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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

DIVISIÓN DE NUEVAS CREACIONES

E. S. D.

Re: Expediente N°. 12-068.980

Solicitud de Patente de Invención titulada: "ANTICUERPO ANTI-TSLP
MODIFICADO POR TÉCNICAS DE INGENIERÍA GENÉTICA"

Solicitante: MERCK SHARP & DOHME CORP.

Nuestra Ref.: P2012/000048

RECURSO DE REPOSICIÓN

CARLOS R. OLARTE, mayor de edad y vecino de Bogotá, D.C., identificado como aparece al pie de mi firma, en mi condición de apoderado de la sociedad solicitante, y dentro del término legal previsto por el artículo 51 del Código Contencioso Administrativo, me permito interponer RECURSO DE REPOSICIÓN en contra de la Resolución No. 13049, notificada por edicto desfijado el 04 de abril de 2014.

1. ANTECEDENTES

- La solicitud de patente ventilada en este expediente fue presentada el 26 de abril de 2012, con Capítulo Reivindicatorio original de 37 reivindicaciones.
- La Superintendencia de Industria y Comercio (SIC) emitió el primer y único examen de fondo fijado en la lista 034 del 27 de agosto de 2013, correspondiente al oficio 15748, objetando el nivel inventivo de la presente invención.
- En respuesta al examen de fondo, el 18 de enero de 2014 se adjunta un Capítulo Reivindicatorio Modificado con un total de 14 reivindicaciones.
- La SIC por medio de la Resolución No. 13049 niega las Reivindicaciones 1 a 14, por supuesta falta de nivel inventivo.

2. OBJETO DEL RECURSO

Que se revoque la resolución recurrida, y en su lugar se conceda el privilegio de patente de invención para las reivindicaciones pendientes de la presente solicitud o aquellas que el Despacho considere cumplen con los requisitos de patentabilidad.

3. FUNDAMENTO DEL RECURSO

Con referencia a los argumentos presentados por la SIC en la Resolución No. 13049 para desvirtuar el nivel inventivo de las Reivindicaciones 1 a 14 de la presente solicitud, difiero respetuosamente del análisis expuesto, y procedo a refutar cada uno de dichos argumentos, exponiendo las motivaciones técnicas que desvirtúan la posición de ese Despacho.

3.1. a- i) La SIC insiste en que la solicitud de patente WO2008076321 (D1) afecta el nivel inventivo de la presente invención.

La SIC argumenta que D1 divulga un anticuerpo que se une específicamente a TSLP humana para el tratamiento de desórdenes inflamatorios (resumen, págs. 25 y 26).

Además, D1 divulga vectores que comprenden el anticuerpo, ácidos nucleicos, el método para producir los anticuerpos, células huésped que comprenden el vector y composiciones de los mismos. D1 además divulga que los anticuerpos pueden tener sustituciones conservativas (página 3, párrafo 3; página 14; página 22; página 24).

En particular, la SIC presenta la siguiente tabla comparando los elementos esenciales entre D1 y la presente invención:

Características esenciales	D1
Un compuesto de unión, caracterizado porque se une específicamente a TSLP humana que comprende:	Un anticuerpo que se une a TSLP humana que comprende (Resumen, Págs. 25 y 26):
HCDR1 con la SEC ID NO: 1, GYIFTDYAMH HCDR2 con la SEC ID NO: 2, TFIPLLDTSYAQKFQG HCDR3 con la SEC ID NO: 3, MGVTHSYVMMA LCDR1 con la SEC ID NO: 4, RASQPISISVH LCDR2 con la SEC ID NO: 5, FASQSI y LCDR3 con la SEC ID NO: 6, QOTFSLPYT	HCDR1 con la SEC ID NO: 1, GYIFTDYAMH HCDR2 con la SEC ID NO: 2, TFIPLLDTSYDYNQNFKG HCDR3 con la SEC ID NO: 3, MGVTHSYVMMA LCDR1 con la SEC ID NO: 4, RASQPISISVH LCDR2 con la SEC ID NO: 5, FASQSI y LCDR3 con la SEC ID NO: 6, QOTFSLPYT

A partir de la anterior tabla, la SIC señala que la diferencia entre la Reivindicación 1 y el anticuerpo divulgado por D1 es que la CDR2 de SEQ ID NO:2 tiene tres aminoácidos diferentes (N, K y K) que reemplazan a (A, K y Q) en el anticuerpo reclamado en D1. El Examinador señala que el

efecto que logra incluir los tres aminoácidos diferentes (N, K y Q) que reemplazan a (A, K y Q) es evitar la desamidación de los anticuerpos.

La SIC argumenta que no hay evidencia de que como consecuencia de la sustitución de tres aminoácidos en la CDR2 se haya logrado algún efecto inesperado, como el efecto de lograr la desaminación, por lo tanto los compuestos de unión reivindicados son obvios para la persona medianamente versada en la materia.

Adicionalmente, este despacho señala que resulta previsible que se hayan realizado las sustituciones de los aminoácidos en la CDR2 de N por K y K por Q, debido a que contemplan sustituciones conservativas en donde los aminoácidos pertenecen al mismo grupo de aminoácidos hidrofílicos; la sustitución de N por A también se considera previsible, teniendo en cuenta que el aminoácido N pertenece al grupo de aminoácidos hidrofílicos y A al grupo de aminoácidos con características intermedias, por lo que las Reivindicaciones 1 y 13 carecen de nivel inventivo.

b- i) En primer lugar me permito señalar que la presente invención se refiere a compuestos de unión que se une específicamente a TSLP humana que comprenden:

- una región variable de cadena pesada de anticuerpo o un fragmento de unión a TSLP del mismo, comprendiendo dicha región variable de cadena pesada: una secuencia de CDR-H1 que comprende SEC ID NO: 1; una secuencia de CDR-H2 que comprende SEC ID NO:2; y una secuencia de CDR-H3 que comprende SEC ID NO: 3; y
- una región variable de cadena ligera de anticuerpo o un fragmento de unión a TSLP del mismo, comprendiendo dicha región variable de cadena ligera: una secuencia de CDR-L1 que comprende SEC ID NO:4; una secuencia de CDR-L2 que comprende SEC ID NO:5; y una secuencia de CDR-L3 que comprende SEC ID NO:6.

ii) Tal y como lo señala el Despacho, las secuencias de la presente invención presenta sustituciones de aminoácidos particulares de la SEQ ID NO: 2 modificando los aminoácidos (N, N, K) por (A, K, Q).

Los cambios mencionados anteriormente permiten evitar el potencial problema de la desamidación de los anticuerpos. La desamidación de Asn (N) es común, y dicha degradación de las proteínas puede afectar significativamente su estructura y función, pudiendo dar lugar a cambios en las

interacciones anticuerpo-antígeno, y por lo tanto representando un problema grave durante el desarrollo de las terapias a base de anticuerpos.

Por lo tanto, es importante evitar estos posibles problemas de desamidación en anticuerpos destinados para uso humano, sin que se produzcan cambios en cualquiera de las características importantes del anticuerpo (tales como la afinidad de unión).

iii) En cuanto al arte previo citado por este despacho, me permito señalar que D1 se refiere a compuestos de unión que se unen específicamente a TSLP humana, para el tratamiento de enfermedades inflamatorias. El anticuerpo de D1 CDR-H2 contiene dos residuos asparaginas (N) en las posiciones 61 y 63 de SEQ ID NO: 4, por lo que podría potencialmente ser desamidado, de ese modo alterando su estructura, lo que puede causar problemas graves que afecte a la seguridad y/o eficacia de dicho anticuerpo.

Adicionalmente, el problema que pretende resolver D1 es la generación de nuevos anticuerpos, sin tener en cuenta el problema de desamidación abordado por la presente invención. D1 divulga que los cambios en los residuos del marco (FR), implicados en el soporte de los bucles de CDR, son requeridos para mantener la afinidad de unión. Adicionalmente, D1 señala que aunque se ha reportado el uso de injertos de CDR y preservación de restos del marco en anticuerpos humanizados, **es difícil predecir si una secuencia particular puede resultar en un anticuerpo con propiedades de unión deseadas al igual que propiedades biológicas buscadas.**

iv) Teniendo en cuenta lo anterior, y contrario a lo señalado por este Despacho, la presente invención tiene diferencias drásticas con D1 que la SIC está considerando superficiales debido al análisis errado que lleva a cabo. Las diferencias se encuentran tanto a nivel estructural como frente al problema técnico que pretenden resolver cada una de las invenciones. De esta forma, incluso D1 señala que no es posible predecir que una estructura de anticuerpo particular pueda tener la afinidad y la actividad biológica deseada.

A continuación me permito señalar las diferencias estructurales de los anticuerpos de la presente invención frente al anticuerpo divulgado por D1:

- a) D1 da a conocer una región variable de cadena pesada que comprende una CDR-H2 con una identidad de sólo **83,1%** con la SEC ID NO: 2 de la presente invención;

- b) de manera similar, D1 difiere de la SEQ ID NO: 7 de la presente invención, puesto que comprende hasta tres residuos de aminoácidos modificados;
- c) no hay enseñanza o sugerencia alguna en D1 que lleve a la persona medianamente versada en la materia a un vector de expresión para un ácido nucleico que codifica una región variable de cadena pesada depositado como PTA-10482;
- d) D1 enseña un anticuerpo que se une a la TSLP, depositado como PTA-7951, lo cual es estructuralmente diferente de la presente invención.

De esta forma, lo que el despacho ve como una simple modificación en residuos de aminoácidos, lleva a una diferencia en la secuencias de aproximadamente 17% en el CDR-H2, y tal como lo menciona D1 **cualquier modificación en los anticuerpos puede llevar a cambios en la afinidad y de la misma forma en la actividad biológica.** Lo anterior muestra que la SIC se encuentra haciendo un análisis limitado y simplista de la presente invención.

v) Frente al argumento de la SIC de que no existe evidencia de que las modificaciones en los aminoácidos lleven a un efecto inesperado, como el efecto de desaminación, me permito respetuosamente, una vez más, llamar la atención del Despacho sobre la figura 1 de la presente invención, la cual muestra una alineación de SEQ ID NO: 11 de la presente solicitud contra SEC ID NO:14 de D1 (es decir, una alineación de las cadena pesadas del anticuerpo reivindicado en esta invención y el anticuerpo descrito en D1).

En la Figura 1, la “Secuencia 1” corresponde a SEC ID NO:11 de la presente solicitud y la “Secuencia 2” corresponde a SEC ID NO:14 de D1. En el anticuerpo reivindicado en esta invención, la **asparagina (N) en posición 61 de SEC ID NO: 14 de D1 fue cambiada por alanina (A)** y la **asparagina en posición 63 de SEC ID NO: 14 fue cambiada a lisina (K)**. Estos cambios fueron realizados para evitar la potencial desamidación de estos residuos. Además, **la lisina (K) en posición 65 de SEC ID NO: 4 de D1 fue cambiada a Glutamina (Q)**. Este cambio fue realizado para **disminuir las posibilidades de crear inmunogenicidad.** Se realizó un cambio adicional en posición 72 de SEC ID NO: 14 de D1, donde una treonina (T) fue cambiada a una alanina (A). Este cambio fue realizado para mejorar la afinidad de unión del anticuerpo.

Lo anterior demuestra la realización de cambios particulares, por medio de los cuales se logró, no solo evitar la desamidación, sino también disminuir las posibilidades de crear inmunogenicidad y mejorar la afinidad de unión del anticuerpo.

vi) De nuevo, la desamidación de los residuos de asparagina es común en la degradación de proteínas y ésta puede cambiar la estructura y la función de péptidos o proteínas, resultando potencialmente en la pérdida en la actividad, así como alteraciones en la farmacocinética y antigenicidad de la proteína.

En el caso de los análogos del factor de liberación de la hormona de crecimiento humano, Lcu²⁷hGRF(1-32)NH₂, la desamidación del residuo 8 de asparagina a isoaspartato lleva a interrupciones de la estructura helicoidal, tal y como se describe en Vlasak¹. Este efecto también ha sido demostrado en la calbidina bovina D_{9k}, en donde la desamidación del residuo 56 de asparagina causa perturbaciones locales en el producto. Más aun, se ha demostrado que los anticuerpos desamidados son menos potentes en su unión con el antígeno comparado con los anticuerpos no degradados, debido a que la desamidación en CDRs puede ser asociada con cambios en las interacciones antígeno-anticuerpo². Por lo tanto resulta necesario determinar si hay sitios de potencial desamidación dentro de un fármaco candidato potencial³.

De acuerdo con lo anterior, el problema que pretende resolver la presente invención es cómo generar un anticuerpo anti-TSLP, que no pueda ser desamidado y por lo tanto, su estructura no se distorsione, evitando serios problemas que afectan la seguridad y la eficacia de los anticuerpos.

La producción de anticuerpos no desamidados dirigidos contra TSLP no puede ser considerado como una rutina de experimentación para una persona medianamente versada en la materia, ya que las enseñanzas disponibles en el área enseñan que la sustitución de residuos de asparagina disminuye la unión de los anticuerpos y por lo tanto hace anticuerpos inefectivos, tal y como se menciona en el artículo “generation of a humanized anti-glypican 3 antibody by CDR grafting and stability optimization”⁴. Este problema ha sido descrito en documentos publicados posteriormente, como se evidencia en el artículo “Identification and multidimensional optimization of an asymmetric bispecific IgG antibody mimicking the function of factor VII cofactor activity”⁵. Adicionalmente, el artículo “Isomerization of an aspartic acid residue in the complementarity-determining regions of a recombinant antibody to human IgE: Identification and effect on binding

¹ Josef Vlasak et al., “Identification and Characterization of Asparagine Deamidation in the Light Chain CDR1 of a Humanized IgG1 Antibody,” *Analytical Biochemistry* 392, no. 2 (September 15, 2009): 145–54, doi:10.1016/j.ab.2009.05.043.

² Ibid.

³ Lihua Huang et al., “In Vivo Deamidation Characterization of Monoclonal Antibody by LC/MS/MS,” *Analytical Chemistry* 77, no. 5 (March 1, 2005): 1432–39, doi:10.1021/ac0494174.

⁴ Kiyotaka Nakano et al., “Generation of a Humanized Anti-Glypican 3 Antibody by CDR Grafting and Stability Optimization,” *Anti-Cancer Drugs* 21, no. 10 (November 2010): 907–16, doi:10.1097/CAD.0b013e32833f5d68.

⁵ Zenjiro Sampei et al., “Identification and Multidimensional Optimization of an Asymmetric Bispecific IgG Antibody Mimicking the Function of Factor VIII Cofactor Activity,” *PloS One* 8, no. 2 (2013): e57479, doi:10.1371/journal.pone.0057479.

affinity”⁶ que es parte del estado de la técnica muestra en la tabla 4 (página 1901) que las sustituciones del residuo de Asp (L32 a Glu) en V104 o el V10 (Asp LK30, L32 o L34 a Ala) disminuyen la unión relativa a IgE.

Por el contrario y de manera sorprendentemente, la presente invención revela anticuerpos mejorados que superan el problema de la desamidación preservando la afinidad por la TSLP humana y evitando problemas futuros de inmunogenicidad.

vii) En resumen, adicional al hecho de que D1 no enseña o sugiere de ninguna manera los cambios específicos que deben ser llevados a cabo sobre los anticuerpos allí descritos para llegar a los de la presente invención, existe arte previo que muestra que el realizar cambios sobre los CDRs, y en especial las sustituciones de residuos de asparagina, se generan disminución en la actividad biológica de los anticuerpos. Es decir, existen enseñanzas que llevarían a la persona medianamente versada en la materia a conclusiones contrarias a las obtenidas en la presente invención.

3.2. a- En este punto vale la pena señalar que de acuerdo con los lineamientos del Instructivo para el examen de solicitud de patentes de invención y modelo de utilidad de la SIC, la presente invención muestra indicios claros de la existencia de nivel inventivo, en cuanto resuelve un problema técnico sin resolver antes de la invención que adicionalmente implica existe una superación de un perjuicio técnico⁷, en tanto que el problema técnico de la desamidación (no abordado por D1), según las enseñanzas del arte previo, no debería resolverse por medio de la sustitución de residuos de asparagina, ya que la persona medianamente versada en la materia estaría necesariamente predispuesta a esperar que tal modificación disminuya la afinidad de unión de los anticuerpos; sin embargo, la presente invención muestra que contrario a lo indicado en el arte previo, sí resulta posible resolver el problema de la desamidación por medio de la sustitución de residuos de asparagina, sin afectar la afinidad de unión de los anticuerpos.

Además, el MAP también establece que las invenciones en el campo de la química tienen nivel inventivo cuando los compuestos tienen una **estructura inesperada** (no derivable por la persona

⁶ J Cacia et al., “Isomerization of an Aspartic Acid Residue in the Complementarity-Determining Regions of a Recombinant Antibody to Human IgE: Identification and Effect on Binding Affinity,” *Biochemistry* 35, no. 6 (February 13, 1996): 1897-1903, doi:10.1021/bi951526c.

⁷ Instructivo Examen de Solicitudes de Patentes de Invención y Modelo de utilidad. Superintendencia de Industria y Comercio. 2012. Sección 2.13. Página 69. (disponible electrónicamente en http://api.sic.gov.co/Documentos/Guia_Examen_Patentes.pdf).

medianamente versada en la materia) o cuando presentan un **efecto técnico inesperado o mejorado impredecible del arte previo**⁸. En el presente caso, los compuestos tienen una estructura no obvia respecto al arte previo, y la actividad presentada por los mismos es sorprendente, lo que es una clara evidencia de nivel inventivo.

b- Por otro lado, frente a la objeción de la SIC referente a que las sustituciones de los anticuerpos de la presente invención resultan obvias, me permito señalar que D1 no enseña o sugiere realizar sustituciones aminoacídicas, por medio de las cuales sea posible obtener los anticuerpos de la presente invención. Más aún, en este caso el Despacho se encuentra haciendo un análisis errado del nivel inventivo, ya que de acuerdo con el instructivo para el examen de solicitudes de patentes de invención y modelos de utilidad es “necesario además de identificar el documento del estado de la técnica más cercano, identificar las diferencias entre la invención y las características técnicas divulgadas en el documento y en el estado de la técnica **debe existir alguna indicación para modificar o adaptar la solución del estado de la técnica más cercano a fin de llegar a la invención reivindicada**”⁹.

Claramente, en D1 no existe ninguna enseñanza o sugerencia que siquiera lleve a la persona medianamente versada en el arte a plantearse el posible problema de la deaminación de anticuerpos, y mucho menos cómo este podría llegar a ser resuelto, incluso cuando D1 muestra explícitamente que cualquier modificación en los CDRs puede llevar a cambios impredecibles en la actividad de los anticuerpos anti-TSLP.

En este punto vale la pena preguntarse ¿de qué manera la persona medianamente versada en el arte, a partir de las enseñanzas de D1 llegaría a determinar que los aminoácidos asparagina (N) de la posición 61 de la SEQ ID NO: 14, la asparagina en la posición 63 y la lisina (K) en la posición 65 fueran modificadas por alanina (A), lisina (K) y glutamina (Q) respectivamente, y de esta forma llegar a la obtención de un anticuerpo mejorado, que mantenga su afinidad de unión y evite la desaminación? La respuesta contundente es que no existen indicaciones particulares de dichas modificaciones en D1, por lo que la persona medianamente versada en la materia no se vería guiada a modificar los anticuerpos de forma tan específica para obtener los anticuerpos de la presente invención y mucho menos esperar que se mantenga la actividad, ya que D1 incluso señala de forma explícita que cualquier variación en los CDR alteraría la actividad de los anticuerpos, ya que dichas

⁸ Manual Andino de Patentes. (2004). Sección 10.8.1 (<http://www.comunidadandina.org/public/patentes.pdf>).

⁹ Instructivo Examen de Solicitudes de Patentes de Invención y Modelo de utilidad. Superintendencia de Industria y Comercio. 2012. Sección 2.13. Página 69. (disponible electrónicamente en http://api.sic.gov.co/Documentos/Guia_Examen_Patentes.pdf).

variaciones llevan a cambios en la conformación de la estructura terciaria y cuaternaria de las proteínas, que es la que determina su actividad.

c- El análisis del nivel inventivo no puede basarse en si la invención era posible de alcanzar, ya que claramente era posible, pues los anticuerpos modificados de la invención de hecho existen. Aplicar un estándar de "posibilidad de alcanzar", haría que ninguna invención fuera susceptibles de patentarse, haciendo inviable el sistema de patentes. Del mismo modo, el análisis de patentabilidad no puede implicar la cuestión de si las técnicas de investigación aplicadas son comunes u obvias, sino más bien si el objeto específicamente reclamado podría haber sido evidentemente derivado del arte previo (sin necesidad de investigación adicional alguna). En otras palabras, para la fecha de prioridad aquí reclamada, la cuestión a determinar es si la persona medianamente versada en la materia, habría realizado la invención, motivado por el conocimiento general y estimulado por el arte previo. Es decir, **la evaluación de nivel inventivo debe basarse únicamente en si existen sugerencias explícitas y suficientes en el arte previo para lograr desarrollar de manera obvia los anticuerpos aquí reclamados, las cuales, como se demostró a lo largo del presente memorial, no existen**. Por consiguiente, aunque en D1 se incluyen anticuerpos anti-TSLP, de ninguna manera se considera el problema de la deaminación de dichos anticuerpos y la manera de cómo este problema puede ser solucionado.

Así las cosas, no existe motivación alguna en el arte previo para que la persona medianamente versada en la materia pueda desarrollar inequívocamente los anticuerpos de la presente invención y esperar una actividad biológica como la aquí reclamada. De hecho la persona medianamente versada en el arte, a partir de D1, ni siquiera hubiera contemplado el problema de la deaminación de anticuerpos y hubiera podido realizar ilimitadas sustituciones de anticuerpos para obtener anticuerpos diferentes, sin solucionar el problema planteado por la presente invención. **La única manera de pensar que dicha modificación puntual podría realizarse y tendría los efectos establecidos en la presente solicitud es que la Examinadora se base en la misma divulgación del Capítulo Descriptivo de la presente invención**. En conclusión, los anticuerpos reclamados en la presente invención revisten nivel inventivo.

d- En este punto vale la pena llamar la atención del Despacho sobre el Instructivo para el Examen de Solicitudes de Patentes de Invención y Modelos de Utilidad de la Superintendencia de Industria y Comercio, donde se establece que:

“La invención no tiene nivel inventivo cuando la combinación de los elementos que proveen la solución está revelada en el estado de la técnica, es decir, no basta con la

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existencia de los elementos de manera aislada o individualmente considerados para concluir la falta de nivel inventivo, sino que ellos en su totalidad e integridad enseñen de manera evidente la manera de llegar a la solución al problema técnico¹⁰ (mi subrayado, mi negrilla)

Si aplicamos las directrices del Instructivo al presente caso se puede concluir que:

- el arte previo citado (D1) **no** enseña los elementos característicos de la presente invención, ni siquiera de manera aislada, pues a lo sumo menciona secuencias de anticuerpos anti TSLP y sin enseñar específicamente las modificaciones específicas necesarias en los aminoácidos: asparagina (N) de la posición 61 de la SEQ ID NO: 14, la asparagina en la posición 63 y la lisina (K) en la posición 65;
- a partir de las enseñanzas de D1, la persona medianamente versada en la materia llegaría a anticuerpos estructuralmente diferentes a los de la presente invención, sin modificaciones en los residuos de asparagina, ya que dichas modificaciones en los CDRs llevaría a cambios en la afinidad de unión de los anticuerpos;
- la divulgación de D1 en su totalidad e integridad **NO** enseña de manera **evidente** cómo llegar a la presente invención y así proporcionar la solución al problema técnico.

3.3. a. En respuesta a mis argumentos en el primer examen de fondo, la SIC señala que a partir de los ejemplos presentados en la presente invención, no se demuestra que el compuesto de unión divulgado tenga menor potencial de desaminación en comparación con el anticuerpo de D1, ni se prueba que como consecuencia de la diferencia estructural con los cambios en los aminoácidos específicos se evite el problema de la desaminación. Tampoco se muestra evidencia del efecto técnico sorprendente, puesto que la actividad de los anticuerpos es similar.

b- Al plantear el anterior argumento, la SIC demuestra una vez más, que realiza un análisis limitado de la presente invención. Es decir, aparentemente la SIC analizara el nivel inventivo simplemente teniendo en cuenta la magnitud subjetiva de las diferencias de la presente invención versus el arte previo. Por lo tanto, la SIC toma un documento que tiene un cierto grado de similitud con los anticuerpos de la presente invención, y aunque ni siquiera planté un problema técnico cercano, lo utiliza para afectar el nivel inventivo, sin sustentar de qué manera resulta evidente la modificación de los residuos de asparaginas y lisina de la secuencia SEQ ID NO: 2.

¹⁰ Instructivo Examen de Solicitudes de Patentes de Invención y Modelo de utilidad. Superintendencia de Industria y Comercio. 2012. Sección 2.13. Página 69. (disponible electrónicamente en http://api.sic.gov.co/Documentos/Guia_Examen_Patentes.pdf).

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En este punto me permito referirme nuevamente los ejemplos de la presente invención, los cuales muestran que los anticuerpos de la presente invención tienen características diferentes a los anticuerpos de D1. En particular, la Tabla 3 muestra las diferencias en los valores de KD para cada uno de los anticuerpos evaluados, en donde se incluyen anticuerpos divulgados por D1, y se evidencia de forma clara que existe una diferencia en la actividad alcanzada:

TABLA 3			
Valores KD Determinados por KinExa			
mAb	TSLP	Expresión	K _D (pM)
Anticuerpo 1	humana	HEK293	1,1
Anticuerpo 2	humana	HEK293	7,7
Anticuerpo 3	humana	HEK293	1,6
Anticuerpo 4	humana	HEK293	3,2

- Anticuerpo 1: Anticuerpo de rata parental 23B12;
- Anticuerpo 2: MAb Anti TSLP hu humanizado 23B12 (que comprende la cadena pesada de SEC ID NO:14 y la cadena ligera de SEC ID NO:16 de D1);
- Anticuerpo 3: mAb 23B12 anti TSLP hu humanizado (que comprende la cadena pesada de SEC ID NO: 14 con una mutación en posición 72 de T a A, y la cadena ligera de SEC ID NO: 16 de D1);
- Anticuerpo 4: mAb 23B12 anti TSLP hu humanizado (que comprende la cadena pesada de SEC ID NO: 11 y la cadena ligera de SEC ID NO: 12 de la presente invención).

