



No. 07-030535- -00002-0000

Fecha: 2007-08-28 16:31:55 Dep. 2020 NULCRACIONE
Tra. 11 PATENTEIYII Eje: 378 FASENACIONALI
Act. 444 RESPUESTAREQUE Folios: 37

Señora

Alix Carmenza Céspedes de Vergel

Jefe de División de Nuevas Creaciones

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

E. _____ S. _____ D. _____

Referencia: Expediente No. 07.030.535

Solicitud de Patente de Invención titulada: "Producción de proteína fusión de TNFR-Ig"

Solicitante: Wyeth Research Ireland Limited

RESPUESTA AL AUTO NOTIFICADO POR FIJACIÓN EN LISTA NO. 269**DEL 27 DE JULIO DE 2007, OFICIO NO. 8882 (EXAMEN DE FORMA)**

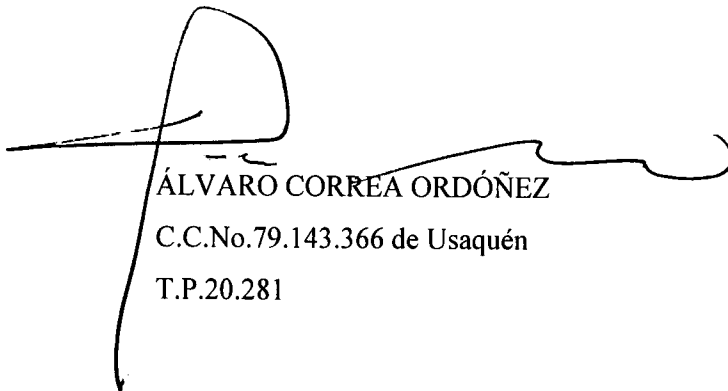
ÁLVARO CORREA ORDÓÑEZ, mayor de edad y vecino de Bogotá D.C., identificado tal como aparece al pie de mi firma, en mi carácter de apoderado de la sociedad en referencia, doy respuesta al auto emanado por ese Despacho dentro del término legal:

1. Presento copia debidamente legalizada del documento en que consta la cesión del derecho a la patente de los inventores a la entidad solicitante. De igual manera presento copia debidamente legalizada del recibo de presentación de la solicitud US11/213,633 en donde se muestra que esta solicitud reclama prioridad de la solicitud 60/605,379.
2. Presento copia debidamente legalizada del Poder de abogado y del documento que acredita la existencia y representación legal de la entidad solicitante.
3. Aprovecho la oportunidad para solicitar la modificación del nombre del inventor LUAN, Yen-Tuang por LUAN, Yen-Tung. Para tal efecto, adjunto formulario de modificación y recibo de la tasa por modificación voluntaria a solicitud en trámite.

3378

100002

Atentamente,



ÁLVARO CORREA ORDÓÑEZ

C.C.No.79.143.366 de Usaquén

T.P.20.281

Anexos: Poder de Abogado
Certificado de Existencia y Representación legal
Documento de Cesión de Inventores
Recibo oficial de presentación de solicitud US11/213,633
Formulario de modificación
Recibo

BOGATM/BOGDMS-86642.DOC

Estado de Massachussets
William Francis Galván
Secretario del Estado

APOSTILLA

1. País: Estados Unidos de América
Este documento público
2. Ha sido firmado por Lisa A. Walsh
3. Quien actúa en calidad de Notario Público
4. Lleva el sello del Estado de Massachussets. Su comisión vence el 29 de mayo de 2009

CERTIFICADO

5. En Boston, Massachussets
 6. El 13 de julio de 2007
 7. Por el Secretario del Estado
 8. N° 1242106
 9. Sello *El Gran Sello del Estado* (Aparece adherido sello dorado con cinta azul)
 10. Firma: (firmado) William Francis Galvin, Secretario del Estado
- 
- 

Hoy 12 de julio de 2007, certifico que el documento anexo es una copia auténtica, exacta, completa e inalterada (3 páginas) hecha por mí de nuestros archivos de un recibo de presentación para el número de serie 11/213.633.

[Firmado] Lisa A. Walsh, Notario Público. Mi comisión vence el 29 de mayo de 2009

339

TRADUCCIÓN OFICIAL N° 2707071 al español de un documento escrito en inglés, efectuada por Jaime Armando Garavito Burgos, c.c. N° 19.130.702 de Bogotá, Traductor e Intérprete Oficial inglés-español-inglés según Resolución N° 034 de enero 11 de 1983 expedida por el Ministerio de Justicia de la República de Colombia

[Aparece sello realizado]

[Aparece impreso un escudo]

OFICINA DE PATENTES Y MARCAS DE LOS ESTADOS UNIDOS

[Aparece dirección postal y página Web]

Solicitud No. 11/213.633
Presentación OR 371
(c) Fecha: Agosto 25, 2005

Unidad de artículo: 1638
Tarifa de presentación recibida: 0.00
Archivo del apoderado No. 2004658-0085 (AM101550L4)
Gráficas: 38
Total reivindicaciones: 108
Reivindicaciones ind.: 11

CONFIRMACIÓN No. 8681

RECIBO DE PRESENTACIÓN

[Aparece código de barras]

OC000000017088375

53170
CHOATE, HALL & STEWART LLP
PATENT GROUP
TWO INTERNATIONAL PLACE
BOSTON, MA 02110

Fecha de envío: Septiembre 23, 2005

Acusamos recibo de esta Solicitud de Patente regular. Será considerada en su orden y se le notificarán los resultados del examen. Sírvase asegurarse de suministrar el NÚMERO DE LA SOLICITUD DE LOS EE.UU., FECHA DE PRESENTACIÓN, NOMBRE DEL SOLICITANTE y TÍTULO DE LA INVENCION cuando averigüe por esta solicitud. Las tarifas transmitidas por cheque o giro están sujetas a cobro. Favor verificar la exactitud de la información presentada en este recibo. **Si se observa algún error en este Recibo de Presentación, favor enviarlo al Comisionado de Patentes P.O. Box 1450 Alexandria Va 22313-1450. Favor suministrar una copia de este Recibo de Presentación**

340

TRADUCCIÓN OFICIAL N° 2707071 al español de un documento escrito en inglés, efectuada por Jaime Armando Garavito Burgos, c.c. N° 19.130.702 de Bogotá, Traductor e Intérprete Oficial inglés-español-inglés según Resolución N° 034 de enero 11 de 1983 expedida por el Ministerio de Justicia de la República de Colombia

con los cambios anotados en él. Si recibió una "Notificación para presentar porciones faltantes" de esta solicitud, sírvanse presentar cualesquier correcciones a este Recibo de Presentación con su respuesta a la Notificación. Cuando la Oficina de Patentes y Marcas de los Estados Unidos tramite la respuesta a la Notificación, generará otro Recibo de Presentación que incluya las correcciones solicitadas (de ser apropiado).

Solicitante(s)

Denis Drapeau, Salem, NH;
Yen-Tung Luan, Chelmsford, MA;
Jim Mercer, Derry, NH;
Wenge Wang, North Chelmsford, MA;
Dan Lasko, Medford, MA;



Cesión de la Solicitud de Patente Publicada
Wyeth, Madison, NJ

Poder: Ninguno

Información de prioridad doméstica reivindicada por el solicitante
Esta solicitud reivindica el beneficio de 60/605.379 Agosto 27, 2004

Solicitudes Extranjeras

Si se requiere, Licencia de Presentación otorgada: Septiembre 13, 2005

El código del país y el número de su solicitud de prioridad que deben usarse para presentación en el exterior bajo la Convención de París es US11/213.633

Fecha proyectada de publicación: Por determinarse – queda pendiente completar las Porciones Faltantes.

[Aparece sello de Recibido del Departamento de Patentes con fecha septiembre 27, 2005]

Sin Solicitud de Publicación: No

Solicitud de Publicación Temprana: No

Título: Producción de TNFR-Ig

Clase preliminar: 800

PROTEGIENDO SU INVENCION FUERA DE LOS ESTADOS UNIDOS

Puesto que los derechos otorgados por una patente de los Estados Unidos se confieren solamente dentro del territorio de los Estados Unidos y no tienen efecto en un país extranjero, un inventor que desee proteger su patente en otro país, debe solicitar una patente en un país específico o en oficinas regionales de patentes. Es probable que los solicitantes deseen considerar la presentación de una solicitud internacional bajo el Tratado de Cooperación de Patentes. Una solicitud internacional generalmente tiene el mismo efecto de una solicitud nacional de patente regular en cada país miembro del Tratado de Cooperación de Patentes. El proceso del Tratado de Cooperación de Patentes **simplifica** la presentación de solicitudes de patente sobre la misma invención en países miembros, pero **no resulta** en el otorgamiento de "una patente internacional" y no elimina la necesidad de que solicitantes presenten documentos adicionales y tarifas en países en los que se desea protección de patente.

Casi todo país tiene su propia legislación concerniente a las patentes y la persona que desee una patente en un país particular debe hacer una solicitud de patente en ese país específico de acuerdo con sus leyes particulares. Puesto que las leyes de muchos países difieren en varios aspectos de la ley de patentes de los Estados Unidos, se recomienda a los solicitantes buscar guía de los países extranjeros específicos para asegurar que los derechos sobre la patente no se pierdan prematuramente.

Jaime A. Garavito

También se recomienda a los solicitantes que para invenciones hechas en los Estados Unidos, el Director de la Oficina de Patentes y Marcas de los Estados Unidos expida una licencia antes de que los solicitantes puedan solicitar una patente en un país extranjero. El recibo de presentación de la solicitud contiene información adicional y guía en cuanto al status de la licencia del solicitante para presentación extranjera.

Es probable que los solicitantes quieran consultar el folleto de la Oficina de Patentes y Marcas de los Estados Unidos, "Información General Concerniente a Patentes" (específicamente, la sección titulada "Tratados y Patentes Extranjeros") para más información sobre los marcos de tiempo y plazos para presentar solicitudes de patente extranjeras. La guía se encuentra disponible en el Centro de Contacto de la Oficina de Patentes y Marcas de los Estados Unidos en el 800-786-9199 o puede ser vista en el sitio en la red de la Oficina de Patentes y Marcas de los Estados Unidos en <http://www.uspto.gov/web/offices/pac/doc/generalindex.html>.

Para información sobre la prevención de hurto de su propiedad intelectual (patentes, marcas y derechos de autor), puede consultar el sitio en la red del gobierno de los Estados Unidos, <http://www.stopfakes.gov>. Parte de una iniciativa del Departamento de Comercio, este sitio en la red incluye kits de herramientas de autoayuda proporcionándoles a los innovadores guía sobre cómo proteger la propiedad intelectual en países específicos, tales como la China, Corea y México. Para preguntas relacionadas con asuntos de exigibilidad

5

Jaime A. Garavito

2007 JUN 11 10:00 AM
U.S. DEPT. OF COMMERCE
OFFICE OF THE SECRETARY
WASHINGTON, D.C. 20501

343

TRADUCCIÓN OFICIAL N° 2707071 al español de un documento escrito en inglés, efectuada por Jaime Armando Garavito Burgos, c.c. N° 19.130.702 de Bogotá, Traductor e Intérprete Oficial inglés-español-inglés según Resolución N° 034 de enero 11 de 1983 expedida por el Ministerio de Justicia de la República de Colombia

de derechos sobre patentes, los solicitantes pueden llamar a la línea del gobierno de los EE.UU. 1-866-999-HALT (1-866-999-4158).

**LICENCIA PARA PRESENTACIÓN EXTRANJERA BAJO
el Título 35, Código de los Estados Unidos, Sección 184
Título 37, Código de Reglamentaciones Federales, 5.11 & 5.15**

OTORGADA

Se ha otorgado al solicitante una licencia bajo el Título 35 del Código de los Estados Unidos, Sección 184, si aparece en este formato la frase "SI SE REQUIERE, LICENCIA DE PRESENTACIÓN EXTRANJERA OTORGADA" seguida por una fecha. Dichas licencias se expiden con respecto a todas las solicitudes cuando se han cumplido las condiciones para la expedición de una licencia, independientemente de si se puede requerir o no una licencia, de acuerdo con lo estipulado en el Título 37 del Código de Reglamentaciones Federales 5.15. El alcance y las limitaciones de esta licencia se estipulan en el Título 37 del Código de Reglamentaciones Federales 5.15(a) a menos que se haya expedido una licencia anterior bajo el Título 37 del Código de Reglamentaciones Federales, 5.15(b). La licencia está sujeta a revocación para lo cual se da notificación escrita. La fecha indicada es la fecha de vigencia de la licencia, a menos que se haya otorgado una licencia anterior de alcance similar bajo el Título 37 del Código de Reglamentaciones Federales, 5.13 ó 5.14.

Esta licencia la tendrá el licenciatario y podrá ser usada en cualquier momento en la fecha de vigencia de la misma o después de ella a menos que sea revocada. Esta licencia se transfiere automáticamente a cual(es)quier

Jaime A. Garavito 6

solicitud(es) presentada(s) bajo el Título 37 del Código de Reglamentaciones Federales 1.53(d). Esta licencia no es retroactiva.

El otorgamiento de una licencia no disminuye de manera alguna la responsabilidad de un licenciatario por la seguridad del objeto según ha sido impuesta por cualquier contrato gubernamental o por las disposiciones de leyes existentes relacionadas con el espionaje y la seguridad nacional o la exportación de datos técnicos. Los licenciarios deben familiarizarse con las reglamentaciones actuales, especialmente con respecto a ciertos países, otros organismos, particularmente la Oficina de Controles Comerciales de Defensa, Departamento de Estado (con respecto a armas, municiones e implementos bélicos (Título 22 del Código de Reglamentaciones Federales 121-128)); la Oficina de Industria y Seguridad, Departamento de Comercio (Título 15 del Código de Reglamentaciones Federales partes 730-774); la Oficina de Control de Activos Extranjeros, Departamento del Tesoro (Título 31 del Código de Reglamentaciones Federales Partes 500+) y el Departamento de Energía.

