virus infection after inoculation with the rHA of the virus [43]. Moreover, two distantly related H1N1 rHA influenza vaccines using the baculovirus-insect cell expression system have also been demonstrated to partially protect mice against the lethal challenge of a recombinant 1918 pandemic influenza virus [24]. In clinical trials, the trivalent rHA vaccine (FluB1Øk) stimulates anti-HA antibody production at least as well as, and in the case of H3 rHA, superior to the traditional egg-based vaccine [19–23]. The 2004/05 influenza season Phase IIB field trial of FluB1Øk that enrolled 460 healthy subjects aged 18–49 further showed that the 45/45/45 dose was 100% efficacious in preventing culture positive influenza illness compared to placebo (Press Release, Protein Sciences Corp., June 14, 2005).

Furthermore, a recombinant protein-based vaccine, such as the FluB1Øk described here, also has other advantages over the traditional egg-based vaccines. It consists solely of three antigens (proteins) stored in sterile phosphate buffered-saline and without preservatives such as thimerosal (a mercury derivative currently used in the egg-based vaccine), antibiotics or adjuvants. Unlike the egg-based vaccines, no live influenza viruses, biocontainment facilities or harsh chemicals such as formaldehyde are used in manufacturing. This may explain why FluB1Øk has shown lower side effects than the licensed vaccines in clinical trials [19–23]. Therefore, a reliable, effective, and affordable recombinant protein-based influenza vaccine can be and should be developed to meet the challenge of a potential influenza pandemic. For pandemic preparedness, developing and stockpiling rHA influenza vaccines against the present H5N1 strain may be a good option to provide some protection for the first response personnel and the population in the hard-hit areas in the case of a pandemic, and to win the precious time for manufacturing of a more specific influenza vaccine.

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## Carbohydrate masking of an antigenic epitope of influenza virus haemagglutinin independent of oligosaccharide size

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**Comparison of the haemagglutinins (HA) of the pathogenic avian influenza viruses A/FPV/Dutch/27 (H7N7) and A/FPV/Rostock/34 (H7N1) revealed 94.7% nucleotide and 93.8% amino acid sequence homologies. Six of the seven *N*-glycosidic oligosaccharides of the Rostock HA are at the same positions as the six carbohydrates of the Dutch strain. The additional oligosaccharide side chain of the Rostock strain, which is of the complex type, is attached to asparagine<sub>149</sub> in antigenic epitope B. The accessibility of this antigenic epitope has been analysed by using rabbit antisera raised against synthetic peptides comprising amino acids 143–162. The carbohydrates of the HA of the Rostock strain have been modified (i) to truncated cores by expression in insect cells using a baculovirus vector, (ii) to oligomannosidic side chains by growth in the presence of the trimming inhibitor methyldeoxynojirimycin and (iii) to a single *N*-acetylglucosamine residue by removal of the oligomannosidic sugar with endo- $\beta$ -*N*-acetylglucosaminidase H. Neither the authentic nor the modified oligosaccharides allowed antibody binding, as indicated by enzyme-linked immunosorbent assay (ELISA) and Western blot analyses. Reactivity was observed, however, after complete removal of the carbohydrate from HA of the Rostock strain by digestion with peptide-*N*-glycosidase F. HA of the Dutch strain was reactive without prior peptide-*N*-glycosidase F treatment. These results demonstrate that a single *N*-acetylglucosamine at asparagine<sub>149</sub> is sufficient to prevent recognition of the peptide epitope.**

**Key words:** carbohydrate-mediated antigenic epitope masking/ influenza virus glycoprotein/*N*-linked oligosaccharides

### Introduction

Glycosylated proteins are standard constituents on the surface of most enveloped viruses and are essential for the initiation of infection, i.e. for receptor binding and for fusion of viral envelopes with cellular membranes (Klenk and Rott, 1988). They are also the major surface antigens eliciting neutralizing antibodies. With most enveloped viruses, the carbohydrates are *N*-glycosidically bound to asparagine and the structures of the carbohydrates are different at each attachment site. Evidence has been obtained that carbohydrates are important for the proper folding and correct oligomerization of polypeptides,

which are both prerequisites for the transport of the glycoproteins from the rough endoplasmic reticulum to the place of virus budding (Gibson *et al.*, 1980; Olden *et al.*, 1985; Deom *et al.*, 1986; Gething *et al.*, 1986; Schuy *et al.*, 1986; Pitta *et al.*, 1989; Vidal *et al.*, 1989; Garten *et al.*, 1992). Studies on influenza virus haemagglutinin (HA) have shown that carbohydrates may also modulate glycoprotein functions by interfering with proteolytic activation (Kawaoka *et al.*, 1984; Ohuchi *et al.*, 1989) and with receptor binding (Robertson *et al.*, 1985). Because the synthesis of *N*-glycosidic carbohydrate side chains by cellular enzymes follows the general pathway for this class of glycans (Kornfeld and Kornfeld, 1985), the oligosaccharides are not recognized as foreign by the immune system and are therefore not antigenic determinants by themselves, but may influence antigenicity by altering polypeptide folding or by modulating the accessibility of polypeptide epitopes to antibodies. Thus, the removal of carbohydrate residues from the sugar side chains can change the antigenic reactivity of viral glycoproteins (Van Eldik *et al.*, 1978; Kaluza *et al.*, 1980; Heinz *et al.*, 1984). Furthermore, escape mutants of influenza virus have been obtained under the pressure of a monoclonal antibody that had lost an oligosaccharide in the respective antigenic epitope (Skehel *et al.*, 1984), and the appearance or disappearance of oligosaccharide side chains has been found to occur naturally during antigenic drift of influenza viruses (Seidel *et al.*, 1991). The acquisition of new carbohydrate side chains may therefore provide a mechanism by which the virus escapes host defence.