Claramente, a pesar de las diferencias estructurales producto de los cambios realizados, los anticuerpos de la presente invención continúan proporcionan afinidad para TSLP humana, tal como se evidencia en la tabla 4 a continuación (Ejemplo 4 que muestra la afinidad de los anticuerpos para TSLP humana y Cyno de la siguiente manera):

TSLP	KD (pM)			
	Anticuerpo 1	Anticuerpo 2	Anticuerpo 3	Anticuerpo 4
humana	141	No determinado	No determinado	172
humana	130	150	128	No determinado
humana	No determinado	No determinado	No determinado	155, 142, 339,170, 153
humana*	No determinado	No determinado	No determinado	339

129

humana*, **	No determinado	No determinado	No determinado	299
cyno	159	No determinado	No determinado	138, 80, 115
cyno	No determinado	No determinado	No determinado	127
cyno **	No determinado	No determinado	No determinado	381

Adicionalmente, las Tablas 5 y 6 en el Ejemplo 5 muestran la capacidad de los diversos anticuerpos anti-TSLP de neutralizar biológicamente TSLP humana y cyno fue evaluada por la aplicación de bioensayos de proliferación a corto plazo que utilizan células las cuales expresan receptores de TSLP humana y cyno recombinante, como se muestra a continuación:

TSLP	EC 50 (µg/ml) Inhibición de Proliferación			
	Anticuerpo 1	Anticuerpo 2	Anticuerpo 3	Anticuerpo 4
humana	0,022	No determinado	No determinado	0,041
humana	0,025	0,092	0,054	No determinado
humana	0,014	No determinado	No determinado	0,024
humana*	0,083	No determinado	No determinado	0,215, 0,137
cyno	0,064	No determinado	No determinado	0,117, 0,077
cyno	0,122	No determinado	No determinado	0,158
cyno	0,054	No determinado	No determinado	0,067

En este orden de ideas, es evidente que la única razón para considerar que la presente invención no es inventiva, es que la SIC no aplicó el método “Problema-solución” y efectuó un análisis retrospectivo, donde a partir de los conocimientos adquiridos al leer la presente solicitud, intenta encontrar en el estado de la técnica fragmentos que demuestren que **teóricamente se podría haber llegado a la invención que aquí se reivindica.**

- c- En conclusión, de acuerdo con la Resolución aquí recurrida, la SIC evidentemente:
- se equivoca al **ignorar los efecto técnicos** que presentan las variantes reivindicadas en la presente solicitud;
 - **realiza un examen post facto o retrospectivo**, donde de manera subjetiva y arbitraria afirma que dado que documento D1 ya divulgaba anticuerpos anti TSLP, era obvio desarrollar los cambios en los aminoácidos específicos;

- no considera los argumentos de la respuesta a la acción oficial, donde se evidencian las diferencias y efectos técnicos inesperados existentes entre los anticuerpos mejorados de la presente invención y los anticuerpos de D1.

Si la SIC hubiera realizado un análisis acertado de las enseñanzas del arte previo y de los argumentos presentados en mi respuesta al primer examen de fondo, habría concluido que una persona medianamente versada en la materia no habría encontrado en D1 la información relevante para desarrollar inequívocamente las modificaciones en los aminoácidos específicos y llegar a los aminoácidos de la presente invención. Una conclusión contraria, como la que realiza la SIC, sólo evidencia un análisis retrospectivo donde no se aplicó o fue incorrecta la aplicación del método problema-solución, pues no se reconoce la existencia de los efectos técnicos inesperados, como se señaló anteriormente.

4. Para terminar, de conformidad con lo establecido en el artículo 59 del Código Contencioso Administrativo, solicito respetuosamente que todos y cada uno de los puntos planteados en el presente recurso sean resueltos de manera individual, completa y motivada, respecto de las razones de hecho, derecho y/o conveniencia que lleven a esa Superintendencia a adoptar la decisión que estime frente a la solicitud aquí formulada.

5. NOTIFICACIÓN

Recibiré notificaciones en mis oficinas ubicadas en la Carrera 5 No. 34-03, de Bogotá.

Atentamente,



CARLOS R. OLARTE

C.C. No. 79.782.747 de Bogotá

T.P. 74.295

ANEXOS:

- ANEXO 1: Josef Vlasak et al., "Identification and Characterization of Asparagine Desamidation in the Light Chain CDR1 of a Humanized IgG1 Antibody," *Analytical Biochemistry* 392, no. 2 (September 15, 2009): 145-54, doi:10.1016/j.ab.2009.05.043.
- ANEXO 2: Lihua Huang et al., "In Vivo Desamidation Characterization of Monoclonal Antibody by LC/MS/MS," *Analytical Chemistry* 77, no. 5 (March 1, 2005): 1432-39, doi:10.1021/ac0494174.

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- ANEXO 3: Kiyotaka Nakano et al., “Generation of a Humanized Anti-Glypican 3 Antibody by CDR Grafting and Stability Optimization,” *Anti-Cancer Drugs* 21, no. 10 (November 2010): 907–16, doi:10.1097/CAD.0b013e32833f5d68.
- ANEXO 4: Zenjiro Sampei et al., “Identification and Multidimensional Optimization of an Asymmetric Bispecific IgG Antibody Mimicking the Function of Factor VIII Cofactor Activity,” *PloS One* 8, no. 2 (2013): e57479, doi:10.1371/journal.pone.0057479.
- ANEXO 5: J Cacia et al., “Isomerization of an Aspartic Acid Residue in the Complementarity-Determining Regions of a Recombinant Antibody to Human IgE: Identification and Effect on Binding Affinity,” *Biochemistry* 35, no. 6 (February 13, 1996): 1897–1903, doi:10.1021/bi951526c.

Identification and characterization of asparagine deamidation in the light chain CDR1 of a humanized IgG1 antibody

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ABSTRACT

Despite technological advances, detection of deamidation in large proteins remains a challenge and the use of orthogonal methods is needed for unequivocal assignment. By a combination of cation-exchange separation, papain digestion, and a panel of mass spectrometry techniques we identified asparagine deamidation in light chain complementarity determining region 1 (CDR1) of a humanized IgG1 monoclonal antibody. The reaction yields both Asp and isoAsp, which were assigned by Edman degradation and by isoAsp detection using protein isoaspartate methyltransferase. The deamidated antibody variants were less potent in antigen binding compared to the nondegraded antibody. Changes in near-UV CD spectra, susceptibility to papain cleavage in an adjacent CDR2 loop, and the tendency of the newly formed isoAsp to undergo isomerization suggest local perturbations in the structure of the isoAsp-containing antibody.

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Deamidation of Asn residues is a common degradation of proteins and it can significantly impact protein structure and function [1–4]. At neutral and basic pH, deamidation proceeds via formation of a five-member ring intermediate—succinimide [5–7]. The reaction is initiated by deprotonation of the peptide bond nitrogen, followed by a nucleophilic attack on the side chain carbonyl, loss of ammonia, and a formation of succinimide. Succinimide is unstable and hydrolyzes into a mixture of two products—isoaspartate (iso-Asp)¹ and Asp (Fig. 1). The ratio between isoAsp and Asp in short peptides is usually 3:1 to 4:1 [5–7]; however, it can be shifted more toward Asp in proteins [8–11], presumably because Asp, as a natural amino acid, can be better accommodated in the protein structure. At acidic pH deamidation occurs as well, but predominantly by direct hydrolysis of the side chain amide, yielding only Asp [7,12]. The rate of deamidation depends on protein sequence and conformation, as well as on external factors such as temperature, pH, and others.

The rate of deamidation as a function of the sequence has been studied in short model peptides and it mainly depends on the amino acid residue following asparagine, Asn-Gly being the most susceptible to deamidation [5,12–15]. Higher order structures usually impose structural constraints, attenuating deamidation rates [8,16–19].

Due to the expanding pharmaceutical use of monoclonal antibodies, assessing fine details of the structure of these large molecules, improving their stability, and understanding of their degradation have received significant attention. Human IgG antibodies consist of two identical heavy and light chains, each assembled from immunoglobulin domains. The heavy chain contains four domains—V_H, C_{H1}, C_{H2}, and C_{H3}. The N-terminal domain is referred to as variable (V_H) due to its different length and sequence among different antibodies. The remaining three domains, referred to as constant (C_H), define individual isotypes (e.g., IgG1). Similarly, the light chain contains one variable domain (V_L) but only one constant domain (C_L). In the folded state, human IgG contain three relatively independent fragments connected via a hinge region—two identical Fab fragments and an Fc fragment. Antigen binding is mediated by the variable domains, mainly by three loops connecting individual β -strands in each domain, called complementarity determining regions (CDR). Depending on the antibody sequence and/or structure, Asn located in the CDRs can undergo deamidation rapidly, presumably due to their relative flexibility and solvent exposure [11,20–22]. In addition, several deamidation-prone Asn residues have been identified in the constant regions of human IgG [19]. Deamidation in the CDRs can be associated with changes in antibody-antigen interactions and therefore represents a

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¹ Abbreviations used: CDR, complementarity determining region; CEX, cation-exchange HPLC; isoAsp, isoaspartate; V_H, variable heavy chain domain; C_H, constant heavy chain domain; ESI-TOF, electrospray-time-of-flight; RP-HPLC, reversed-phase high-performance liquid chromatography; L_C, light chain; F_d, heavy chain V_H/C_{H1} domains; L₁D, laser induced decomposition; LT2, light chain tryptic peptide 2; PTH-AA, phenylthiohydantoin-amino acids; 4-MUP, 4-methylumbelliferyl phosphate; EC₅₀, effective concentration for achieving 50% binding; SAH, S-adenosyl-L-homocysteine; DSC, differential scanning calorimetry; CD, circular dichroism; MS, mass spectrometry; PIMT, protein L-isoaspartyl O-methyltransferase.

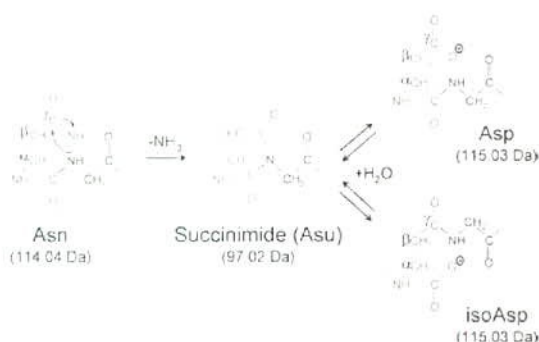


Fig. 1. The mechanism of asparagine deamidation and aspartic acid isomerization via succinimide intermediate. Theoretical monoisotopic masses of Asn, Asp, and isoAsp residues and of the succinimide intermediate are indicated.

serious concern during the development of antibody-based therapeutics [11,20].

Materials and methods

Antibody

The IgG1 monoclonal antibody was manufactured at Merck & Co., Inc., and formulated in a liquid formulation at pH 6.5. Unless stated otherwise, forced degradation studies were carried out in the same formulation.

Cation-exchange HPLC and fraction purification

Cation-exchange HPLC (CEX) was performed using a ProPac WCX-10 4 × 250 mm column (Dionex, Sunnyvale, CA) and a gradient elution of 0–35% B in 50 min at a flow rate of 0.5 ml/min (mobile phase A was 10 mM sodium phosphate, pH 6.5; mobile phase B was 10 mM sodium phosphate, 0.5 M NaCl, pH 6.5). For fraction purification, a larger 9 × 250-mm column was used with 2 ml/min flow rate and a gradient adjusted to fit the different preparation purposes. IgG variants (A1, A2, and M) or the corresponding Fab-fragment variants (FabB, FabD, and FabM) were isolated using CEX. In order to obtain enough material, the degradation was accelerated by incubating the antibody or its Fab fragment at 45 °C. For MS analysis, Fab-fragment variants were isolated from Fab fragments obtained by papain digestion of IgG after forced degradation. To avoid the loss of FabD (isoAsp) due to papain cleavage in LC CDR2, for other analyses, Fab-fragment variants were obtained from a Fab fragment sample after forced degradation.

Papain digestion

Monoclonal antibodies were digested with immobilized papain (Fab Preparation kit, Pierce, Rockford, IL) following the manufacturer's instructions; the digestion was carried out at 37 °C overnight. The Fab and Fc fragments were separated using a Protein A column included in the kit.

LC-ESI-TOF analysis of CEX-isolated Fab fragments

Fab-fragment samples were diluted with water (1/2) and analyzed on a ZORBAX 300SB-C8 reversed-phase column 2.1 × 150 mm, 3.5 μm (Interchim, France), using an Alliance liquid chromatography system coupled to a LCT electrospray-time-of-flight (LCT ESI-TOF) mass spectrometer (Waters, France). Reversed-phase HPLC conditions involved a gradient using two mobile phases.

Solvent A consisted of acidified water (0.05% TFA) and solvent B of *n*-propanol/water (90/10) containing 1% formic acid and 0.02% TFA. The gradient stayed for 19 min at 0% B, increased from 0 to 50% B in 1 min, and was followed by isocratic elution at 50% B during 10 min with a flow rate of 0.25 ml/min. The column temperature was kept at 60 °C and the elution was monitored between 210 and 300 nm. The LCT ESI-TOF was set to run in W positive ion mode with a capillary voltage of 3800 V, sample cone voltage of 150 V, *m/z* range of 900–3000 at 1 s per scan, mass resolution of 10000, and was calibrated using 2 μg/μl solution of cesium iodide in 2-propanol/water solution (50/50). The samples were diluted with water to reach a concentration between 0.5 and 2 mg/ml and 4 to 10 μg were injected. Control Fab (FabCtr) was injected at the beginning and the end of each set of Fab-fragment samples. To avoid cross-contamination a blank run was performed between each sample. The MaxEnt algorithm (Waters, France) was used to determine molecular masses.

LC-ESI-TOF analyses of reduced Fab fragments

Fab-fragment samples (10 μg) in 10 mM sodium phosphate, pH 6.5, + NaCl obtained after CEX purification were lyophilized and solubilized in 30 μl of 100 mM Tris-HCl, 2 mM EDTA, 6 M guanidine HCl, pH 8.0, to a final concentration of 0.33 mg/ml. Samples were flushed with N_2 . Disulfide reduction was performed by incubating Fab solution with 20 mM DTT for 2 h at 37 °C. The reaction was quenched by adding acetic acid (final concentration 10%) and *n*-propanol (final concentration 20%). The resulting light chains (LC) and heavy-chain fragments (Fd or V_HC_{H1}) were then separated on a ZORBAX 300SB-C8 reversed-phase column, 2.1 × 150 mm, 3.5 μm (Interchim, France). Reversed-phase HPLC conditions involved gradient using two mobile phases. Solvent A consisted of acidified water (0.05% TFA) and solvent B of *n*-propanol/water (90/10) containing 1% formic acid and 0.02% TFA. The gradient was increased from 2 to 35% B in 30 min, from 35 to 80% in 1 min, followed by isocratic elution at 80% B for 4 min with a flow rate of 0.25 ml/min. Column temperature was 60 °C and the elution was monitored between 210 and 300 nm. The LCT ESI-TOF was set to run in W positive ion mode with a capillary voltage of 3400 V, sample cone voltage of 150 V, *m/z* range of 900–2500 at 1 s per scan, mass resolution of 10000, and was calibrated using 2 μg/μl solution of cesium iodide. Cesium iodide solution (0.1 μg/μl) solubilized in acetonitrile/water (50/50) was introduced into the lockspray electrospray unit and the ion at *m/z* = 1172.1837 was used to perform mass correction. The reduced samples were diluted with water to the half and an aliquot of 2.5 μg was injected at each run. Reduced control Fab samples were injected upfront and at the end of each series of analyses.

Reduction and alkylation of CEX-isolated Fab fragments for identification of the deamidation site

The Fab samples (300 μg) in 10 mM sodium phosphate, pH 6.5, and NaCl obtained after CEX purification were lyophilized and solubilized in 200 μl 100 mM Tris-HCl, 2 mM EDTA, 6 M guanidine HCl, pH 8.0, buffer to a final concentration of 1.5 mg/ml. Samples were flushed with N_2 . Disulfide reduction was performed by incubating Fab solutions with 20 mM DTT (final concentration) for 2 h at 37 °C. Iodoacetamide was then added to a final concentration of 25 mM and the reaction was allowed to proceed in the dark for 1 h at room temperature. The reaction was quenched by the addition of acetic acid (final concentration 10%) and *n*-propanol (final concentration 20%).

Light chain (LC) and heavy chain V_HC_{H1} domains (Fd) separation

Alkylated LC and Fd fragment were separated on a ZORBAX 300SB-C8 reversed-phase column 4.6 × 150 mm, 3.5 μm (Inter-

chim, France). RP-HPLC conditions involved gradient using two mobile phases. Solvent A consisted of acidified water (0.05% TFA) and solvent B was *n*-propanol/water (90/10) containing 0.1% formic acid. The gradient was increased from 20 to 35% B in 30 min, from 35 to 80% in 1 min, and was followed by isocratic elution at 80% B for 5 min with a flow rate of 1 ml/min. The column temperature was 60 °C and the elution was monitored between 210 to 300 nm. Fd and LC fractions were collected using UV detection and then freeze-dried.

LC-ESI-TOF analyses of trypsin-digested light chains

Reduced and alkylated LC (~60 µg) were solubilized with 50 µl of water and sonicated. Then 200 µl of 50 mM Tris/Tris-HCl, 1 mM CaCl₂, pH 7.5, buffer; 25 µl of acetonitrile, and 10 µl trypsin in water solution (0.5 µg/µl, enzyme/substrate ratio of 1/12 w/w) were added. Samples were incubated at 37 °C for 7 h. The reaction was quenched by adding 0.5 µl of TFA. LC-ESI-MS analyses of peptide mixtures obtained by enzymatic digestion were carried out using an Alliance system coupled to a LCT ESI-TOF mass spectrometer. RP-HPLC conditions involved gradient using two mobile phases. Solvent A consisted of acidified water (0.1% TFA) and solvent B was acetonitrile containing 0.08% TFA. The gradient stayed for 5 min at 0% B, and then increased from 0 to 60% B in 60 min, from 60 to 80% in 5 min, followed by isocratic elution at 80% B for 5 min. Peptides were separated on a reversed-phase Xbridge BEH 300 C18, 3.5 µm, 2.1 × 150 mm (Waters, France) with a flow rate of 0.25 ml/min and were detected at 214 nm (extracted from a range of 200–300 nm) by a Waters 996 photodiode array detector. The LCT ESI-TOF was set to run in W positive ion mode with a capillary voltage of 1000 V, sample cone voltage of 35 V, *m/z* range of 200–2000 at 1 s per scan, mass resolution of 10000. The mass spectrometer was calibrated with a 0.005% solution of H₃PO₄ in water/acetonitrile (50/50). (Glu)-Fibrinopeptide B, human solution (500 fmol/µl) solubilized in acetonitrile/water (50/50) with 0.1% formic acid, was introduced into the lockspray electrospray unit. The ion at *m/z* 785.8421 was used to perform mass correction.

MALDI-TOF-TOF analysis of peptides obtained from trypsin-digested light chains

Samples were prepared by standard dried-droplet preparation on stainless-steel MALDI, target-cyano-4-hydroxycinnamic acid as matrix. MS and Ultraflex-MS-MS spectra were acquired with a Bruker Ultraflex TOF/TOF with gridless ion optics under control of Flexcontrol 2.0. This instrument, equipped with the Scout (Bruker Bremen, Germany) high-resolution optics with X-Y multisample probe and gridless reflector, was used at a maximum accelerating potential of 20 kV and was operated in the reflector mode. Ionization was accomplished with a 337 nm beam from a nitrogen laser with a repetition rate of 3 Hz. The output signal from the detector was digitized at a sampling rate of 2 GHz. External calibration of MALDI mass spectra was carried out using singly charged monoisotopic peaks of a mixture of human angiotensin II (*m/z* 1046.542), substance P (*m/z* 1349.643), bombesin (*m/z* 1619.823), and ACTH 18–39 (*m/z* 2465.199). Monoisotopic peptide masses were automatically annotated using the XMASS 5.1.2 NT software package. Collision cell and the Ultraflex device providing the second ion source was accelerated to 7 kV and the postacceleration after Ultraflex was 19 kV. In the standard mode, the Ultraflex TOF/TOF uses laser-induced decomposition (LID) for the generation of MS-MS fragments. Their masses were analyzed after ion reflector passage. Ultraflex data were processed using the *de novo* analysis module of Biotools protein sequence analysis package (Version 2.2).

EDMAN degradation

LT2 peptides were isolated using UV detection and the collected peptide solutions were diluted with water in order to keep an acetonitrile concentration below 15%. The N-terminal sequences of purified peptides were determined by automatic EDMAN degradation on an Applied Biosystems cLC Procise microsequencer. The PVDF membrane (ProSorb Cartridge) was wetted with 10 µl of methanol. LT2 peptide solution was added on the PVDF membrane and dried. Phenylthiohydantoin-amino acids (PTH-AA) were identified by chromatography on a C18 column (PTH C-18, 0.8 × 200 mm) at 269 nm.

Determination of the binding activity

The binding activity was determined by ELISA. Plates were coated with the antigen, blocked, and then incubated with a series of dilutions of each sample. Antigen-antibody complexes were detected with monoclonal mouse anti-human IgG Fc fragment antibody (clone H2) conjugated with alkaline phosphatase (Southern Biotechnology Associates Inc., Birmingham, AL), using a fluorogenic substrate SuperPhos, 4-methylumbelliferyl phosphate (4-MUP) (Virolabs, Chantilly, VA). The fluorescence was excited at 355 nm and detected at 460 nm. Four-parameter logistic fit was applied to each dilution series and the effective concentration for achieving 50% binding (EC50) was obtained by comparison of the fitted curve with a –70 °C standard.

ISOQUANT

Samples were diluted 1:1 into RapiGest (Waters Corporation, Milford, MA). DTT was added to a final concentration of 1 mM, and the solutions were incubated for 5 min at 100 °C. The samples were then digested by Trypsin (Sigma, Saint Louis, MO), 1:20 (w/w Trypsin/Fab) at 37 °C for 2 h. The amount of isoAsp was measured using the ISOQUANT isoaspartate detection kit (Promega, Madison, WI). The detection of 5'-adenosyl-L-homocysteine (SAH) was achieved by reversed-phase HPLC using Thermo Electron-Hypersil BetaBasic-C18 column, 150 × 4.6 mm, particle size 3 µm (Thermo Scientific, Waltham, MA); detection was at 260 nm; mobile phase A: 3% acetonitrile in 0.01 M potassium phosphate pH 6.2; mobile phase B: 100% acetonitrile; gradient: 0–30% B in 5 min. The isoAsp content was calculated by comparing the area of the SAH peak in the sample with a standard curve generated from a dilution series of SAH standard [23].

Differential scanning calorimetry (DSC)

DSC measurements were performed on a VP-Capillary DSC Platform (MicroCal, LLC, Northampton, MA) at 0.35 mg/ml protein concentration and scan rates of 0.25 and 0.5 °C/min. The protein was in 10 mM phosphate buffer, pH 6.5. DSC profiles were calculated using the Origin 7.0 software. Buffer background was subtracted and thermograms were normalized to the molar concentration of the protein. The final excess heat capacity thermogram was obtained by interpolating a cubic baseline in the transition region. The thermograms were analyzed using the equations for a "two-state irreversible model" described in Kurganov et al. [24] and the software TableCurve 2D (Systat Software, Inc.).

Circular dichroism (CD) spectroscopy

CD measurements were performed on a Jasco-810 spectropolarimeter. The near-UV CD of samples in 10 mM phosphate, pH 6.5, and protein concn 1 mg/ml was measured at 20 °C using 1-cm cells

and the following data acquisition parameters: bandwidth 2 nm, response time 4 s, speed 10 nm/min, accumulation 5.

Results and discussion

Cation-exchange HPLC resolves acidic variants A1 and A2

Following storage at elevated temperatures the humanized IgG1 monoclonal antibody described in this report shows significant heterogeneity detected by CEX (Fig. 2A). The chromatogram consists of three fractions designated A1, A2, and M; each containing additional heterogeneity. Basic peaks in each fraction represent lysine variants of the antibody (labeled as K and KK in Fig. 2A). The acidic peaks were attributed to changes in the Fc fragment and will be discussed elsewhere. Here we describe the identification of the degradation responsible for the formation of A1 and A2 and also present characterization of these two degradation variants.

To aid the identification of the degradation, we and others [11,25] have used papain digestion to generate Fab and Fc fragments. The Fab fragment of A1 contains two CEX peaks of equal intensity—a nondegraded Fab peak, FabM, and an acidic peak FabB (Fig. 2A, inset). In the case of the Fab fragment prepared from A2, a more complex chromatogram was observed due to unexpected pa-

pain cleavages in the V_L domain (discussed later in the paper). However, not considering papain digestion artifacts, the Fab fragment from the A2 fraction would contain (similarly to A1) a nondegraded Fab peak, FabM, and an acidic peak FabD. Despite the significant heterogeneity of the M fraction in the stressed sample (Fig. 2A), the Fab fragment of the M fraction contains only one peak (FabM) corresponding to the nondegraded molecule (not shown). This observation together with the fact that Fab fragments exposed to elevated temperatures show formation of homogeneous degradation peaks FabB and FabD (Fig. 2B) suggests that the heterogeneity within A1, A2, and M is located in the Fc fragment.

The presence of two Fab peaks in A1 and A2 (one degraded and one intact) in a 1:1 ratio shows that only one Fab is degraded in the whole IgG, which is in agreement with the kinetics of degradation (Ionescu and Vlasak, manuscript in preparation). Since FabB and FabD are homogeneous fractions (lacking the nondegraded Fab and also the Fc part) compared to their A1 and A2 counterparts, they are more amenable to experimentation and therefore the identification of the chemical nature of this degradation and some characterization experiments were carried out using Fab fragments. To obtain sufficient amounts of FabB and FabD, the degradation was accelerated by incubating the IgG at 45 °C. Fab fragments were then prepared from the IgG by papain digestion. Alternatively, due to the unexpected papain cleavage of the A2 variant, a Fab fragment was first prepared from a fresh material and then subjected to forced degradation at 45 °C, which led to accumulation of FabB and FabD (Fig. 2B).

Mass spectrometry analyses of intact and reduced FabB and FabD suggest deamidation in the light chain

CEX-isolated Fab fragments were sequentially analyzed by LC-MS following “top-down/bottom-up” strategy [26–28]. First, LC-ESI-TOF mass spectrum was recorded for FabB, FabD, and FabM isolated from a sample stored for approximately 1 month at 45 °C (Fig. 2A), as well as for a Fab fragment prepared from a non-stressed material (FabCtr). Then, the Fab fragments were reduced and alkylated and analyzed by LC-ESI-TOF mass spectrometry (Supplementary Fig. 1). The experimental mass of FabCtr (48096 Da) was in a good agreement with the calculated mass (48096 Da). As expected, after reduction and alkylation two fragments were generated and well separated by RP-HPLC. They were identified by online MS as the light chain (LC) (calc. mass = 24063.0 Da, exp. mass = 24063.0 Da) and the heavy chain V_HC_H1 domain (also referred to as the Fd fragment, calc. mass = 24043.0 Da, exp. mass = 24043.0 Da), respectively. As anticipated, similar profiles and masses were observed for FabM from the stressed sample (exp. mass = 48096 Da, LC exp. mass = 24063.2 Da, Fd exp. mass = 24042.7 Da). For FabB, a mass increase of 1 Da was observed (48097 Da) and also recorded for the corresponding LC (24064.0 Da), indicating a possible deamidation in the LC. The mass of the FabB Fd fragment had the expected value (24043.0 Da). For FabD, a 0.8 Da increase in mass was detected for the LC, but not for the whole Fab (48096 Da) or the Fd fragment (24042.8 Da). Considering that the precision of mass measurements in the present study was 20 ppm, a mass difference of 1 Da could be detected with a good confidence for a 25 kDa protein (e.g., LC or Fd fragment), but not for a 50-kDa protein like the Fab fragment. Based on the MS data obtained for LC and Fd from CEX-isolated Fab-fragment variants, a bottom-up approach was performed by peptide mapping of the LC.