NO OTORGADA

No se ha otorgado en este momento licencia bajo el Título 35 del Código de los Estados Unidos, Sección 184 si NO aparece en el formato la frase "SI SE REQUIERE, LICENCIA DE PRESENTACIÓN EXTRANJERA". Aún así, el solicitante podrá solicitar una licencia bajo el Título 37 del Código de Reglamentaciones Federales, 5.12, si se desea una licencia antes del vencimiento de los 6 meses siguientes a la fecha de presentación de la solicitud. Si han transcurrido 6 meses después de la fecha de presentación de esta

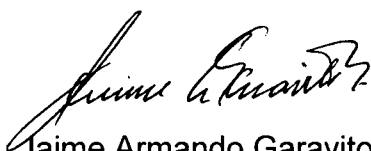
Jaime A. Garavito
Jaime A. Garavito
Traductor e Intérprete Oficial
Inglés-Español-Inglés
C.C. 19.130.702 de Bogotá

345

TRADUCCIÓN OFICIAL N° 2707071 al español de un documento escrito en inglés, efectuada por Jaime Armando Garavito Burgos, c.c. N° 19.130.702 de Bogotá, Traductor e Intérprete Oficial inglés-español-inglés según Resolución N° 034 de enero 11 de 1983 expedida por el Ministerio de Justicia de la República de Colombia

solicitud y el licenciatario no ha recibido ninguna indicación de una orden de secreto bajo el Título 35 del Código de los Estados Unidos, Sección 181, el licenciatario podrá presentar la solicitud extranjera de conformidad con el Título 37 del Código de Reglamentaciones Federales, 5.15.

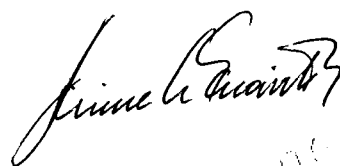
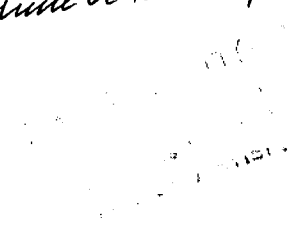
La anterior es traducción fiel y completa de la cual se deja copia en esta oficina para su confrontación.



Jaime Armando Garavito Burgos
c.c. N° 19.130.702 de Bogotá

Traductor e Intérprete Oficial según Resolución N° 034 de enero 11 de 1983 expedida por el Ministerio de Justicia

BOGDM5 # 84157

346
The Commonwealth of Massachusetts

William Francis Galvin
Secretary of the Commonwealth

APOSTILLE

(Convention de La Haye du 5 octobre 1961)

1. Country: United States of America

This public document

2. has been signed by: Lisa A. Walsh

3. acting in the capacity of: Notary Public

4. bears the seal/stamp of: the State of Massachusetts
whose commission expires on: May 29, 2009

Certified

5. at: Boston, Massachusetts

6. the: 13 July, 2007

7. by: the Secretary of the Commonwealth

8. No.: 1242106

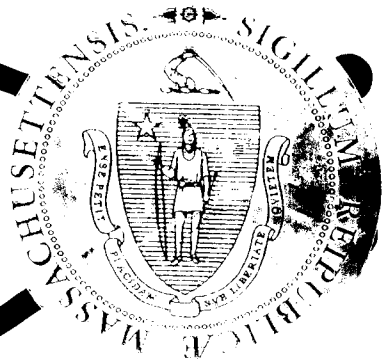
9. Seal/stamp:

Great Seal of the Commonwealth

10. Signature:



William Francis Galvin
Secretary of the Commonwealth




Lisa A. Walsh
Notary Public
My Commission expires May 29, 2017

Notary Public

My Commission expires May 29, 2009



UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
 United States Patent and Trademark Office
 Address: COMMISSIONER FOR PATENTS
 P.O. Box 1450
 Alexandria, Virginia 22313-1450
 www.uspto.gov

APPL NO.	FILING OR 371 (c) DATE	ART UNIT	FIL FEE REC'D	ATTY. DOCKET NO	DRAWINGS	TOT CLMS	IND CLMS
11/213,633	08/25/2005	1638	0.00	2004658-0085 (AM101550L4)	38	108	11

Wyeth

CONFIRMATION NO. 8681

53170
 CHOATE, HALL & STEWART LLP
 PATENT GROUP
 TWO INTERNATIONAL PLACE
 BOSTON, MA 02110

FILING RECEIPT



OC000000017088375

Date Mailed: 09/23/2005

Receipt is acknowledged of this regular Patent Application. It will be considered in its order and you will be notified as to the results of the examination. Be sure to provide the U.S. APPLICATION NUMBER, FILING DATE, NAME OF APPLICANT, and TITLE OF INVENTION when inquiring about this application. Fees transmitted by check or draft are subject to collection. Please verify the accuracy of the data presented on this receipt. If an error is noted on this Filing Receipt, please mail to the Commissioner for Patents P.O. Box 1450 Alexandria Va 22313-1450. Please provide a copy of this Filing Receipt with the changes noted thereon. If you received a "Notice to File Missing Parts" for this application, please submit any corrections to this Filing Receipt with your reply to the Notice. When the USPTO processes the reply to the Notice, the USPTO will generate another Filing Receipt incorporating the requested corrections (if appropriate).

Applicant(s)

Denis Drapeau, Salem, NH;
 Yen-Tung Luan, Chelmsford, MA;
 Jim Mercer, Derry, NH;
 Wenge Wang, North Chelmsford, MA;
 Dan Lasko, Medford, MA;

Assignment For Published Patent Application

Wyeth, Madison, NJ

Power of Attorney: None

Domestic Priority data as claimed by applicant

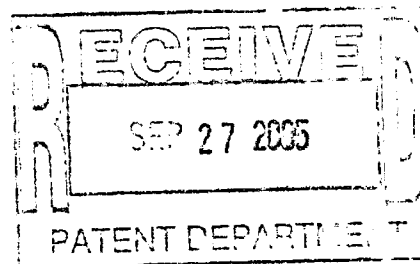
This appln claims benefit of 60/605,379 08/27/2004

Foreign Applications

If Required, Foreign Filing License Granted: 09/13/2005

The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US11/213,633**

Projected Publication Date: To Be Determined - pending completion of Missing Parts



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non-

Non-Publication Request: No**Early Publication Request: No****Title**

Production of TNFR-Ig

Preliminary Class

800

PROTECTING YOUR INVENTION OUTSIDE THE UNITED STATES

Since the rights granted by a U.S. patent extend only throughout the territory of the United States and have no effect in a foreign country, an inventor who wishes patent protection in another country must apply for a patent in a specific country or in regional patent offices. Applicants may wish to consider the filing of an international application under the Patent Cooperation Treaty (PCT). An international (PCT) application generally has the same effect as a regular national patent application in each PCT-member country. The PCT process **simplifies** the filing of patent applications on the same invention in member countries, but **does not result** in a grant of "an international patent" and does not eliminate the need of applicants to file additional documents and fees in countries where patent protection is desired.

Almost every country has its own patent law, and a person desiring a patent in a particular country must make an application for patent in that country in accordance with its particular laws. Since the laws of many countries differ in various respects from the patent law of the United States, applicants are advised to seek guidance from specific foreign countries to ensure that patent rights are not lost prematurely.

Applicants also are advised that in the case of inventions made in the United States, the Director of the USPTO must issue a license before applicants can apply for a patent in a foreign country. The filing of a U.S. patent application serves as a request for a foreign filing license. The application's filing receipt contains further information and guidance as to the status of applicant's license for foreign filing.

Applicants may wish to consult the USPTO booklet, "General Information Concerning Patents" (specifically, the section entitled "Treaties and Foreign Patents") for more information on timeframes and deadlines for filing foreign patent applications. The guide is available either by contacting the USPTO Contact Center at 800-786-9199, or it can be viewed on the USPTO website at <http://www.uspto.gov/web/offices/pac/doc/general/index.html>.

For information on preventing theft of your intellectual property (patents, trademarks and copyrights), you may wish to consult the U.S. Government website, <http://www.stopfakes.gov>. Part of a Department of Commerce initiative, this website includes self-help "toolkits" giving innovators guidance on how to protect intellectual property in specific countries such as China, Korea and Mexico. For questions regarding patent enforcement issues, applicants may call the U.S. Government hotline at 1-866-999-HALT (1-866-999-4158).

LICENSE FOR FOREIGN FILING UNDER**Title 35, United States Code, Section 184****Title 37, Code of Federal Regulations, 5.11 & 5.15****GRANTED**

The applicant has been granted a license under 35 U.S.C. 184, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" followed by a date appears on this form. Such licenses are issued in all applications where the conditions for issuance of a license have been met, regardless of whether or not a license may be required as

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ve date thereof

of the subject

REIGN FILING

351
000 3

TRADUCCIÓN OFICIAL N° 230807 al español de un documento escrito en inglés, efectuada por Jaime Armando Garavito Burgos, c.c. N° 19.130.702 de Bogotá, Traductor e Intérprete Oficial inglés-español-inglés según Resolución N° 034 de enero 11 de 1983 expedida por el Ministerio de Justicia de la República de Colombia

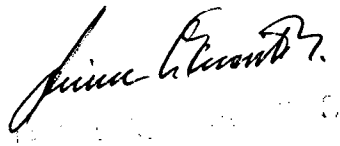
Cesión de Invención a Wyeth Research Ireland Limited

Estado de Massachussets
William Francis Galván
Secretario del Estado

APOSTILLA

1. País: Estados Unidos de América
Este documento público
2. Ha sido firmado por Lisa A. Walsh
3. Quien actúa en calidad de Notario Público
4. Lleva el sello del Estado de Massachusetts. Su comisión vence el 29 de mayo de 2009

CERTIFICADO

5. En Boston, Massachussets
 6. El 02 de julio de 2007
 7. Por el Secretario del Estado
 8. N° 1239300
 9. Firmado bajo el Gran Sello del Estado (Aparece adherido sello dorado con cinta azul)
 10. Firma: [firmado] William Francis Galvin, Secretario del Estado
- 

Hoy 2 de julio de 2007, certifico que el documento anexo es una copia auténtica, exacta, completa e inalterada (5 páginas) hecha por mí de nuestros archivos de una cesión, Serie No. 11/213.633 a Wyeth.

352

TRADUCCIÓN OFICIAL N° 230807 al español de un documento escrito en inglés, efectuada por Jaime Armando Garavito Burgos, c.c. N° 19.130.702 de Bogotá, Traductor e Intérprete Oficial inglés-español-inglés según Resolución N° 034 de enero 11 de 1983 expedida por el Ministerio de Justicia de la República de Colombia

[Firma ilegible] Lisa A. Walsh
Notario Público
Mi comisión vence el 29 de mayo de 2009

[Aparece sello notarial realzado que dice: "Lisa A. Walsh, Notario Público.

Estado de Massachusetts. Mi comisión vence el 29 de mayo de 2009"]

[Aparece en el encabezamiento de las páginas referentes a las cesiones:
"Archivo No. 2004658-0085 (AM10155004 EE.UU. Producción de TNFR-Ig")]
Solicitud de Patente No. No. 11/213.633

(Aparece en el pie de página de las dos primeras hojas referentes a la cesión:
"Cesión de Invención
Solicitud No Provisional")

Cesión de Invención

En consideración del pago por la CESIONARIA a la CEDENTE de una
contraprestación válida y suficiente cuyo recibo se acusa por medio del presente,
los CEDENTES:

- (1) Nombre Completo del Inventor: Denis Drapeau
Residencia: 55 Old Farm Road
Salem, New Hampshire 03079
Ciudadanía: Estados Unidos
- (2) Nombre Completo del Inventor: Yen-Tung Luan
Residencia: 3 Armand Drive
Chelmsford, Massachusetts 01824
Ciudadanía: Estados Unidos
- (3) Nombre Completo del Inventor: James R. Mercer
Residencia: 226 Hampstead Road
Derry, New Hampshire 03038
Ciudadanía: Estados Unidos
- (4) Nombre Completo del Inventor: Wenge Wang
Residencia: 1 Hollowridge Road
North Chelmsford, Massachusetts 01863
Ciudadanía: China



359

TRADUCCIÓN OFICIAL N° 230807 al español de un documento escrito en inglés, efectuada por Jaime Armando Garavito Burgos, c.c. N° 19.130.702 de Bogotá, Traductor e Intérprete Oficial inglés-español-inglés según Resolución N° 034 de enero 11 de 1983 expedida por el Ministerio de Justicia de la República de Colombia

- (5) Nombre Completo del Inventor: Daniel R. Lasko
Residencia: 19 Gleason Street
Medford, Massachusetts 02155
Ciudadanía: Estados Unidos

por medio del presente vende, cede y transfiere a la CESIONARIA:

Wyeth Research Ireland Limited
una sociedad de Irlanda con oficina registrada en:
Little Connell
Newbridge
County Kildare
Ireland

y los sucesores, CESIONARIOS y representantes legales de la CESIONARIA, la totalidad del derecho, título e intereses para los Estados Unidos y sus posesiones territoriales y en todos los países extranjeros, incluyendo todos los derechos a reivindicar prioridad con respecto a la invención titulada:

PRODUCCIÓN DE TNFR-Ig

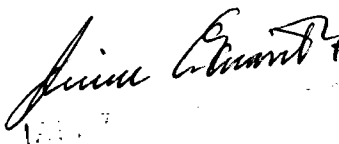
e inventada a menos en parte por los CEDENTES y que se encuentra en la Solicitud Provisional de los EE.UU. No. 11/213.633 presentada el 25 de agosto de 2005

- [] Los CEDENTES que abajo firman, por medio del presente autorizan y solicitan insertar arriba de número de la solicitud y fecha de presentación, cuando se conozcan

[Verifique además si se están cediendo también solicitud(es) extranjera(es)]

- [X] y cualquier equivalente legal de las mismas en un país extranjero

incluyendo el derecho a invocar prioridad, incluyendo todas y cada una de las mejoras divulgadas en la misma y en todas las patentes obtenidas para dicha



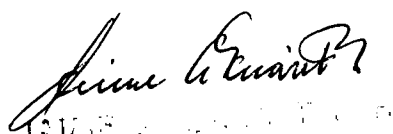
TRADUCCIÓN OFICIAL N° 230807 al español de un documento escrito en inglés, efectuada por Jaime Armando Garavito Burgos, c.c. N° 19.130.702 de Bogotá, Traductor e Intérprete Oficial inglés-español-inglés según Resolución N° 034 de enero 11 de 1983 expedida por el Ministerio de Justicia de la República de Colombia

invención a través de la anterior solicitud y cualquier solicitud provisional, no provisional, continuación, divisional, renovación o sustitución de la misma presentada posteriormente y en cuanto a la patente, cualquier re-expedición o nuevo examen de la misma.