Since it is well known that the structures of oligosaccharide side chains of viral glycoproteins vary, depending on the glycosylation site on the polypeptide (Keil *et al.*, 1985) and on the host cell, it was of interest to find out whether the structure and, in particular, the size of an oligosaccharide have an influence on antigenic epitope masking. We have analysed these questions in the present study on the HA of fowl plague virus (FPV). The structure of the HA oligosaccharides of the Rostock variant of this virus had been elucidated previously, and it was known that one of these side chains was attached to antigenic epitope B (Keil *et al.*, 1985). This oligosaccharide is missing in the Dutch variant of FPV and we have analysed here the reactivity of epitope B with specific peptide antisera depending on the presence of the side chain. We have also modified the oligosaccharide by using a trimming inhibitor, by expressing the HA in insect cells and by using endoglycosidases. We have found that the antisera reacted with the epitope only when it was completely devoid of carbohydrate.

### Results

#### *Comparison of the HA of the FPV strains Dutch and Rostock*

Sequence analysis reveals that the HA gene of FPV strain Dutch has the same length of 1742 nucleotides as found with FPV strain Rostock (Porter *et al.*, 1979; Garten *et al.*, 1985).

The HA of both strains differ in 86 nucleotides: 51 of these exchanges are silent mutations and 35 nucleotide exchanges result in 29 exchanged amino acids, six of which are double mutations (Figure 1).

The number and positions of all glycosylation sites are conserved, except for the attachment site at asparagine<sub>149</sub>, which cannot be glycosylated with the Dutch strain because serine at position 151 has been replaced by alanine (Figure 1). This site is localized on a loop forming antigenic epitope B on the tip of the HA molecule (Figure 8). That all attachment sites on the HA of the Dutch strain are glycosylated has been shown by the controlled removal of the oligosaccharides by endoglucosaminidase H (endo H). To obtain uniform oligosaccharides, virus was grown in the presence of the trimming inhibitor *N*-methyl-1-deoxynojirimycin (MdN) prior to enzyme incubation (Figure 2). Five distinct HA<sub>1</sub> (large subunit of influenza virus HA) and three HA<sub>2</sub> (small subunit of influenza virus HA) bands were found after labelling with [<sup>35</sup>S]methionine, indicating that HA<sub>1</sub> and HA<sub>2</sub> have four and two side chains, respectively. Figure 2 shows also that, when grown in the absence of the trimming inhibitor, HA<sub>1</sub> is largely insensitive to endo H treatment, whereas HA<sub>2</sub> has only one endo H-sensitive oligosaccharide. These results are similar to those obtained with the Rostock strain, except for an additional endo H-resistant oligosaccharide present on Rostock HA<sub>1</sub>. Thus, it could be demonstrated that the Dutch HA differs in its glycosylation pattern from the Rostock HA mainly by the absence of the complex oligosaccharide at asparagine<sub>149</sub>.

#### *The oligosaccharide at asparagine<sub>149</sub> prevents antibody binding at antigenic epitope B*

Since asparagine<sub>149</sub> is located in antigenic epitope B, it was of interest to find out if the differential glycosylation observed in this position with strains Rostock and Dutch had an influence on the reactivity of the epitope with specific antibodies. For this purpose, we have raised antisera in rabbits against peptides homologous to amino acids 143–162 of both strains. These sequences, differing only in two positions, one of which is essential for the glycosylation site (Figure 1), form a linear epitope at the tip of the HA spike (Figure 3).

Prior to examining the binding abilities of the anti-peptide antisera to native virions, it was necessary to demonstrate that the two rabbits immunized with either Dutch or Rostock peptide 143–162 coupled to keyhole limpet haemocyanin (KLH) produced a specific anti-peptide response. Antisera obtained from the two rabbits bound both peptides at comparable levels, whereas little binding to a control peptide (insulin) was observed, as determined by enzyme-linked immunosorbent assay (ELISA) (Figure 4B). The titre of the anti-Dutch peptide antiserum was ~1:50 000, expressed as the dilution required to give 50% maximum optical density reading at 492 nm, and that of the anti-Rostock peptide antiserum 1:6000, irrespective of the peptide used for coating the plates. Thus, both antisera developed a specific anti-peptide response and the differences within the amino acid sequences did not influence antigenic reactivities.

When the peptide antisera were tested for their binding capacities to virions, they were coated to the solid phase under mild conditions to ascertain that the HA is present in undenatured form. Although the response of the antisera to virus was less efficient than their response to the peptide antigens, the results

show that both antisera recognize virions of the Dutch strain much better than virions of the Rostock strain (Figure 4A).

We then analysed the reactivity of the anti-Dutch peptide antiserum with both HA in an immunoblot assay, and assayed the effect of glycosidase-mediated carbohydrate removal from HA on immune recognition. As shown in Figure 5, the HA<sub>1</sub> subunit of the Dutch strain was immunostained, whereas the anti-peptide antiserum failed to recognize that of the Rostock strain. However, after complete removal of the oligosaccharide by treatment with peptide *N*-glycosidase F (glyco F), HA<sub>1</sub> of the Rostock strain could be detected in the immune reaction. On the other hand, apart from reducing the electrophoretic mobility, deglycosylation had no effect on the immune reactivity of Dutch HA<sub>1</sub>. As indicated by the immunoblot with anti-virus antiserum also shown in Figure 5, equivalent amounts of HA<sub>1</sub> of both strains have been used in this experiment. These observations indicate that the complex oligosaccharide attached to asparagine<sub>149</sub> prevents the binding of site-specific antibodies to the HA.

#### *Epitope shielding by modified oligosaccharide side chains*

The complex oligosaccharides found on HA from vertebrate hosts are replaced by small truncated side chains of the structures Man<sub>3</sub>GlcNAc<sub>2</sub> and Man<sub>3</sub>[Fuc]GlcNAc<sub>2</sub> when the HA is expressed in insect cells (Kuroda *et al.*, 1990). To test the effect of a trimannosyl core at asparagine<sub>149</sub> on the binding of the anti-peptide antisera, we have expressed the HA of the Rostock strain in SF9 cells using a baculovirus vector. Comparative immunoblots of Rostock HA obtained from insect cells, and from Rostock and Dutch HA originating from chick embryo cells (CEC), are shown in Figure 6.