Identification of deamidation in light chain Asn33

Light chains of different Fab-fragment isolates (FabCtr, FabB, FabD, and FabM) were separated from their Fd fragments by

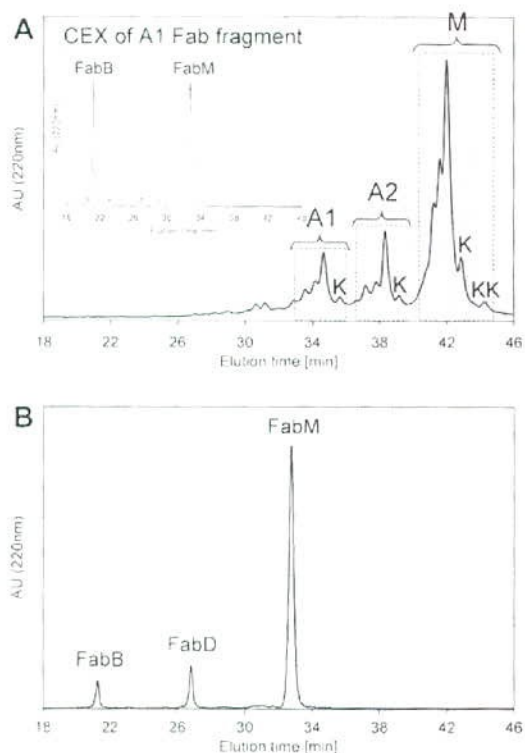


Fig. 2. (A) CEX profile of a stressed antibody and the Fab fragment of the A1 acidic variant (inset). The antibody was stored for approximately 1 month at 45 °C. Two acidic fractions A1 and A2 with significant CEX separation from the nondegraded fraction M are indicated in gray boxes. Each of the three fractions (A1, A2, and M) has corresponding lysine variants (K and KK), due to incomplete heavy chain C-terminal Lys removal (determined by carboxypeptidase B treatment as previously described [38]) and additional acidic variants due to degradation in the Fc fragment. Due to the low signal of A1 and A2, their KK variant is not apparent. Inset: CEX of Fab fragments prepared from purified A1 fraction by papain digestion. FabM, the nondegraded Fab fragment; FabB, Fab fragment containing the degradation. (B) CEX profile of a stressed Fab fragment. The Fab fragment was exposed to the same conditions as the IgG in panel A. Note the homogeneity of peaks FabB and FabD compared to IgG fractions A1 and A2 due to absence of the Fc-related heterogeneity.

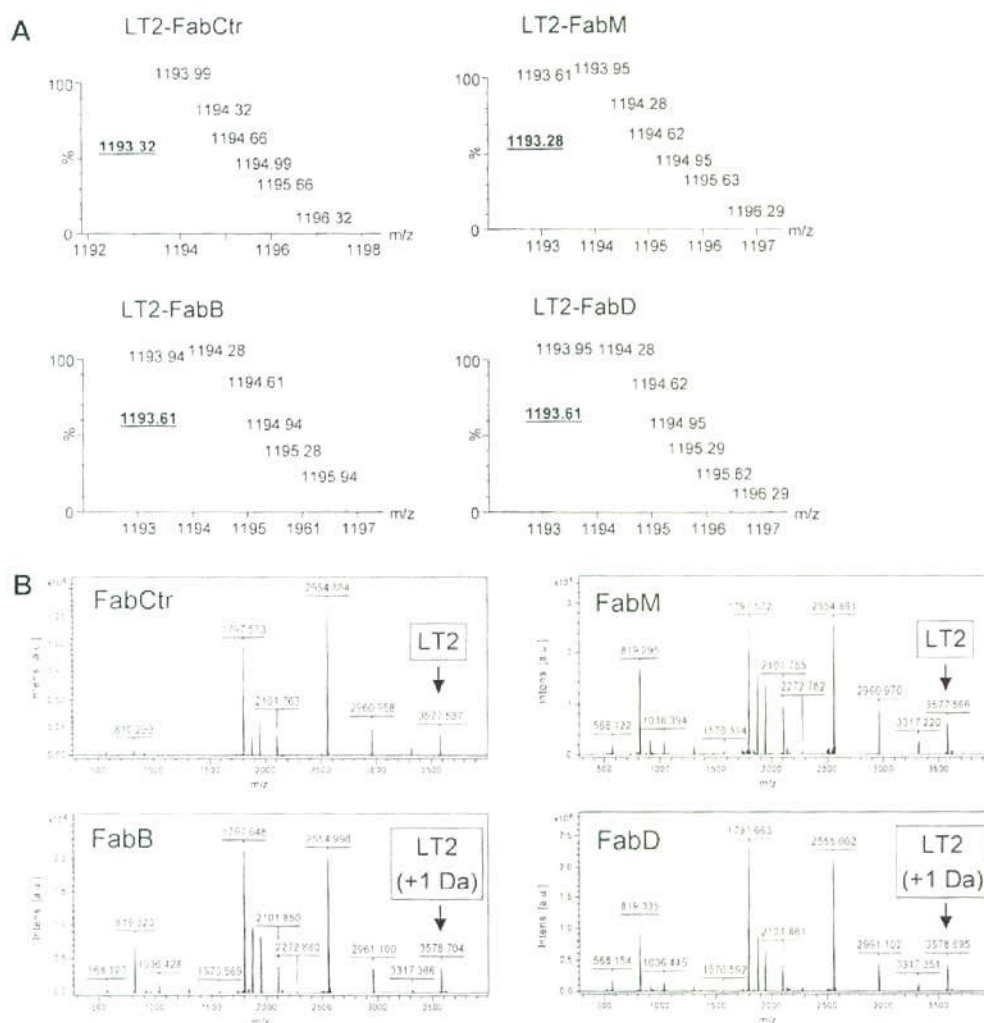


Fig. 3. (A) ESI-MS isotopic distributions $[M+3H]^+$ of LT2 peptides. (B) MALDI-MS analysis of trypsin digested LC from FabCtr, FabB, FabD, and FabM.

RP-HPLC and then subjected to digestion with trypsin to confirm and localize the presumed deamidation. The resulting peptide maps were analyzed by two complementary MS methods, LC-ESI-TOF and MALDI-TOF (Fig. 3). By LC-ESI-TOF, no detectable changes in retention times were observed in the RP-HPLC peptide maps of the LC (Supplementary Fig. 2) but approximately a 1 Da mass increase was observed for the second tryptic peptide (LT2) derived from both FabB and FabD (+0.3 Da for $M+3H^+$) compared to the calculated isotopic profile and to LT2 from FabCtr or FabM (Fig. 3A). For all four Fab fragments, the remaining peptides showed masses corresponding to their theoretical values, indicating that the only degradation that occurred in LC was deamidation in the LT2 peptide. Similarly, by MALDI-TOF (Fig. 3B) a mass increase of approximately 1 Da was observed for the LT2 peptide of FabB (3578.70 Da, $M+H^+$) and FabD (3578.69 Da) compared to FabCtr (3577.54 Da), FabM (3577.57 Da), and to the calculated mass (3577.84 Da), confirming the LC-ESI-TOF data. LC-ESI-TOF peptide mapping was also carried out with isolated Fd fragments. In agreement with the intact-mass measurements (above), no differences were observed in the Fd-fragment maps from FabCtr, FabB, FabD, and FabM; both in the retention times of the individual peptides and in their isotopic envelopes (not shown).

LT2 (residues 25–55) is a 31-amino acid peptide with two Asn located at positions 33 and 35 (...N₃₃GN₃₅T...). For each trypsin digestion sample (FabCtr, FabM, FabB, and FabD), LT2 peptides were identified and fragmented by MALDI-TOF/TOF (not shown). The results showed that deamidation occurred only at position 33 and not at position 35. Asn35 deamidation was only observed under harsh sample preparation conditions (not shown), which confirmed that our MS analytical conditions were able to detect this deamidation. In summary, MS measurements showed that both FabB and FabD and thus their corresponding IgG acidic variants A1 and A2, respectively, have been formed by deamidation of LC-Asn33, which is located in the first complementarity determining region (CDR1). CDR localization and the fact that Asn33 is followed by a glycine support our observation that this Asn is a deamidation hotspot.

Several reports described Asn deamidation in CDRs of monoclonal antibodies [11,20–22]. The occurrence of deamidation (and other degradations as well) in CDRs is presumed to be related to their greater flexibility and solvent exposure compared to other regions of the antibody. Antibodies generally differ in the sequence of their CDRs, which determines their antigen specificity. However, the same CDR motifs can be found in many antibodies, mainly

those derived from the same germline gene. This is particularly true for CDR1 and CDR2, which, unlike CDR3, are coded in the germline. Indeed, the CDR1 Asn-Gly-Asn-Thr motif (found in murine germline genes of the VK1 and VK2 families) is present in other antibodies. In nimotuzumab, no susceptibility to deamidation was noted [29]. In contrast, a hybridoma-expressed murine monoclonal antibody with similar CDR1 was found to contain only Asp33, presumably due to deamidation induced during production [30]. It is therefore plausible that the mouse-germline derived Asn-Gly-Asn-Thr CDR1 motif could be a deamidation hotspot in other antibodies as well.

Detection of isoAsp33 in FabD by EDMAN degradation and ISOQUANT

Considering that FabB and FabD are resolved by CEX yet they contain deamidation of the same residue, LC-Asn33, it is likely that they represent the two deamidation products—Asp and isoAsp. Separation of Asp and isoAsp antibody variants by CEX has been shown for other antibodies [11]. In order to discriminate between Asp33 or isoAsp33, and as another means to confirm the deamidation, LT2 peptide was isolated by RP-HPLC from the tryptic digest of the reduced/alkylated LC of each Fab sample (FabCtr, FabB, FabD, and FabM). Isolated peptides were applied onto a PVDF membrane and subjected to microsequencing by EDMAN degradation for 14 cycles (upfront microsequencing analysis, PTH-AA standards were injected). The expected native sequence was observed for the first 14 amino acids of the LT2 peptide obtained from FabCtr and FabM. For the LT2 peptide of FabB, Asp was found at position 33 instead of Asn and the presence of an Asn at position 35 was confirmed. For FabD LT2 peptide, the EDMAN degradation was stopped after amino acid at position 32. Based on the MS/MS data for this peptide and to explain differences observed in CEX retention of FabB and FabD, this was expected and consistent with an isoAsp residue at position 33.

Additional evidence for the presence of LC-isoAsp33 in FabD was obtained by measuring the overall isoAsp content in FabB and FabD using the ISOQUANT kit, which utilizes the ability of protein *L*-isoaspartyl *O*-methyltransferase (PIMT) to selectively recognize isoAsp residues in proteins. Similar levels of isoAsp (mol isoAsp/mol protein) were found in FabB (0.53) and FabM (0.37). In contrast, FabD (1.25) contained about 1 mol more than FabB or FabM, confirming that FabD is the isoAsp and FabB is the Asp product of LC-Asn33 deamidation.

Changes in antigen binding of A1 and A2

To assess the impact of LC-Asn33 deamidation on antibody function, the binding activity (measured as relative EC₅₀) of IgG acidic variants A1 and A2 was determined using an ELISA assay. A1 (LC-Asp33/Asn33), A2 (LC-isoAsp33/Asn33), and M (LC-Asn33/Asn33) were purified from a sample stored for 3 months at 37 °C. This sample had reduced binding to 76% (±3.2) compared to an un-stressed control. Both A1 and A2 variants had low activities—39% (±7.2) and 60% (±3.3), respectively (standard deviation in parenthesis is derived from two independent experiments). The M fraction from the stressed sample was almost fully active (96%, ±1.8), despite only 76% activity of the whole stressed sample. Together, these results demonstrate that deamidation variants A1 and A2 are less potent in antigen binding and that the loss of binding activity following temperature stress is caused by the increase in A1 and A2 concentration. Since the activity was determined using IgG variants A1 and A2, which still contain one nondegraded Fab fragment, it is not clear whether the deamidation completely abolished binding or just reduced affinity.

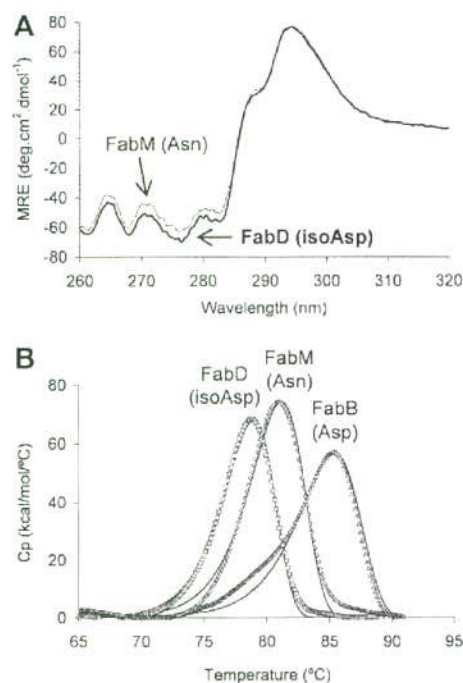


Fig. 4. (A) Near-UV CD spectra of FabM (LC-Asn33) and FabD (LC-isoAsp33). (B) DSC thermograms. Buffer-corrected heat capacity normalized to protein concentration is shown as symbols. Experimental data were fitted using nonlinear least-square fitting (TableCurve 2D software, version 5.01) to the two-state irreversible model (lines) using equation described by [24]. The samples were in 10 mM phosphate, pH 6.5, scan rate of 0.5 °C/min, concentration 0.35 mg/ml.

Circular dichroism spectroscopy

The impact of LC-Asn33 deamidation on the antibody secondary and tertiary structure was evaluated by far-UV and near-UV CD, respectively. No differences between the native Fab fragment and its LC-Asp33 and LC-isoAsp33 variants were observed in far-UV CD spectra, indicating that these variants have comparable secondary structures. Near-UV CD spectra (Fig. 4A) showed subtle but reproducible decrease in ellipticity (in the 260–280 nm region) of the isoAsp33-containing Fab compared to the native Fab (sufficient amount of FabB (LC-Asp33) was not available for the measurement). The near-UV CD spectrum is affected by the microenvironment around aromatic residues and disulfides and is sensitive to the tertiary structure of the protein. Therefore, the observed changes in the near-UV CD spectrum suggest alterations of the tertiary structure on replacement of LC-Asn33 with isoAsp.

Differential scanning calorimetry

The thermal stability of the Fab fragment and its two deamidation products was evaluated by DSC. One transition corresponding to the unfolding of the Fab fragment was observed in all samples (Fig. 4B). FabB (LC-Asp33) had an additional shoulder in front of the main transition, suggesting changes in the unfolding cooperativity of the four immunoglobulin domains in the Fab fragment. The apparent melting temperatures (in 10 mM phosphate at pH 6.5, 0.5 °C/min scan rate) for FabB, FabM, and FabD were 85.3, 80.8, and 78.8 °C, respectively. A similar trend of decreasing apparent melting temperature in the order FabB–FabM–FabD was observed under different conditions: different buffers (phosphate or histidine), with and without 2 M GuHCl, and at two scan rates 0.25 and 0.5 °C/min (only 0.5 °C/min was tested for FabB due to a

limited amount of material). The DSC profiles were highly reproducible; in duplicate scans the variability between T_m was less than 0.1 °C. Comparable results were obtained for the corresponding IgG variants A1, A2, and M, although the differences were less pronounced, presumably due to averaging between the degraded and the intact Fab in each IgG molecule.

Overall, DSC measurements showed nonnegligible and highly reproducible differences in thermal unfolding between the intact Fab fragment and both of its LC-Asn33 deamidation variants. Due to the irreversibility of Fab-fragment unfolding, equilibrium thermodynamics analysis cannot be performed. Instead, a quantitative comparison can be obtained from models which take into account kinetic processes such as aggregation. For many proteins irreversible denaturation monitored by DSC can be described by a relatively simple "two-state irreversible model" [24,31–33], where the native protein (N) undergoes irreversible denaturation to a final product (D) with a first-order rate constant (k) that changes with temperature according to the Arrhenius equation. This model is in reasonably good agreement with our experimental data (Fig. 4B). Small deviations from the model (as reflected by differences between the experimental and the calculated thermograms) and the dependence of the activation energy (E_a) on the scan rate suggest that the unfolding does not strictly follow the one-step irreversible model and that additional steps are involved [24]. Parameters derived from the fitting show differences between the three variants in both kinetic (represented by T^* , the temperature where the rate constant of denaturation equals 1 min^{-1}) and energetic (represented by E_a , the activation energy of unfolding) factors of irreversible denaturation. At the same scan rate (0.5 °C/min), there is a systematic decrease in E_a from FabD–FabM–FabB (121–115–104) and a systematic increase in T^* (354.7–357.2–362.2). These trends show that both the activation energy of unfolding and the aggregation properties are affected by deamidation and that formation of Asp (FabB) or isoAsp (FabD) has the opposite impact on both E_a or T^* relative to the unaltered protein (FabM).

Limited proteolysis with papain

In addition to the two deamidation products FabB and FabD, two other acidic peaks (FabA and FabC) were observed by CEX in Fab fragments prepared from the IgG by papain digestion (not shown). Examination of Fab fragments from IgG fractions A1, A2, and M revealed that these two peaks originated only from the A2 fraction, i.e., the LC-isoAsp33-containing variant. FabA and FabC were identified by mass spectrometry as LC-Asn33 deamidation variants with additional fragmentations in the LC (Supplementary Figs. 3 and 4). FabA LC was clipped at Ser57-Asn58 and Tyr61-Gly62 bonds (located in CDR2 loop), while the LC of FabC was

clipped at the Tyr61-Gly62 bond only. The clipping was not detected in an undigested antibody, and, when a Fab fragment was subjected to temperature stress, accumulation of FabB and FabD but not FabA or FabC was observed (Fig. 2B). These results suggested that FabA and FabC were introduced artificially during papain digestion. Further confirmation was obtained by direct digestion of FabB, FabD, and FabM. Following incubation with papain, FabB (Asp) and FabM (Asn) remained mostly unchanged; however, the intensity of FabD (isoAsp) decreased significantly and two new peaks—FabA and FabC appeared in the sample (Fig. 5). The susceptibility of the LC CDR1 isoAsp33 variant to cleavage in the adjacent CDR2 loop suggests differences in the local conformation compared to the native or the LC-Asp33-containing antibody.

Evidence for isomerization of LC-isoAsp33 to Asp33

Following the time course of the degradation, the ratio between A2/FabD (isoAsp) and A1/FabB (Asp) was gradually changing in favor of A1/FabB, suggesting that A2/FabD (isoAsp33) was undergoing isomerization. To obtain more direct evidence for the isomerization, purified FabB, FabD, and FabM were set at accelerated stability at 45 °C (Fig. 6). As anticipated, deamidation of FabM led to the formation of FabB and FabD. FabB (LC-Asp33) was relatively stable showing only a small increase of more acidic species of unknown nature. In contrast, FabD (LC-isoAsp33) was converting rapidly into a peak with the same retention time as FabB, and it also showed additional degradations represented by a number of small, more acidic, peaks. Further evidence for isomerization was obtained by measuring isoAsp content (using the ISOQUANT kit) in FabB, FabD, and FabM following storage at 45 °C. At time zero, FabB, FabD, and FabM contained 0.53, 1.25, and 0.37 mol isoAsp/mol of antibody, respectively. After 3 months at 45 °C (Fig. 6) the level of isoAsp changed to 0.91, 1.26, and 0.97, respectively; i.e., in FabB and FabM it increased by approximately 0.5 mol isoAsp/mol of antibody but it remained the same for FabD. We interpret this observation as follows—during the extended storage at 45 °C, degradations elsewhere in the molecule lead to accumulation of isoAsp in all three Fab fragments; however, the increase of isoAsp in FabD is compensated by a loss of isoAsp33 due to isomerization. Interestingly, no conversion of FabB (Asp) into FabD (isoAsp) was observed. This implies that the isomerization is practically unidirectional and that at equilibrium Asp would be the only product.

pH dependence of LC-Asn33 deamidation

To assess the impact of pH on LC-Asn33 deamidation, the antibody was formulated in 100 mM sodium phosphate (with varying

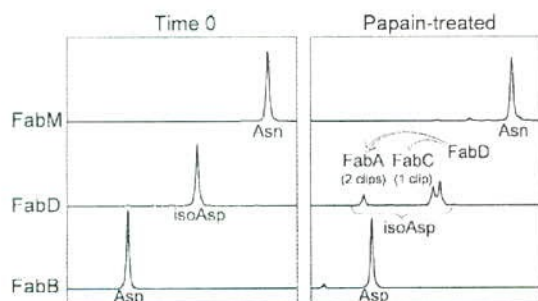


Fig. 5. Limited proteolysis with papain. CEX of FabM (LC-Asn33), FabD (LC-isoAsp33), and FabB (LC-Asp33) (left panel); after incubation with papain (right panel). FabB, FabD, and FabM were obtained by CEX from a Fab fragment stored for 39 days at 45 °C.

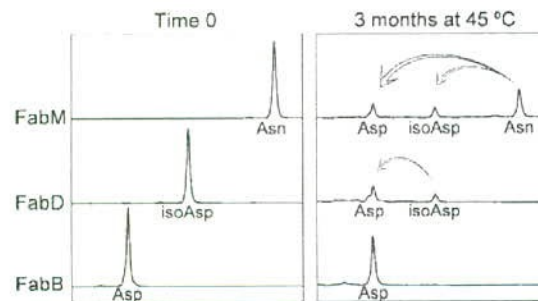


Fig. 6. Isomerization of LC-isoAsp33. CEX of FabM (LC-Asn33), FabD (LC-isoAsp33), and FabB (LC-Asp33) (left panel); after incubation at 45 °C for 3 months (right panel). FabB, FabD, and FabM in the left panel were obtained by CEX from a Fab fragment stored for 39 days at 45 °C.

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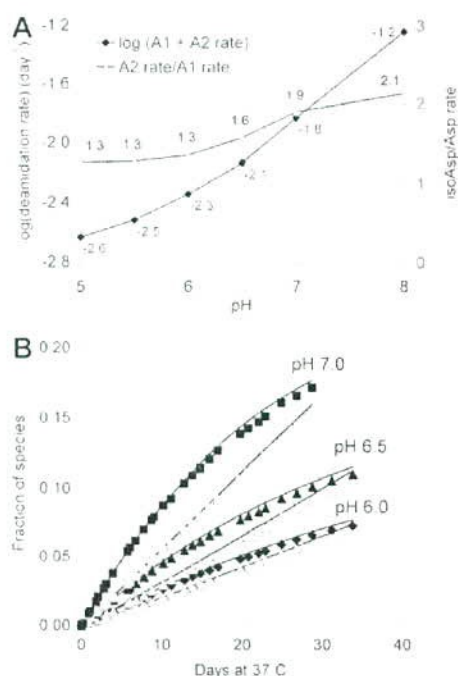


Fig. 7. (A) The pH dependence of deamidation rates and of the A2/A1 rates ratio. (B) The time course of degradation at pH 6.0, 6.5, and 7.0. Filled symbols, A2 (isoAsp33-containing IgG variant); open symbols, A1 (Asp33-containing IgG variant); squares, pH 7.0; triangles, pH 6.5; diamonds, pH 6.0. Solid lines represent fitting results using a model describing first-order accumulation of A1 and A2, and also considering A2-to-A1 isomerization [Ionescu and Vlasak, manuscript in preparation]. The isomerization rate was set to a constant value for all conditions.

pH) and stored at 37 °C. The accumulation of A1 and A2 was monitored by CEX. The reaction rate was minimal at the lowest pH tested—pH 5 (below pH 5 the level of clipping and aggregation hindered the analysis) and significantly accelerated with increasing pH, as observed for deamidation in model peptides [6,7] and other proteins [34] (Fig. 7A). At 37 °C, the half-life was about 2 weeks at pH 8 and almost 1 year at pH 5. To determine whether the observed pH dependence was due to intrinsic properties of deamidation (i.e., base-catalyzed reaction) or whether it was also a contribution of changes in protein structure, we measured near-UV and far-UV CD spectra for antibodies formulated in the tested buffers (Supplementary Fig. 5). No differences were seen by far-UV CD, indicating that the secondary structure remained the same across the pH range. Near-UV CD spectrum was slightly different for the sample formulated at pH 8 compared to the other samples, suggesting subtle changes in protein tertiary structure at pH 8. In addition, DSC measurements with the protein in different buffers presented a shift to higher values of the apparent melting temperatures as the pH was increased (data not shown). Therefore, although protein stability is slightly impacted across the pH range studied, this is not accompanied by significant changes in protein structure and this data suggest that the pH dependence of LC-Asn33 deamidation is likely due to intrinsic properties of deamidation reaction and not due to variations in protein conformation.

In addition to the significant impact on deamidation rate, pH also slightly affected the ratio of A2 (isoAsp) and A1 (Asp) rates (Fig. 7A). At pH 8 the ratio between A2/A1 rates was 2.1, gradually decreasing with pH and reaching a value of 1.3 at pH 5. Similar pH dependence of the ratio of isoAsp/Asp has been observed in peptides [6,7].

Examples of experimental data for pH 6.0, 6.5, and 7.0 (Fig. 7B) illustrate the impact of pH on A2 and A1 rates, on their ratio, and also

show the existence of A2 (isoAsp)-to-A1 (Asp) isomerization. The slope of the two curves (A1 and A2) is steeper at higher pH due to higher deamidation rates. At higher pH, the two curves (A1 and A2) initially move away from each other at a larger angle than at lower pH, due to a higher rate of A2 formation compared to the A1 rate. As the degradation proceeds, the curves come again closer to each other (and will eventually cross) as a result of A2-to-A1 isomerization. The position of the crossover point depends on the difference between A1 and A2 rates, which drives the divergence of the curves and, at the same time, on the A2-to-A1 isomerization rate, which brings them together. Accurate determination of the isomerization rate is difficult because isomerization becomes an important factor only after significant levels of A2 had accumulated. At that stage of degradation, other, relatively slower degradations could become significant, preventing reliable quantification of the isomerization rate. For the modeling presented in Fig. 7B, the isomerization rate was set to a constant value (0.013 days⁻¹) for all conditions. The good agreement between the observed and the predicted values of A1 and A2 fractions suggests that the isomerization rate is relatively constant across the pH range studied. Data analysis using the isomerization rate as an additional parameter did not improve significantly the goodness of the fit, but reduced the precision in retrieving the A1 and A2 rates.

In the case of rhuMab HER2 [11], deamidation of light chain Asn30 to Asp30 (isoAsp30 was not detected) was observed at pH 7.2–7.4, but not at pH 5. Interestingly, although Asn30 was found stable at low pH, the population of Asp30 was depleted in time at pH 5 presumably by formation of succinimide. For the antibody presented in this paper, we did not observe so different outcomes of the degradation of LC-Asn33 at low and high pH. Although the rate of deamidation was significantly slower as pH was decreased, the formation of both Asp33 and isoAsp33 was observed across the entire pH range studied.

Summary of the impact of LC isoAsp33 on the antibody molecule

Deamidation of Asn33 located in LC CDR1 region was detected as acidic charge variants by CEX. The separation of Asp and iso-

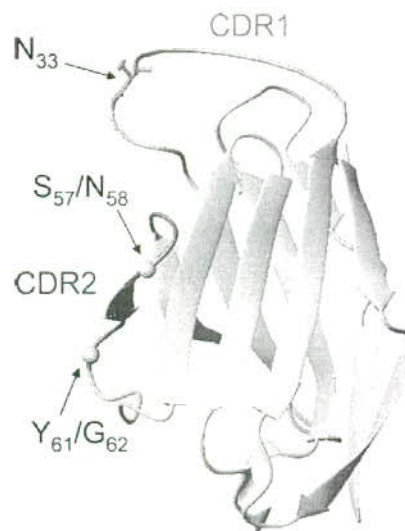


Fig. 8. The crystal structure of a VL domain (pdb file 1CLY). 1CLY LC has significant sequence homology with the LC of the antibody examined in this report. CDR1 loop is indicated in red; Asn33 is shown in green. CDR2 is indicated in blue; the two cleavage sites in CDR2 are shown as yellow balls. The numbering and the residues shown are those of the investigated antibody and not of 1CLY. This figure was generated using the program MOLMOL [39].