Los CEDENTES por medio del presente acuerdan que no se ha hecho ni se hará o suscribirá ninguna cesión, venta, acuerdo o gravamen que pueda estar en conflicto con esta cesión.

Los CEDENTES autorizan a la CESIONARIA para que haga solicitudes y para que reciban patentes para dicha invención en cualquiera de los mencionados países en su propio nombre, o en el nombre de la CEDENTE o a su elección.

Los CEDENTES acuerdan suscribir u obtener cualquier seguridad adicional necesaria del título a dicha invención y cualquier patente que pueda ser expedida para la misma y a entregar en cualquier momento y a solicitud y expensas de la CESIONARIA cualquier testimonio en cualquier derecho preexistente que impida el patentamiento de la invención, litigio o procedimiento relacionado y a suscribir todos los documentos que puedan ser necesarios o convenientes para perfeccionar el título a dicho invento o cualquier patente que pueda ser otorgada para el mismo a la CESIONARIA, sus sucesores, CESIONARIOS u otros representantes legales y a suscribir, en cualquier momento, a solicitud y expensas de la CESIONARIA, cualquier continuación, continuación parcial, divisional, renovación o sustitución de la misma y en cuanto a patentes a re-expedir o re-examinar las mismas, o cualesquier otras solicitudes


JAIME A. GARAVITO
TRADUCTOR E INTÉRPRETE OFICIAL
INGLÉS-ESPAÑOL-INGLÉS
N° 19.130.702

TRADUCCIÓN OFICIAL N° 230807 al español de un documento escrito en inglés, efectuada por Jaime Armando Garavito Burgos, c.c. N° 19.130.702 de Bogotá, Traductor e Intérprete Oficial inglés-español-inglés según Resolución N° 034 de enero 11 de 1983 expedida por el Ministerio de Justicia de la República de Colombia

adicionales de patentes para dicho invento o cualquier parte del mismo, todas las cuales y cualquier patente que se expida se ceden por medio del presente a la CESIONARIA y prestará todos los juramentos legales o declaraciones y hará todos los actos legales requeridos para adquirirlos, sin compensación adicional, pero a expensas de la CESIONARIA, sus sucesores, cesionarios u otros representantes legales.

Los CEDENTES autorizan y solicitan al Comisionado de Patentes expedir todas y cada una de las patentes de los Estados Unidos para dicho invento, que resulte de cualquiera de las solicitudes a su CESIONARIA.

(Firmado)
Denis Drapeau

Enero 18, 2006
Fecha

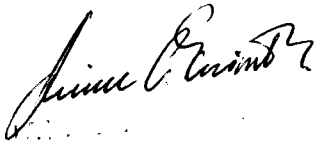
RECONOCIMIENTO

Estado de Massachusetts)
Condado de Essex) Declaración juramentada

El 18 de enero de 2006, **Denis Drapeau** compareció personalmente ante mí, a quien demostró mediante identificación satisfactoria, a saber su licencia de conducción de Massachusetts, ser la persona describa en el anterior instrumento y quien suscribió el mismo y reconoció haberlo suscrito en forma libre y espontánea para los fines consignados en él.

(Firmado) Diane R. Driscoll, Notario Público

[Sello en tinta que dice: "Diane R. Driscoll, Notario Público. Estado de Massachusetts. Mi comisión vence el 21 de mayo de 2010"]



356

Enero 18, 2006
Fecha

Fecha

Declaración juramentada

El 18 de enero de 2006, **Yen-Tung Luan** compareció personalmente ante

Massachusetts. Mi comisión vence el 21 de mayo de 2010”]

Enero 18, 2006
Fecha

Fecha

Declaración juramentada

El 18 de enero de 2006, **James R. Mercer** compareció personalmente

6

357

Родина

(Firmado) Wenge Wang

Enero 18, 2006
Fecha

Estado de Massachusetts)
 Condado de Essex) Declaración juramentada

(Firmado) Diane R. Driscoll, Notario Público

[Sello en tinta que dice: "Diane R. Driscoll, Notario Público. Estado de Massachusetts. Mi comisión vence el 21 de mayo de 2010"]

RECONOCIMIENTO

Estado de Massachusetts)
) Declaración juramentada
Condado de Essex)

7

258

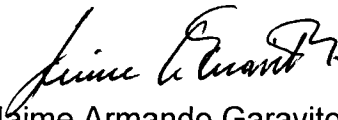
TRADUCCIÓN OFICIAL N° 230807 al español de un documento escrito en inglés, efectuada por Jaime Armando Garavito Burgos, c.c. N° 19.130.702 de Bogotá, Traductor e Intérprete Oficial inglés-español-inglés según Resolución N° 034 de enero 11 de 1983 expedida por el Ministerio de Justicia de la República de Colombia

El 18 de enero de 2006, **Daniel R. Lasko** compareció personalmente ante mí, a quien demostró mediante identificación satisfactoria, a saber su licencia de conducción de Massachusetts, ser la persona describa en el anterior instrumento y quien suscribió el mismo y reconoció haberlo suscrito en forma libre y espontánea para los fines consignados en él.

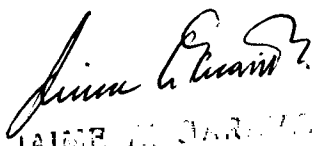
(Firmado) Diane R. Driscoll, Notario Público

[Sello en tinta que dice: "Diane R. Driscoll, Notario Público. Estado de Massachusetts. Mi comisión vence el 21 de mayo de 2010"]

La anterior es traducción fiel y completa de la cual se deja copia en esta oficina para su confrontación.


Jaime Armando Garavito Burgos
c.c. N° 19.130.702 de Bogotá
Traductor e Intérprete Oficial según Resolución N° 034 de enero 11 de 1983 expedida por el Ministerio de Justicia

BOGDM5 # 86736


JAIME A. GARAVITO B.
C.C. 19.130.702
BOGOTÁ, D.C.
034 DE ENERO 11 DE 1983
MINISTERIO DE JUSTICIA

359

The Commonwealth of Massachusetts

William Francis Galvin
Secretary of the Commonwealth

APOSTILLE

(Convention de La Haye du 5 octobre 1961)

1. Country: United States of America
This public document
2. has been signed by: Lisa A. Walsh
3. acting in the capacity of: Notary Public
4. bears the seal/stamp of: the State of Massachusetts
whose commission expires on: May 29, 2009

Certified

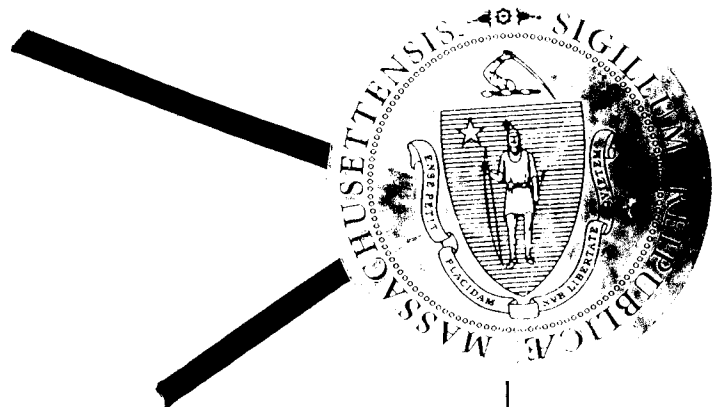
5. at: Boston, Massachusetts
6. the: 2 July, 2007
7. by: the Secretary of the Commonwealth
8. No.: 1239300
9. Seal/stamp:

Great Seal of the Commonwealth

10. Signature:

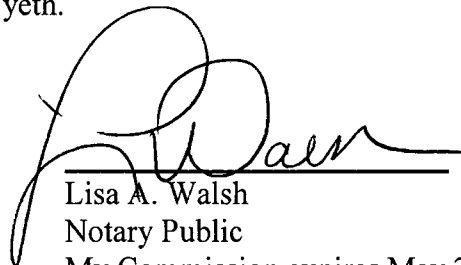


William Francis Galvin
Secretary of the Commonwealth



360

On this 2nd day of July, 2007, I certify that the attached document is a true, exact, complete, and unaltered copy (5 pages) made by me from our files of an assignment, Serial No. 11/213,633 to Wyeth.

A handwritten signature in black ink, appearing to read 'Lisa A. Walsh', written over a horizontal line.

Lisa A. Walsh

Notary Public

My Commission expires May 29, 2009

Assignment of Invention

In consideration of the payment by ASSIGNEE to ASSIGNOR of good and valuable consideration, the receipt of which is hereby acknowledged,
ASSIGNOR:

- (1) Full Name of Inventor: Denis Drapeau
Residence: 55 Old Farm Road
Salem, New Hampshire 03079
Citizenship: United States

- (2) Full Name of Inventor: Yen-Tung Luan
Residence: 3 Armand Drive
Chelmsford, Massachusetts 01824
Citizenship: United States

- (3) Full Name of Inventor: James R. Mercer
Residence: 226 Hampstead Road
Derry, New Hampshire 03038
Citizenship: United States

- (4) Full Name of Inventor: Wenge Wang
Residence: 1 Hollowridge Road
North Chelmsford, Massachusetts 01863
Citizenship: China

- (5) Full Name of Inventor: Daniel R. Lasko
Residence: 19 Gleason Street
Medford, Massachusetts 02155
Citizenship: United States

hereby sells, assigns and transfers to ASSIGNEE:

Wyeth Research Ireland Limited
a corporation of Ireland whose registered address is:
Little Connell
Newbridge
County Kildare
Ireland

and the successors, assigns and legal representatives of the ASSIGNEE the entire right, title and interest for the United States and its territorial possessions and in all foreign countries, including all rights to claim priority, in and to the invention entitled:

and invented at least in part by ASSIGNOR:

and which is found in U.S. Application No. 11/213,633 filed on: August 25, 2005

- ☐ The ASSIGNOR signing below, hereby authorizes and requests insertion above of the application number and filing date, when they become known,

[also check if foreign application(s) is(are) also being assigned]

- ☒ and any legal equivalent thereof in a foreign country,

including the right to claim priority, including any and all improvements disclosed therein, and in and to all Letters Patent to be obtained for said invention by the above application or any subsequently filed provisional, nonprovisional, continuation, divisional, renewal, or substitute thereof, and as to Letters Patent any reissue or re-examination thereof.

ASSIGNOR hereby covenants that no assignment, sale, agreement or encumbrance has been or will be made or entered into which would conflict with this assignment.

ASSIGNOR authorizes ASSIGNEE to make applications for and to receive Letters patent for said invention in any of said countries in its own name, or in ASSIGNOR's name, at its election.

ASSIGNOR covenants and agrees to execute or procure any further necessary assurance of the title to said invention and any Letters Patent which may issue therefore and to, at any time, upon the request and at the expense of ASSIGNEE deliver any testimony in any interference, litigation or proceeding related thereto and execute all papers that may be necessary or desirable to perfect the title to said invention or any Letters Patent which may be granted therefore in ASSIGNEE, its successors, assigns or other legal representatives, and that to, at any time, upon the request and at the expense of ASSIGNEE execute any continuation, continuation-in-part, divisional, renewal or substitute thereof, and as to Letters Patent and reissue or re-examination thereof, or any other additional applications of Letters Patent for said invention or any part thereof, all of which applications and any Letters Patent issuing thereon are hereby assigned to ASSIGNEE, and will make all rightful oaths or declarations, and do all lawful acts requisite for procuring the same therein, without further compensation, but at the expense of ASSIGNEE, its successors, assigns or other legal representatives.

ASSIGNOR authorizes and requests the Commissioner of Patents to issue any and all Letters Patent of the United States for said invention, resulting from any of the aforesaid applications to its ASSIGNEE.

Denis Drapeau
Denis Drapeau

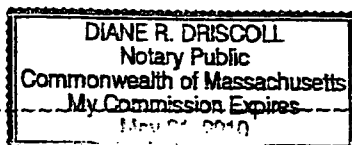
18 JAN 06
Date

ACKNOWLEDGMENT

State of Massachusetts }
County of Essex } ss:

On the 18th day of January 2006, before me, personally appeared **Denis Drapeau**, proved to me through satisfactory evidence of identity which was NH driver's licence to be the person whose name is signed on the preceding or attached document, and acknowledged that he/~~she~~ executed the same, of his/~~her~~ own free will and for the purposes set forth.