The results show that the immunoreactivity of the Rostock strain is enhanced after carbohydrate removal by glyco F, regardless of whether the HA has been derived from SF9 cells or CEC. In contrast, Dutch HA<sub>1</sub> obtained from CEC reacted with the antiserum without prior glyco F treatment. Thus, the presumptive trimannosyl core (Kuroda *et al.*, 1990) interferes with antiserum binding at asparagine<sub>149</sub>, as does the complex oligosaccharide side chain.

When FPV is grown in the presence of the trimming inhibitor (MdN), HA is assembled into trimers, transported to the cell surface and incorporated into virus particles, but its carbohydrate side chains persist in the unprocessed oligomannosidic form (Romero *et al.*, 1983). These oligosaccharides, among which the structures Man<sub>6-9</sub>GlcNAc<sub>2</sub> prevail, are susceptible to cleavage by endo H which leaves the proximal *N*-acetylglucosamine residue bound to the polypeptide. It was therefore possible to analyse the effects of oligomannosidic side chains and of a single *N*-acetylglucosamine on epitope shielding.

FPV strains Dutch and Rostock were grown in CEC in the absence or presence of the trimming inhibitor. Purified virus grown in the presence of MdN was treated with endo H and, for complete deglycosylation, virus grown in the absence of MdN was incubated with glyco F. Enzyme-treated or untreated virus samples were subjected to SDS-PAGE, and HA was visualized by immunoblotting with anti-Dutch peptide antiserum (Figure 7). The results obtained with Rostock strain show that anti-peptide antiserum recognized HA grown in the presence of the trimming inhibitor MdN neither before nor after endo H treatment, but did recognize HA after complete carbohydrate removal by glyco F. In contrast, irrespective of

## H7 HEMAGGLUTINININS OF THE INFLUENZA VIRUS STRAINS

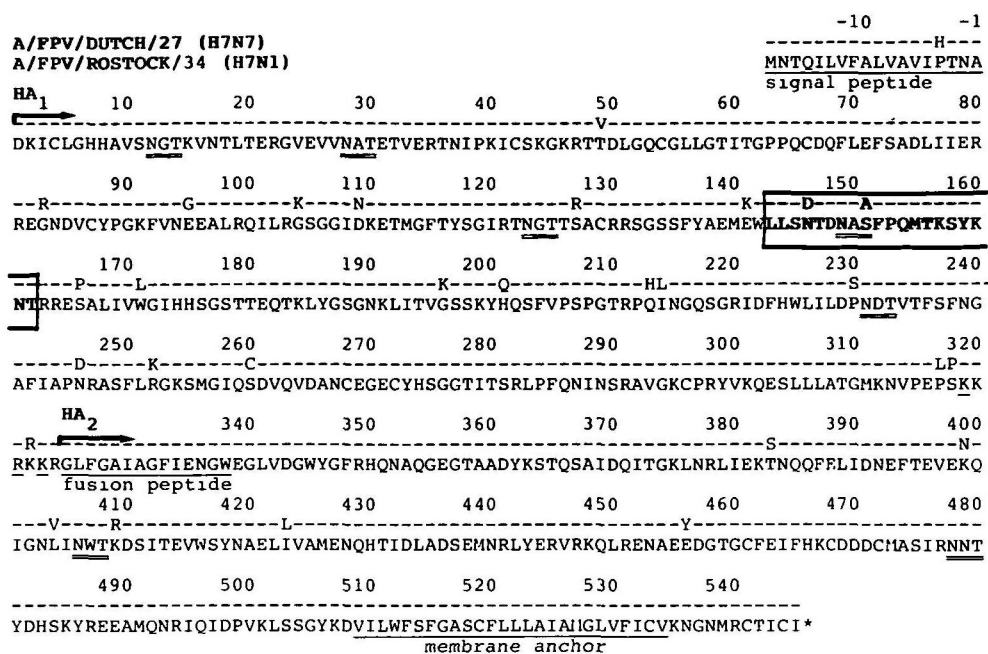


Fig. 1. Amino acid sequences of the HA of the strains A/FPV/Rostock/34 (H7N1) and A/FPV/Dutch/27 (H7N7). The hydrophobic domains (signal, fusion and membrane anchor peptides) are underlined, the attachment sites for *N*-glycosylated carbohydrates are indicated by double lines and the amino acids connecting HA<sub>1</sub> with HA<sub>2</sub> by a dashed line. The sequences of the synthetic peptides used for immunization are framed.