Asp-bearing antibodies, which have identical net charge, would suggest some level of structural differences between these two variants. This was supported by other independent measurements. LC CDR1-isoAsp variant (and not the Asp variant) can be cleaved by papain in an adjacent CDR2 loop (Fig. 8), indicating that the conversion of Asn33 residue into isoAsp33 impacted not only the residue itself but also surrounding regions. Susceptibility to proteolysis is commonly used to probe conformational changes in proteins [35]. The sites of proteolysis typically exhibit greater flexibility and the scission often occurs across several peptide bonds in the more flexible region [36]. It is therefore conceivable that the flexibility of CDR2 and/or its exposure to solvent is increased in the CDR1-isoAsp33 variant.

The isoAsp residues formed by Asn deamidation showed a tendency to gradually isomerize to Asp. Similar observation was reported for RNase A [9]. In RNase A, deamidation of Asn67 yields both Asp and isoAsp and their initial ratio is determined by the rates of succinimide hydrolysis. As the degradation proceeds, the ratio becomes shifted toward the Asp variant as a result of a thermodynamic control on the reaction. Higher free energy of the isoAsp variant (due to its impact on RNase A structure) compared to its Asp counterpart drives the isoAsp-to-Asp isomerization. The rates of isoAsp-to-Asp and Asp-to-isoAsp isomerization define Asp/isoAsp ratio at equilibrium. In parallel with RNase A, our observations indicate that the isoAsp-containing antibody is in a thermodynamically unfavorable state compared to the Asp variant.

Spectroscopic methods such as CD, fluorescence, and second-derivative UV absorbance did not show significant differences between the IgG1 variants A1 (LC-Asp33/Asn33), A2 (LC-isoAsp33/Asn33), and M (LC-Asn33/Asn33) (not shown). However, when the contribution from the nondegraded Fab fragment and the Fc fragment (and thus signal averaging) was removed by analyzing purified Fab-fragment variants we could detect differences in near-UV CD spectra between the nondegraded FabM and the LC-isoAsp33-bearing FabD, indicating changes in the tertiary structure on replacement of LC Asn33 with isoAsp.

Together the aforementioned observations demonstrate that the replacement of LC-Asn33 with isoAsp has greater impact on the antibody structure than the replacement with Asp. Changes in protein structure following deamidation and particularly due to the presence of isoAsp have been observed in other proteins. In the case of an analog of human growth hormone releasing factor, Leu²⁷ hGRF(1–32)NH₂, the substitution of Asn8 with isoAsp (but not with Asp) led to local disruption of the helical structure [37]. Another example is bovine calbindin D_{9k}, where deamidation of Asn56 caused local perturbations in the isoAsp product but not in the Asp product [4]. The most detailed assessment of the impact of deamidation on the protein has been conducted for Asn67 of RNase A (noted above). As for LC-Asn33 in the antibody described here, Asn67 of RNase A is followed by glycine and it is also located in an external loop. The crystal structure of isoAsp67 RNase A showed that the presence of isoAsp breaks up the hydrogen bond network that makes the loop rigid and that it also changes the overall orientation of the Asn67-containing loop with respect to the protein core [3].

In conclusion, our data demonstrate that deamidation of an Asn residue in a CDR loop can have significant impact on various properties of the antibody, including chromatographic behavior, antigen binding, and antibody structure.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ab.2009.05.043.

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In Vivo Deamidation Characterization of Monoclonal Antibody by LC/MS/MS

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The spontaneous nonenzymatic deamidation of glutaminyl and asparaginyl residues of peptides and proteins has been observed both in vitro and in vivo. Deamidation may change the structure and function of a peptide or protein, potentially resulting in decreased bioactivity, as well as alterations in pharmacokinetics and antigenicity of the protein pharmaceutical. Therefore, it is necessary to monitor the effect of storage and formulation conditions on deamidation of a protein drug candidate. Of particular interest is the investigation of in vivo deamidation mechanisms of protein drug candidates. Several methods are available to characterize the deamidation of peptides and proteins. We present here a LC/MS/MS method used to evaluate the deamidation of an antibody after in vivo administration. A humanized monoclonal IgG1 antibody (MAb) has several "hot spots" for spontaneous deamidation. One site, amino acid residue Asn55 located in the CDR2 region of the heavy chain, is of particular interest since deamidation at this site greatly decreases the binding activity. MAb was administered to cynomolgus monkeys by intravenous and subcutaneous routes. At various times after dosing, monkey serum was prepared and MAb captured by the immobilized antigen or a goat anti-human IgG Fc γ antibody. The captured MAb was treated with trypsin followed by endoproteinase Glu-C. The digests were separated on RP-HPLC and analyzed by MS/MS on Q-ToF Global mass spectrometer. Using this method, we were able to determine the deamidation half-life of amino acid residue Asn55 in vivo and the ratio of the deamidated derivatives, i.e., isoAsp55 and Asp55. The method is rapid and sensitive with low-nanogram quantities of protein detected in the biological matrix.

Nonenzymatic deamidation of asparagine (Asn) or glutamine (Gln) is a major degradation pathway of peptides or proteins and can occur spontaneously during manipulation, purification, and long-term storage. Deamidation can cause structurally and biologically important alterations in peptides and proteins through the introduction of unfavorable negative charge. For example, de-

amidation of an Asn residue has been shown to cause a 25–500-fold reduction in the potency of human growth hormone releasing factor.¹ Studies^{2–5} have shown that there may be a close relationship between several disease states and in vivo deamidation of proteins, such as prion disease, Alzheimer's disease, and cataract formation. Robinson and Robinson⁶ suggested in vivo deamidation of Asn residues of proteins as a molecular timer of biological events and as a mechanism for postsynthetic production of unique proteins of biological significance.

Nonenzymatic deamidation of proteins and peptides involves the formation of a succinimide intermediate.^{7,8} The deamidation rate is dependent on the solution pH, temperature, solvent dielectric constant, ionic strength, primary sequence, local polypeptide conformation, and tertiary structure.^{2,9–11} With regard to the primary sequence, deamidation rates depend on the amino acid residues adjacent to Asn and Gln in the peptide chain. Glycine (Gly) and serine (Ser) are found to be the most destabilizing C-terminal amino acids for deamidation of Asn. Serine, and to a lesser extent threonine (Thr) and lysine (Lys), also appeared to favor deamidation of Asn when they are located at its N-terminal side.^{7,12} Although Gln residues can also be deamidated, the rate of deamidation is much slower than the Asn residue under the same circumstances. Deamidation half-times of Asn and Gln in synthetic pentapeptide chains at neutral pHs and 37 °C are in the range of 1–500 days for Asn and 100–5000 days for Gln.⁹ Deamidation of Asn residues of proteins and peptides results in the formation of both isomeric α - and β -aspartyl residues, with the β -derivatives typically predominate in a ~3:1 ratio.^{2,7}

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Table 1. Concentration of MAb in Cynomolgus Monkey Serum Samples Introduced by Intravenous (IV) or Subcutaneous (SC) Injection

time (h)	concentration ($\mu\text{g/mL}$) of MAb					
	IV injection			SC injection		
	monkey 1	monkey 2	monkey 3	monkey 1	monkey 2	monkey 3
0.25	28.3	28.7	31.4			
4				8.2	11.9	9.3
24	10.7	11.7	12.3			
48				14.2	14.3	18.6
120	3.4	2.9	3.9	7.1	7.5	6.2
312	0.43	0.2	1.1	0.76	1.06	0.39

Antibodies are the first line of defense of the adaptive immune response and are found in the blood plasma and extracellular fluids. Many monoclonal antibodies are marketed and being tested in clinical trials as therapeutics in autoimmune and inflammatory diseases, oncology, and other therapeutic areas.^{13,14} Typically, therapeutic monoclonal antibodies have in vivo half-lives of days to weeks, staying in the circulation much longer than therapeutic growth factors, cytokines, and peptides. Hence, in vivo deamidation of a therapeutic monoclonal antibody may potentially have an impact on its efficacy, especially, when deamidation occurs in the binding regions. Characterization of in vivo deamidation of therapeutic monoclonal antibody could aid in development of more robust protein and antibody therapeutics.

Many methods are available to detect and quantify deamidation of Asn residues. Deamidation introduces negative charge and changes the protein mass (NH_2 vs OH, $\Delta = 1$ Da) and hydrophobicity. Separation techniques including electrophoretic (IEF, cIEF,¹⁵ urea gel electrophoresis¹⁶), and chromatographic (reversed-phase,¹⁷ ion exchange,^{18,19} and hydrophilic interaction²⁰) methods can be used to separate or isolate deamidated forms. Identification of deamidated forms can readily be achieved using mass spectrometry,^{21,22} and N-terminal sequencing, as well as enzymatically using protein L-isoaspartyl/D-aspartyl carboxyl methyltransferase.²³ In this paper, we present a sensitive and rapid LC/MS/MS method for the evaluation of in vivo deamidation in therapeutic monoclonal antibody (MAb).

EXPERIMENTAL SECTION

Materials. MAb, mutants, and human antigen were recombinant products of Eli Lilly and Co. Trypsin, TPCK treated, was

bought from Worthington Biochemical Corp. (Freehold, NJ), and endoproteinase Glu-C was a product of Roche Diagnostics Corp. (Indianapolis, IN). CNBr-activated Sepharose 4 Fast Flow was bought from Amersham Pharmacia Biotech AB (Uppsala, Sweden). AffiniPure goat anti-human IgG, Fc γ is a product of Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). The immobilized antigen (2 mg/mL resin) or goat anti-human IgG, Fc γ (10 mg/mL resin) resin was prepared according to the standard procedure provided by the vendor. All other chemicals were analytical grade and commercially available.

Experiments. Cation Exchange Chromatogram of MAb. Sterile-filtered MAb samples, at 1 mg/mL in PBS (10 mM sodium phosphate, 150 mM sodium chloride buffer, pH 7.4), were kept at 4 °C and 37 °C for one week and analyzed by cation exchange chromatography (CEX). Prior to loading, MAb samples were exchanged into 20 mM sodium phosphate buffer at pH 6.5 (pump A buffer) using a Millipore centrifugal filter with 10 000 MWCO membrane. Samples were run on a Dionex Propac WCX-10 column with a flow rate of 1 mL/min using a linear gradient from 0 to 45% of 20 mM sodium phosphate, 250 mM NaCl, pH 6.5 (pump B buffer).

Monkey pK Study of MAb. MAb was administered to male cynomolgus monkeys ($n = 2/\text{group}$) as a single intravenous bolus or subcutaneous administration of 1 mg/kg. Blood samples were collected over a 4-week period and serum prepared. Concentrations (see Table 1 for sample information) of MAb in serum samples were determined by an antigen-capture ELISA method.

MAb in Vitro Experiment. One-milliliter aliquots of monkey serum were spiked with 1 or 10 μg of MAb, respectively, and the samples were then incubated at 37 °C for 96 h. The serum was either directly subjected to capture and LC/MS/MS analysis or stored at -20 °C for later analysis.

MAb Capture from Monkey Serum. Approximately 200 μL of serum from each monkey, collected during the monkey pK study, was mixed with ~ 2 μL of immobilized antigen or anti-human IgG Fc γ antibody resin and the solution was then incubated at ambient temperature, gently shaking for 2 h. The resin was separated by centrifugation, and the supernatant was removed. The resin was washed with PBS buffer containing 0.2% Tween 20 followed with three PBS buffer washes. The antibody was released from the resin by treatment with 15 μL of 0.15% formic acid aqueous solution at ambient temperature for 15 min. The supernatant containing the captured antibody was lyophilized to dryness using a centrifugation vacuum system.

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Enzymatic Digestions of Captured Monoclonal Antibody. The lyophilized material of each sample was reconstituted in 2 μ L of 8 M urea and 0.5 M Tris buffer, pH 8.0, with 2.5 mg/mL dithiothreitol (DTT), and the solution was then incubated at 37 $^{\circ}$ C for 30 min. To the solution, 2 μ L of a solution containing 10 mM ammonium bicarbonate buffer and 10 mg/mL iodoacetamide was added and the sample was incubated at ambient temperature in the dark for 10 min. The solution was diluted with 35 μ L of 10 mM ammonium bicarbonate buffer and treated with 1 μ L of 0.1 mg/mL trypsin solution at 37 $^{\circ}$ C for 90 min, followed by treatment with 1 μ L of 0.1 mg/mL Glu-C solution at 37 $^{\circ}$ C for an additional 60 min. The purpose of subjecting the sample to a subsequent Glu-C digestion was to further reduce the peptide size thereby facilitating the separation of native and deamidated peptides and enhancing the sensitivity of LC/MS/MS method. The enzymatic activity was quenched by adding 1 μ L of 10% acetic acid solution, and the samples were either immediately analyzed by LC/MS/MS or stored at -20° C until LC/MS/MS analysis.

Enzymatic Digestion of Stressed MAb. Deamidated MAb was generated *in vitro* by thermally stressing the protein at pH 7.4. The protocol subjected a 50- μ L antibody sample at 1 mg/mL to 37 $^{\circ}$ C for one week in PBS (10 mM sodium phosphate, 150 mM sodium chloride buffer, pH 7.4). The sample was subsequently denatured and reduced by the addition of 50 mg of urea, 1 μ L of 50 mg/mL DTT, and 20 μ L of 3 M Tris buffer, pH 8.0. The resulting solution was incubated at 37 $^{\circ}$ C for 30 min. The protein was alkylated by adding 6 μ L of 50 mg/mL iodoacetamide solution and incubating at room temperature in the dark for 15 min. The solution was desalted on a 1-mL spin column packed with P-6 resin (Bio-Rad; Hercules, CA). The desalting columns were washed and eluted with 0.025 M NH_4HCO_3 buffer. About 250 μ L of protein fraction was collected for each sample from the column. Each protein fraction was mixed with 1 μ L of 1 mg/mL trypsin solution, and then the mixture was incubated at 37 $^{\circ}$ C for $\sim$ 3 h. A 5- μ L aliquot of 0.1 mg/mL endoproteinase Glu-C solution was added to a $\sim$ 100- μ L aliquot of the tryptic digests, and the mixtures were incubated at 37 $^{\circ}$ C for 2 h. The reaction was quenched by adjusting the solution pH with acetic acid.

Reversed-Phase HPLC Chromatography. A Waters Alliance HPLC system, model 2960, was used to separate the enzymatic digests of antibody starting material or captured material from monkey serum. The HPLC column was a Zorbax 300SB C18, 2.1 $\times$ 150 mm, 5-mm particle size column. The flow rate was 0.2 mL/min and the column was at ambient temperature. The mobile phase was as follows: solution A, 0.15% (v/v) formic acid aqueous solution; solution B, 0.12% formic acid in acetonitrile. Prior to sample injection, the column was equilibrated at 12% solution B for 15 min. After the injection of sample, a gradient elution was performed from 12 to 25% solution B over 12 min, then to 90% solution B over 1 min, held at 90% solution B for 1 min, and then rapidly returned to initial conditions. An Agilent 1100 HPLC system was used to analyze the enzymatic digests of the stressed samples with the same conditions except a different gradient was used. The column was equilibrated at 5% solution B, and elution achieved by increasing solution B to 30% over 50 min, then 90% over 3 min, and subsequently held at 90% solution B for 1 min before being rapidly returned to initial conditions.

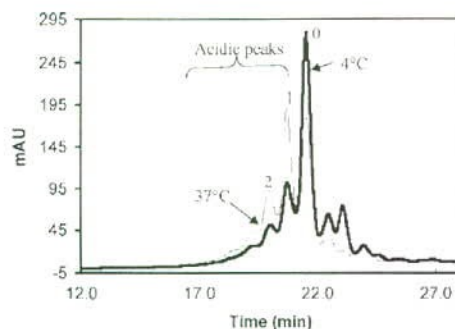


Figure 1. Cation exchange chromatography of MAb. Control sample at 4 $^{\circ}$ C vs a stressed sample kept at 37 $^{\circ}$ C for 7 days in PBS buffer at pH 7.4.

Mass Spectrometry. An Applied Biosystem Sciex API 150EX (Thornhill, ON, Canada) single-quadrupole mass spectrometer equipped with a nebulizer-assisted electrospray source was used for LC/MS peptide map analysis of thermally stressed MAb samples. The single-quadrupole mass spectrometer was operated in the positive-ion mode with a mass scan range of 300–1500 Da, using a declustering potential of 30 V, focusing potential of 250 V, and scan rate of 2 s/scan. LC/MS/MS was performed on a Micromass Q-TOF Ultima Global with the following settings: a capillary voltage of 3.46 kV, a cone voltage of 100 V, a collision energy of 22 eV, a mass range of 100–2000, a desolvation temperature of 400 $^{\circ}$ C, and a desolvation gas flow of 600 L/h. For single-quadrupole mass spectrometry, the HPLC stream was split $\sim$ 1:4 with the smaller fraction of the stream diverted into the mass spectrometer. For the Micromass Q-TOF mass spectrometer, the HPLC effluent was introduced directly into the mass spectrometer without splitting the stream.

RESULTS AND DISCUSSION

Nonenzymatic deamidation of Asn or Gln residues of a peptide or protein is greatly affected by the amino acid residues near the Asn and Gln in the peptide chain. Gly and Ser located C-terminally to the deamidation site were found to be the most destabilizing.^{7,12} The MAb investigated in this study contains three Asn-Gly sequences. One of these sequences, Asn55 of the heavy chain, is in the binding region (CDR2), while the other two are in the Fc region. Figure 1 shows the CEX chromatograms of the thermally stressed MAb, in PBS buffer at 37 $^{\circ}$ C for one week, relative to a control sample at 4 $^{\circ}$ C. The main peak (peak 0) corresponds to antibody lacking Lys residues at the C-terminus of both heavy chains. The acidic peaks (peaks 1 and 2) were greatly increased after a one-week incubation at 37 $^{\circ}$ C and thus were attributed to deamidation in the antibody.

Identification of Deamidation at Asn55 of MAb. To confirm that the increases in acidic peaks were due to Asn deamidation, LC/MS was used to analyze the enzymatic digest of the thermally stressed samples. The total ion chromatograms (TICs) of the control and thermally stressed MAb samples are shown in Figure 2. Based on mass analysis, the three peaks identified correspond to peptide masses (1444.8 for peak 1, 1445.7 for peak 2, and 1445.8 for peak 3), which are consistent with the native form and deamidated derivatives of heavy-chain peptide 51–63 ($_{51}$ ILPGNG-NINYNK $_{63}$). As shown in Figure 2, the deamidated

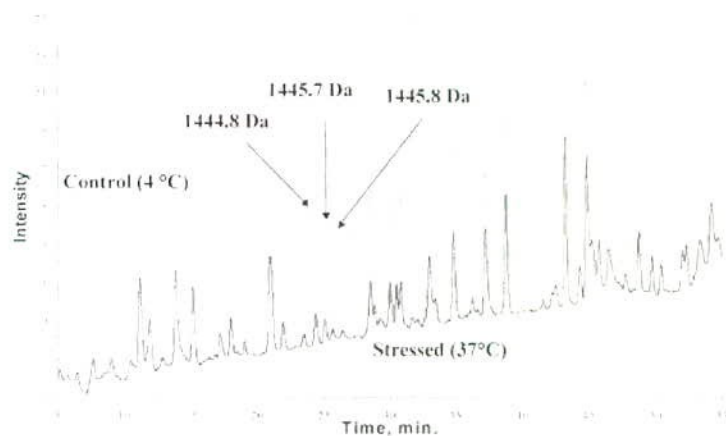


Figure 2. TICs of the digests of the control and stressed MAb.

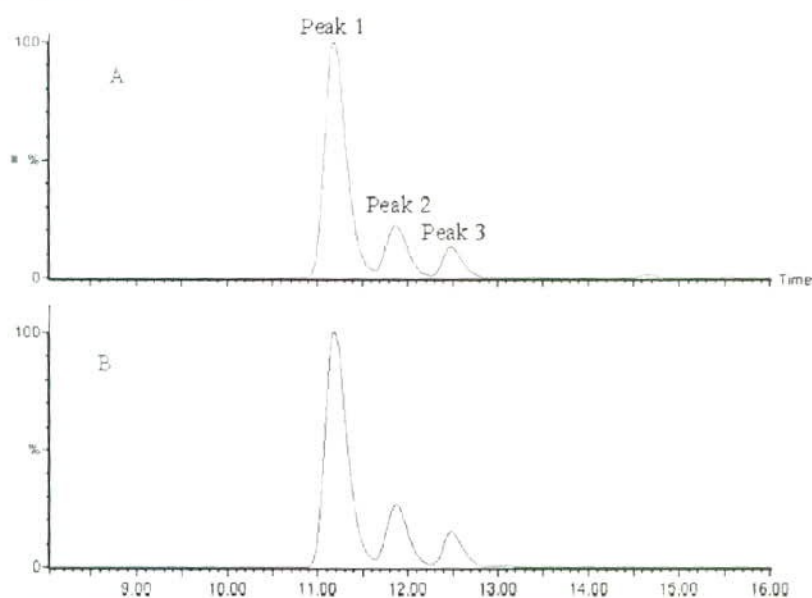


Figure 3. LC/MS/MS TIC (A) with the set mass (m/z) at 723 (equal to the doubly charged ion of peptide 51–63) and (B) the XIC from the ion at m/z 610–613, which covers the doubly charged ion of the fragment (y_{11}) of peptide 51–63.

derivatives were more abundant in the thermally stressed sample. The half-life of deamidation in PBS at 37 °C was estimated to be ~6 days by CFX analysis. BIAcore analysis for the deamidated form of the MAb or the MAb mutein (N55D), in which Asn55 was recombinantly replaced with Asp55, indicated that deamidation at Asn55 greatly reduced the binding affinity to antigen by 14 $\times$ (data not shown). Asn residues in the other two Asn-Gly sites in the Fc region were also deamidated, but at a much slower rate compared with that of the Asn55 residue; however, the deamidation in the Fc region is not expected to influence the binding affinity to antigen. In this paper, the focus is solely on Asn deamidation in the Fab region. Nonetheless, the method discussed is generally applicable to the characterization of Asn deamidation in the Fc region or in other proteins or peptides.

Heavy-chain peptide 51–63, which contains the Asn55 residue, was obtained by tryptic digestion followed by treatment with endoproteinase, Glu-C. The native peptide 51–63 and its deamidated derivatives were separated into three peaks by reversed-

phase HPLC. Figure 3 is the LC/MS/MS TIC for the doubly charged ion of peptide 51–63 and its derivatives. MS/MS spectra for each peak are shown in Figure 4. The y-series of ions are the predominant fragments. From these fragment (y-series) ions, peaks 1–3 contain the same y-series (≤ 8) of ion for low-mass fragments, indicating that the C-terminal amino acid residues between Gly56 and Lys 61 are the same for all three peaks; but the y_9 – y_{11} ions from peaks 2 and 3 are 1 Da higher than the corresponding ions from peak 1. The mass difference between the y_9 and y_8 is 114 Da for peak 1, matching an Asn residue, and 115 Da for peaks 2 and 3, 1 Da higher than Asn residue mass, thus corresponding to either deamidated derivative, Asp or IsoAsp of the Asn residue. Asp and IsoAsp possess isobaric mass and are generally indistinguishable by mass spectrometry. However, the area of peak 2 is twice that of peak 3. Under neutral or alkaline pH, a deamidation derivative ratio of IsoAsp/Asp of ~3/1 has been observed.^{2,7} Assuming this ratio of IsoAsp/Asp holds for this study and that the ionization efficiency of the peptides are not altered

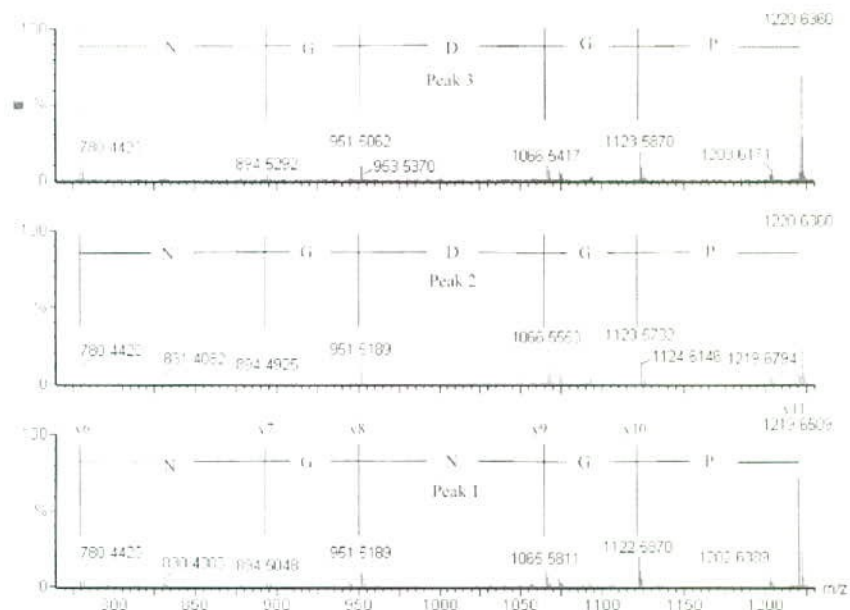


Figure 4. MS/MS spectra of peptide 51–63 peaks 1–3.

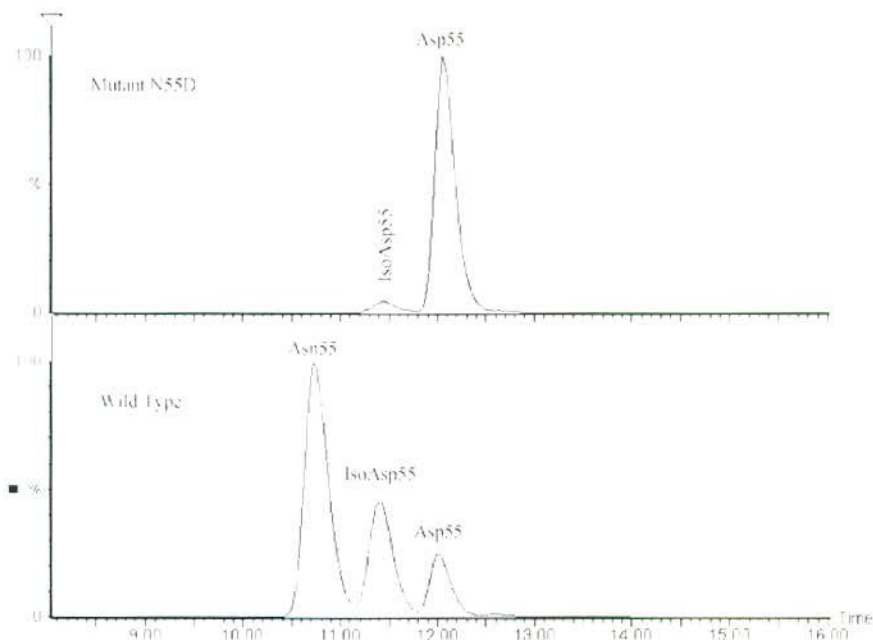


Figure 5. XICs of the double-charged ion of the fragment (y_{11}) (the ion from m/z 610 to 613), of peptide 51–63 of wild-type MAb and its Asn55Asp mutant.

by the deamidation, peak 2 is assigned as the IsoAsp derivative and peak 3 the Asp derivative. To unambiguously assign the peaks and assess the assumptions made regarding the deamidation derivative ratio and ionization efficiencies, LC/MS/MS analysis of N55D mutin was used to confirm peak 3 as the Asp derivative (Figure 5) and thus by deduction, peak 2 is the IsoAsp derivative of the peptide 51–63.

Method Qualification for Use in *In Vivo* Sample Analysis.