Diane R. Driscoll
Notary Public



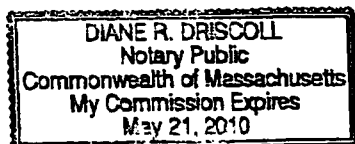
Yen-Tung Luan
Yen-Tung Luan

Jan 18, 2006
Date

ACKNOWLEDGMENT

State of Massachusetts }
County of Essex } ss:

On the 18th day of January 2006, before me, personally appeared **Yen-Tung Luan**, proved to me through satisfactory evidence of identity which was MA driver's license to be the person whose name is signed on the preceding or attached document, and acknowledged that he/she executed the same, of his/~~her~~ own free will and for the purposes set forth.



Diane R. Driscoll
Notary Public

364

James R Mercer
James R. Mercer

18 JAN 06
Date

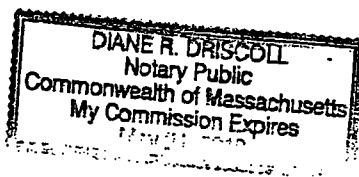
ACKNOWLEDGMENT

State of Massachusetts }

ss:

County of Essex }

On the 18th day of January 2006, before me, personally appeared **James R. Mercer**, proved to me through satisfactory evidence of identity which was MA driver's license to be the person whose name is signed on the preceding or attached document, and acknowledged that he/~~she~~ executed the same, of his/~~her~~ own free will and for the purposes set forth.



Diane R. Driscoll
Notary Public

Wenge Wang
Wenge Wang

Jan 18, 2006
Date

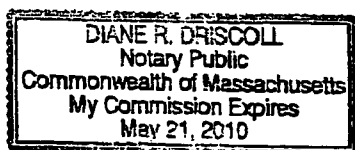
ACKNOWLEDGMENT

State of Massachusetts }

ss:

County of Essex }

On the 18th day of January 2006, before me, personally appeared **Wenge Wang**, proved to me through satisfactory evidence of identity which was MA driver's license to be the person whose name is signed on the preceding or attached document, and acknowledged that he/~~she~~ executed the same, of his/~~her~~ own free will and for the purposes set forth.



Diane R. Driscoll
Notary Public

365

Daniel R. Lasko
Daniel R. Lasko

18 JAN 2006
Date

ACKNOWLEDGMENT

State of Massachusetts }

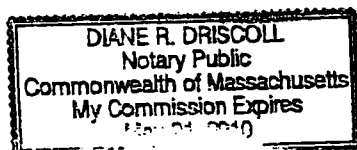
County of Essex } ss:

On the 18th day of January

2006, before me, personally appeared

Daniel R. Lasko, proved to me through satisfactory evidence of identity which was

MA driver's licence to be the person whose name is signed on the preceding or attached document, and acknowledged that he/she executed the same, of his/~~her~~ own free will and for the purposes set forth.



Diane R. Driscoll
Notary Public

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TRADUCCIÓN OFICIAL N° 1006071 al español de un documento escrito en inglés, efectuada por Jaime Armando Garavito Burgos, c.c. N° 19.130.702 de Bogotá, Traductor e Intérprete Oficial inglés-español-inglés según Resolución N° 034 de enero 11 de 1983 expedida por el Ministerio de Justicia de la República de Colombia

PODER OTORGADO POR WYETH RESEARCH IRELAND LIMITED

APOSTILLA

1. PAÍS: ESTADOS UNIDOS DE AMÉRICA
2. ESTE DOCUMENTO PÚBLICO HA SIDO FIRMADO POR:
MICHELLE L MC GOWAN
3. QUIEN ACTÚA EN CALIDAD DE NOTARIO PÚBLICO DE NUEVA JERSEY
4. LLEVA EL SELLO DE MICHELLE L MC GOWAN, NOTARIO

CERTIFICADO

5. EN TRENTON, NUEVA JERSEY
6. EL 18 DE MAYO DE 2007
7. POR BRADLEY ABELOW, TESORERO DE NUEVA JERSEY
8. N° A292099
9. SELLO (APARECE ADHERIDO SELLO DORADO)
10. FIRMA (FIRMADO) BRADLEY ABELOW

Jaime A. Garavito B.
JAIME A. GARAVITO B.
N° 19.130.702
BOGOTÁ, D.C.
MINISTERIO DE JUSTICIA

Texto en inglés y en español mediante el cual **WYETH RESEARCH IRELAND LIMITED**, domiciliada en los Estados Unidos de América otorga poder a **ALVARO CORREA ORDOÑEZ y/o OLGA GEORGETTE OTERO y/o RICARDO METKE y/o JUAN PABLO CONCHA**, todos miembros de la firma de abogados Raisbeck, Lara, Rodríguez & Rueda domiciliada en Bogotá, Colombia.

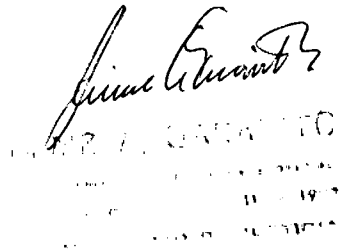
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10032

TRADUCCIÓN OFICIAL N° 1006071 al español de un documento escrito en inglés, efectuada por Jaime Armando Garavito Burgos, c.c. N° 19.130.702 de Bogotá, Traductor e Intérprete Oficial inglés-español-inglés según Resolución N° 034 de enero 11 de 1983 expedida por el Ministerio de Justicia de la República de Colombia

Solicitud de Patente en Colombia titulada: **"PRODUCCIÓN DE PROTEÍNA DE FUSIÓN TNRF-1G"**.

Dado y firmado el 16 de mayo de 2007

(Firma ilegible)
Paul J. Jones, Director



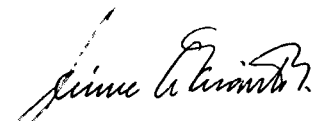
[No se traduce el texto de la Certificación Notarial por estar en inglés y en español]

(Firmado) Michelle L. McGowan, Notario Público

(Sello en tinta que dice: "Michelle L. McGowan, Notario Público, Estado de Nueva Jersey. No. 2108903. Calificado en el Condado de Morris. Comisión vence el 09 de febrero de 2011")

[Sello notarial realzado que dice: "Michelle L. McGowan, Notario Público, Nueva Jersey"]

La anterior es traducción fiel y completa de la cual se deja copia en esta oficina para su confrontación.



Jaime Armando Garavito Burgos
c.c. N° 19.130.702 de Bogotá
Traductor e Intérprete Oficial según Resolución N° 034 de enero 11 de 1983 expedida por el Ministerio de Justicia

BOGDM5 # 82486

368

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APOSTILLE

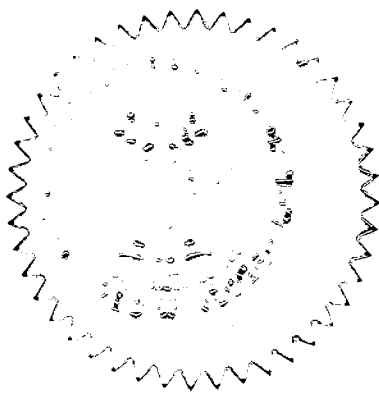
(CONVENTION DE LA HAYE DU 5 OCTOBRE 1961)

1. COUNTRY: UNITED STATES OF AMERICA
2. THIS PUBLIC DOCUMENT HAS BEEN SIGNED BY:
MICHELLE L MC GOWAN
3. ACTING IN THE CAPACITY OF:
NOTARY PUBLIC OF NEW JERSEY
4. BEARS THE SEAL/STAMP OF:
MICHELLE L MC GOWAN, NOTARY

CERTIFIED

5. AT TRENTON, NEW JERSEY
6. THE 18TH DAY OF MAY 2007
7. BY: Bradley Abelow, NEW JERSEY TREASURER
8. NO. A292099
9. SEAL/STAMP:
10. SIGNATURE

Bradley Abelow



Raisbeck, Lara, Rodríguez & Rueda
Av. 82 No. 10 - 62 6th Floor
Bogotá, Colombia

NIT: 860.068.840-3
Tel: +57 1 634 1500
Fax: +57 1 376 2211
www.bakernet.com

POWER OF ATTORNEY

The undersigned.

WYETH RESEARCH IRELAND LIMITED

with offices at

Little Connell
Newbridge
County Kildare, Ireland

declares that it hereby grants to

**ALVARO CORREA-ORDOÑEZ and/or
OLGA GEORGETTE OTERO and/or
RICARDO METKE and/or
JUAN PABLO CONCHA**

all members of the law firm Raisbeck, Lara, Rodriguez & Rueda, with domicile at Av. 82 No. 10 - 62, 6Th floor, of Bogotá D. C., Colombia, and for so long as they remain members of the law firm, a full and sufficient power of attorney with respect to Colombian patent application(s) based on PCT/US2005/030439 and entitled "PRODUCTION OF TNFR-Ig FUSION PROTEIN" and to obtain patent protection thereof, for which purpose I (we) authorize the said attorneys to represent me (us) before any authorities or persons, with or without jurisdiction, specially those administrative, administrative contentious and judicial, as well as before Arbitration Court, in all matters concerning the following: a) Obtainment of a patent(s) based upon said patent application(s), including filing of assignments, change of name or address, voluntary cancellation; and defending oppositions, cancellations, nullity, writ mandamus, and in general the civil, penal, an administrative processes relating to those rights.

I hereby ratify all lawful actions previously taken on behalf of this company by the aforementioned attorneys with respect to the mentioned matters.

Done and subscribed in

On the 16th day of May of 2007

Signature of the person issuing the Power

WYETH RESEARCH IRELAND LIMITED


Paul J. Jones
Director

PODER DE ABOGADO

La abajo firmada,

WYETH RESEARCH IRELAND LIMITED

con oficinas en

Little Connell
Newbridge
County Kildare, Irlanda

declara por el presente que otorga a

**ALVARO CORREA-ORDOÑEZ y/o
OLGA GEORGETTE OTERO y/o
RICARDO METKE y/o
JUAN PABLO CONCHA**

Todos miembros de la firma de abogados Raisbeck, Lara, Rodríguez & Rueda, con domicilio en la Av. 82 No. 10 - 62 Piso 6 en Bogotá D. C., Colombia, y hasta tanto ellos continúen siendo miembros de la firma, poder especial amplio y suficiente con respecto a la solicitud(es) de patente en Colombia basada(s) en PCT/US2005/030439 y titulada "PRODUCCION DE TNFR-Ig"

y para obtener la protección de patente de la misma, a cuyo efecto autorizo (amos) a los dichos apoderados para que me (nos) representen ante cualesquiera autoridades o personas, ejerzan o no jurisdicción, especialmente ante las del orden administrativo, contencioso-administrativo y judicial, así como los tribunales de arbitramento, en todo lo concerniente a los siguientes asuntos: a) Obtención de la(s) patente(s) de invención basada(s) en la mencionada solicitud de patente, incluyendo presentación de traspasos, cambio de nombre o dirección, cancelación voluntaria; y defensa en oposiciones, cancelaciones, nulidades, la tutela, y en general procesos civiles, penales, administrativos relacionados con estos derechos.

Igualmente, ratifico todo lo actuado en derecho hasta el momento en nombre de esta compañía por cualquiera de los mencionados apoderados en los casos mencionados.

Dado y firmado en

A los 16 días del mes de mayo de 2007

Firma de la persona que firma el Poder

NOTARIAL CERTIFICATION

On this 16th day of may
2007, the undersigned Notary

CERTIFIES

1. That **Paul J. Jones**

set his hand on the foregoing document.

2. That the grantor is to me personally known and that he/she has legal capacity to execute the instrument.

3. That the grantor has executed the foregoing document as

Director

of the judicial person **WYETH RESEARCH IRELAND LIMITED**

4. That the judicial person **WYETH RESEARCH IRELAND LIMITED**

having its home office in

Little Connell
New Bridge
County Kildare, Ireland

is duly organized, has present legal existence and that the purposes for which the power of attorney is granted are within the scope of its corporate purposes.

5. That the grantor has in fact the referred to authority and that his/her representation is legal.

6. That I have based certifications 3, 4 and 5 above on the following authentic documents for such purpose exhibited to me and which I mention specifically, giving their dates and their origin or source:
Minutes of the Board of the Director's meeting dated May 19, 2005

CERTIFICACION NOTARIAL

A los 16 días del mes de mayo
de 2007, el suscrito Notario

DA FE

- 1 Que **Paul J. Jones**

suscribió de su puño y letra el documento que antecede.

2. Que conozco al otorgante y que éste tiene capacidad legal para el otorgamiento.

3. Que el otorgante ha suscrito el referido documento en su carácter de

Director

de la persona jurídica

WYETH RESEARCH IRELAND LIMITED

4. Que la persona jurídica **WYETH RESEARCH IRELAND LIMITED**

con sede en

Little Connell
New Bridge
County Kildare, Irlanda

está legalmente constituida, que tiene existencia legal actual y que el acto para el cual se otorga el poder está comprendido entre los que constituyen su objeto social.

5. Que el otorgante tiene efectivamente la representación que ostenta y que ésta es legítima.

6. Que he basado las certificaciones 3, 4 y 5 anteriores en los siguientes documentos auténticos que al efecto se me exhibieron y que menciono específicamente con expresión de sus fechas y de su origen o procedencia:

Acta de la Junta Directiva fechada a 19 de mayo de 2005

Michelle L. McGowan
Notary Public

MICHELLE L. MCGOWAN
Notary Public, State of New Jersey
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FORMULARIO UNICO DE CORRECCIONES Y MODIFICACIONES

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SOLICITUD DE

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Corrección de Error Material

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Modificación de una solicitud

2

SOLICITANTE

Nombre: WYETH RESEARCH IRELAND LIMITED

Dirección: Little Connell, Newbridge, County Kildare, República de Irlanda

Nacionalidad o Domicilio: República de Irlanda

Lugar de Constitución: República de Irlanda

Teléfono:

Fax:

E-Mail

IDENTIFICACION

C.C.