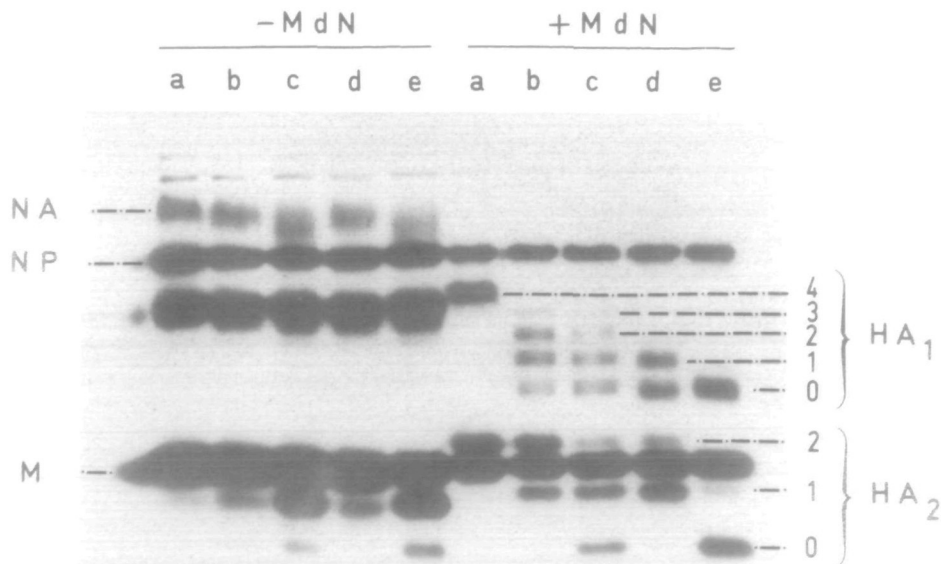


Fig. 2. Controlled removal of oligosaccharides from the HA of strain A/FPV/Dutch/27. Virus labelled with [<sup>35</sup>S]methionine was grown in the absence (-M d N) or in the presence (+M d N) of the trimming inhibitor. After purification, native or denatured (96°C, 2 min, 0.2% SDS and 1% mercaptoethanol) virus was incubated with endo H. (a) Control virus not incubated with endo H. (b) Virus incubated with endo H for 15 min. (c) Denatured virus incubated with endo H for 15 min. (d) Native virus incubated with endo H for 3 h. (e) Denatured virus incubated with endo H for 3 h. The viral proteins were separated by SDS-PAGE and analysed by fluorography. The HA bands (HA<sub>1</sub> and HA<sub>2</sub>) with the number of carbohydrate side chains are indicated. Neuraminidase (NA), nucleoprotein (NP) and matrix protein (M) of influenza virus are also shown.

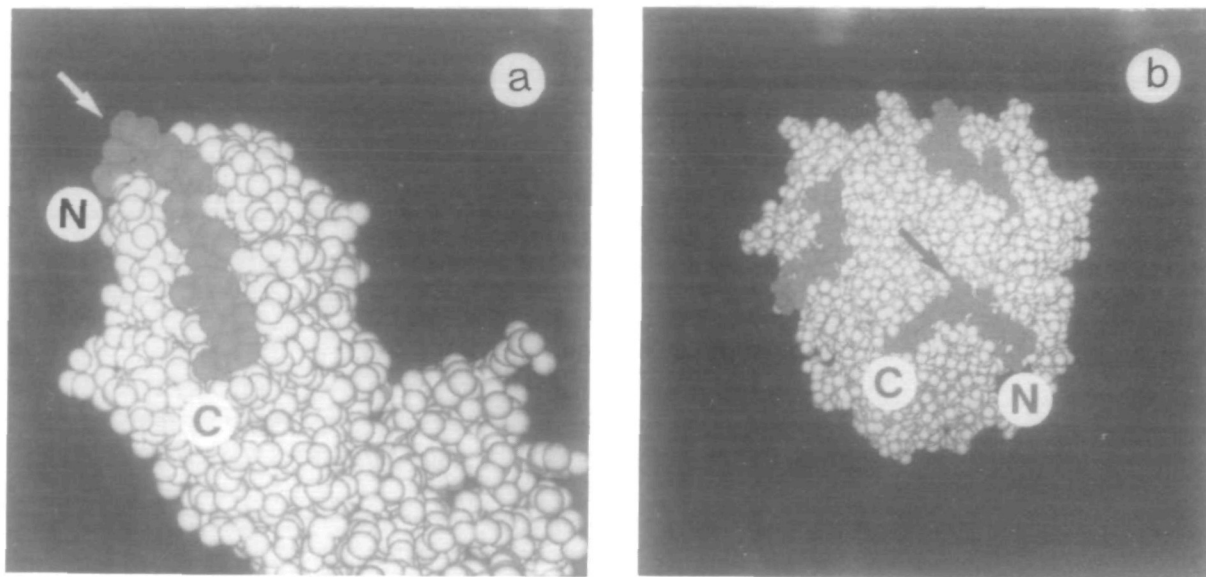


**Fig. 3.** The location of the peptide segment 143–162 within the globular domain of the HA. (a) HA monomer, (b) HA trimer. The oligosaccharide attachment site at asparagine<sub>149</sub> in the HA of strain Rostock (arrow) and the N- and C-terminal orientation of the peptide segment used for immunization (dark atoms) are indicated.