The MAb immunoprecipitated from monkey serum was not very pure, particularly in samples with very low MAb concentration

(for example, $<1 \mu\text{g/mL}$). Consequently, peptides from the contaminating proteins could interfere with the quantitative determination of the TIC from LC/MS/MS. Since the doubly charged ion of the y_{11} (data not shown) is the dominant fragment in the MS/MS spectrum of the peptide 51–63, the quantitative results of deamidation in the peptide 51–63 can be determined from the extracted ion chromatogram (XIC) of the y_{11} ion (see Figure 3b). Based on LC/MS/MS analysis for a standard sample, i.e., the MAb spiked into monkey serum, the quantitative results obtained from TIC and XIC for the deamidation of the Asn55 are

Table 2. LC/MS/MS Data Obtained for the Peptide 51–63 at Different Concentrations of MAb

amount (ng) injected	peak area from XIC			relative %			IsoAsp/Asp
	Asn55	IsoAsp55	Asp55	Asn55	IsoAsp55	Asp55	
10	211.3	65.9	29.2	69.0	21.5	9.5	2.3
50	911.7	278.1	117.1	69.8	21.3	9.0	2.4
100	1505.6	457.1	217.2	69.1	21.0	10.0	2.1
500	7089.2	2292.1	1088.3	67.7	21.9	10.4	2.1
av				68.9	21.4	9.7	2.2
SD				0.9	0.4	0.6	0.1
RSD, %				1	2	6	6

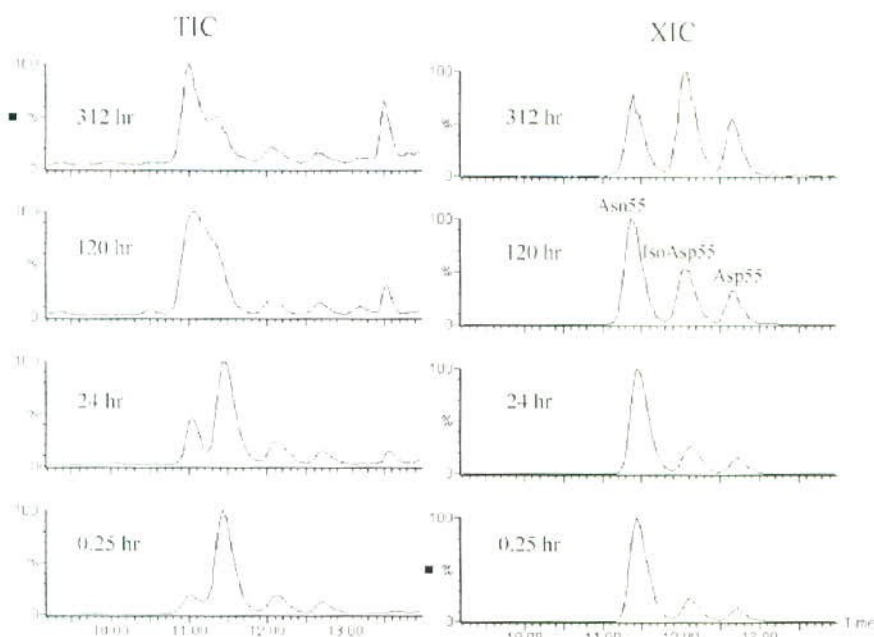


Figure 6. TICs of the double charged ion of peptide 51–63 and XICs of the double-charged ion of y11 fragment of peptide 51–63 from monkey serum samples at different time points.

very similar (data not shown). Furthermore, the quantitative results obtained by LC/MS/MS are consistent with those obtained by the other methods, such as CEX and capillary isoelectric focusing for a standard sample. Again, the results suggested that the ionization efficiency of both native and deamidated peptide 51–63 was very similar.

To evaluate the limit of quantitation and linearity of the LC/MS/MS method for deamidation characterization of peptide 51–63, a series of digests with different concentrations of MAb were analyzed. The area for each of three peaks was integrated from XIC to calculate the relative percentage of each peak. The results are shown in Table 2. Linear regression analysis for the data indicated that the response is linear ($R^2 > 0.99$) from 10 to 500 ng/injection of MAb for the native peptide 51–63 and its deamidated derivatives. In fact, quantitative determination of deamidation of peptide 51–63 only requires relative percent and not absolute value. Data shown in Table 2 demonstrate that the percent of deamidation of peptide 51–63 is independent of the amount injected over the range studied. These results show that LC/MS/MS can accurately determine the relative percent of deamidation of peptide 51–63 when > 10 -ng amounts of MAb are available.

Analysis of in Vivo Monkey Serum Samples. Serum samples were obtained from cynomolgus monkeys at various time points after subcutaneous or intravenous administration of the MAb and were analyzed by LC/MS/MS to determine the extent of deamidation of amino acid residue Asn 55 of the heavy chain. Most of the time points had more than enough antibody ($> 1 \mu\text{g/mL}$) for LC/MS/MS analysis. However, several of the later time point samples had MAb concentrations as low as 200 ng/mL, and only 200 μL of serum was available for analysis. However even under these circumstances, if the capture efficiency is sufficient, enough antibody is present to determine deamidation by LC/MS/MS. Although we did not determine the capture efficiency, the quantitative results (see below) indicated that enough MAb could be captured from the serum for quantitative analysis. Figure 6 is the TIC with the set mass (m/z) equal to 723 (which equals the doubly charged ion mass of the peptide 51–63) and XIC from the ion (m/z) 610–613 (which covers the doubly charged fragment 53–63 (i.e., y11)) at time points 0.25, 24, 120, and 312 h. For the 312- and 120-h time points, an impurity peak dominated the TIC tracing; thus, it was difficult to quantify the extent of deamidation at Asn55 from the TICs. However, only three peaks, all related to peptide 51–63, were present in the XICs. The XICs

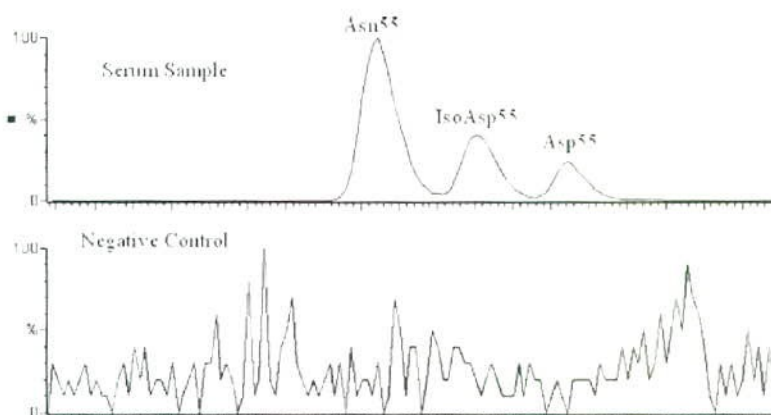


Figure 7. XICs of monkey serum sample of MAb and negative controls (vehicle serum).

for monkey serum MAb sample and the negative control (vehicle serum) are shown in Figure 7. No significant peaks for vehicle serum were detected, indicating that the method is specific for peptide 51–63. For the monkey serum MAb sample, the area of each peak was determined from the XIC and the calculated relative percent of native peptide 51–63 and its deamidated derivative ascertained. The results are summarized in Table 3. As shown in the table, the extent of deamidation was ~25% in the time 0.25- and 4-h samples. This is the amount of deamidation occurred during the protein production and storage.

With increasing circulation time of the antibody in monkey blood, the percent of deamidation at Asn55 increased, but at all time points the ratio of IsoAsp/Asp derivative is consistent, 2:1. Several conclusions can be drawn from these data. The MAb continuously deamidates in monkey blood, as expected, through a process consistent with a nonenzymatic mechanism. Moreover, the ratio of IsoAsp:Asp remains constant during the time course studied (IsoAsp:Asp = 2:1), suggesting that the clearance rates for IsoAsp derivative and Asp derivative are similar. If this were not the case, the ratio of IsoAsp/Asp would change with increasing circulation time.

Rate of Asn55 Deamidation in Vivo. Assuming that the in vivo clearance of native Mab (Asn55) and its deamidated derivatives (IsoAsp55 and Asp55) are similar, the deamidation rate of MAb in vivo could be calculated from the relative percent of deamidation. Nonenzymatic deamidation of Asn and Gln residue is a first-order reaction, i.e., the rate $[R]_d$ of deamidation derivative formed is proportional to the concentration of the native form;

$$[R]_d = -d[D]/dt = -d[N]/dt = k[N]$$

$$d[N]/([N] + [D])/dt = -k[N]/([N] + [D])$$

$$d[N]_r/dt = -k[N]_r$$

$$[N]_r = A \exp(-kt)$$

$$\ln[N]_r = -kt + C \quad (1)$$

where $[N]_r$ is relative percent of native form, $[N]$ and $[D]$ are concentrations of native and deamidated derivatives, respectively. Equation 1 was used to fit the data to obtain parameters of k (rate constant) and C ($\ln(\text{percent of native MAb})$) at time zero.

Table 3. LC/MS/MS Data Obtained from Analysis of Monkey Serum Samples

time (h)	% deamidation for IV				% deamidation for SC			
	M 1	M 2	M 3	IsoAsp/Asp	M 1	M 2	M 3	IsoAsp/Asp
0.25	25.5	24.6	25.8	1.9				
4					25.6	26.1	26.0	1.9
24	28.7	30.8	25.7	2.0				
48					35.7	36.2	35.3	1.9
120	45.5	46.5	40.6	1.9	44.7	44.3	45.0	2.0
312	67.9	55.5	64.0	2.1	68.0	66.7	62.5	2.1

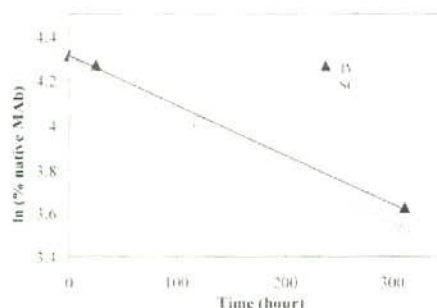


Figure 8. Logarithm of relative native peptide of monkey serum sample vs time for in vivo PK study.

The plot of the logarithm of relative percent of native MAb as a function of time is linear and shown in Figure 8. The linear regression fit predicts that slope ($-k$) equals -0.0022 and -0.0025 for IV and SC administration, respectively, and the intercept (C) equals 4.31 and 4.30 for IV and SC administration, respectively. From these data, the half-life of deamidation of the Asn55 of MAb in monkey is ~277 h for both IV and SC. However, since antibodies are dimers possessing two heavy chains, i.e., two Asn55 residues, and assuming the deamidations at these two Asn55 are independent of each other, the half-life of the deamidation of intact MAb is half of that ascribed to the Asn55 peptide, or 140 h or 6 days in monkey. The rate of deamidation does not appear to be dependent on the route of administration (SC vs IV).

As discussed above, this LC/MS/MS methodology enabled the quantitative determination of Asn and Gln deamidation in a peptide or protein from a complex biological matrix. In this methodology, it is beneficial that the chromatographic separation

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be of high enough resolution to resolve amidated and deamidated forms of the same peptide. If the amidated and deamidated forms coelute, the single mass unit increase due to deamidation will only be observable, in most instruments, by an alteration in the isotopic distribution pattern. The methodology also requires that a sufficient number of MS/MS spectra for a specific parent ion be acquired for quantitation. This may preclude simultaneous quantitation of the relative extent of deamidation at multiple sites within a mixture of proteins in a single LC/MS/MS run. In some cases it may also be advantageous to add an alternate enzymatic digestion protocol to generate suitable sized peptides before LC/MS/MS analysis.

CONCLUSION

Nonenzymatic deamidation of Asn or Gln of a peptide or protein is one of the major degradation pathways observed *in vivo* as well in the manufacturing and storage of biopharmaceuticals. Deamidation of a protein or peptide introduces additional negative charge, which may cause structural changes in the molecule. These structural changes may further introduce changes in biological function, leading to a decrease in activity or function.

Deamidation of a protein or peptide introduces an unnatural amino acid residue, IsoAsp, and may change its antigenicity. Hence, deamidation of a protein or peptide biopharmaceutical must be carefully monitored. There are many available methods to characterize and quantify deamidation of protein or peptide. However, these methods require pure materials or large quantities of samples and cannot handle complex mixtures.

As we discussed in this paper, by combining HPLC/MS/MS and microscale immunopurification, it is possible to characterize deamidation *in vivo* with low-picomole or high-femtomole amounts of material. Specifically, we have described a LC/MS/MS method to characterize deamidation of Asn55 of the heavy chain of a monoclonal antibody, MAb, *in vivo*. The method is sensitive and rapid with analysis accomplished in less than one day. To our knowledge, this is the first paper describing a rapid and sensitive method to characterize *in vivo* deamidation of a protein or peptide pharmaceutical.

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Generation of a humanized anti-glypican 3 antibody by CDR grafting and stability optimization

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Glypican 3 (GPC3), a glycosylphosphatidylinositol-anchored heparan sulfate proteoglycan, is expressed in a majority of hepatocellular carcinoma tissues. The murine monoclonal antibody GC33 that specifically binds to the COOH-terminal part of GPC3 causes strong antibody-dependent cellular cytotoxicity against hepatocellular carcinoma cells and exhibits strong antitumor activity in the xenograft models. To apply GC33 for clinical use, we generated a humanized GC33 from complementarity-determining region grafting with the aid of both the hybrid variable region and two-step design methods. The humanized antibody bound to GPC3 specifically and induced antibody-dependent cellular cytotoxicity as effectively as a chimeric GC33 antibody. To improve stability of the humanized GC33, we further optimized humanized GC33 by replacing the amino acid residues that may affect the structure of the variable region of a heavy chain. Substitution of Glu6 with Gln in the heavy chain significantly improved the stability under high temperatures. GC33 also has the risk of deamidation

of the -Asn-Gly- sequence in the complementarity-determining region 1 of the light chain. As substitution of Asn diminished the antigen binding, we changed the neighboring Gly to Arg to avoid deamidation. The resulting humanized anti-GPC3 antibody was as efficacious as chimeric GC33 against the HepG2 xenograft and is now being evaluated in clinical trials. *Anti-Cancer Drugs* 21:907–916 © 2010 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Glypican 3 (GPC3) is a member of the glypican family of heparan sulfate proteoglycans that are attached to the cell surface by a glycosylphosphatidylinositol anchor [1]. Loss-of-function mutations of GPC3 in humans cause Simpson–Golabi–Behmel syndrome, an X-linked condition characterized by prenatal and postnatal overgrowth [2]. GPC3 regulates cell growth positively and negatively in a cell type-dependent manner. Overgrowth of Simpson–Golabi–Behmel syndrome and also of GPC3 knockout mice suggests a negative regulation of cell growth by GPC3 [3]. In contrast, GPC3 stimulates the growth of hepatocellular carcinoma cells (HCC) *in vitro* and *in vivo* by activating the canonical Wnt signaling [4].

GPC3 expression has been shown to be markedly elevated in HCCs at both the mRNA and protein levels [5–8]. GPC3 immunostaining was observed in more than 70% of the HCC patients. We recently found that GPC3 membrane expression might be involved in the increase of macrophages in human HCC [9,10]. In addition, Shirakawa *et al.* [11] reported that GPC3 expression is correlated with a

poor prognosis in HCC patients. As there are detectable levels of GPC3 in the sera of HCC patients, soluble GPC3 levels may be used as a prediction marker for prognosis [12–14]. Furthermore, elevated levels of GPC3 have also been reported in melanoma [15,16] and gastric cancer [17].

Earlier, we generated a monoclonal antibody (mAb) against the COOH-terminal part of GPC3, which induced antibody-dependent cellular cytotoxicity (ADCC) against the GPC3-positive human HCC cells but not the GPC3-negative cells [18]. The mAb, designated as GC33, exhibited marked tumor growth inhibition of subcutaneously transplanted HepG2 and HuH-7 xenografts that expressed GPC3 [19]. To apply GC33 for clinical use, we generated a humanized version. This study describes the humanization of GC33 in detail.

Methods

Construction of chimeric GC33

GC33 hybridoma was obtained in the earlier study by immunizing soluble GPC3 [18]. Total RNA was prepared from hybridoma cells using the RNeasy Plant Mini Kit

(Qiagen, Chatsworth, California, USA). The variable regions of the heavy chain (VH) and the light chain (VL) cDNAs were obtained by rapid amplification of cDNA ends using a SMART RACE cDNA amplification kit (Clontech, Palo Alto, California, USA) and the 3'-end primers designed for each chain of immunoglobulin (κ , γ 2a). The PCR products were cloned into pGEM-T Easy Vector (Promega, Madison, Wisconsin, USA) and the sequences of VH and VL for GC33 were determined. The framework regions (FRs) and complementarity-determining regions (CDR) were determined as reported [20]. To produce chimeric GC33, in which the mouse variable regions were linked to the human IgG1 and κ constant regions, the cDNA for the VH region was cloned at the HindIII/BamHI sites of HEF-VII-gy1 [21] that carries the constant region of human IgG1, whereas the VL region was cloned at the HindIII/BamHI sites of HEF-VI-gk [21] harboring the constant region of the human κ chain. The resulting plasmid DNA was transfected into CHO cells by electroporation. The mAbs in the culture media were purified by protein A-Sepharose affinity chromatography followed by dialysis against PBS.

Construction of humanized GC33

The mAb GC33 was humanized using the CDR-grafting method as described earlier [21–23]. The first versions (hGC33VHA, hGC33VLA) of the humanized GC33 VH and VL regions were generated using 14 overlapping oligonucleotides ranging from 36 to 50 bp, with 20 bp of complementarity, in a PCR-based protocol. The PCR primers for hGC33VLA and hGC33VHA are shown in Tables 1 and 2, respectively. The signal sequence of the accession number of S40357 that has a high homology with AB064105 was used as the signal sequence of the VL chain. The DNA fragments were cloned at the HindIII/BamHI sites of the HEF expression vectors. CHO cells expressing humanized GC33 were established and the humanized GC33 was purified by protein A-Sepharose affinity chromatography followed by size-exclusion chromatography (SEC). The hybrid variable regions, in which some FRs of the humanized variable regions had been

Table 1 Sequence of the overlapping PCR primers for humanized antibody light chain variable region hGC33VLA

Primers	Sequence
hVLthin	5'-ttcaagcttcaccatgaatacctattgcctacggca-3'
hVLrbam	5'-ttgggacccactcactgtttgatctccagcttggtcc-3'
hVL1	5'-atgaatactatttgctacggcagcgctggaattgtattactcgggc-3'
hVL2	5'-tgagtcacacacatcgccatggcggctgggcccgcagtaataacaa-3'
hVL3	5'-gatgttgatgaactcagctccactctccctgccgtccacccctggaga-3'
hVL4	5'-ctctgactagatctcaggagatggaggccggctctcaggggtgacggg-3'
hVL5	5'-tcagatctagtcagagcctgtacacagtaattggaacacctattaca-3'
hVL6	5'-tgtggagactgcccctgctctcagggtaccaatgtaaalagggtttcc-3'
hVL7	5'-ccagggcagctccacagctcctgatctataaagtccaacccgatttc-3'
hVL8	5'-gatccactgccaactgaacctgtcagggaccacagaaatcggttgaac-3'
hVL9	5'-ttcagtgccagtggtgagcagcagattttacactgaaacacagag-3'
hVL10	5'-gagcagtaataaaccccaacatcctcagcctcactctgctgatttcag-3'
hVL11	5'-gggggtttattactgctctcaataacatgttctcctactgttggcca-3'
hVL12	5'-ttgatctccagcttggtccctggcccaacagtaggag-3'

VL, light chain.

Table 2 Sequence of the overlapping PCR primers for humanized antibody heavy chain variable region hGC33VHA

Primers	Sequence
hVHthin	5'-ttcaagcttcaccatggactggacctggaggttctc-3'
hVHrbam	5'-ttgggacccactcactcctgaggagcgggtgaccagg-3'
hVH1	5'-atggactggactggaggttctcttgggtggcagcagctacaggtgt-3'
hVH2	5'-gctccagactccaccagctgacactgggactggacacctgtagctgtc-3'
hVH3	5'-ctgggtggagctggagctgaggtgaagaagcctggggcctcagtgagg-3'
hVH4	5'-tcgggtgaaggtglatccagaagccttgcaggagacctcactgagggccc-3'
hVH5	5'-ggatacaacttcaccagctatgaatgactgggtgcagagccctgg-3'
hVH6	5'-ggatcaagagctccactccactcaagccttgcaggggcctgtgcac-3'
hVH7	5'-atgggagctcttgatctaaactgggtgatacctcactcagcagaagti-3'
hVH8	5'-gattctcgcgggtaactgactctgccctgaactctgactgaggg-3'
hVH9	5'-attaccgggacgaatccacgagcagcctacatggagctgagcagcct-3'
hVH10	5'-cgcacagtaatacagcggcgtgtcctcagatctcaggtctcagctcca-3'
hVH11	5'-ccgtgtattactgtgcgagattactctactactctggggccaggga-3'
hVH12	5'-tgaggagacgggtgaccagggttccctggccccagtaag-3'

VH, heavy chain.

replaced with mouse FRs, were constructed by overlap extension PCR. Additional changes of amino acids were introduced into the humanized GC33 VH and VL by site-directed mutagenesis using a QuikChange kit (Stratagene, La Jolla, California, USA).

Enzyme-linked immunosorbent assay

Soluble human GPC3 core protein (sGPC3 Δ heparan sulfate, 1–563, mutated Ser495 and Ser509 to Ala) was prepared as described earlier [18]. Microtiter plates were coated with 1 μ g/ml of sGPC3 HS and blocked with 1% BSA. The plates were then incubated with mAbs at the required dilutions. Bound mAbs were detected with alkaline phosphatase-labeled anti-human Ig VL κ (Sigma, Woodlands, Texas, USA). Absorbance at 405 nm was measured with a microplate reader and the results were analyzed using GraphPad Prism (GraphPad Software, San Diego, California, USA). Competition enzyme-linked immunosorbent assay (ELISA) was performed with biotinylated mouse GC33 that had been prepared using a Biotin Labeling Kit (Roche Diagnostics, Penzberg, Germany). sGPC3 Δ HS coated microtiter plates were incubated with biotinylated mouse GC33 in the presence of various concentrations of humanized GC33. After washing, the binding of biotinylated GC33 was detected using alkaline phosphatase-conjugated streptavidin (Invitrogen, Carlsbad, California, USA).

Antibody-dependent cellular cytotoxicity assay

ADCC from the humanized GC33 was determined by a ^{51} Cr-release assay [18]. SK-03, an SK-HEP-1 derived transfectant expressing human GPC3, was used as the target cell line [19]. As the effector cells, human peripheral blood mononuclear cells were purified from whole blood samples of healthy volunteers using Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden). Target cells were labeled with 0.1 mCi of ^{51} Cr-sodium chromate (New England Nuclear, Boston, Massachusetts, USA) at 37°C for 1 h. The ^{51} Cr-labeled cells were incubated with serial dilutions of antibodies in the presence of effector

cells at an E:T ratio of 50:1. After incubation at 37°C for 4 h, the supernatants were collected and the radioactivity of each supernatant was counted with a γ -counter. Specific cell cytotoxicity was calculated using the following formula: cell cytotoxicity (%) = (A-C)/(B-C), in which A, B, and C represent the radioactivities of the supernatants of the target cells incubated with the antibody plus effector cells, cells lysed with 2% nonidet P-40, and nontreated cells, respectively.

Accelerated stability study

The humanized antibodies were diluted in the formulation buffer (20 mmol/l sodium acetate, 150 mmol/l NaCl at pH 6.0) at a concentration of 0.5 mg/ml and stored for 0–2 weeks at 60°C. SEC was then carried out on an Alliance (2690/5) HPLC system (Waters, Milford, Massachusetts, USA) equipped with a TSKgel SuperSW3000 column (Tosoh, Tokyo, Japan). The column was equilibrated with 50 mmol/l sodium phosphate pH 7.0, 300 mmol/l NaCl. Samples were injected onto the column and eluted isocratically at a flow rate of 0.3 ml/min with the equilibration buffer. Protein elution was monitored at 280 nm. For quantification purposes, all species eluted before and after the main peak were integrated, and represent high molecular weight species and low molecular weight species, respectively.

Animals and drug administration

The human hepatoblastoma cell line HepG2 was obtained from ATCC and the cells were cultured in Eagle's minimal essential medium (Sigma) supplemented with 10% fetal bovine serum, nonessential amino acids, and 1 mmol/l sodium pyruvate. Six-week-old male, CB-17 severe combined immunodeficient mice were intraperitoneally injected with 200 μ g of anti-asialo GM1 antibody (Wako, Osaka, Japan) and then subcutaneously inoculated with 5×10^6 cells of HepG2 suspended in 50% Matrigel (Becton Dickinson, San Jose, California, USA). When the tumor volume reached approximately 200 mm³, the mice were intravenously administered the indicated doses of the anti-GPC3 antibody once a week for 3 weeks. The control mice received the same volume (10 ml/kg) of PBS. Tumor volume was estimated using the equation $V = ab^2/2$, in which a and b are the tumor length and width, respectively.

Results

Humanization of GC33

The cDNAs coding for the mouse GC33 VH and VL regions were isolated by PCR and 5'-rapid amplification of cDNA ends, respectively. The deduced amino acid sequences from the cDNAs showed typical mouse VL and VH variable regions belonging to κ VL subgroup II and VH subgroup II (A) (Fig. 1). A chimeric GC33, in which the VL region was linked to the human κ constant region and the VH region to the human IgG1 constant

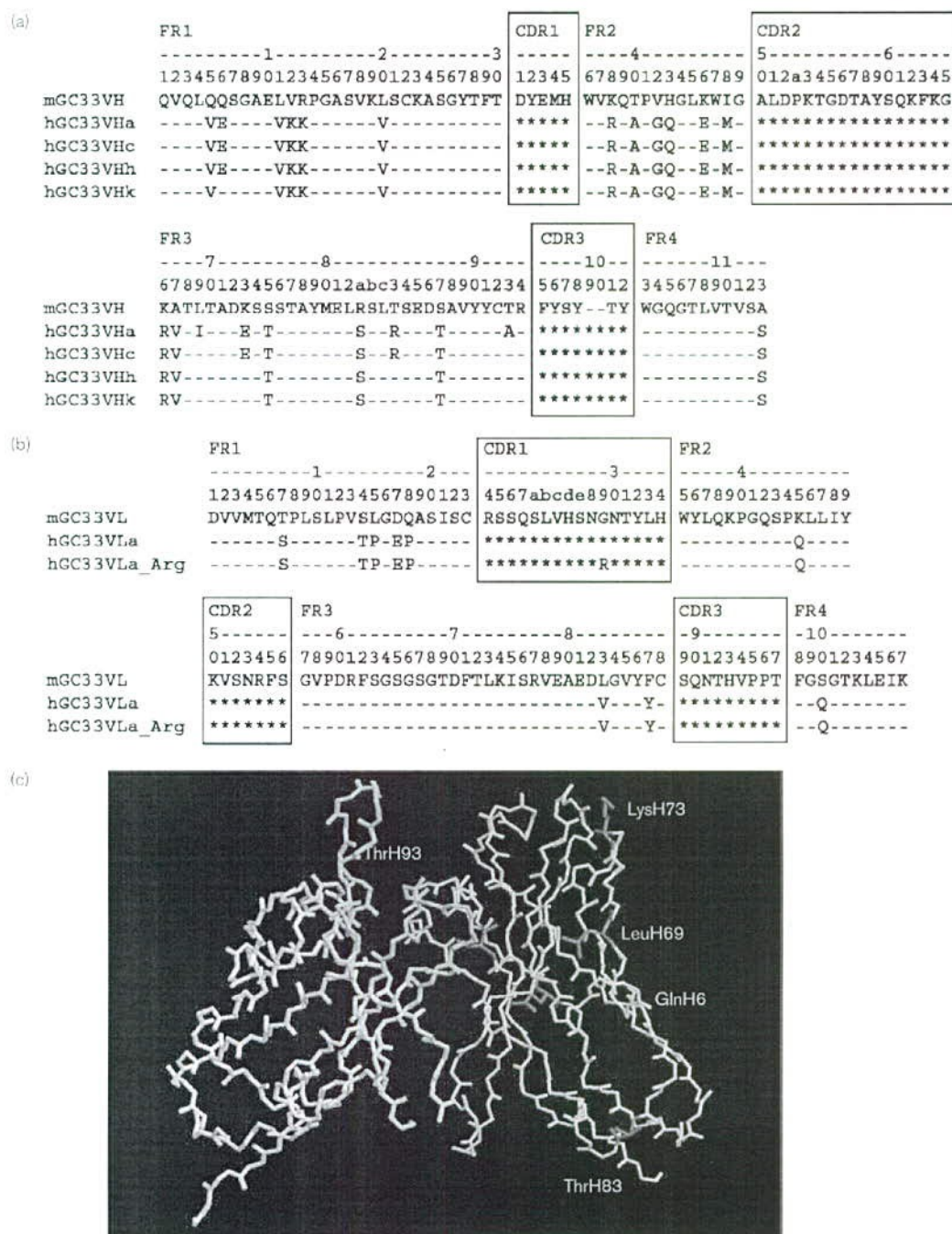
region, was cloned into an expression vector and used as a positive control for the evaluation of the humanized antibodies.