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NIT

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C.E.

☐

Otro

☐

Cual

Número

3

REPRESENTANTE
O APODERADO

Nombre: ALVARO CORREA ORDOÑEZ

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IDENTIFICACION

C.C.

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Número 79.143.366

TP 20.281

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Numero de expediente

07.030.535

5

Descripción de la corrección o modificación solicitada

El solicitante presenta corrección de nombre un inventor de

LUAN, Yen-Tuang a LUAN, Yen-Tung

6

Comprobante de Pago No.

07 - 53347

Fecha

29-junio-2007

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Anexos

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Comprobante de pago de la tasa.

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Poderes, si fuere el caso.

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

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NOMBRE ALVARO CORREA ORDOÑEZ

FIRMA

C.C. 79.143.366

T.P. 20.281

Instrucciones para el diligenciamiento del presente formulario, ver reverso de esta página.

2000-F03

Industria y Comercio
SUPERINTENDENCIA

DIRECCIÓN DE NUEVAS CREACIONES

RADICACIÓN EXPEDIENTE No. PCT 07-30535

EL EXTRACTO DE LA PRESENTE SOLICITUD DE **PATENTE DE INVENCION**

FUE PUBLICADO EN LA GACETA DE PROPIEDAD INDUSTRIAL No **595** DE

FECHA **29 DE AGOSTO DE 2008** EL TÉRMINO HÁBIL SEÑALADO POR LA

LEY EXPIRA EL **26 DE NOVIEMBRE DE 2008**

NOTA: Dentro del plazo de los seis (6) meses siguientes a partir de la fecha de publicación en la gaceta de Propiedad Industrial, deberá solicitar y cancelar el examen de patentabilidad so pena de que la solicitud caiga en abandono, de acuerdo con lo establecido en el Artículo 44 de la Decisión 486/2000.

SE PRESENTARON OPOSICIONES POR PARTE DE TERCEROS.

SI _____

NO X

Ammonium Alters N-Glycan Structures of Recombinant TNFR-IgG: Degradative Versus Biosynthetic Mechanisms

Martin Gawlitzek,* Thomas Ryll, Jim Lofgren, Mary B. Sliwowski

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Received 8 August 1999; accepted 25 December 1999

Abstract: The effect of ammonium on the glycosylation pattern of the recombinant immunoadhesin tumor necrosis factor-IgG (TNFR-IgG) produced by Chinese hamster ovary cells is elucidated in this study. TNFR-IgG is a chimeric IgG fusion protein bearing one N-linked glycosylation site in the Fc region and three complex-type N-glycans in the TNF-receptor portion of each monomer. The ammonium concentration of batch suspension cultures was adjusted with glutamine and/or NH_4Cl . The amount of galactose (Gal) and N-acetylneuraminic acid (NANA) residues on TNFR-IgG correlated in a dose-dependent manner with the ammonium concentration under which the N-linked oligosaccharides were synthesized. As ammonium increased from 1 to 15 mM, a concomitant decrease of up to 40% was observed in terminal galactosylation and sialylation of the molecule. Cell culture supernatants contained measurable β -galactosidase and sialidase activity, which increased throughout the culture. The β -galactosidase, but not the sialidase, level was proportional to the ammonium concentration. No loss of N-glycans was observed in incubation studies using β -galactosidase and sialidase containing cell culture supernatants, suggesting that the ammonium effect was biosynthetic and not degradative. Several biosynthetic mechanisms were investigated. Ammonium (a weak base) is known to affect the pH of acidic intracellular compartments (e.g., *trans*-Golgi) as well as intracellular nucleotide sugar pools (increases UDP-N-acetylglucosamine and UDP-N-acetylglucosamine). Ammonium might also affect the expression rates of β 1,4-galactosyltransferase (β 1,4-GT) and α 2,3-sialyltransferase (α 2,3-ST). To separate these mechanisms, experiments were designed using chloroquine (changes intracellular pH) and glucosamine (increases UDP-GNAc pool [sum of UDP-GlcNAc and UDP-GalNAc]). The ammonium effect on TNFR-IgG oligosaccharide structures could be mimicked only by chloroquine, another weak base. No differences in N-glycosylation were found in the product synthesized in the presence of glucosamine. No differences in β 1,4-galactosyltransferase (β 1,4-GT) and α 2,3-sialyltransferase (α 2,3-ST) messenger RNA (mRNA) and enzyme levels were observed in cells cultivated in the presence or absence of 13 mM NH_4Cl . pH titration of endogenous CHO α 2,3-ST and β 1,4-GT revealed a sharp optimum at pH 6.5, the reported *trans*-Golgi pH. Thus, at pH 7.0 to 7.2, a likely *trans*-Golgi pH range in the presence of 10 to 15 mM ammonium, activities for both

enzymes are reduced to 50% to 60%. Consequently, ammonium seems to alter the carbohydrate biosynthesis of TNFR-IgG by a pH-mediated effect on glycosyltransferase activity. © 2000 John Wiley & Sons, Inc. *Biotecnol Bioeng* 68: 373–386, 2000

Keywords: ammonium; N-glycosylation; recombinant glycoprotein; Chinese hamster ovary (CHO)

INTRODUCTION

The development and production of recombinant proteins for human therapeutic use has increased dramatically in the last decade. Many of these products are glycoproteins expressed in mammalian cells; for example, tissue plasminogen activator, factor VIII, erythropoietin, DNase, interleukin-2 (IL-2), interferon- β (IFN- β), immunoadhesins, and immunoglobulins. Physiological and pharmacological properties of glycoproteins, including biological activity, solubility, immunogenicity, and in vivo clearance, are known to be affected by their covalently bound oligosaccharide moieties (Varki, 1993). Numerous bioprocess parameters were shown to have a significant influence on protein glycosylation. These parameters include serum content, culture pH, growth phase, and ammonium concentration (for review, see Goochee et al., 1991; Jenkins et al., 1996). The mechanisms that lead to altered oligosaccharide structures are not well understood. Product consistency, including glycosylation consistency, is a prerequisite for regulatory approval of glycoprotein therapeutics. To achieve this goal, there is a need to identify factors that might change protein glycosylation during cell culture production and to understand the biological mechanisms involved.

Ammonium, which usually accumulates in mammalian cell cultures, has long been identified as a detrimental factor (reviewed in Schneider et al., 1996). Ammonium is mainly a byproduct of glutamine metabolism and the thermal degradation of glutamine to pyrrolidonecarboxylic acid. Glutamine is the main nitrogen source and a major energy source for mammalian cells cultivated in vitro (Reitzer, et al., 1979; Zielke et al., 1984). Depending on the concentration of glutamine in the cell culture medium, ammonium can accumulate to concentrations of up to 10 mM and higher (Gawlitzek et al., 1998). Numerous studies reported negative effects of elevated ammonium concentrations on cell growth and productivity of different cell lines (Schneider et al., 1996).

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Several mechanisms have been proposed to explain the observed effects, including intracellular pH changes and disturbance of the mitochondrial energy metabolism (Schneider et al., 1996). We (Ryll et al., 1994) reported a dramatic increase of UDP-GNAc as a rapid response to elevated ammonium concentrations, accompanied by a transient depletion of UTP. A similar increase in these activated aminosugars has been observed immediately before and during the phase of reduced growth (Moore et al., 1997; Ryll and Wagner, 1992).

Varied effects of ammonium on protein glycosylation have been described. Thorens and Vassalli (1986) reported that sialylation of immunoglobulin secreted from plasma cells was completely inhibited in the presence of 10 mM NH_4Cl . The extent of N-glycosylation of mouse placental lactogen I expressed by Chinese hamster ovary (CHO) cells was inhibited by increasing levels of ammonium chloride in the medium (Borys et al., 1994). Ammonium concentrations ranging from 0 to 10 mM significantly reduced the sialylation of O-glycans on granulocyte colony-stimulating factor produced by recombinant CHO cells (Andersen and Gooch, 1995). Most recently, Zanghi et al. (1998a,b) reported that 10 mM NH_4Cl decreased the polysialic acid content of the neural-cell adhesion molecule (NCAM), a cell-surface protein expressed in CHO cells, to 10% of the content synthesized by control cells. In previous studies, we showed that increasing ammonium levels leads to more complex N-glycan structures of IL-2 N-glycosylation mutants synthesized by baby hamster kidney cells (BHK-21). Higher branching was shown to correlate with increased UDP-GNAc pools (Gawlitczek et al., 1998).

In the studies reported here, we used tumor necrosis factor receptor-immunoglobulin G (TNFR-IgG) as a model glycoprotein (Chamow and Ashkenazi, 1996) to investigate the effect of ammonium on protein glycosylation in CHO cell cultures. The extent of N-glycosylation has been implicated in clearance of TNFR-IgG (Ryll et al., 1999). N-linked oligosaccharides produced under various ammonium concentrations were analyzed. Ammonium decreased galactosylation and sialylation of TNFR-IgG N-glycans. Different biosynthetic and degradative mechanisms were investigated.

MATERIALS AND METHODS

Cells

Experiments were performed using CHO cells derived from a dihydrofolate reductase-minus (*dhfr*⁻) CHO DUKX B11 host (Urlaub and Chasin, 1980). Cells were genetically engineered to secrete TNFR-IgG (under the control of a SV40 promoter) using a *dhfr*-methotrexate selection method similar to that used by Kaufman and Sharpe (1982).

Bioreactor Experiments

All experiments were performed in stirred-tank bioreactors (Applikon, Foster City, CA) with marine vortexing impel-

lers. Reactors were equipped with calibrated dissolved oxygen, pH, and temperature probes. Dissolved oxygen was controlled on-line through sparging with air and/or oxygen at $60 \pm 5\%$ air saturation. pH was maintained through on-line addition of CO_2 or Na_2CO_3 at $\text{pH } 7.2 \pm 0.1$. Vessels were equipped with temperature jackets and cells were grown at 37°C for the first 48 h. After 2 days, and for the remainder of the culture, the cultivation temperature was reduced to 31°C to delay the onset of apoptotic cell death (Moore et al., 1997). Cell viability was assessed by trypan blue exclusion and by measurement of total and supernatant lactate dehydrogenase (LDH) (Goergen et al., 1993). All cell culture experiments were performed in serum-free medium based on DMEM/F12. Cells were inoculated at a concentration of $1.2 \times 10^6/\text{mL}$. To ensure equal starting conditions for different cases, the experiment was started in a 10-L reactor with an 8.5-L working volume. On day 4, cells were harvested by centrifugation at 150g for 10 min. Cells were resuspended at a concentration of about $5 \times 10^6/\text{mL}$ in 3-L bioreactors (1.8-L working volume) using different media compositions. Starting osmolality in different cases was adjusted with NaCl to guarantee similar conditions. Various ammonium concentrations were obtained by using medium containing different amounts of glutamine (0 mM vs. 11 mM) and/or NH_4Cl (0 to 13 mM). Several experiments were run and similar results were obtained. In most cases, data from only one representative experiment are shown. To investigate different biosynthetic mechanisms, cells were cultivated in glutamine-free medium supplemented with either of the following substances: (1) 13 mM NaCl (control); (2) 13 mM NH_4Cl ; (3) 5 mM glucosamine + 10.5 mM NaCl; or (4) 165 μM chloroquine + 13 mM NaCl. Cultures were terminated on day 9 or 10 and accumulated TNFR-IgG was purified.

Ammonium Measurement

The ammonium concentration in cell culture samples (assay range 0 to 20 mM) was measured by flow-injection analysis based on a system described earlier (Forman et al., 1991). Deionized water (Milli-Q system, Millipore) was used for preparation of all solutions. Reagents were of ACS grade. Analyzer streams were water (carrier), 2 M sodium hydroxide (base), and 0.1 mM phenol red in 1 mM sodium phosphate at pH 7.2 (indicator). NH_3 increases the pH in the receiving stream (indicator) and causes a color change, which was detected by absorbance at 590 nm.

β -Galactosidase Activity Assay

β -Galactosidase activity in the CHO cell culture supernatant was measured using 4-methylumbelliferyl β -D-galactoside (4-MU-Gal; Sigma) as a fluorometric substrate (Gramer and Gooch, 1993; Potier et al., 1979). The assay was performed in 96-well (round-bottom) plates (Corning-Costar, Acton, MA) incubating 10 μL of 1 M Na-acetate/HCl (pH 4.0), 65 μL of cell culture supernatant, and 25 μL of 4 mM

4-MU-Gal for 30 min at 37°C. The reaction was stopped by adding 200 μ L of 0.2 M glycine NaOH (pH 10.4). Fluorescence of samples was measured at 360-nm excitation and 460-nm emission using a Cytofluor-2 fluorescence plate reader (PerSeptive Biosystems, Framingham, MA). For β -galactosidase activity calculations, fluorescence was compared with standard 4-methylumbelliferyl dilutions. β -Galactosidase activity days were calculated by integrating the area under the curve of β -galactosidase activity over culture time.