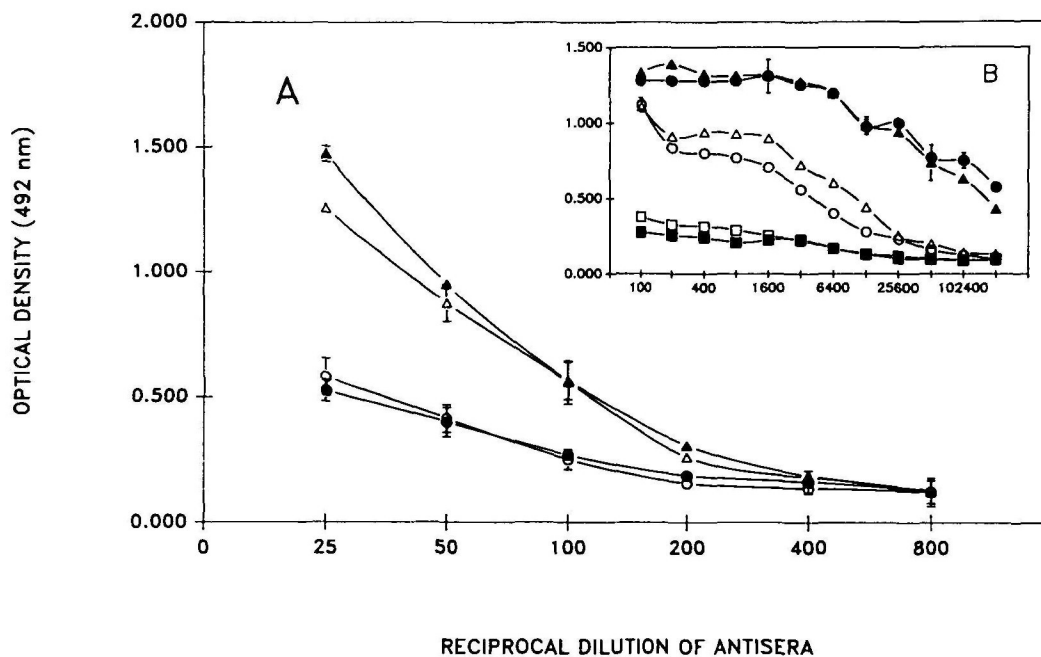


**Fig. 4.** Specificity of peptide antisera. Antisera raised in rabbits against synthetic peptides linked to KLH were analysed at the dilutions indicated in an ELISA for their reactivity with virions and peptides. (A) Reactivity with virions. FPV/Dutch virions (triangles) and FPV/Rostock virions (circles) were used as antigens. The binding curves of the antisera raised against the Dutch peptide (filled symbols) and against the Rostock peptide (open symbols) are shown. When preimmune sera were used, only background binding was observed at dilutions of 1:25 (data not shown). (B) Reactivity with peptides. The following peptides were used to coat the solid phase: Dutch peptide (triangles), Rostock peptide (circles) and insulin (rectangles) as control. The binding curves of the antisera raised against the Dutch peptide (filled symbols) and against the Rostock peptide (open symbols) are shown.

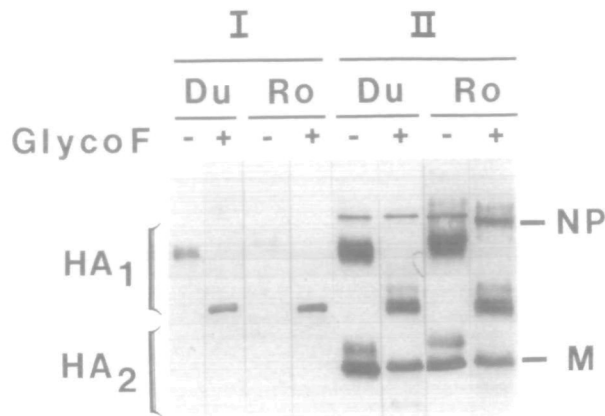


Fig. 5. Effect of HA deglycosylation with glyco F on the reactivity with the anti-Dutch peptide antiserum. Strains A/FPV/Rostock/34 (Ro) and A/FPV/Dutch/27 (Du) were grown in embryonated eggs. The immune reactivity was assessed against purified virus, which was either deglycosylated (+) by glyco F or untreated (-), subjected to SDS-PAGE and subsequently blotted onto nitrocellulose. I Reactivity of anti-Dutch-peptide antiserum with HA<sub>1</sub>. II Reactivity of antiserum raised against virus A/FPV/Rostock/34 with HA<sub>1</sub>, HA<sub>2</sub>, NP and M.

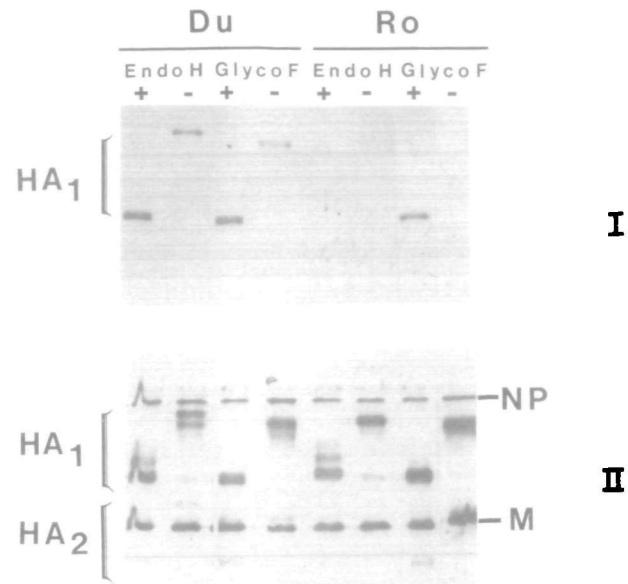


Fig. 7. Effect of HA deglycosylation with endo H on the reactivity with the anti-Dutch peptide antiserum. Strains A/FPV/Rostock/34 (Ro) and A/FPV/Dutch/27 (Du) were grown in CEC in the presence and absence of the trimming inhibitor MdN. Purified virus grown in the presence of MdN was incubated with (+) or without (-) endo H. Purified virus grown in the absence of MdN was incubated with (+) or without (-) glyco F. Samples were subsequently analysed with the anti-peptide antiserum (I) and as a control with antibodies directed against influenza virus FPV/Rostock/34 (II).

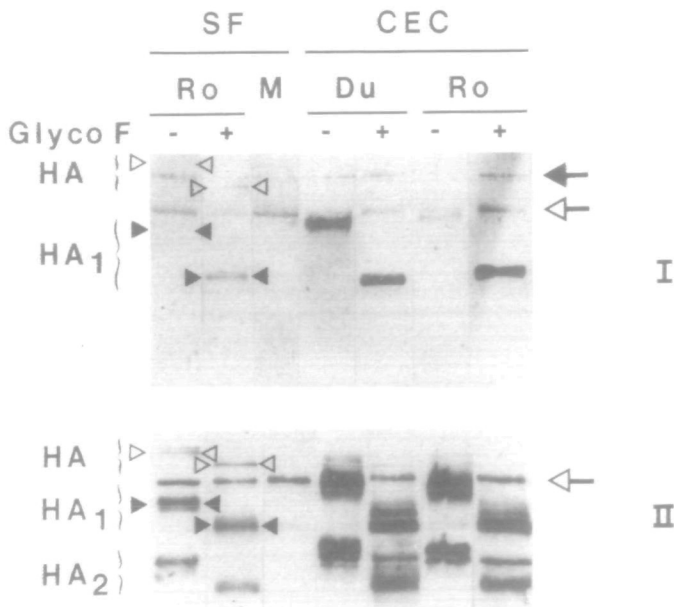


Fig. 6. Reactivity of anti-Dutch peptide antiserum with HA expressed in insect cells. Rostock HA (Ro) expressed in *S. frugiperda* (SF) cells, and HA of strains Rostock (Ro) and Dutch (Du) obtained from fowl plague virions, grown in CEC, were immunoprecipitated using the monoclonal antibody HC2 before treatment with glyco F. Treated (+) and untreated (-) samples of immunoprecipitated HA were subjected to SDS-PAGE, transferred to nitrocellulose and incubated with anti-Dutch peptide antiserum (I) or antiserum raised against influenza virus A/FPV/Rostock/34 (II). SF cells infected with wild-type baculovirus AcNPV are also shown (M). Uncleaved HA is indicated by open triangles, subunit HA<sub>1</sub> by filled-in triangles. Two non-specific bands are marked by open and filled arrows.