MAb GC33 was humanized using CDR grafting [21–23]. To select suitable human variable domains to serve as framework donors, the amino acid sequences of GC33 VH and VL were independently aligned against the entire repertoire of human antibody sequences contained in the Kabat Database (<http://ftp.ebi.ac.uk/pub/databases/kabat/>) and the ImMunoGeneTics Database (IMGT, <http://imgt.cines.fr/>). Canonical residues are key residues in the CDR and/or framework that determine the conformation of the CDR loop. Residues 24, 26, 27, 29, 71, 94 of the VH are part of the canonical structure [24], as are residues 2, 48, 64, 71 of the VL [25]. We searched the antibody sequences that matched these canonical residues with GC33. The FRs of the VH of human antibody DN13 [26] were 75% identical to the GC33 VH and the FRs of the human antibody K64 (GenBank accession number AB064105) were 89% identical to the GC33 VL. We selected the variable regions of these two human antibodies as frameworks for the humanized GC33 antibody VH and VL, respectively.

First, three CDRs from the GC33 VL or VH were directly grafted onto the human antibody VL or VH frameworks to generate humanized antibody genes. The CDR-grafted antibody VL (hGC33VL_a) and VH (hGC33VH_a) were synthesized using an overlap extension method. The resultant humanized *VH* gene and *VL* gene were cloned into expression vectors and were coexpressed with the GC33 chimeric antibody VH (cGC33VH) or VL (cGC33VL) expression vector in COS7 cells, yielding a humanized antibody (hGC33VH_a/hGC33VL_a), hemihumanized antibodies (hGC33VH_a/cGC33VL_a, cGC33VH/hGC33VL_a), and a chimeric antibody (cGC33VH/cGC33VL). These antibodies in the COS7 cell culture supernatant were quantified by sandwich ELISA and the binding activity was evaluated by soluble GPC3-coated ELISA. The results showed a three-fold decrease in the antigen binding activity of the humanized GC33 VH compared with the chimeric GC33 VH (data not shown) and additional alterations to the human frameworks were required. The humanized GC33 VL bound to the antigen as effectively as the chimeric antibody (data not shown) and so no additional versions of the humanized VL regions were required.

To identify which mouse FR was required to create a functional antigen-binding site, hybrid variable regions were constructed [22]. When the FR3 of hGC33VH_a was replaced by the mouse FR3, the binding activity was comparable with the chimeric antibody (data not shown). Thus, some amino acids in the mouse FR3 are thought to be critical in recreating a functional antigen-binding site. For the design of a second set of humanized GC33 VH regions, a structure model of the mouse GC33 variable regions was built using the molecular operating environment

Fig. 1



Amino acid sequence of humanized GC33 heavy chain (VH) (a) and light chain (VL) (b) variable regions. mGC33VH and mGC33VL, respectively, indicate VH and VL variable regions of murine GC33 mAb. The VH of the human antibody DN13 was chosen as the framework for the humanized VH and the VL of human antibody K64 was chosen for the humanized VL. hGC33VHa, hGC33VHc, hGC33VHh, and hGC33VHk are different versions of the humanized VH variable region. hGC33VL_a and hGC33VL_a_Arg are different versions of the humanized VL variable region. Dashes represent amino acids that are the same as the corresponding residues in mouse GC33. Amino acids are numbered according to Kabat database. (c) Structural model of GC33 antibody. Complementarity-determining regions (CDRs) of light chains are shown in dark gray. CDRs of heavy chains are shown in light gray. The five amino acid substitutions are highlighted in red. FR, framework region.

software (Montreal, Canada) (Fig. 1c), and five amino acid residues in FR3 (positions 66, 67, 69, 73, and 93) were selected to create a Vernier zone to adjust the CDR structure and fine-tune the fit to the antigen [21,27–30]. Additional versions were sequentially constructed by combining various amino acid residues of interest, and, as a result, two additional alterations, Ile69 to Leu (I69L) and Ala93 to Thr (A93T), were found to retain the full antigen binding activity with minimum alterations (Fig. 2a); the resultant humanized antibody was designated as hGC33VHc. In competitive binding assays, the humanized antibody hGC33VHc showed the same ability as chimeric GC33 to inhibit the binding of biotin-labeled murine GC33 to soluble GPC3 (Fig. 2b). The data were fitted to an equation for simple competition to provide the IC_{50} , yielding relative binding affinities (Fig. 2). To evaluate the antitumor activity, the humanized antibody and the chimeric antibody were subjected to ADCC assays using the GPC3-transfected cell line SK-03 with human peripheral blood mononuclear cells used as the target and effector cells, respectively. The chimeric antibody effectively induced ^{51}Cr release from the SK-03 cells in a dose-dependent manner. The humanized GC33 killed GPC3-expressing cells as effectively as the chimeric GC33 (Fig. 2c). These results indicate that the framework CDR in the humanized GC33 preserve the native CDR conformations.

In contrast, to remove any artificial FR sequences in the humanized antibody, two-step design methods were carried out [23]. The hGC33VHc antibody was subjected to a homology search of the databases. The first half of the FR3 region (positions 66–82) of AB067018 and the last half of the FR3 region (positions 79–94) of AJ506422 showed higher similarity to humanized GC33VHc. Therefore, the humanized GC33 VII region was further engineered by introducing the alteration of Glu73 to Lys and Arg86 to Thr, which resulted in amino acid residues that matched the mosaic of the two FR3 sequences derived from AB067018 and AJ506422 (Fig. 1). This humanized antibody designed second, designated as hGC33VHh/hGC33VLh, and in which all the FRs were from naturally processed human FRs, bound to the antigen as effectively as the humanized antibody designed first (Fig. 2).

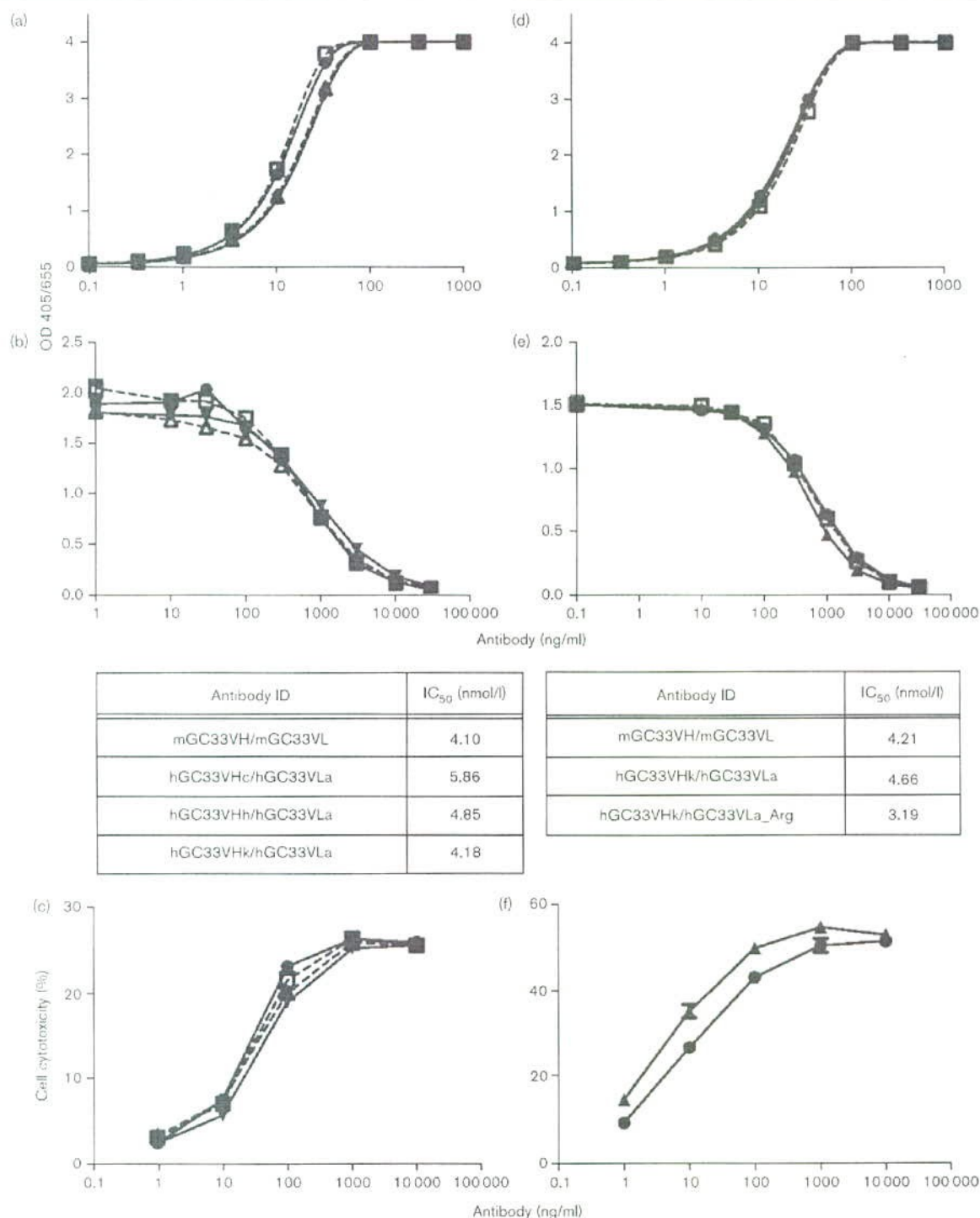
Optimization of humanized GC33

We performed further optimization of humanized GC33 using knowledge attained from earlier publications. First, we searched for amino acid residues with a risk of interfering with stable conformation. Jung *et al.* [31] reported that VII domain frameworks can be grouped into four distinct types, depending on the main chain conformation of framework 1. They established rules for the combinations of amino acids allowed at positions H6, H17, and H19 that maintain the stability of antibodies. The

buried side chain of H6, which can be either a glutamate or a glutamine residue in almost all VII sequences, affects the stability of single chain variable fragment, and Glu in position H6 requires either a proline or a glycine residue in position H19. The VII domain of murine GC33 belongs to the structural subtype III (6Q7S9A), but the substitution of Gln6 to Glu occurred in the humanized GC33. This alteration causes a deviation from the combinations of amino acids allowed and may have a negative effect on the stability of the antibody. To assess this possibility, we made the alteration of Glu6 to Gln and the resulting hGC33VHk retained 100% of the antigen binding activity and ADCC activity (Fig. 2). Next, we compared the stability of humanized antibodies using an accelerated stability study. Three versions (hGC33VHc/hGC33VLh, hGC33VHh/hGC33VLh, and hGC33VHh/hGC33VLh) of humanized GC33 were formulated at a concentration of 0.5 mg/ml and stored at 60°C for 0, 1, and 2 weeks. The fragments and aggregates of the antibodies were detected by SEC. Figure 3a shows the rates of high molecular weight species and low molecular weight species. In the case of hGC33VHc/hGC33VLh, the rate of monomer antibodies decreased to 51% after incubation at 60°C for 2 weeks. In contrast, the monomer rate for hGC33VHh/hGC33VLh was greater than for either hGC33VHc/hGC33VLh or hGC33VHh/hGC33VLh. The test samples of the accelerated stability study were further analyzed for their binding activity against sGPC3. hGC33VHh/hGC33VLh retained the binding activity after incubation at 60°C for 2 weeks, whereas hGC33VHc/hGC33VLh and hGC33VHh/hGC33VLh showed about a two-fold decrease in the antigen binding activity (Fig. 3b). We concluded that the substitution of Glu6 with Gln improved the stability of the antibody.

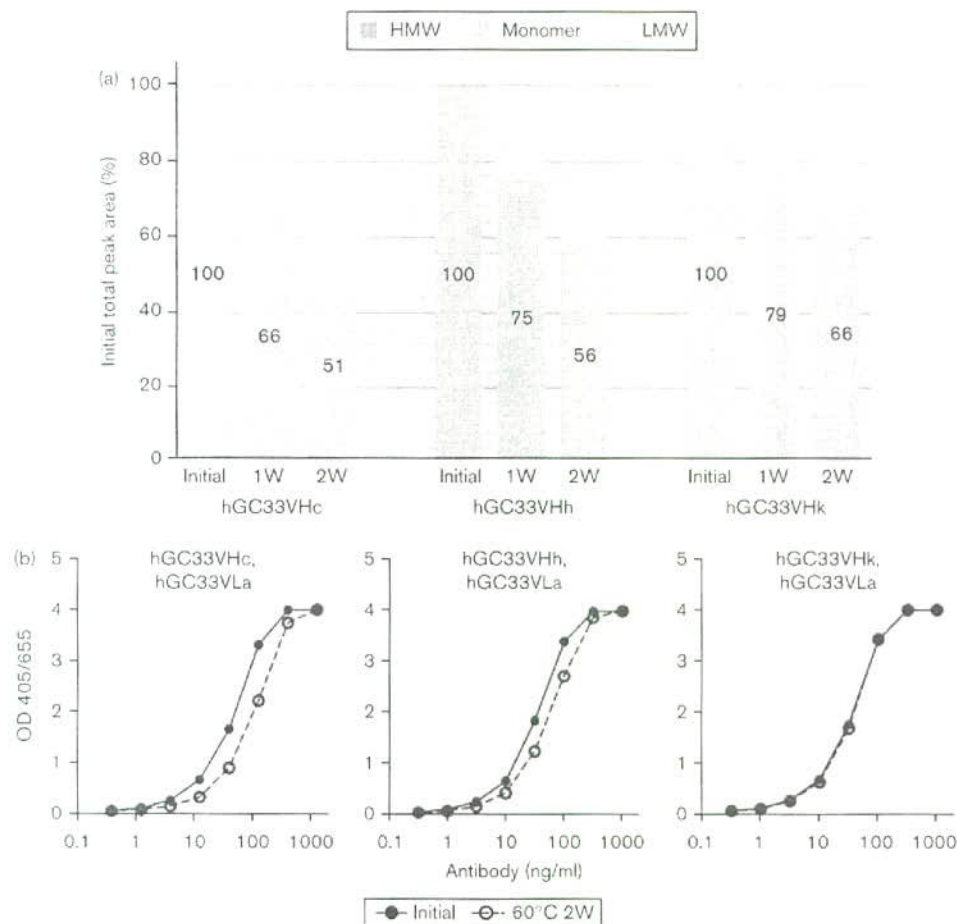
Next, we evaluated the risk of degradation from the amino acid residues of the antibodies. Nonenzymatic deamidation of Asn or Gln is a major degradation pathway of peptides and proteins and can occur spontaneously during manipulation, purification, and long-term storage. The reaction is initiated by deprotonation of the peptide bond nitrogen, followed by a nucleophilic attack on the side chain carbonyl, loss of ammonia, and the formation of succinimide. Succinimide is unstable and is hydrolyzed into a mixture of two products: isoaspartate and Asp [32]. Deamidation can cause structurally and biologically important alterations in proteins through the introduction of an unfavorable negative charge. Some mAbs have been reported to lose activity because of deamidation or isomerization in the CDR regions [33]. With regard to the primary sequence, deamidation rates depend on the amino acid residues adjacent to Asn in the peptide chain. Ser and Gly are found to be the most destabilizing C-terminal amino acids for the deamidation of Asn [34]. The humanized GC33 contains three Asn–Gly sequences. One of these sequences, Asn28 of the VL, is

Fig. 2



Analysis of chimeric and humanized GC33. (a and d) Binding of antibodies to soluble human glypican 3 (sGPC3) as measured by enzyme-linked immunosorbent assay. (b and e) Competitive binding assay measuring the ability of humanized and chimeric GC33 to compete with biotin-labeled murine GC33. The IC₅₀ values are shown. (c and f) Antibody dependent cellular cytotoxicity. ⁵¹Cr-labeled SK-03 was incubated with human peripheral blood mononuclear cells at an E:T ratio of 50:1 in the presence of the indicated concentration of the antibody. (a-c): hGC33VHc/hGC33VL (▲), hGC33VHh/hGC33VL (●), mGC33VH/mGC33VL (□). (d-f): hGC33VHk/hGC33VL (●), hGC33VHk/hGC33VL_Arg (▲), mGC33VH/mGC33VL (□). OD, optical density; VH, heavy chain; VL, light chain.

Fig. 3



(a) Accelerated stability test of humanized GC33. Percentage of species with high molecular weight (HMW), low molecular weight (LMW), and monomer antibodies calculated from the peak of the size-exclusion chromatography of humanized antibodies (hGC33VHc/hGC33VLc, hGC33VHh/hGC33VLh, hGC33VHk/hGC33VLk) over 2 weeks of storage at 60°C, at 0.5 mg/ml formulated in 20 mmol/l sodium acetate, and 150 mmol/l NaCl at pH 6.0. (b) Binding activity of humanized GC33 after the accelerated stability test. (●): Binding activity after incubation at 4°C. (○): Binding activity after incubation at 60°C. OD, optical density; VH, heavy chain; VL, light chain.

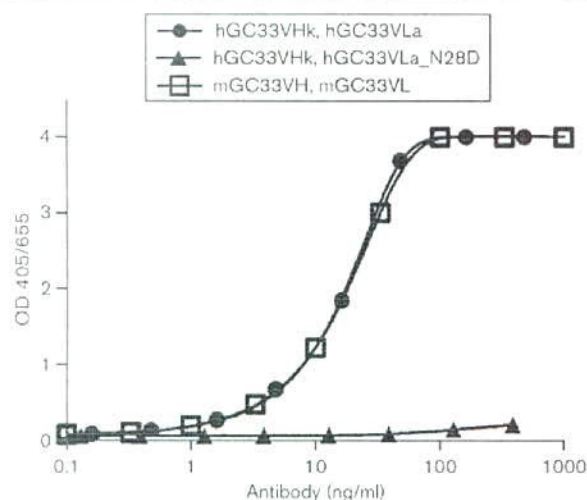
in the binding region (CDR1), whereas the other two are in the immunoglobulin constant region.

To study the effect of deamidation on the activity of antibodies, we replaced Asn28 with Asp by site-directed mutagenesis (N28D), and found that mutant N28D greatly reduced the binding affinity to the antigen (Fig. 4). We concluded that Asn28 has a risk of deamidation that would result in the loss of binding activity. Site-directed mutagenesis to improve the stability of the mAbs by avoiding deamidation at 'hot-spot' residues such as Asn28 is not always feasible because this method may cause significant reduction in mAb potency. Therefore, we substituted the neighboring Gly29 by other amino acids to get rid of the deamidation. The mutant hGC33VLc_Arg, in which Gly29 of the VL

was replaced with Arg, bound to the antigen as effectively as the chimeric antibodies (Fig. 2d and e) and showed ADCC activity as strong and as effective as hGC33VLc (Fig. 2f).

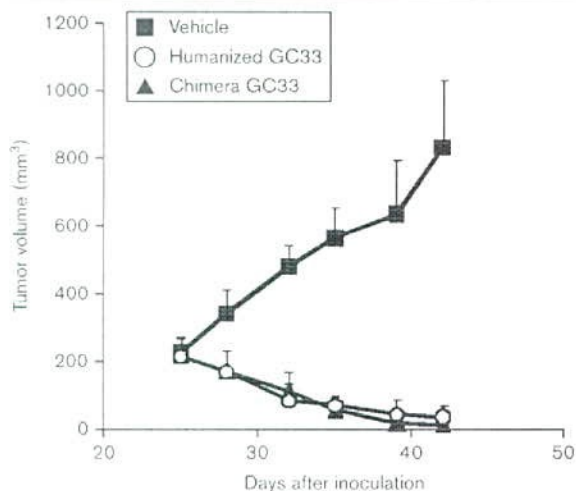
The final version of humanized GC33 (hGC33VHk, hGC33VLc_Arg) specifically bound to GPC3 and induced ADCC as effectively as chimeric GC33. The binding affinity of humanized GC33 was determined by Scatchard analysis, in which increasing concentrations of radioactive iodine-labeled antibodies were incubated with human hepatocellular carcinoma HuH-7 cells. The dissociation constant (or K_d value) of humanized GC33 for GPC3 was calculated to be 0.673 ± 0.024 nmol/l (mean $\pm$ standard deviation) from three independent experiments. When administered to the severe combined immunodeficient

Fig. 4



Antigen binding assays for humanized GC33 and N38D mutant that mimic the effect of deamidation. Serial dilutions of chimeric GC33 (mGC33VH/mGC33VL), humanized GC33 (hGC33VHk/hGC33VLα), and the deamidation mimic mutant (hGC33VHk/hGC33VLα_N28D) were added to soluble human glypican 3-coated wells. Bound antibodies were detected with alkaline phosphatase-labeled anti-human Ig light chain κ . OD, optical density; VH, heavy chain; VL, light chain.

Fig. 5



Antitumor activity of humanized GC33 and chimeric GC33 in HepG2 xenograft models. Severe combined immunodeficient mice were inoculated subcutaneously with 5×10^6 cells of HepG2. When the tumors reached an average volume of 200 mm³, the mice were administered 5 mg/kg of humanized GC33 (○), chimera GC33 (▲) or only PBS (vehicle, ■). GC33 and PBS were administered once a week for 3 weeks. Tumor volume is indicated. Each data point is the mean value of five animals; bars, standard deviation. Arrows indicate the days on which GC33 was administered.

mice transplanted with HepG2 cells, 5 mg/kg of the humanized GC33 significantly inhibited tumor growth. The antitumor activity of the humanized GC33 was almost the same as chimeric GC33 (Fig. 5).

Discussion

To reduce the immunogenicity of murine mAbs, humanized antibodies have been developed by grafting the CDRs of a mouse antibody onto the corresponding regions of a human antibody. However, the transfer of murine CDRs alone usually results in a significant loss of antigen binding activity because certain FRs are critical for the preservation of the CDR conformation. A number of alterations in human FRs are required to recreate a functional antigen-binding site. The number of changes in the human FRs depends heavily upon human FR selection and should be minimized to avoid immunogenicity in humans. As a general approach, key residues in FRs are identified by comparing canonical structures [35,36] with packing residues and consensus amino acid sequences for the subgroups of the variable regions [30,37] and by carefully examining a structural model of the variable regions. In many cases, however, a random mutation approach is still required.

To assist in this process, we have devised a useful method based on the construction of mouse-human hybrid variable regions [22]. In the case of the humanized GC33, the use of a hybrid of variable region enabled us to identify the importance of the Ile69 and Ala93 in FR3, which was found to be critical for FR residues to yield 100% antigen binding activity. To remove the artificial FR sequences in the humanized antibody, we have also devised a useful method based on redesigning the modified FRs [23]. In the case of the humanized GC33, the FR3 of hGC33VHc, for which two amino acid substitutions were required for optimal antigen bindings, was redesigned. We constructed hGC33VHh, which comprised all the sequences found in the naturally processed human FRs. Although naturally processed human variable regions have somatic mutations with respect to the germ-line segments, their immunogenicity is apparently less than that of artificially generated FR sequences.

In addition, using a knowledge-based approach, we optimized the stability of humanized GC33. From data of earlier humanized antibodies obtained in-house and from public sources, we realized that certain residues were important for stability and therefore selected some amino acid residues for modification. According to Jung *et al.* [31], the marked structural variability of the VH framework 1 region is caused by three residues (H6, H7, H9), and deviation from the allowed combinations of these amino acids may lead to severely reduced stability. They generated six mutants of single chain variable fragment forms, and the thermodynamic stability was

compared after equilibrium denaturation with urea. In the case of GC33, we found the rules can be applied to the stability of IgG forms. Our data may not directly indicate long-term stability with normal storage conditions; however, we can conclude that substitution of amino acids is effective in improving the stability of antibodies.

Next, we substituted the amino acid residue at risk of deamidation in CDR1 of the VL. We found that the substitution of Asn28 with Asp, which mimics deamidation, greatly reduced the binding activity to the antigen. In this study, we successfully avoided the risk of the deamidation of Asn28 by substituting the neighboring Gly with Arg. Site-directed mutagenesis of Asn28 is not always feasible because this method may cause significant reduction in mAb potency. However, hGC33VL_{Arg} retained the binding activity and the ADCC activity (Fig. 2). The parental humanized GC33 (hGC33VHk/hGC33VL_{Arg}) did not cause a reduction in the binding activity even at 60°C for 2 weeks, so we have yet to prove whether this alteration (Gly29 to Arg) can reduce the rate of deamidation.

Humanized anti-GPC3 antibodies are expected to be a potent immunotherapy for HCC patients. The antitumor activity and immunogenicity of this humanized antibody in human patients will be tested in clinical studies.

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Identification and Multidimensional Optimization of an Asymmetric Bispecific IgG Antibody Mimicking the Function of Factor VIII Cofactor Activity

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Abstract

In hemophilia A, routine prophylaxis with exogenous factor VIII (FVIII) requires frequent intravenous injections and can lead to the development of anti-FVIII alloantibodies (FVIII inhibitors). To overcome these drawbacks, we screened asymmetric bispecific IgG antibodies to factor IXa (FIXa) and factor X (FX), mimicking the FVIII cofactor function. Since the therapeutic potential of the lead bispecific antibody was marginal, FVIII-mimetic activity was improved by modifying its binding properties to FIXa and FX, and the pharmacokinetics was improved by engineering the charge properties of the variable region. Difficulties in manufacturing the bispecific antibody were overcome by identifying a common light chain for the anti-FIXa and anti-FX heavy chains through framework/complementarity determining region shuffling, and by pl engineering of the two heavy chains to facilitate ion exchange chromatographic purification of the bispecific antibody from the mixture of byproducts. Engineering to overcome low solubility and deamidation was also performed. The multidimensionally optimized bispecific antibody hBS910 exhibited potent FVIII-mimetic activity in human FVIII-deficient plasma, and had a half-life of 3 weeks and high subcutaneous bioavailability in cynomolgus monkeys. Importantly, the activity of hBS910 was not affected by FVIII inhibitors, while anti-hBS910 antibodies did not inhibit FVIII activity, allowing the use of hBS910 without considering the development or presence of FVIII inhibitors. Furthermore, hBS910 could be purified on a large manufacturing scale and formulated into a subcutaneously injectable liquid formulation for clinical use. These features of hBS910 enable routine prophylaxis by subcutaneous delivery at a long dosing interval without considering the development or presence of FVIII inhibitors. We expect that hBS910 (investigational drug name: ACE910) will provide significant benefit for severe hemophilia A patients.