Sialidase Measurement

The concentration of sialidase in the CHO cell culture supernatant was measured using a sialidase enzyme-linked immunoassay (ELISA). Ninety-six-well microtiter plates (Nunc Immulon Maxisorp, Nunc A.S., Roskilde, Denmark) were coated overnight at 4°C with 100 μ L well of polyclonal antibody to recombinant CHO sialidase diluted in 0.05 M sodium carbonate buffer (pH 9.6). The excess antibody was removed and plates were blocked with 150 μ L well of phosphate-buffered saline (PBS) containing 0.5% (w/v) bovine serum albumin (BSA) for 1 h at room temperature on an orbital shaker (Bellco Glass, Inc., Vineland, NJ). The plates were then washed three times with PBS containing 0.05% (v/v) Tween-20 (wash buffer). Recombinant CHO sialidase calibrators and samples were diluted in PBS containing 0.5% BSA + 0.05% Tween-20 (assay buffer). After dilution, 100 μ L of the calibrators and samples were added to each well, and the plates were incubated for 2 h at room temperature on an orbital shaker. The plates were then washed as described earlier, and 100 μ L of biotinylated sialidase polyclonal antibody diluted in assay buffer was added per well. The plates were incubated for 1 h at room temperature on an orbital shaker, and washed as indicated earlier. Then, 100 μ L well of streptavidin horseradish peroxidase diluted in assay buffer was added, and plates were incubated for 1 h at room temperature on an orbital shaker. For colorimetric development, the plates were washed as indicated and 100 μ L of tetramethylbenzidine was added to each well. After sufficient color development was attained (5 to 10 min) at room temperature, the reaction was stopped by the addition of 100 μ L well of 1 M phosphoric acid. The absorbance was measured at 450 nm using an automated plate reader (Molecular Devices, Palo Alto, CA). Data reduction was done with a standard four-parameter fit using SOFTMAX (Molecular Devices, Inc.) software. Sialidase activity was measured using 4-methylumbelliferyl-N-acetylneuraminic acid (Sigma) as a substrate (Gramer and Gooch, 1993). A linear correlation between sialidase activity and sialidase concentration in supernatants was found throughout the culture duration (data not shown).

Sialyltransferase Activity Assay

α 2,3-Sialyltransferase activity in CHO cells was determined using an assay based on the incorporation of fluorescently

labeled N-acetylneuraminic acid into an asialo-acceptor glycoprotein (Gross et al., 1990). Asialo-TNFR-IgG was produced by a sialylation-negative CHO clone. Samples of 5-mL cell suspension (1 to 7×10^6 cells/mL) were centrifuged at 150g for 5 min. Cells were washed with 1 mL of cold PBS, pelleted again at 150g for 5 min, and lysed at a concentration of 1×10^7 cells/mL in 0.2 M NaCl, 1 mM ethylene-diamine tetraacetic acid (EDTA), and 1% (v/v) NP-40 for 30 min at 4°C. The lysate was frozen at -80°C . To measure α 2,3-ST activity, the lysate was thawed, centrifuged at 16,000g for 15 min (4°C), and filtered (0.2 μ m, Millipore). Twenty microliters of lysate (corresponding to about 1×10^6 cells) was incubated at 37°C for 60 min in 20 μ L of 0.15 M sodium cacodylate buffer (pH 6.5), 10 μ L of 75 μ M CMP-9-fluoresceinyl-AcNeu (Boehringer Mannheim, Indianapolis, IN), and 10 μ L of 6.75 mg/mL asialo-TNFR-IgG in a total volume of 60 μ L. The reaction was stopped by adding 8 μ L of 0.1 M cytidine triphosphate (CTP). To determine the incorporation of fluorescently labeled N-acetylneuraminic acid (NANA) into the acceptor glycoprotein, free CMP-9-fluoresceinyl-AcNeu was separated from glycoprotein by protein A chromatography using high-performance liquid chromatography (HPLC, HP1090, Hewlett Packard, Mountain View, CA). Separation was done using a gradient of buffer A (0.018 M Tris-HCl, 1.9 mM Tris-Base, 0.5 tetramethylammonium chloride in H_2O) and buffer B (2% [v/v] HOAc, 0.3 M MgCl_2 in H_2O) at 1 mL/min, 0 to 2.5 min, 0% B; 2.5 to 2.7 min, 0% to 100% B; 2.7 to 3.7 min, 100% B; 3.7 to 3.9 min, 100% to 0% B; 3.9 to 8.0 min, 0% B. The acceptor glycoprotein was detected by UV (280 nm, total TNFR-IgG) and fluorescence (HP1046-A fluorescence detector, excitation 490 nm, emission 520 nm, fluorescently labeled TNFR-IgG) spectroscopy. α 2,3-ST activity was calculated by integrating peak areas and comparing them to calibration curves.

Galactosyltransferase Activity Assay

Cell suspension samples containing 1×10^7 cells were centrifuged at 150g for 5 min. Cells were washed with 1 mL of cold PBS, pelleted again at 150g for 5 min, and lysed in 1 mL of lysis buffer (0.5 M Tris-HCl, pH 6.5; 0.5% [v/v] Triton X-100; 0.15 M NaCl) for 40 to 60 min at 4°C. The lysate was frozen at -80°C . β 1,4-Galactosyltransferase activity in CHO cells was measured by using a solid-phase assay with BSA containing covalently attached GlcNAc residues (BSA-GlcNAc) as a substrate (Zatta et al., 1991).

Northern Hybridization

Total RNA was extracted from CHO cells using Trizol reagent as described by the manufacturer (Gibco BRL, Gaithersburg, MD). Poly A mRNA was isolated using a Poly Atract 1 mRNA isolation system according to the manufacturer's recommendations (Promega, Madison, WI). Two micrograms of mRNA per sample was denatured in 50% formamide, 3% formaldehyde, 1 $\times$ MAE buffer (40 mM

MOPS, 10 mM Na-acetate, 1 mM EDTA, pH 7.0) at 55° to 60°C for 15 min and electrophoresed in a 1% agarose gel containing 0.66 M formaldehyde. RNA was then blotted into nylon membranes (Max-S Nytran, Schleicher & Schuell, Keene, NH) and crosslinked to the membrane using UV irradiation (Stratalinker, Stratagene, La Jolla, CA). The membranes were hybridized to nick-translated ³²P-labeled cDNA probes specific for either the β 1,4-GT or α 2,3-ST transcripts using a ³²P-nick translation kit (specific activity >108 cpm/g; Boehringer Mannheim, Germany) according to the manufacturer's recommendations. Hybridized probe was detected using autoradiography.

Nucleotide and Nucleotide Sugar Extraction and Analysis

Intracellular nucleotides and sugar nucleotides were extracted from CHO cells with perchloric acid and analyzed by ion-pair reversed-phase HPLC as described previously (Gawlitze et al., 1999; Ryll et al., 1991).

Purification of TNFR-IgG

The cell suspension was harvested, cells were removed by low-speed centrifugation (200g for 10 min), and the cell-free supernatant was filtered (0.2 μ m). TNFR-IgG was purified by protein-A chromatography using fast-protein liquid chromatography (FPLC; Pharmacia LKB, Uppsala, Sweden). Flow rates were 4 mL/min for equilibration, load, and wash steps and 2 mL/min for elution. A ProSep Protein-A column (1-mL bed volume; Bioprocessing, Ltd., Durham, UK) was equilibrated with PBS (pH 7.0) and an appropriate volume of supernatant was loaded. The column was washed with PBS until baseline was reached and recombinant protein was eluted with sodium citrate (60 mM, pH 3.0). Afterwards, protein A was regenerated with a buffer containing 20 mM Tris and 2 M guanidine HCl (pH 7.5).

N-Glycan Release and Desialylation

N-glycan release by PNGase F digest from protein-A-purified TNFR-IgG and the subsequent oligosaccharide isolation were performed as described previously (Gawlitze et al., 1999). For matrix-assisted laser desorption/ionization/time-of-flight mass spectrometry (MALDI/TOF-MS) analysis, isolated carbohydrates were desialylated by treatment with 10% glacial acetic acid at 80°C for 3 h. Following desialylation, sialic acid was removed with 0.25 mL of AG1-X2 resin, acetate form (Bio-Rad).

MALDI-TOF/MS Analysis of Desialylated N-Glycans

Desialylated N-glycans were analyzed by MALDI/TOF-MS using a Voyager Elite mass spectrometer (PerSeptive Bio-

systems) as described earlier (Gawlitze et al., 1999; Papac et al., 1996).

Monosaccharide Composition Analysis by High-pH Anion-Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD)

The monosaccharide composition of TNFR-IgG N-glycans was analyzed by HPAEC-PAD using a Dionex BioLC System (Dionex, Sunnyvale, CA) equipped with a CarboPac PA-1 column (4 $\times$ 250 mm) and a pulsed amperometric detector (detector potentials and pulse durations: E_1 = +50 mV, t_1 = 400 ms; E_2 = +75 mV, t_2 = 200 ms; E_3 = -150 mV, t_3 = 400 ms).

Neutral and amino sugars. Protein-A-purified TNFR-IgG was desalted on an NAP-5 column (Pharmacia, Piscataway NJ). Aliquots of 90 μ g were lyophilized followed by N-glycan hydrolysis in 4N trifluoroacetic acid (TFA) for 1 h at 120°C. Samples were evaporated to dryness in a SpeedVac concentrator and reconstituted in 150 μ L of H₂O. Forty-microliter samples were subjected to HPAEC-PAD. Monosaccharides were eluted at a flow rate of 1 mL/min using buffers A (0.1 M NaOH), B (H₂O), and C (0.2 M NaOH) and the following gradient: 0 to 20 min 87% B; 0% C, 20 to 21 min 0% to 100% C; 21 to 31 min 100% C; 31 to 32 min 0% to 13% A; 0% to 87% B; 32 to 42 min 13% A, 87% B.

N-acetylneuraminic acid. Sialic acid was released by incubating 50- μ g samples of desalted and lyophilized TNFR-IgG in 0.2 M TFA for 1 h at 80°C. Free sialic acid was separated at a flow rate of 1 mL/min using buffers A (0.1 M NaOH) and B (0.1 M NaOH, 0.5 M Na-acetate) and the following gradient: 0 to 1 min 20% B; 1 to 11 min 20% to 70% B; 11 to 11.5 min 70% B; 11.5 to 12 min 20% B; 12 to 19 min 20% B. Monosaccharides were identified based on the comparison of retention times of reference sugars.

Sialic Acid Content by O-Phenylenediamine-2HCl (OPD) Derivatization

Sialic acid content of harvested TNFR-IgG was also quantified using the OPD-derivatization method described by Anumula (1995). Hydrolyzed sialic acids from purified TNFR-IgG were derivatized with OPD (Aldrich Chemical Co., Milwaukee, WI) and analyzed by reversed-phase HPLC (HP1090) on C₁₈ (Ultrasphere ODS, 250 $\times$ 4.5 mm; Beckman Instruments, Inc., Fullerton, CA). Fluorescently labeled monosaccharide derivatives were detected by fluorescence spectroscopy (HP1046-A, excitation 230 nm, emission 425 nm).

RESULTS

Effect of Ammonium on Carbohydrate Composition of TNFR-IgG

CHO cells producing TNFR-IgG were cultivated in the presence of various ammonium concentrations achieved by

adjusting glutamine and NH_4Cl levels. Primary 10-L bioreactors were used to grow cells to the desired cell concentration before inoculating duplicate 3-L bioreactors for each ammonium level studied (see Materials and Methods and Fig. 1). Ammonium concentrations were measured throughout the cell culture time course (Fig. 2). In the growth phase of the cultures, the ammonium concentration increased from a starting level of $\sim 0.9 \text{ mM}$ to $\sim 6.5 \text{ mM}$ on day 4 (data not shown). Ammonium levels remained fairly stable and reflected the medium used after cells were transferred into 3-L bioreactors on day 4. By using this experimental setup, we could examine the effect of a range of biotechnologically relevant ammonium concentrations (~ 1 to 15 mM) on the N-glycans of TNFR-IgG. No detrimental effect of high ammonium levels on cell growth or viability was observed. Cell-specific productivity of TNFR-IgG was similar in all cases. The cell culture process was terminated on day 10 and secreted TNFR-IgG was purified by protein-A chromatography for N-glycan analysis.

The monosaccharide composition of PNGase F-released N-glycans from TNFR-IgG produced in the presence of different ammonium concentrations was analyzed by HPAEC-PAD. The content of galactose (Gal) and N-acetylneuraminic acid (NANA) residues on TNFR-IgG correlated in a dose-dependent manner with the ammonium concentration under which the N-linked oligosaccharides were synthesized. Galactose and sialic acid content decreased as the ammonium levels increased (see Fig. 3). The contents of N-acetylglucosamine (GlcNAc), mannose (Man), and fucose (Fuc) remained unchanged and suggest that N-glycan site occupancy was not affected. These results were confirmed by MALDI-TOF-MS (data not shown). No

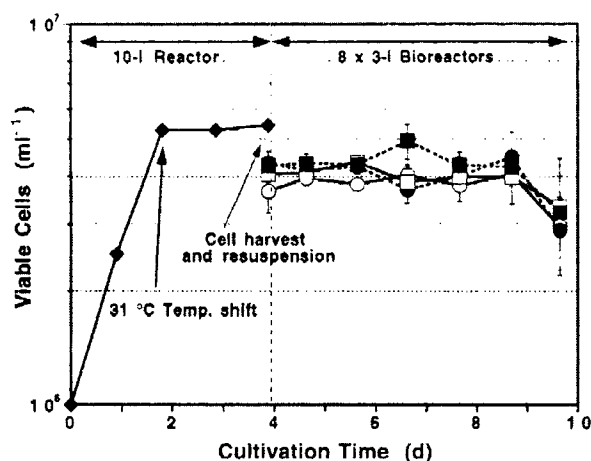


Figure 1. Viable cell concentration of CHO cells cultivated under different ammonium concentrations. Cells were grown for 4 days in a 10-L bioreactor, then harvested and distributed into eight 3-L bioreactors, as described in Materials and Methods. The cultivation temperature was reduced to 31°C on day 2. Ten-liter reactor ($\diamond$); $-\text{Gln}$ ($\circ$); $-\text{Gln} + 13 \text{ mM } \text{NH}_4\text{Cl}$ ($\bullet$); $+11 \text{ mM } \text{Gln}$ ($\square$); $+11 \text{ mM } \text{Gln} + 13 \text{ mM } \text{NH}_4\text{Cl}$ ($\blacksquare$). Culture conditions were changed on day 4, as described in Materials and Methods. Viable cell concentrations are average values from duplicate runs ($n = 2$). Error bars indicate one standard deviation.