treatment with MdN, endo H or glyco F, HA of the Dutch strain was always recognized. Similar results were obtained when an anti-Rostock antiserum was used for immunoblotting (data not shown). A control blot with anti-FPV antiserum also shown in Figure 7 demonstrates that equal amounts of viral

proteins had been used in all lanes. These data indicate that a single *N*-acetylglucosamine residue at asparagine<sub>149</sub> prevents the binding of the anti-peptide antibodies, as is the case with carbohydrates of the complex and the oligomannosidic type, and with the truncated oligosaccharide cores obtained in invertebrate cells.

## Discussion

The HA of the Dutch and Rostock strains of FPV are closely related in their structural and biological properties. They have 94.7% homology at the nucleotide level and 93.8% homology at the amino acid level. Homology includes glycosylation sites at asparagine residues 12, 28, 123, 231 and 478, which all contain complex oligosaccharides, as well as an attachment site at asparagine<sub>406</sub> that is utilized by an oligomannosidic side chain. The Rostock HA has a seventh glycosylation site at asparagine<sub>149</sub> which is occupied by a complex side chain. This oligosaccharide is missing in the Dutch strain, in which the consensus sequence Asn-Ala-Ser has been altered to Asn-Ala-Ala. As pointed out above, it has been shown before that variable oligosaccharides, such as the one at asparagine<sub>149</sub>, mask antigenic peptide epitopes and thus enable the virus to escape immune defence by specific antibodies (Alexander and Elder, 1984; Skehel *et al.*, 1984; Caust *et al.*, 1987; Davis *et al.*, 1987). This concept is supported by the observation that the epitope B-specific antisera used in the present study recognize the HA of the Dutch, but not of the Rostock strain. It was also of interest to find out if structure and size of the oligosaccharide have an effect on its masking capacity. We have therefore extended our studies by modifying the oligosaccharide