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Introduction

Hemophilia A is caused by an X-linked inherited dysfunction of coagulation factor VIII (FVIII). Patients with severe hemophilia A, who have plasma FVIII levels of less than 1% of normal, typically experience bleeding events several times a month [1]. Routine supplementation with exogenous human FVIII to maintain FVIII levels at 1% of normal or above is effective for reducing joint bleeding events and improving joint status and health-related quality of life in hemophilia A patients [2]. However, there are two major drawbacks to this prophylactic usage of exogenous FVIII. The first drawback is the necessity of frequent intravenous administration: three intravenous injections weekly of FVIII are necessary because of its low subcutaneous bioavailability and its short plasma half-life [3,4,5]. The second drawback is the

development of inhibitory anti-FVIII alloantibodies, known as "inhibitors" [6]. Once FVIII inhibitors have developed, routine supplementation with exogenous FVIII will be no longer effective and the usage of exogenous FVIII for treating on-going bleeds is restricted. In such cases, alternative agents, such as activated factor VII and activated prothrombin complex concentrate, which are more expensive and have less stable hemostatic effects, need to be used to control bleeding [7,8]. Therefore, a new agent that resolves these drawbacks of exogenous FVIII is awaited in the field of the bleeding prophylaxis of severe hemophilia A.

Monoclonal antibodies have become an important therapeutic option in numerous diseases and are expected to play a greater role in the future of disease treatment [9,10]. Various monoclonal antibodies have been generated [11]; these not only include those with antagonistic activity but also those with agonistic activity [12].

catalytic activity [13], and allosteric activity [14]. Antibody engineering technologies to generate bispecific antibodies have been extensively studied due to the huge potential of these antibodies for therapeutic applications [15]. Bispecific antibodies can be applied to simultaneously target two disease related antigens, retarget effector cells against the target cell [16], and coligate two different antigens on the same cell [17].

FVIII is cleaved by thrombin or factor Xa (FXa), and the resultant factor VIIIa (FVIIIa) presents a heterotrimeric structure consisting of the A1 subunit, the A2 subunit, and the light chain [18]. Simultaneous binding of FVIIIa to FIXa and FX by the light chain and the A2 subunit, and by the A1 subunit, respectively, contributes to FVIII cofactor activity which places FIXa and FX into proximity, and also allosterically enhances the catalytic rate constant of FIXa [19,20,21,22,23] (Fig. 1A).

Considering this function of FVIII and the versatility of antibodies, we hypothesized that a bispecific antibody recognizing FIXa with one arm and FX with the other arm could mimic the FVIII cofactor activity by placing FIXa and FX in spatially appropriate positions, and by allosterically enhancing the catalytic activity of FIXa (Fig. 1B) [23]. We have recently reported a recombinant humanized bispecific antibody to FIXa and FX, termed hBS23, which exerted coagulation activity in FVIII-deficient plasma, even in the presence of FVIII inhibitors, and showed *in vivo* hemostatic activity in a cynomolgus monkey model of acquired hemophilia A, and had high subcutaneous bio-

availability and a 2-week half-life in cynomolgus monkey [23]. Although the pharmacological concept of FVIII-mimetic bispecific antibody was clearly demonstrated in this report, the detail of this anti-FIXa/FX asymmetric bispecific IgG antibody identification was not described, and moreover, it required further optimization in several ways before the clinical use of such an agent in humans.

For therapeutic development, optimization of the bispecific antibody by molecular engineering to enable large-scale manufacturing of the bispecific antibody at clinical grade would be required. Although a variety of molecular formats for bispecific antibodies have been studied, including single-chain diabody, tandem scFv, IgG-scFv, DVD-Ig, CrossMab, and dual-binding Fab [24], we selected an asymmetric bispecific IgG format because it is the only format that can recognize FIXa and FX with each arm and has a long half-life and a native IgG structure. However, recombinant production of this format is challenging in comparison to the other formats because it consists of two different heavy chains and two different light chains which would result in the secretion of a mixture of ten different combinations of heavy and light chains [25]. Purification of one desired bispecific antibody from a mixture including nine miss-paired byproducts is nearly impossible. Engineering technologies to resolve such a difficulty have been previously reported. First, identification of a common light chain by phage display as the partner of the two heavy chains can reduce the number of heavy and light combinations to three: one heterodimeric bispecific antibody and two homodimeric

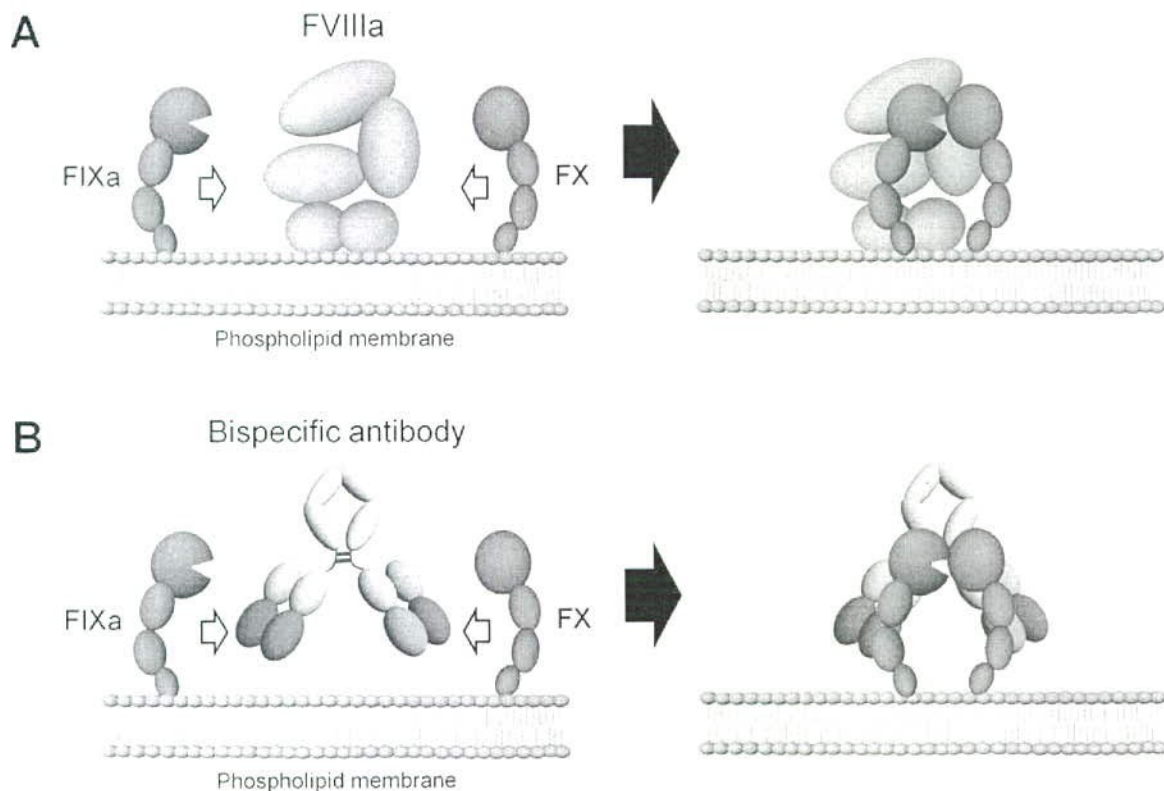


Figure 1. Schematic illustrations of cofactor actions of FVIIIa and a bispecific antibody promoting the interaction between FIXa and FX. (A) FVIIIa forms a complex with FIXa and supports the interaction between FIXa and FX through its binding ability to both factors on the phospholipid membrane. (B) A bispecific antibody binding to FIXa and FX would promote the interaction between FIXa and FX and exert FVIII-mimetic activity on the phospholipid membrane.

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monospecific antibodies [26]. However, selection of a common light chain with potent FVIII-mimetic activity based on the binding affinity by phage display was not feasible for our bispecific antibody, because higher binding affinities was considered not to lead to higher activity. To date, alternative approach to obtain common light chain has not been reported. Second, engineering the C_H3 domain to facilitate Fc heterodimerization can minimize the amount of homodimeric byproducts [25,26]. Nevertheless, the efficiency of heterodimerization is not complete; therefore, a small amount of homodimeric byproduct is formed, which needs to be removed in the downstream purification process. However, because the biophysical properties of the homodimeric byproducts are often similar to those of the target bispecific antibody, purifying the target bispecific antibody on a large scale for clinical applications is still challenging. To date, technologies to address this issue have not been reported. Therefore, improving the manufacturability of this type of FVIII-mimetic antibody by molecular engineering is required for therapeutic application.

Moreover, for maximizing the therapeutic potential of FVIII-mimetic bispecific antibody, optimization to increase the FVIII-mimetic activity of the bispecific antibody, prolong the half-life, improve the physicochemical properties of the antibody and reduce the immunogenicity of the humanized antibody would be required. This would enable more effective and long-term prophylaxis with stronger hemostatic effect for hemophilia A patients by a subcutaneous formulation with a longer dosing interval.

In this paper, we report molecular identification and multidimensional optimization of a FVIII-mimetic bispecific antibody which can be used for clinical application. At the start of this study, we examined bispecific combinations of large number of monoclonal anti-FIXa and anti-FX antibodies, and identified the lead anti-FIXa/FX bispecific IgG antibody having FVIII-mimetic activity. Then, this lead bispecific antibody was subjected to multidimensional optimization processes [27] to improve both its therapeutic potential and manufacturability. We successfully generated a humanized bispecific IgG antibody having sufficient FVIII-mimetic activity for prophylactic use even in the presence of FVIII inhibitors, high subcutaneous bioavailability with an approximately 3-week plasma half-life in cynomolgus monkeys, and minimal immunogenicity risk. In addition, molecular engineering enabled purification on a large manufacturing scale and formulation into a liquid formulation of 150 mg/mL for subcutaneous delivery. We expect that this anti-FIXa/FX bispecific antibody mimicking FVIII cofactor activity will provide significant benefit for managing bleeding events in severe hemophilia A patients.

Results

Research Flow of Identification and Multidimensional Optimization of Lead FVIII-mimetic anti-FIXa/FX Bispecific Antibody

Figure 2 shows the flow of the screening process to identify the lead bispecific antibody with a common light chain (BS15). BS15 was generated by combinatorial screening of bispecific antibodies composed of anti-FIXa and anti-FX antibodies derived from immunization, followed by screening of common light chains and then framework/complementarity determining region (FR/CDR) shuffling.

Figure 3 represents the multidimensional optimization flow to generate the bispecific antibody with the most appropriate properties for clinical application (hBS910) from the lead bispecific antibody (BS15). BS15 was firstly humanized to generate hBS1,

followed by engineering to improve FVIII-mimetic activity (hBS106), improve pharmacokinetics (hBS128 and hBS228), enable purification of target bispecific antibody (hBS366 and hBS376), improve solubility (hBS560), remove deamidation site (hBS660), and reduce immunogenicity risk (deimmunization) to generate a multidimensionally optimized bispecific antibody (hBS910). Through this multidimensional optimization process, the numbers of variable region variants that we have generated for anti-FIXa heavy chain, anti-FX heavy chain and common light chain were approximately 500, 300 and 400, respectively, and the number of bispecific IgG antibodies that we have prepared and evaluated is approximately 2,400. Supplementary table S1 represents the number of mutations which were introduced into hBS1 to generate bispecific antibodies described in this report.

Identification of Lead Anti-FIXa/FX Bispecific Antibody with FVIII-mimetic Activity

Approximately 200 monoclonal antibodies against FIXa or FX were obtained from animals immunized with either human FIXa or FX. Approximately 40,000 bispecific IgG antibodies that comprised different combinations of anti-FIXa and anti-FX antibodies in each arm were expressed. Although the expression product of two different heavy chain and two different light chain genes consists of a mixture of ten different species with different heavy and light chain combinations, including the one desired bispecific antibody and the nine miss-paired antibodies (miss-paired antibody includes two homodimeric antibodies with a correct heavy and light chain pair), Fc heterodimerization mutations would theoretically enable expression of an antibody mixture containing at least approximately 20% of the target bispecific antibody (see methods for detail) [25]. A total number of 94 bispecific antibodies, or combinations of anti-FIXa heavy chain and anti-FX heavy chain, which had FVIII-mimetic activity were successfully identified by an enzymatic assay. Heavy chain combinations were selected from the point of high FVIII-mimetic activity, not from the point of the similarity of the cognate light chain.

Next, in order to identify a common light chain for the different heavy chains to FIXa and FX, the selected heavy chain combinations were expressed with either one of cognate light chains. Out of 188 light chain commonized bispecific antibodies, the most potent one, termed c1, which consisted of rat anti-FIXa V_H and mouse anti-FX V_H chimerized with human IgG₄ and rat anti-FIXa V_L, chimerized with human κ (c1L), was selected for the next step.

Finally, in order to design a more potent common light chain, we performed FR/CDR shuffling. Since the cognate light chain for the selected anti-FX V_H (c2L) was not effective as a common light chain at all (data not shown), we tried to seek another effective common light chain with a high homology to c2L. From our antibody source, we identified a light chain (c3L) whose CDR sequence had >85% homology to that of c2L, and found out that it was effective as a common light chain for the selected two heavy chains. Therefore, we decided to use c3L for FR/CDR shuffling, too. Then, CDRs of c1L, c2L and c3L were shuffled among each other and grafted onto the FRs of c1L and c3L to generate twenty four light chain variants (supplementary Fig. S2A). Twenty four light chain variants were expressed with the two heavy chains, and the most potent common light chain, BS15L, a mouse-rat hybrid V_L chimerized with human κ, was identified (supplementary Fig. S2B). Thus, the light chain commonized bispecific antibody with BS15L was selected as the lead chimeric bispecific IgG antibody (termed BS15).

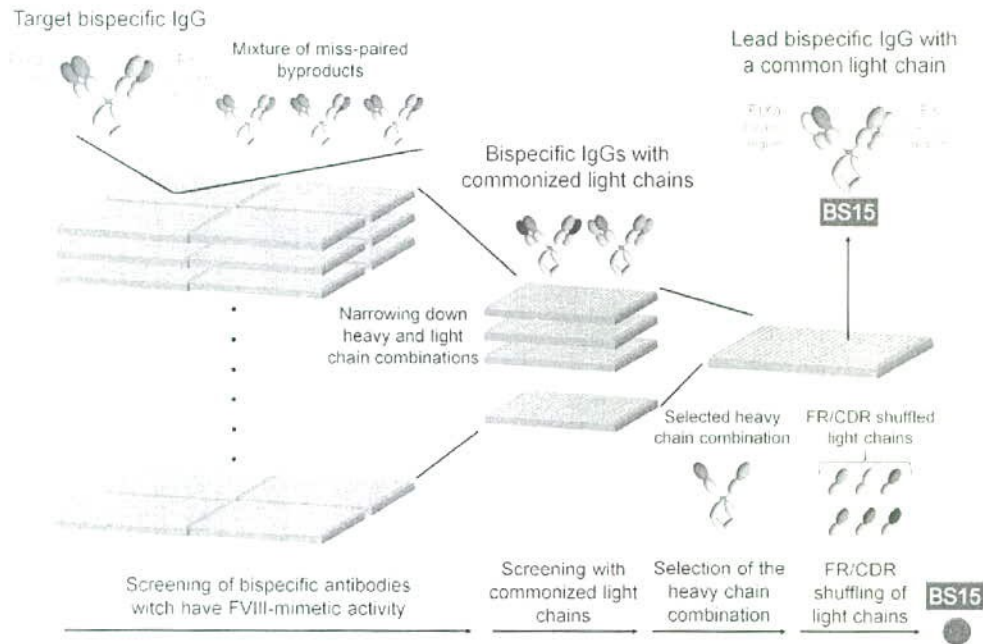


Figure 2. Flow of process to identify the lead bispecific antibody (BS15). BS15 was identified by combinatorial screening of bispecific antibodies, followed by screening of common light chains and then FR/CDR shuffling.
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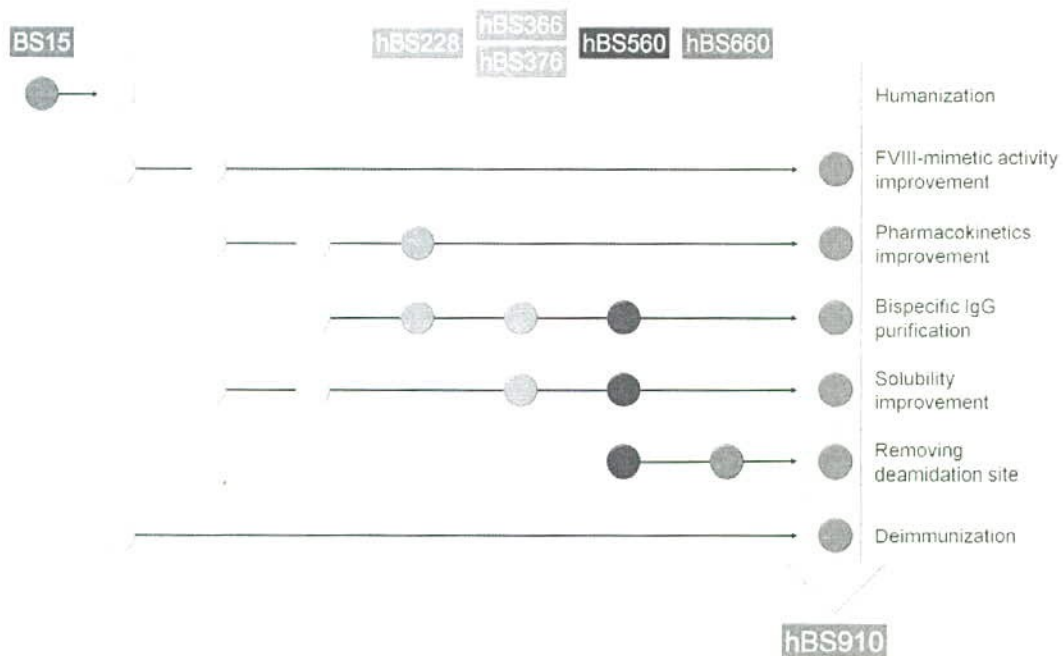


Figure 3. Multidimensional optimization flow to generate the bispecific antibody with most appropriate properties (hBS910). hBS910 was generated through multidimensional optimization with various antibody engineering technologies.
doi:10.1371/journal.pone.0057479.g003

Humanization of Lead Chimeric Bispecific Antibody

The lead chimeric bispecific IgG antibody, BS15, was subjected to humanization. CDRs of the anti-FIXa heavy chain, the anti-FX heavy chain and the common light chain were grafted onto homologous human antibody FRs, which were the FRs of V_H3, V_H1 and V_κ1 subfamilies respectively, by using a conventional CDR grafting approach [28]. A humanized bispecific IgG₄ antibody, termed hBS1, was successfully generated while maintaining FVIII-mimetic activity (supplementary Fig. S3).

Improving FVIII-mimetic Activity of the Bispecific Antibody

Although the humanized bispecific antibody hBS1 enhanced FX activation dose-dependently, demonstrating FVIII-mimetic activity, its therapeutic potential was marginal and its FVIII-mimetic activity needed to be improved for therapeutic application. Therefore, we explored mutations in the CDRs of hBS1 to improve the FVIII-mimetic activity, and we identified several effective mutations in the CDRs of the three chains. Following extensive studies to identify effective combinations of mutations that additively or synergistically improved FVIII-mimetic activity, we successfully generated hBS106. hBS106 demonstrated marked improvement of FVIII-mimetic activity over hBS1, including maximum activity, in the enzymatic assay (Fig. 4A).

During the course of subsequent optimization of hBS106 from the point of other aspects, FVIII-mimetic activity was monitored for each mutation so that the mutation would not compromise the activity. Moreover, further screening for mutations to further improve the activity was performed in parallel with other optimizations. Finally, we successfully generated hBS910, whose activity was even higher than that of hBS106 (Fig. 4A).

Improving Pharmacokinetics of the Bispecific Antibody

To assess the pharmacokinetics of hBS106, the variant with improved FVIII-mimetic activity, the time course of the plasma concentration of this antibody (Fig. 4B) and its pharmacokinetic parameters (supplementary Table S2) were determined in mice. Clearance of hBS106 (67 mL/day/kg) was unexpectedly larger than the clearance of human IgG, antibody reported in mice after subcutaneous injection (3–20 mL/day/kg) [29].

A homology model of hBS106 was used to explore the molecular features responsible for the poor pharmacokinetics, and a positive charge cluster was identified on the surface of Fv of the anti-FIXa arm (supplementary Fig. S4). To remove this cluster, we initially attempted subjecting lysine and arginine residues in the cluster to mutagenesis. However, these residues were found to be indispensable for the FVIII-mimetic activity (data not shown). Therefore, we explored the introduction of negatively charged residues near the cluster to neutralize the positive charge, and we identified a Tyr30Glu mutation in the common light chain that achieved this without any reduction in FVIII-mimetic activity. With hBS128, a variant of hBS106 with the single Tyr30Glu mutation, we observed improved plasma concentration and an approximately 4-fold improvement in the clearance compared to hBS106. Furthermore, the isoelectric point (pI) of hBS128 was lowered by introducing multiple mutations in the variable regions. hBS228, a variant of hBS128 with lowered pI, demonstrated further improved plasma concentration and approximately 2-fold improvement in clearance compared to hBS128 (Fig. 4B, supplementary Table S2). During the course of subsequent optimization of hBS228, we constantly made an effort to further improve the pharmacokinetics of the bispecific antibody.

Isoelectric Point Engineering to Facilitate Purification of the Target Bispecific Antibody

Having a common light chain reduces the number of pairs of heavy and light chains to three, and engineering the C_H3 domain enables preferential secretion of heterodimerized heavy chains. However, it is still difficult to completely prevent miss-paired homodimerization in large-scale production. Therefore, a downstream purification process to remove homodimeric byproducts is essential for pharmaceutical development. Ion exchange chromatography (IEC) is the major purification process by which to remove impurities after Protein A purification. The retention of IgG antibodies by IEC is determined by the electrostatic charge of the antibody molecule, which can be measured as its pI. Therefore, the pIs of hBS128 and hBS228, variants with improved pharmacokinetics, and pIs of their homodimeric byproducts were determined by cIEF (Fig. 5A). For both hBS128 and hBS228, the pIs of the bispecific antibody and the homodimeric byproducts

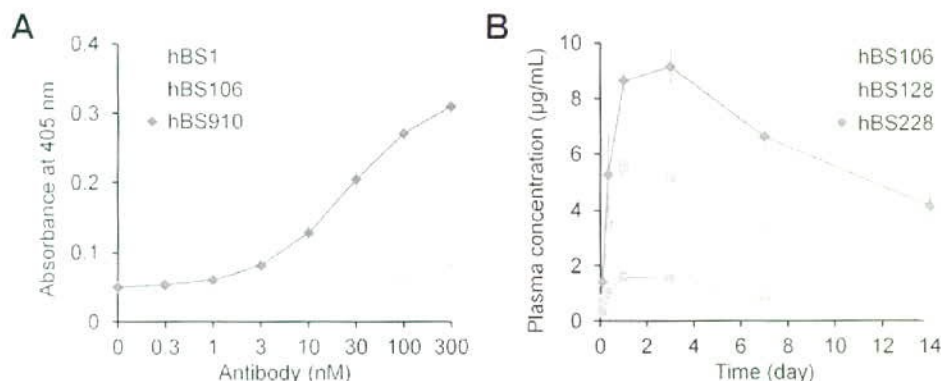


Figure 4. Improvement of therapeutic potential of the bispecific antibody. (A) Improving FVIII-mimetic activity of the bispecific antibody. Effect of hBS1 (circles), hBS106 (squares), and hBS910 (diamonds) on FX activation in the presence of FIXa, FX, and synthetic phospholipid is shown. The Y-axis indicates the absorbance at 405 nm of the chromogenic substrate assay (in many cases, the bars depicting s.d. are shorter than the height of the symbols). (B) Improving pharmacokinetics of the bispecific antibody. Time profiles of plasma concentration of hBS106 (circles), hBS128 (squares), and hBS228 (diamonds) in mice after subcutaneous injection at a dose of 1 mg/kg are shown. All the data were collected in triplicate and are expressed as mean $\pm$ s.d.

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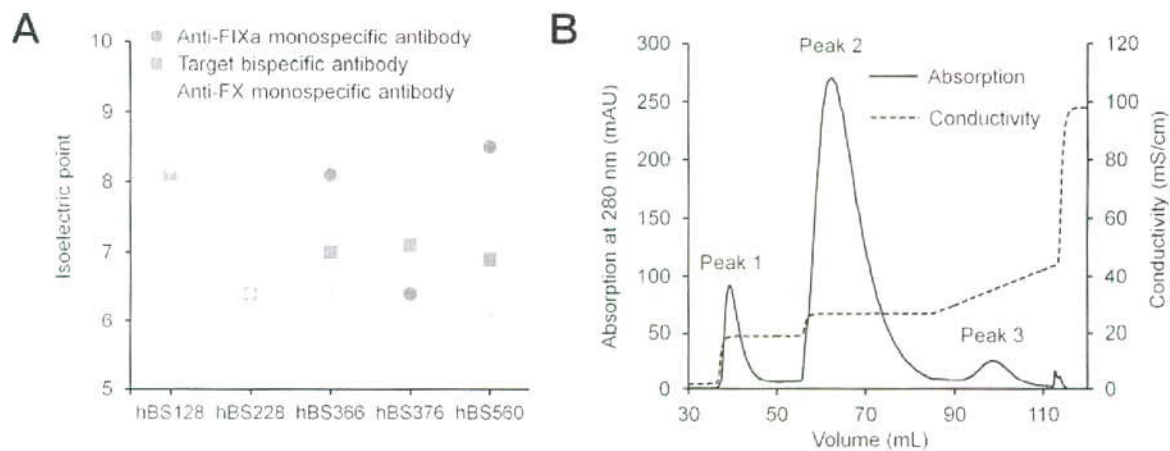


Figure 5. Isoelectric point engineering to facilitate purification of the target bispecific antibody. (A) Isoelectric points of target bispecific (squares) antibodies and homodimeric byproducts (anti-FIXa monospecific antibodies (circles) and anti-FX monospecific antibodies (diamonds)) determined by cIEP. (B) Cation exchange purification chromatogram of the target bispecific antibody of hBS560 from its homodimeric byproducts with step-wise elution with different NaCl concentrations. Peak 1, anti-FX homodimeric antibody; Peak 2, target bispecific antibody; Peak 3, anti-FIXa homodimeric antibody. Each peak area of peak 1, peak 2 and peak 3 was 9.9%, 85.7% and 4.4%, respectively. doi:10.1371/journal.pone.0057479.g005

were very close to each other, indicating that purification of the bispecific antibody hBS128 or hBS228 from the mixture of homodimeric byproducts is not feasible.

To facilitate the purification of the bispecific antibody, we implemented pI engineering into either one of the heavy chain variable regions to increase the pI difference between the bispecific antibody and the homodimeric byproducts. We successfully generated two variants, hBS366 (pI of the anti-FX heavy chain is lowered) and hBS376 (pI of the anti-FIXa heavy chain is lowered), in which FVIII-mimetic activity was maintained. The difference in the pI between the bispecific antibody and homodimeric byproducts markedly increased (Fig. 5A).

hBS366 was further optimized from the point of solubility (detail in the next paragraph), to generate hBS560. The target molecular form of hBS560 (heterodimeric bispecific antibody) was well separated from the two homodimeric byproducts by using cation exchange chromatography with step-wise elution (Fig. 5B). During the course of subsequent optimization of hBS560, the pI difference between the two heavy chains was carefully maintained. The multidimensionally optimized variant hBS910 was capable of being purified on a large production scale (2500-liter fermentation).