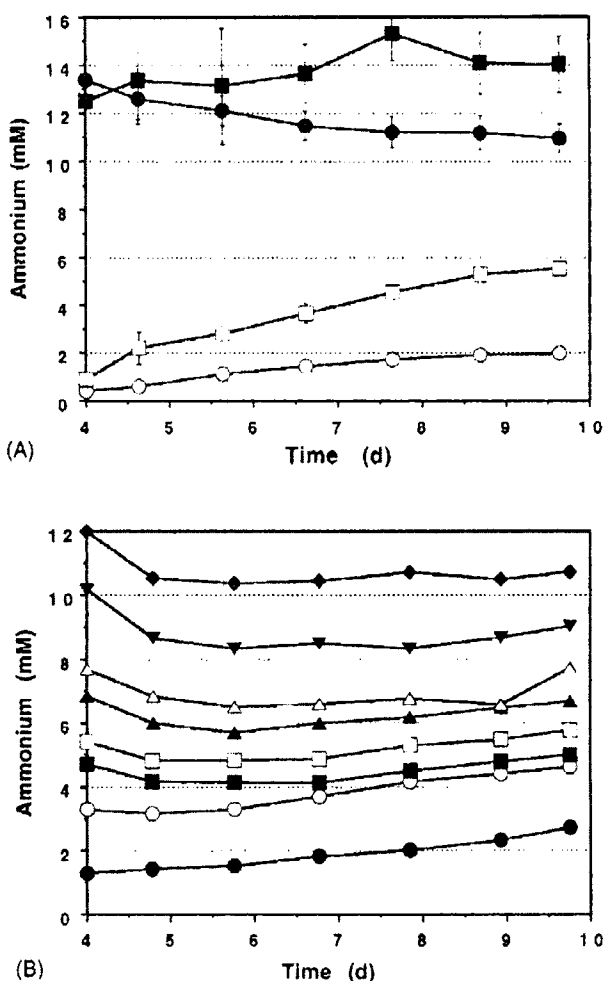


Figure 2. Supernatant ammonium concentrations of cultures supplemented with different concentrations of NH_4Cl . Time shown is after resuspension and distribution into 3-L bioreactors, as described in Materials and Methods. Different NH_4Cl and glutamine concentrations were used to obtain a broad range of ammonium levels. (A) $-\text{Gln}$ ($\circ$); $+\text{Gln} + 13 \text{ mM } \text{NH}_4\text{Cl}$ ($\bullet$); $+11 \text{ mM } \text{Gln}$ ($\square$); $+11 \text{ mM } \text{Gln} + 13 \text{ mM } \text{NH}_4\text{Cl}$ ($\blacksquare$). Ammonium concentrations are average values from duplicate runs ($n = 2$). Error bars indicate one standard deviation. (B) (All cases without glutamine) $1 \text{ mM } \text{NH}_4\text{Cl}$ ($\bullet$); $3 \text{ mM } \text{NH}_4\text{Cl}$ ($\circ$); $4 \text{ mM } \text{NH}_4\text{Cl}$ ($\blacksquare$); $5 \text{ mM } \text{NH}_4\text{Cl}$ ($\square$); $6 \text{ mM } \text{NH}_4\text{Cl}$ ($\blacktriangle$); $7 \text{ mM } \text{NH}_4\text{Cl}$ ($\triangle$); $10 \text{ mM } \text{NH}_4\text{Cl}$ ($\blacktriangledown$); $13 \text{ mM } \text{NH}_4\text{Cl}$ ($\diamond$).

differences in oligosaccharide branching were observed. To elucidate the mechanism(s) by which ammonium alters the N-glycosylation pattern of TNFR-IgG, potential degradative and biosynthetic mechanisms were explored.

Possible Degradative Mechanisms

Glycosidases present in cell culture supernatants have the potential to degrade oligosaccharide structures of recombinant glycoproteins (Ferrari et al., 1994; Gramer and Goochee, 1993; Gramer et al., 1995). Ammonium is known to disrupt the intracellular pH of acidic compartments leading to secretion of lysosomal degradative enzymes

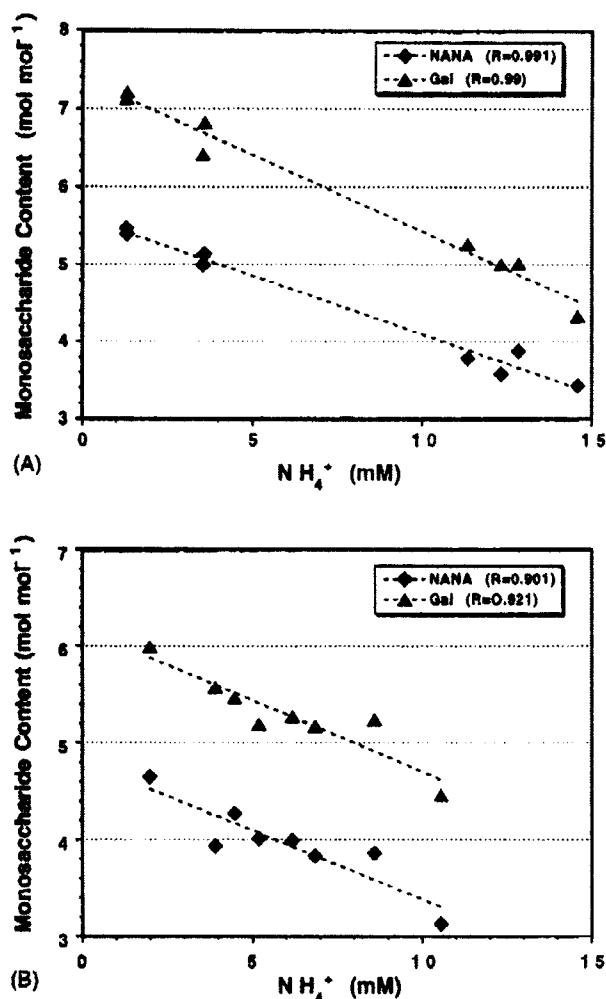


Figure 3. Galactose (▲) and sialic acid (◆) contents of TNFR-IgG plotted over average ammonium concentrations in two different experiments.

(Lit). Increased β -galactosidase and sialidase activity may be responsible for the decreased galactose and *N*-acetylneuraminic acid content of TNFR-IgG observed in the presence of ammonium. β -Galactosidase activity and sialidase concentration in supernatants were monitored over time in the cell culture experiments (data from two experiments shown in Figs. 4 and 5). β -Galactosidase activities correlated positively with the ammonium concentration of the culture. Increasing ammonium levels resulted in an increased release of β -galactosidase (Fig. 4A). No correlation between ammonium and sialidase release was found in our experiments (Fig. 4B). However, sialidase concentration correlated linearly with supernatant lactate dehydrogenase (SN-LDH; Fig. 5B), a marker for cell lysis, indicating that sialidase is released by cell lysis, consistent with previous observations (Gramer et al., 1995). In contrast, there was no linear correlation between β -galactosidase activity and SN-LDH. β -Galactosidase was mainly released when cells were still viable, indicating that this lysosomal glycosidase was primarily released by active secretion (Fig. 5A). Because

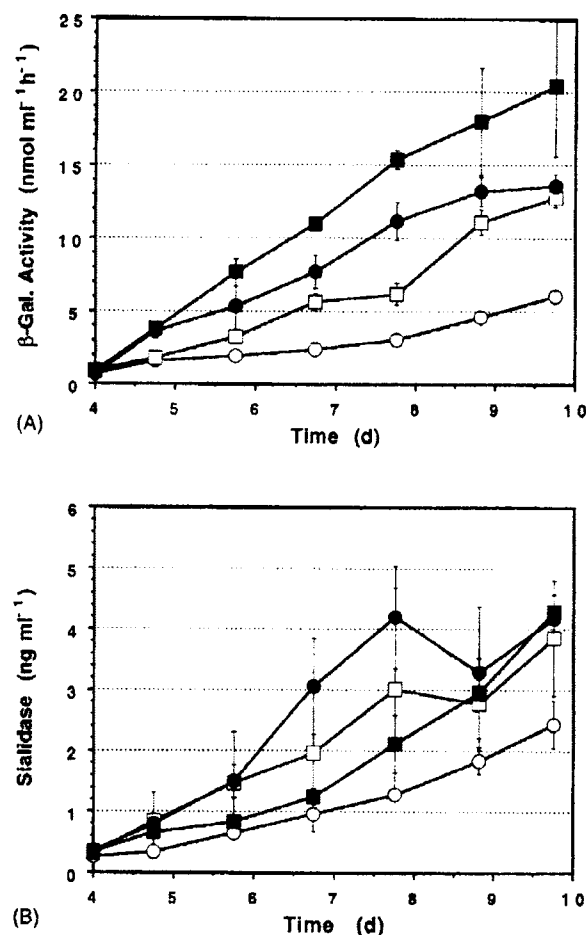


Figure 4. Time course of β -galactosidase activity (A) and sialidase concentration (B) under different ammonium concentrations. Time shown is after resuspension and distribution into 3-L bioreactors, as described in Materials and Methods. Average values for cases 1 to 4 ($n = 2$) are plotted with one standard deviation: -Gln (○); -Gln/-13 mM NH_4Cl (●); 11 mM Gln (control, □); 11 mM Gln/-13 mM NH_4Cl (■).

β -galactosidase activity correlated with the ammonium concentration and with the lack of galactose on TNFR-IgG, incubation experiments were performed to determine if this glycosidase is able to cleave terminal galactose from recombinant glycoprotein substrates. Several recombinant glycoproteins (TNFR-IgG, asialo-TNFR-IgG and two monoclonal antibodies of the IgG1 subclass) were incubated in different β -galactosidase-containing CHO cell culture supernatants (with β -galactosidase activities ranging from 15 to 120 nmol/mL $\cdot$ h⁻¹) for 7 to 21 days under cell-culture-like conditions (37°C and pH 7.2). β -Galactosidase activity was monitored throughout the experiment and remained stable for at least 7 days. After incubation, glycoproteins were purified and N-glycans were analyzed (data not shown). No change in the glycosylation profile (e.g., galactose content) of incubated glycoproteins was found. Thus, our results suggest that CHO-cell-released β -galactosidase is not the cause for the decreased galactosylation of TNFR-IgG produced in the presence of elevated ammonium concentrations.

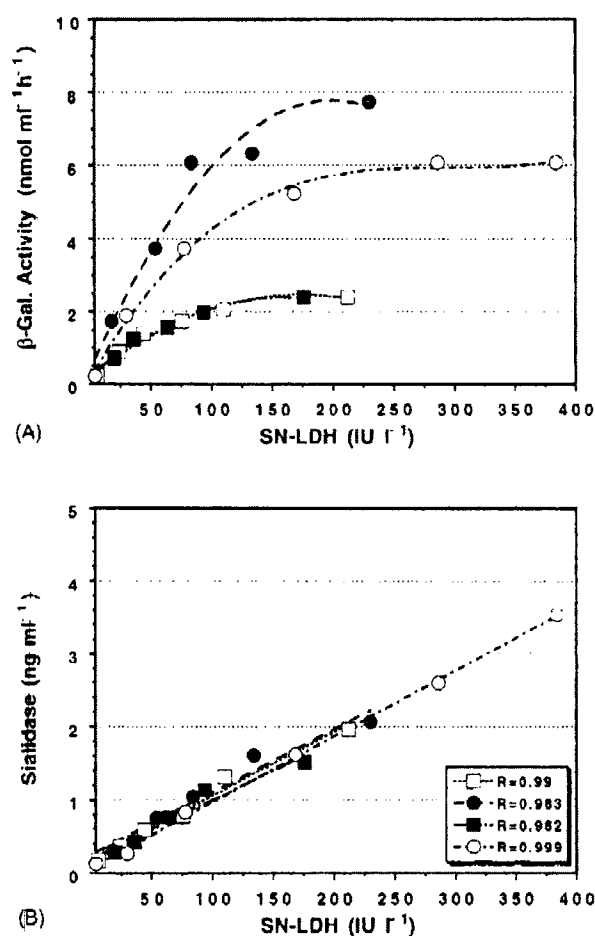


Figure 5. Correlation between β -galactosidase activity (A) or sialidase concentration (B) and supernatant LDH (SN-LDH; 1 IU = 1 μ mol/min) under different culture conditions: control ($\square$); 13 mM NH₄Cl ($\bullet$); 5 mM glucosamine ($\blacksquare$); 165 μ M chloroquine ($\circ$).

Possible Biosynthetic Mechanisms

To investigate further how ammonia alters terminal glycosylation, different potential biosynthetic mechanisms were examined.

Effect of Ammonium on Intracellular mRNA Levels and Enzyme Activity of β 1,4-Galactosyltransferase and α 2,3-Sialyltransferase

β 1,4-Galactosyltransferase and α 2,3-sialyltransferase are the enzymes responsible for galactosylation and sialylation, respectively, of N-linked oligosaccharides in CHO cells. To determine whether ammonia has a direct effect on enzyme expression, intracellular mRNA levels and enzyme activities of both glycosyltransferases were monitored over time in cells cultivated under control and high ammonium conditions. Similar mRNA levels (Fig. 6) and enzyme activities (Fig. 7) were found when comparing cells cultivated under low or high ammonium concentrations.