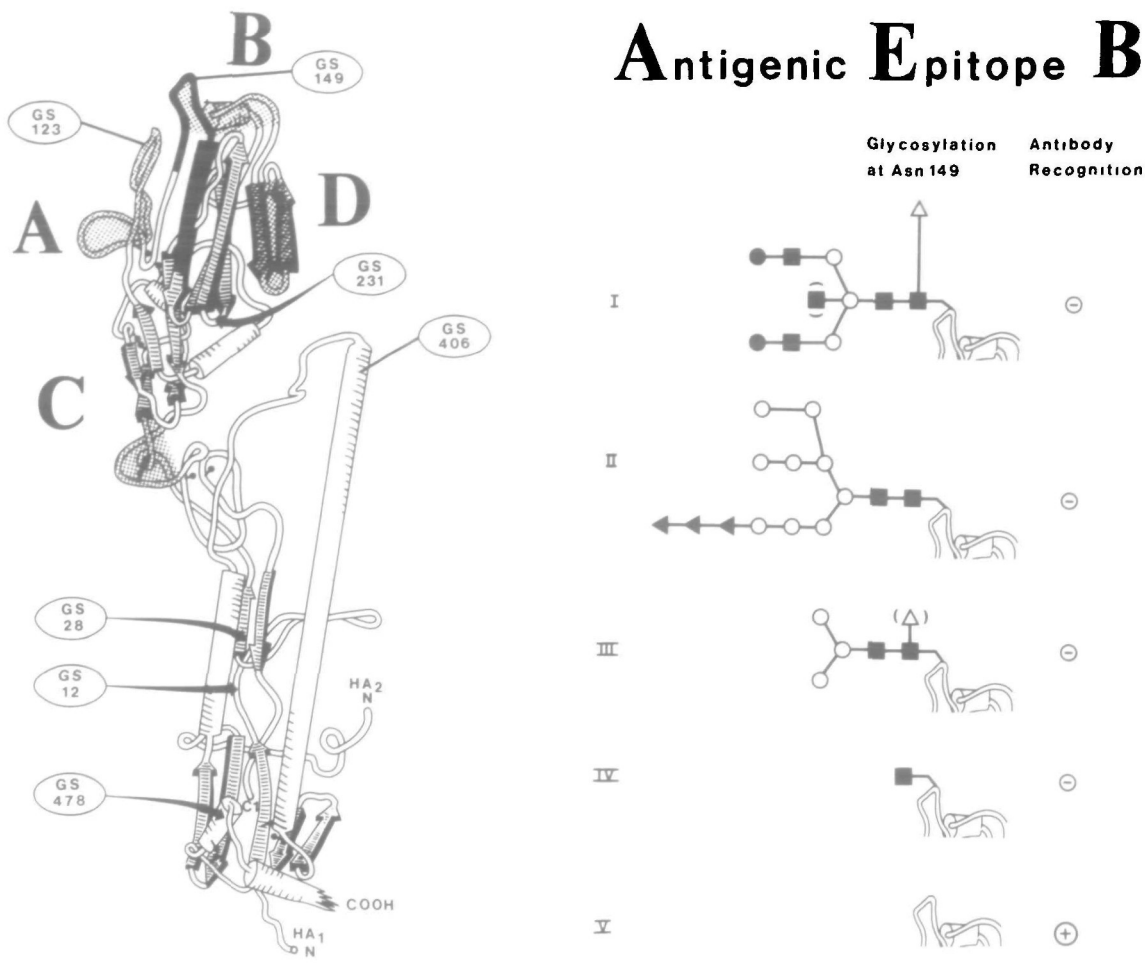


**Fig. 8.** Influence of different oligosaccharide structures on antibody binding to antigenic epitope B. The three-dimensional structure of the H3 HA molecule with the antigenic epitopes A, B, C and D (Wiley *et al.*, 1981; Wilson *et al.*, 1981) and the glycosylation sites (GS) as determined for the HA of A/FPV/Rostock/34 (H7N1) (Keil *et al.*, 1985) are shown on the left. The peptide segment to which the antisera are directed is drawn in black. Recognition of antigenic epitope B by the site-specific antisera, as depending on the structure of the oligosaccharide attached to asparagine<sub>149</sub>, is shown on the right. The following oligosaccharide side chains have been analysed: I, complex biantennary oligosaccharide present on Rostock HA derived from chick embryo cells; II, oligomannosidic found on Rostock HA grown in the presence of the trimming inhibitor MdN; III, truncated oligosaccharide present on Rostock HA expressed in insect cells; IV, *N*-acetylglucosamine retained after endo H treatment of structure III; V, complete absence of carbohydrate observed on Rostock HA after glyco F treatment or on Dutch HA; (■) *N*-acetylglucosamine; (○) mannose; (●) galactose; (△) fucose; (▲) glucose.

at position asparagine<sub>149</sub> of the Rostock HA. The results, which are summarized in Figure 8, indicate that the structure of the oligosaccharide is not important and that a single *N*-acetylglucosamine residue is sufficient for antigen masking.

Antisera directed against epitope B were prepared by immunizing rabbits with a site-specific synthetic peptide. It was known from a previous study, employing antisera against a series of subtype H3-specific peptides, that the sole requirement for the generation of anti-peptide antibodies reactive with native molecules is the presentation of the peptide on the surface of the proteins (Green *et al.*, 1982). As shown in Figure 3, peptide 143–162 fulfils this requirement. The model also reveals that this peptide has a linear configuration on the HA surface. It is therefore not surprising that antibodies raised against both synthetic peptides are also recognized by native HA. Furthermore, it is reasonable to assume that unfolding of the HA, occurring during Western blot analysis, does not drastically alter the antigenicity of this epitope. Since the same results were obtained with antisera raised against the Dutch and Rostock specific peptides, it is clear that only the variations in glycosyl-

ation, but not the amino acid exchanges at positions 146 and 151, account for the differential reactivities of the antisera with both HA.

Because viruses use the glycosylation machinery of the cell for carbohydrate synthesis, the glycosylation patterns of viral glycoproteins are determined to a large extent by the host. Such host-specific changes in glycosylation are quite dramatic when a glycoprotein of vertebrate origin is expressed in insect cells by a baculovirus vector. Under these conditions, the complex oligosaccharides present on the FPV HA derived from vertebrate cells are replaced by truncated side chains (Kuroda *et al.*, 1990). Because of the reduced size of these side chains, only 10% of the HA surface is covered with carbohydrate, as compared to 20% on HA derived from mammalian cells. It was therefore interesting to see in the present study that, at least for epitope B, antibody accessibility was not enhanced by reduction of oligosaccharide size. Even more surprisingly, antibody reactivity was abolished by a single glucosamine residue. An explanation for these unexpected results may be provided by the information available on the chemistry of antibody binding.

The main factors supporting the antigenicity of a polypeptide are high mobility, low packing density, convex shape, and significant negative electrostatic potential at the molecular surface (Geysen *et al.*, 1987). Our data suggest that two of these factors, the electrostatic potential and the flexibility of the polypeptide chain, are affected by the variations in glycosylation. The immunogenic loop of epitope B of the Rostock HA contains three charged amino acids. Two of them are localized close to the C-terminus of the synthetic peptide and may play a minor role in eliciting an immune response because the peptide was C-terminally coupled to the carrier protein for immunization, whereas the majority of antibodies obtained from rabbits are assumed to be directed against the N-termini of synthetic peptides (Dyrberg and Oldstone, 1986). However, the third charged amino acid, aspartate<sub>148</sub>, appears to be directly affected by the carbohydrate. Since it is immediately adjacent to the glycosylation site at asparagine<sub>149</sub>, it is not difficult to understand that a truncated oligosaccharide core or even a single *N*-acetylglucosamine is sufficient to mask the charged residue. Only a complete lack of carbohydrate would then allow antibody-antigen complex formation at an increased rate by promoting facilitated diffusion and stabilizing a final antigen-antibody contact. The second factor interfering with the antigenicity of the underlying peptide appears to be the relative rigidity of the trimannosyl core and of the single *N*-acetylglucosamine residue, which are both less flexible than the ordinary biantennary complex oligosaccharide (Montreuil, 1984). Rigidity of the carbohydrate might then prevent side chain rearrangement of the local amino acids which is assumed to be necessary for antibody binding to a protein. Finally, when compared to the regular biantennary complex oligosaccharide, the partially fucosylated trimannosyl core has an increased hydrophobicity (Montreuil, 1984). Hydrophobicity, well known to be incompatible with antigenicity, may therefore be another factor contributing to diminished antibody binding. Thus, there are at least three mechanisms that could explain why carbohydrate side chains retain their capacity to mask antigenic epitopes even when they are drastically reduced in size. It therefore appears that steric hindrance may not be the only mechanism by which carbohydrates modulate the accessibility of a peptide epitope to antibody, but that they also accomplish this by imposing conformational changes on the protein structure.