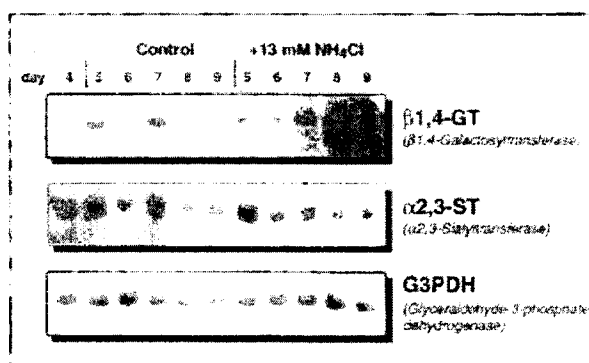


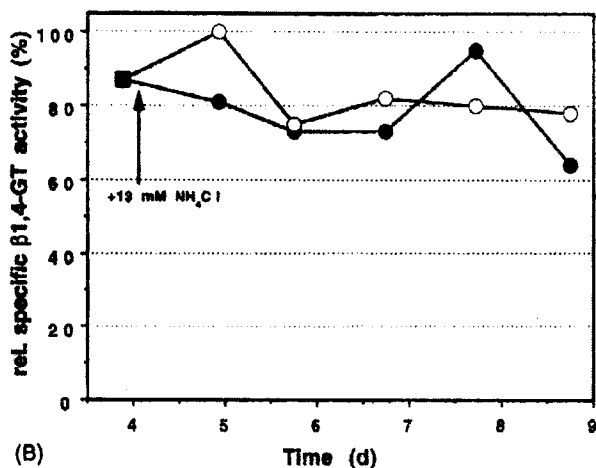
Figure 6. Northern blot analysis of β 1,4-galactosyltransferase and α 2,3-sialyltransferase mRNA in CHO cells cultivated under control (+Gln) and high-ammonium (+Gln + 13 mM NH₄Cl) conditions (see Materials and Methods for details). mRNA levels of glycosyltransferases compared with those of the housekeeping enzyme, glyceraldehyde-3-phosphate dehydrogenase.

Nucleotide Pools vs Intracellular pH Effect

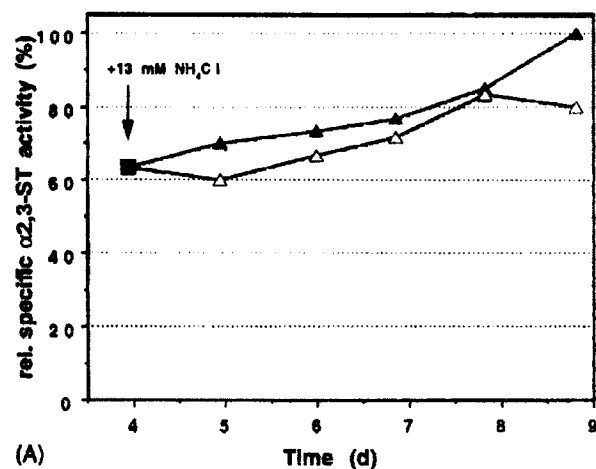
Ammonium and other weak bases are known to increase the pH of acidic compartments (e.g., *trans*-Golgi; Kim et al., 1996; Seksek et al., 1995; von Figura and Hasilik, 1986). Ammonium has also been shown to increase the intracellular levels of UDP-activated hexosamines and to transiently reduce the UTP pool (Ryll et al., 1994). Both mechanisms could potentially affect galactosylation and sialylation. Chloroquine mimics the intracellular pH effect of ammonium (Andersen and Gooch, 1995; Thorens and Vassalli, 1986) presumably without affecting intracellular nucleotide pools. In contrast, glucosamine increases the UDP-GNAc pool, similar to ammonium (Gawlitze et al., 1998; Zanghi et al., 1998), but has presumably no effect on intracellular pH. To study these effects individually, cells were cultivated in the presence of ammonium, glucosamine, or chloroquine. Nucleotide pools were monitored over time in all cases. Ammonium and glucosamine caused a significant and similar increase in UDP-GNAc concentrations compared with control or chloroquine cultures (Table I). β -Galactosidase activity in the cell culture supernatant was used as an indicator for intracellular pH changes. Lysosomal enzymes such as β -galactosidase have been shown to be secreted in response to increased lysosomal pH (Hasilik and Neufeld, 1980a,b). Supernatant β -galactosidase activity was found to be significantly and similarly increased in ammonium and chloroquine cultures, indicating an elevated lysosomal pH (Fig. 5A and Table I). However, similar activities were measured in control and glucosamine cultures.

N-glycan analysis revealed that galactosylation and sialylation were decreased in TNFR-IgG produced in the presence of ammonium and chloroquine. However, no differences in terminal glycosylation (NANA and Gal content) were found in TNFR-IgG synthesized under glucosamine conditions when compared with control material (Table I).

The pH optima of β 1,4-GT and α 2,3-ST are shown in Figure 8. Both enzymes revealed their highest activity at pH



(B)



(A)

Figure 7. Enzyme activities of β 1,4-galactosyltransferase and α 2,3-sialyltransferase in CHO cells cultivated under control (–Gln) and high-ammonium (–Gln/+13 mM NH_4Cl) conditions. On day 4, 13 mM NH_4Cl was added after the enzyme samples were taken.

6.5, the assumed *trans*-Golgi pH. Significantly reduced enzyme activity was observed at pH 7.2, the likely *trans*-Golgi pH under high-ammonium conditions (Barasch et al., 1991; Kim et al., 1996; Seksek et al., 1995).

DISCUSSION

Ammonium in mammalian cell cultures has been shown to affect the N- and O-glycosylation of proteins. Several mechanisms have been implicated for the effect of ammonium on protein glycosylation. An increase in the *trans*-Golgi pH mediated by ammonium chloride or chloroquine was suggested to be the reason for incomplete sialylation of immunoglobulins in plasma cells (Thorens and Vassalli, 1986) and for significantly decreased polysialylation of the neural cell adhesion molecule (NCAM) on the cell surface of CHO cells cultivated in the presence of elevated ammonium concentrations (Zanghi et al., 1998a,b). Waldman and

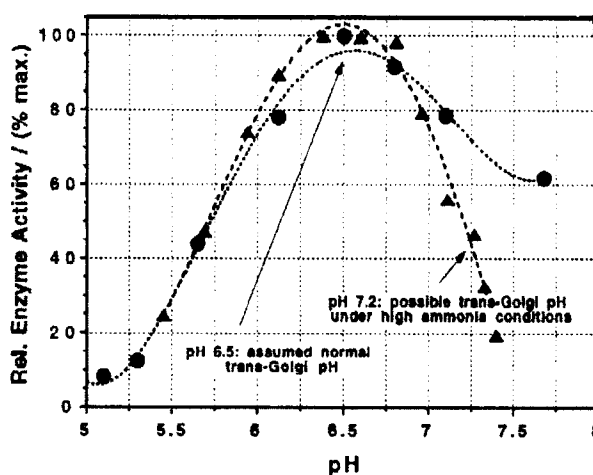


Figure 8. The pH profile of CHO α 2,3-ST ($\blacktriangle$) and β 1,4-GT ($\bullet$) activity.

Rudnick (1990) proposed that nucleotide sugar transport into the Golgi apparatus might be regulated by the pH (acidification) of this compartment. Increased branching of IL-2 N-glycosylation variants produced in BHK-21 cells was found to correlate with increased levels of UDP-GNAc as a response to ammonium or glucosamine and uridine (Gawlitczek et al., 1998; Grammatikos et al., 1998). Using ^{15}N -labeled ammonium chloride we have recently shown that ammonium is directly incorporated into GlcNAc and NANA of N-glycan structures (Gawlitczek et al., 1999), presumably via incorporation into glucosamine-6-phosphate by glucosamine-6-phosphate isomerase (Ryll et al., 1994). Pels-Rijcken et al. (1995) suggested that elevated UDP-GNAc levels (caused by glucosamine and/or uridine feed in their experiments) might impair the transport of CMP-NANA into the *trans*-Golgi compartment, resulting in reduced sialylation.

The results presented here strongly suggest that ammonium inhibits galactosylation and sialylation of TNFR-IgG N-glycans by pH-regulated mechanisms. We hypothesize that ammonium decreases α 2,3-sialyltransferase and β 1,4-galactosyltransferase activities by increasing the pH of the *trans*-Golgi compartment. A significant decrease in the content of galactose and N-acetylneuraminic acid was also observed in the presence of chloroquine, a weak base that increases the pH of acidic compartments (e.g., *trans*-Golgi, lysosomes), similar to ammonium (Hasilik and Neufeld, 1980a). Because chloroquine is known to induce many cellular effects, the possibility cannot be excluded that factors other than an increase in the *trans*-Golgi pH might also contribute to altered glycosylation. In contrast, glucosamine, which elevates UDP-GNAc levels in a manner similar to ammonium, without affecting the intracellular pH, had no effect on terminal glycosylation of TNFR-IgG. The observation that β 1,4-GT and α 2,3-ST both have a slightly acidic pH optimum (pH 6.5), and showed significantly reduced activity at pH 7.2, further supports our hypothesis.

Table 1. Effect of control, ammonium, glucosamine, and chloroquine conditions on β -galactosidase activity in supernatant, the cellular UDP-GNAc content of differentiated CHO cells, and the terminal glycosylation (sialic acid content and galactosylation) of harvested TNFR-IgG.

Cell culture addition	Average NH_4^+ conc., $n = 6$ (mM)	β -Gal activity days (nmol $\cdot$ d $\cdot$ mL $^{-1}$ $\cdot$ h $^{-1}$)	Average cell-specific UDP-GNAc content, $n = 5$ (fmol)	Sialic acid content (nmol/mol)	Terminal galactosylation (%)
Control (no addition)	1.5 (± 0.43)	7.26 (± 0.00)	4.29 (± 1.91)	4.8 (± 0.1)	75.0
NH_4Cl (13 mM)	13.16 (± 1.73)	21.65 (± 0.12)	7.64 (± 1.19)	3.6 (± 0.04)	58.9
Glucosamine (5 mM)	1.52 (± 0.58)	6.70 (± 0.24)	7.34 (± 1.7)	4.7 (± 0.18)	73.2
Chloroquine (165 μM)	1.35 (± 0.35)	19.87 (± 0.1)	3.22 (± 2.2)	4 (± 0.1)	66.4

^aSialic acid content analyzed by OPD and the degree of galactosylation determined on desialylated TNFR-IgG by MALDI-TOF MS (see Materials and Methods for details). Average values are given with one standard deviation. Galactosylation data by MALDI-TOF MS represents the average of 256 laser shots of a single sample.

It is noteworthy that the equal changes in sialylation and galactosylation (Fig. 3) caused by ammonium could be the result of reduced galactosylation alone or in combination with reduced sialylation. Our findings are consistent with several other reports. Andersen and Gooch (1995) suggested that an ammonium-induced increase in the *trans*-Golgi pH is responsible for decreased sialylation of granulocyte colony-stimulating factor (G-CSF) O-glycans. Barasch et al. (1991) reported that defective acidification of intracellular organelles (e.g., *trans*-Golgi) in cells from cystic fibrosis patients (nasal polyp epithelial cells) resulted in reduced sialylation of glycoproteins and lipids. Reduced sialyltransferase activity due to the alkalization of the compartment is believed to be the cause for the decreased sialylation.

Several different glycosidases have been found in CHO cell culture supernatant, including sialidase, β -galactosidase, and fucosidase (Gramer and Gooch, 1993). Gramer et al. (1995) reported that extracellular CHO sialidase originates from the cytosol of the cell and is released to the cell culture supernatant as a result of damage to the cellular membrane. These investigators also showed that extracellular sialidase is active toward a variety of CHO-cell produced glycoproteins, and can hydrolyze sialic acid from the recombinant glycoprotein gp-120 in cell culture supernatant. Extracellular sialidase activity was also detected in all of our cell culture experiments and could have contributed to decreased sialylation. However, supernatant sialidase did not correlate with the lack of sialic acid on TNFR-IgG nor with the ammonium concentration of the respective case. Release of sialidase did correlate with supernatant LDH activity in our studies, confirming its cytosolic origin and release via cell lysis. Our results suggest that sialidase was not the cause of altered sialylation induced by ammonium under the conditions used here. However, we found

that extracellular β -galactosidase correlated with ammonium concentration (and with chloroquine) in the supernatant and also with the lack of galactose on TNFR-IgG. β -Galactosidase did not correlate with supernatant LDH and was primarily released early in the culture when cell viability was high. These results indicate that β -galactosidase was mainly released by active secretion. This phenomenon of misorting of lysosomal enzymes is caused by weak bases (e.g., ammonium, chloroquine), which disturb the mannose-6-phosphate recycling process by elevating the lysosomal pH (Hasilik and Neufeld, 1980a,b; Stryer, 1995; Tapper and Sundler, 1990). β -Galactosidase did not cleave terminal galactose from any of several recombinant glycoprotein substrates in incubations that mimicked cell culture conditions. The pH optimum of β -galactosidase was between pH 3.5 and 4.0 and the enzyme showed little activity ($<10\%$ of maximum) at neutral pH (Gramer and Gooch, 1993). This might explain why β -galactosidase did not cleave terminal galactose in our incubation experiments, nor presumably in cell culture. The inaccessibility of terminal galactose residues for β -galactosidase is another possible explanation.

In contrast to previous results (Gawlitze et al., 1998; Grammatikos et al., 1998), we did not observe changes in N-glycan branching as a response to elevated ammonium (or glucosamine) levels. Differences in the cell line used (CHO vs. BHK), the glycoprotein produced (e.g., protein-specific glycosylation, accessibility of glycosylation site), and in the cell culture process (batch vs. perfusion culture) might be responsible for the different results.

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