## Materials and methods

### Propagation and purification of virus

The Dutch and Rostock strains of FPV [A/FPV/Dutch/27(H7N7) and A/FPV/Rostock/34 (H7N1)] were grown in 11-day-old embryonated hen eggs and subsequently purified by sucrose gradient centrifugation after adsorption to and elution from chicken erythrocytes. In some experiments, virus was propagated in primary chick fibroblasts in the absence or presence of MdN (2 mM) (Rott *et al.*, 1984) and purified by equilibrium centrifugation in sucrose density gradients. Virus was metabolically radiolabelled with [<sup>35</sup>S]methionine (10 µCi/ml). The recombinant *Autographa californica* nuclear polyhedrosis virus (AcNPV-HA) was propagated in *Spodoptera frugiperda* (SF9) cells (Kuroda *et al.*, 1986).

### Preparation of viral RNA and nucleotide sequencing

Viral RNA was obtained from egg-grown FPV, Dutch strain. The HA gene was analysed by the primer extension method (Daniels *et al.*, 1983; Schuy *et al.*, 1986) using synthetic oligonucleotide primers complementary to the nucleotide sequences 4–17, 205–215, 427–437, 573–583, 721–730, 996–1005, 1315–1323, 1444–1457 and 1597–1607 of the HA gene of FPV (Porter *et al.*, 1979; Garten *et al.*, 1985). Computer-assisted sequence analyses were performed using the microGenie system (Beckmann, Palo Alto, CA).

### Synthetic peptide antigens and anti-peptide antibodies

Peptides comprising amino acids 143–162 of the FPV HA were synthesized by the solid phase method (Merrifield, 1963). Peptide (15 mg) was incubated with 15 mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (Sigma, Deisenhofen, FRG) for 20 min at 0°C in 200 µl 1 mM HCl (pH 3.0). KLH (30 mg; Calbiochem, Frankfurt/Main, FRG) solubilized in water was mixed with the activated peptide in a final volume of 1 ml, adjusted to pH 9.0 by the addition of triethylamine and the mixture was stirred at 20°C for 3 h. The C-terminal KLH-coupled peptide was desalted by chromatography through a prepacked Sephadex G-25 column (Pharmacia, Freiburg, FRG) which was equilibrated with phosphate-buffered saline (PBS). Amounts of 5 mg KLH-peptide conjugates were mixed with complete Freund's adjuvant and s.c. injected into rabbits. Injections were repeated twice at intervals of 4 weeks. The rabbits were bled 4 weeks after the last booster injection.

### ELISA

Virus was plated at 100 µl/well of 0.5 µg protein/ml in PBS (calcium and magnesium free) and incubated overnight at 4°C. Using radioactively labelled virions, equal amounts (~65%) of both strains were found to be absorbed to the plastic. Synthetic peptides, as solid-phase substrates, were prepared by drying 0.5 µg peptide in distilled water on 96-well plates (Nunc Immulon, Wiesbaden, FRG) overnight at 37°C (Vaux *et al.*, 1988). Non-specific sites were blocked by preincubation with PBS containing 0.05% Tween 20 and 0.2% bovine serum albumin (BSA) for 1 h at 37°C. For detection, biotin-donkey antibody to rabbit Ig and avidin-conjugated horseradish peroxidase (Amersham-Buchler, Braunschweig, FRG), each at dilutions of 1:600 in blocking solution, were used. Peroxidase activity was determined with *o*-phenylenediamine (Abbott, Wiesbaden, FRG) as substrate. The binding of the rabbit antisera was quantitated spectrophotometrically at 495 nm.

### Digestion with endo H and glyco F

For controlled removal of oligosaccharides, labelled virus samples [20 haemagglutinating units (HAU)] were incubated with endo H (EC 3.2.1.96), *Streptomyces phycatus* recombinant from *Escherichia coli* (0.5 mU) (Boehringer Mannheim, FRG) in 20 µl Tris-HCl buffer (0.1 M, pH 6.8) at 37°C or had been denatured by boiling for 2 min in the presence of 0.2% SDS and 1% mercaptoethanol prior to enzyme incubation (Rott *et al.*, 1984). For the other deglycosylation experiments, virus samples or immunoprecipitated HA (Kuroda *et al.*, 1989) were suspended in 20 µl sodium acetate buffer (0.1 M, pH 7.0; 50 mM EDTA) containing 0.1% SDS, 0.5% mercaptoethanol and 0.5% *n*-octylglycoside. After boiling for 5 min, samples were incubated for 15 min at 37°C with endo H (0.2 mU) and glyco F (40 mU) [(EC 3.2.2.18), from *Flavobacterium meningosepticum*] (Boehringer Mannheim, FRG), respectively. The virus proteins of the immunoprecipitated samples were clarified by centrifugation prior to enzyme incubation.

### Immunoblotting

Samples were analysed on 12% gels by SDS-PAGE (Laemmli, 1970) and subsequently blotted onto nitrocellulose (Kyhse-Andersen, 1984). The blots were visualized with peptide or virus-specific rabbit antisera which were used at a dilution of 1:500. After washing, the blots were incubated with biotinylated donkey antibody to rabbit immunoglobulins (Amersham-Buchler, Braunschweig, FRG). After incubation with streptavidin-peroxidase complex (Amersham-Buchler), the blots were developed with 4-chloro-1-naphthol (Sigma, Deisenhofen, FRG) and H<sub>2</sub>O<sub>2</sub>.

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## Abbreviations

CEC, chick embryo cells; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; endo H, endo- $\beta$ -*N*-acetylglucosaminidase H; FPV, fowl plague virus; glyco F, peptide *N*-glycosidase F; HA<sub>1</sub>, haemagglutinin of influenza virus, large subunit; HA<sub>2</sub>, haemagglutinin of

influenza virus, small subunit; HAU, haemagglutinating units; KLH, keyhole limpet haemocyanin; M, matrix protein of influenza virus; MdN, methyldeoxynojirimycin; NP, nucleoprotein of influenza virus; PBS, phosphate-buffered saline; SF9, *Spodoptera frugiperda* cell line 9.

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