Improving Solubility Properties of the Bispecific Antibody

hBS106, a variant with improved FVIII-mimetic activity, had unexpectedly low solubility, exhibiting either precipitation or liquid liquid phase separation [30] (supplementary Fig. S5). This lack of solubility was partially due to the positive charge cluster, since the Tyr30Glu mutation described above markedly improved the solubility. However, for hBS376, the variant whose anti-FIXa heavy chain pI was lowered, at concentrations of 4 and 40 mg/mL, precipitation and phase separation still occurred in phosphate buffer of pH 5.5 to 7.0 and NaCl of 100 mM or less (Fig. 6A).

To improve the solubility of hBS376, mutations in the variable regions of hBS376 were explored. Several effective mutations including substitutions of hydrophobic residues into hydrophilic residues were identified, and their combinations successfully generated hBS560. Precipitation and phase separation of

hBS560, the variant with improved solubility, was markedly suppressed and occurred only below 40 mM NaCl (Fig. 6A). The solubility of hBS560 was more than 100 mg/mL. During the course of subsequent optimization of hBS560, the effect of mutations on the solubility was constantly monitored and we made further efforts on improving the solubility. We successfully generated hBS910, a multidimensionally optimized variant, which did not exhibit precipitation or phase separation under the conditions tested (Fig. 6A). Furthermore, hBS910 could be concentrated up to at least 200 mg/mL.

Removing Deamidation Site in the CDR of the Bispecific Antibody

An accelerated stability study revealed that hBS560 exhibited asparagine deamidation in the third complementarity-determining region of the heavy chain (HCDR3) (Asn99) of the anti-FIXa arm after incubation at 40 °C for 2 weeks, as shown by the increase in the acidic peak in the cation exchange chromatography analysis (Fig. 6B) [31], and reduction of FVIII-mimetic activity was observed (data not shown). A single mutation of Asn99 to another amino acid to remove this deamidation site was not feasible due to the loss of FVIII-mimetic activity and solubility. Subsequently, a double mutation was explored, and hBS660, in which a His98Arg and Asn99Glu double mutation was introduced to hBS560, was identified to maintain the FVIII-mimetic activity. hBS660 showed no increase in the acidic peak after incubation, demonstrating that the deamidation site was removed (Fig. 6B). During the course of subsequent optimization of hBS660, deamidation was carefully monitored, and successfully generated hBS910, a multidimensionally optimized variant, which did not exhibit deamidation.

Deimmunization of Humanized Bispecific Antibody by Removing T-cell Epitopes

During the course of multidimensional optimization of hBS1, the effects of each mutation on immunogenicity were evaluated by Epibase (Lonza), an *in silico* T-cell epitope prediction system [32]. Any mutation that was predicted to increase the potential

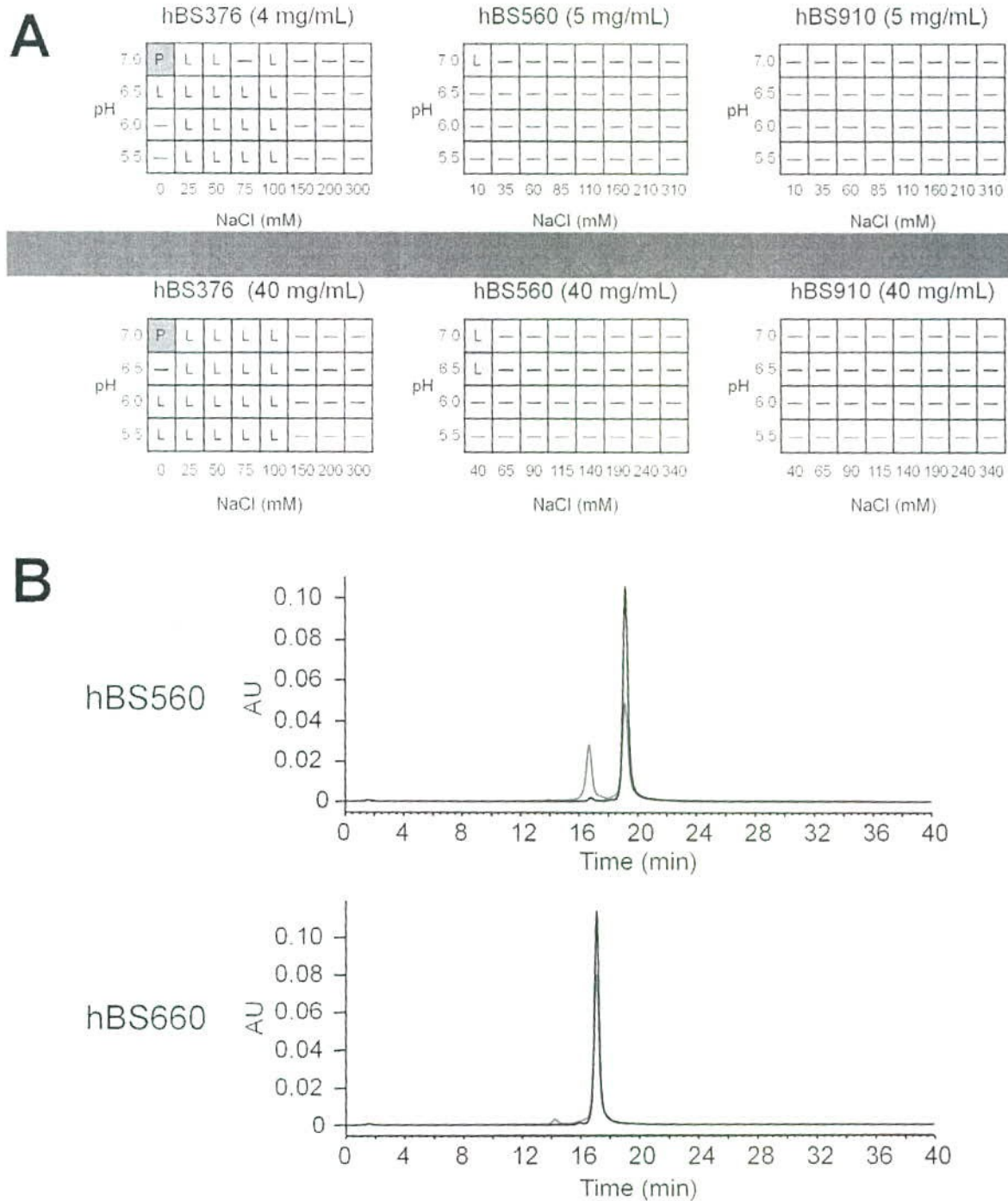


Figure 6. Improvement of pharmaceutical properties of bispecific antibodies. (A) Antibody solution profiles of hBS376 and hBS560 at different antibody concentrations, pH, and NaCl concentrations. The antibody solution under each condition was photographed and the state determined (P, precipitation; L, liquid-liquid phase separation; —, clear liquid). (B) Cation exchange chromatography of hBS560 and hBS660 before (black) and after incubation at 40°C for 2 weeks (red). Acidic peak indicating deamidation at HCDR3 increased after incubation at 40°C for 2 weeks for hBS560. No marked increase of acidic peak was observed for hBS660.
doi:10.1371/journal.pone.0057479.g006

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immunogenicity risk was avoided as much as possible. Simultaneously, to generate a bispecific antibody with minimum immunogenicity risk, any mutation that was predicted to decrease the potential immunogenicity risk by reducing the number of T-cell epitopes was screened.

The immunogenicity risk score of hBS910, the multidimensionally optimized variant, was markedly decreased compared to the lead chimeric antibody (BS15) and its humanized version (hBS1), and was comparable to trastuzumab and palivizumab which are non-immunogenic in clinical (supplementary Fig. S6A). Moreover, EpiMatrix (EpiVax), another *in silico* immunogenicity prediction system [33,34], also predicted that the sequence of hBS910 was minimally immunogenic (supplementary Fig. S6B).

Therapeutic Potential of the Multidimensionally Optimized Bispecific Antibody, hBS910

To examine the therapeutic potential of hBS910, its FVIII-mimetic activity was compared with human FVIII by using thrombin generation assay (TGA) [23,35,36] in commercially available human FVIII-deficient plasma which was derived from a single donor with severe hemophilia A without FVIII inhibitors (Fig. 7A). hBS910 dose-dependently increased peak height (defined as the peak concentration of free thrombin) in the same manner as recombinant human FVIII (rhFVIII). The thrombin generation activity of hBS910 was also observed even in plasma of a hemophilia A donor who has FVIII inhibitors, whereas 1 IU/mL of rhFVIII did not exhibit any effects (data not shown). On the other hand, while polyclonal anti-idiotypic antibodies to the anti-FIXa Fab or anti-FX Fab of hBS910 completely inhibited the activity of hBS910, they did not interfere with rhFVIII activity at all (data not shown).

To assess the potential for subcutaneous delivery with a long dosing interval, hBS910 was intravenously and subcutaneously administered to cynomolgus monkeys, and the time course of the antibody plasma concentration and pharmacokinetic parameters were obtained (Fig. 7B, supplementary Table S3). The subcutaneous bioavailability was sufficiently high (86%) and the plasma half-life was approximately 3 weeks. Moreover, hBS910 could be formulated into a 150 mg/mL liquid formulation for subcutaneous delivery in the clinical setting without any significant aggregation or degradation during storage.

Discussion

The lead bispecific antibody was identified from approximately 40,000 different bispecific antibodies. Bispecific antibodies meeting the criteria for FVIII cofactor activity were extremely rare (<0.3%). This seems reasonable since such a bispecific antibody requires simultaneous binding to the appropriate epitope of both FIXa and FX in order to place these two factors into a spatially appropriate position and precisely bring the catalytic site of FIXa close to the cleavage site of FX. Requirement of simultaneous binding to FIXa and FX by a single bispecific antibody was supported by the fact that only a bispecific antibody, and neither monospecific antibodies nor a mixture of them, exhibited FVIII-mimetic activity (supplementary Fig. S7).

Generally, the biological activity of antagonistic antibodies can be improved by increasing the binding affinity to the target antigen [37,38]. The biological activity of agonistic antibodies, on the other hand, is reported to be inversely correlated with the affinity to the antigen, presumably due to the necessity to dissociate from the antigen to repeatedly induce agonistic signals to the target [39]. In the case of our bispecific antibody, the antibody needs to bind to both FIXa and FX with sufficient affinity to promote the

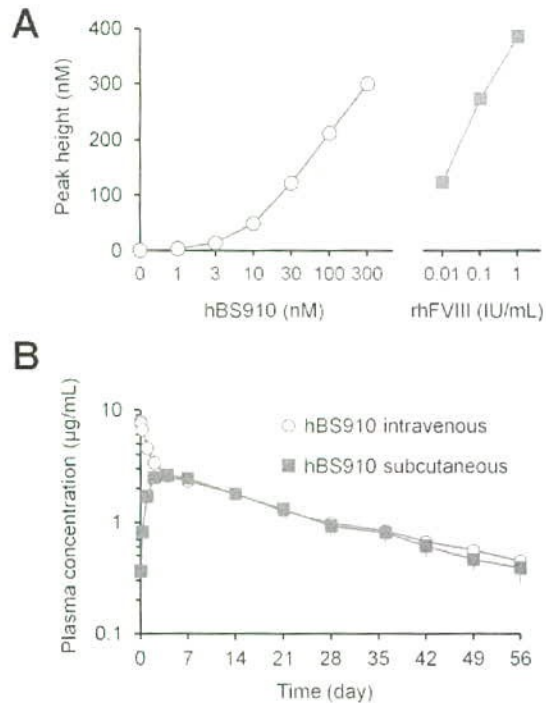


Figure 7. Therapeutic potential of multidimensionally optimized bispecific antibody, hBS910. (A) FVIII-mimetic activity of hBS910 in thrombin generation assay (TGA). Effect of hBS910 (circles) or recombinant human FVIII (squares) on thrombin generation in FVIII-deficient plasma is shown. The reaction was triggered by FIXa, synthetic phospholipid, and Ca^{2+} . The Y-axis indicates the peak height, a thrombin generation parameter (in many cases, the bars depicting s.d. are shorter than the height of the symbols). Data were collected in triplicate for each plasma lot and are expressed as mean $\pm$ s.d. (B) Pharmacokinetics of hBS910 in cynomolgus monkeys. Time profiles of plasma concentration of hBS910 after intravenous (circles) or subcutaneous (squares) injection are shown.

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interaction between the factors, while after FX activation by FIXa, FIXa needs to be rapidly dissociated from the antibody to proceed to the subsequent coagulation reaction and to enable the antibody to turn over. Therefore, we assumed that a simple increase in the binding affinity to both FIXa and FX would not necessarily improve the FVIII-mimetic activity. Consequently, generation of variants that had improved activity required screening of a large number of variants in which mutations had been introduced via structure-based or random mutagenesis. These mutations were mainly introduced to the residues that were predicted to directly or indirectly contact the antigen by the homology modeling of the lead antibody or experimentally identified to affect FVIII-mimetic activity.

To gain insights into the mechanisms underlying the improvement in activity, kinetic analyses of hBS1, hBS106, and hBS910 (bispecific antibodies each with different FVIII-mimetic activity) binding to FIXa and FX were performed by SPR analysis (supplementary Fig. S8A, B). However, because the kinetic parameters of these antibodies were differently affected by the fitting conditions of the sensorgrams, we were not able to obtain meaningful kinetic parameters with which to compare these antibodies. Nevertheless, the binding properties of these antibodies

were obviously different, and these changes might have contributed to the improvement in activity. It appears that the binding affinity of hBS910 to FIXa is weaker than hBS1 and hBS106, and hBS910 has faster association and dissociation rate for FX. Although this tendency might result in rapid turnover of FX activation by the bispecific antibody and explain the highest FVIII-mimetic activity of hBS910, it does not explain the large difference of FVIII-mimetic activity between hBS1 and hBS106. Since bispecific antibody needs to strictly place catalytic site of FIXa to the cleavage site of FX and may require allosteric effect on FIXa in order to mimic the function of FVIII, it can be postulated that the improved FVIII-mimetic activity of hBS106 compared to hBS1 may be derived not only from the changes in the binding kinetics but also from the subtle changes in the binding epitope, binding angle or allosteric effect caused by the mutations introduced.

The FVIII-mimetic activity of 30 nM hBS910 was equivalent to rhFVIII activity at 0.01 IU/mL (1% of normal level), and the activity of 300 nM hBS910 was greater than rhFVIII activity at 0.1 IU/mL (10% of normal level) in the TGA using human FVIII-deficient plasma. The activity was also observed even in the presence of FVIII inhibitors, which is reasonable considering the lack of homology between the sequences of hBS910 and FVIII. Recently, we have demonstrated that FVIII-mimetic bispecific antibody, termed hBS23, exerts hemostatic activity *in vivo* in acquired hemophilia A model using cynomolgus monkey, which was considered sufficient for routine prophylaxis [23]. hBS23 is one of the FVIII-mimetic activity improved variants obtained during the multidimensional optimization to generate hBS910, and has similar FVIII-mimetic activity as hBS106. Kinetic analysis [23] of hBS23 and hBS910 demonstrated that hBS910 showed two times the effect on increasing k_{cat}/K_m compared to hBS23 (unpublished data). In human FVIII-deficient plasma, hBS910 required only two third of plasma concentration to exhibit the equivalent activity to hBS23 in the TGA, suggesting that hBS910 has 1.5 times the activity to promote thrombin burst (unpublished data). When compared with FVIII, this *in vivo* hemostatic effect was consistent with its *in vitro* activity in TGA. Thus, the above results clearly demonstrate that hBS910, which is more potent than hBS23, could exert FVIII-mimetic activity in hemophilia A patients sufficient to achieve routine prophylaxis regardless of the presence of FVIII inhibitors. The amino acid sequence of hBS23 is described in our patent as an antibody name Q153-G4k/J142-G4h/L180-k, and that of hBS910 is described in the patent [Igawa T, Sampei Z, Kojima T, Soeda T, Muto A, et al. Multi-specific antigen-binding molecule having alternative function to function of blood coagulation factor VIII. WO/2012/067176].

It is highly desirable that our bispecific antibody be able to be administered subcutaneously with a long interval between doses. The poor pharmacokinetics of hBS106 in mice was partially attributed to the large positive charge cluster, and the single Tyr30Glu mutation to neutralize this charge cluster markedly improved the pharmacokinetics. Such a charge cluster may increase non-specific binding to the extracellular matrix which would increase the clearance of the molecule. Recently, it was reported that antibodies with lower pI have better pharmacokinetics, and engineering antibodies to lower the pI improved their pharmacokinetics [29]. This approach was successfully applied to our bispecific antibody. hBS910 exhibited a plasma half-life of approximately 3 weeks in cynomolgus monkeys, which is longer than the half-lives of hBS23 (approximately 2 weeks) [23] and other humanized or fully human IgG antibodies [40,41], presumably due to the benefit of pI engineering. Since the half-lives of IgG antibodies are generally longer in humans than in cynomolgus

monkeys [42], we expect that hBS910 would have a half-life of at least 3 weeks in humans, which is overwhelmingly longer than that of exogenous FVIII (0.5 days) [4]. Furthermore, hBS910 exhibited 86% subcutaneous bioavailability, consistent with that of other IgG antibodies [24,43,44], providing a huge advantage over exogenous FVIII which requires intravenous administration [5]. This high subcutaneous bioavailability and long half-life of hBS910 strongly supports the feasibility of routine prophylaxis by subcutaneous administration with a long dosing interval.

We utilized an *in silico* T-cell epitope prediction system, EpiBase, to minimize the number of T-cell epitopes present in the bispecific antibody. The immunogenicity risk of hBS910 was predicted not only by EpiBase but also by EpiMatrix to be comparable with that of non-immunogenic antibodies. Considering that up to 30% of patients develop inhibitors against exogenous FVIII [45], immunogenicity is an important issue in the routine prophylaxis of hemophilia A. Although the true immunogenicity of hBS910 needs to be evaluated clinically, it is noteworthy that two different *in silico* systems predicted hBS910 to be non-immunogenic. However, the possibility that a small number of patients could develop anti-hBS910 antibodies cannot be ruled out. In case hBS910 becomes ineffective owing to development of anti-hBS910 antibodies, it is important that they do not cross-react with FVIII so that exogenous FVIII treatment remains as an alternative. In addition to the fact that there is no homology between the amino acid sequences of hBS910 and FVIII, the risk of such cross-reactivity was found to be negligible because polyclonal anti-idiotypic antibodies against hBS910 did not inhibit the thrombin generation activity of FVIII. This demonstrates that the development of anti-hBS910 antibodies would not compromise the use of exogenous human FVIII therapy.

No recombinant bispecific IgG antibody has yet reached the market. One of the reasons is the difficulty in large-scale manufacturing at clinical grade. Identification of a common light chain is an important step for manufacturing asymmetric bispecific IgG antibodies. A previous study utilized a phage display library to identify a common light chain for the two heavy chains against different antigens [26]. However, since high-affinity binding to FIXa or FX would not necessarily result in high FVIII-mimetic activity, selection of a common light chain with potent activity based on the binding affinity by phage display was not feasible. We successfully identified a potent common light chain by a novel FR/CDR shuffling approach. Since we were able to obtain the common light chain (BS15L) that had much better potency than the parental light chain (c11) from the initial twenty four light chain variants, we did not perform further shuffling of FRs. Because residues in the FR often affects antigen binding of the antibody, we propose that shuffling of both CDRs and FRs would be an efficient and general approach to identify potent common light chains for bispecific antibodies. This novel approach enables incorporation of the beneficial residues from each CDR and FR into a common light chain. Although it might be not suitable for screening common light chain against large panels of heavy chains, this approach can be generally applicable for identification of a common light chain for the selected pair of heavy chains without using a library display system. In our other asymmetric bispecific antibodies with the same molecular format, common light chains for two different heavy chains could be successfully identified by this approach. In some cases, it was possible to directly identify humanized common light chain by performing CDR shuffling on the human FRs. This could be a more efficient approach since it abbreviates the following humanization process. However, since FR residues are often important for antigen binding property, we took more cautious approach in this FVIII-

mimetic bispecific antibody, and performed CDR shuffling on its cognate FRs to identify a potent common light chain, followed by humanization of the identified common light chain.

Although the heavy chain heterodimerization efficiency depended on the expression balance of each heavy chain, our Fc heterodimerization mutations achieved approximately 85% efficiency for hBS560 in the system of unoptimized expression balance (Fig. 5B). Nevertheless, expression of a small amount of monospecific homodimeric antibodies is inevitable even with Fc heterodimerization mutations. In the case of our bispecific antibody, byproducts are anti-FIXa and anti-FX bivalent monospecific antibodies. These byproducts are not simply impurities with no activity, but they have the potential to competitively inhibit the activity of the bispecific antibody by bivalent binding to the factors. Therefore, homodimeric byproducts need to be removed as much as possible by a downstream process. However, the only molecular difference between a homodimeric byproduct and the bispecific antibody is the heavy chain variable region of one arm. Since antibody variable regions generally have similar sequences except for the CDRs, it was assumed that separation of such byproducts from the bispecific antibody by IEC would be difficult. Therefore, we took the advantage of pI engineering, and engineered the heavy chain variable region to increase the pI difference between the bispecific antibody and the byproducts, thereby improving the separation by IEC. pI engineered hBS910 could be actually purified from 2500-liter fermentation by Protein A and IEC using conventional antibody purification processes. Such a novel pI engineering approach would be generally applicable to facilitate the purification of bispecific IgG antibodies.

To realize subcutaneous delivery in a clinical setting, bispecific antibodies need to be formulated into a high concentration (i.e., >100 mg/mL) since the volume that can be subcutaneously injected is generally limited to less than 1.5 mL [46]. However, hBS376 exhibited phase separation even at 4 mg/mL. Phase separation of antibody solutions into an upper phase with low antibody concentration and a lower phase with high antibody concentration not only precludes high concentration formulation but also makes downstream purification processes difficult [30]. It has been recently reported that low solubility of antibodies has been overcome by introducing specific mutations into the molecular surface [47], but mutations to prevent phase separation have not been reported. We demonstrated that phase separation of hBS376 could also be eliminated by introducing multiple mutations into the molecular surface. hBS910 exhibited no phase separation under the conditions tested, and could be concentrated up to at least 200 mg/mL without any issue.

Since FVIII is unstable under liquid formulation, all the marketed FVIII agents are distributed as lyophilized formulation, and therefore require reconstitution before injection. Liquid formulation allows injection without this process, and thus would be much more convenient for the patients. However, monoclonal antibodies stored in aqueous solution often undergo deamidation of the asparagine residues in the CDRs resulting in reduction of the biological activity of the antibody, which was the case for hBS560 [31]. Although the general strategy is to remove the deamidation site by mutating the asparagine residue itself to another amino acid, this strategy was not feasible in the case of hBS560. A simultaneous double mutation approach enabled storage of hBS910 at 40°C for 2 weeks without reduction of activity. Consequently, hBS910 could be stably stored in a patient-friendly 150 mg/mL liquid formulation, which would enable approximately 3 mg/kg subcutaneous delivery in humans (1.5 mL injection for a 75 kg patient). Considering the FVIII-mimetic activity determined by TGA and the pharmacokinetics in

cynomolgus monkeys, such a formulation would provide effective prophylaxis by subcutaneous delivery with a long dosing interval.

In conclusion, we have generated a novel humanized anti-FIXa/FX bispecific IgG antibody, hBS910, through a process of identifying the lead candidate from approximately 40,000 bispecific combinations, followed by a multidimensional optimization process to improve both the therapeutic potential and the manufacturability. hBS910 overcomes the two major drawbacks of routine prophylaxis by exogenous FVIII. First, while exogenous FVIII requires frequent intravenous administration, hBS910 can be subcutaneously administered with a long dosing interval. Second, while the development of FVIII inhibitors is a critical issue for exogenous FVIII, our study suggests that hBS910 can be used without fear of developing FVIII inhibitors and can be used in patients who have already developed FVIII inhibitors. We believe that hBS910, with its multidimensionally optimized profile, will provide significant improvement in the quality of life of hemophilia A patients by reducing not only bleeding but also the burden on the patients themselves, their parents, and all medical staff. Potential of hBS910 (investigational drug name: ACE910) in hemophilia A patient is currently being evaluated in clinical study.

Materials and Methods

Ethics Statement

Animal studies were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals at Chugai Pharmaceutical Co., Ltd. under the approval of the company's Institutional Animal Care and Use Committee. The company is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (<http://www.aalac.org>). Details of cynomolgus monkey care and maintenance, including shelter and availability of food, water, and environmental enrichment are as follows. Identification of individuals; individual animals were identified by microchip number. Each cage was identified by a cage number indicated on the cage rack, cage type; a stainless steel cage, housing density; 1 animal/cage, temperature; 18°C to 28°C, relative humidity; 35% to 75%, air change frequency; at least 10 times per h, illumination timing; 12 h per day, from 7:00 am to 7:00 pm, feed; cynomolgus monkeys were daily provided approximately 100 g of solid chow (Certified Primate Diet 5048, Japan SLC) and supplementary foods (1/2 peeled banana or 50 g of sweet potato) and drinking water; animal room tap water, provided ad libitum using the automatic water supply system. The studies involved injection of the agents and collection of blood samples which did not require procedures that would cause more than slight or momentary pain or distress to the animals. All injections and blood collection were conducted by trained and qualified primate therapeutic staff in Chugai Pharmaceutical. Provisions were made in the approved protocol for veterinary intervention in the case of any distress or morbidity from the injected agent; however no adverse events occurred during the studies or during the recovery period. Therefore, no scarification of the animals was conducted.

Generation of Anti-FIXa/FX Bispecific Antibodies

Approximately 200 monoclonal antibodies to FIXa or FX were obtained from FIXa or FX immunized mice, rats, and rabbits. The V_H and V_L of those antibodies were then combined with engineered human IgG₂ or IgG₁ that included mutations to facilitate Fc heterodimerization. These engineered human IgG₂ or IgG₁, which included the knobs-into-holes mutations [26], were generated by introducing the same substitutions as those of IgG₁ knobs-into-holes to the corresponding positions of IgG₂ and IgG₁

heavy chains, and were used through the screening for the lead identification. Anti-FIXa/FX bispecific were generated by HEK293 cells co-transfected with mixture of four plasmids encoding anti-FIXa heavy and light chain and anti-FX heavy and light chain (supplementary Fig. S1). At the screening step for lead identification, we used knobs-into-holes mutations which could achieve at least 90% efficiency of the two heavy chain heterodimerization. If the assembly of a heavy chain with two light chains occurs with equal probability, 25% of heavy chain heterodimeric antibodies will have the correct heavy and light chain pair [25]. Theoretically, in the supernatant of transfected cells, this would result in at least approximately 20% ($90\% \times 25\% = 22.5\%$) of antibodies to be the target bispecific antibody, and less than 3% ($10\% \times 25\% = 2.5\%$) of antibodies to be homodimeric antibodies with a correct heavy and light chain pair. Transfected cells were cultured in 96-well culture plates, and either filtrated culture supernatants or Protein A purified antibodies were used for evaluation. We also generated bispecific antibodies with a common light chain, anti-FIXa monospecific IgG consisting of anti-FIXa heavy chain and a common light chain, and anti-FX monospecific IgG consisting of anti-FX heavy chain and a common light chain by the method described above. The engineered human IgG₄ including the knobs-into-holes mutations was used during the early stage of the multidimensional optimization, and a different engineered IgG₄ including electrostatic steering mutations [25] was used during the late stage optimization. By using the engineered IgG₄, approximately 85–95% of the Protein A purified antibody would be the target bispecific antibody and the rest would be homodimeric antibodies. For the detailed characterization of hBS910, homodimeric antibodies were removed by ion exchange chromatography.

Screening Bispecific Antibodies for FVIII-mimetic Activity (Enzymatic Assay)

The ability of each antibody to enhance FIXa-catalyzed FXa generation was evaluated in an enzymatic assay with purified human coagulation factors (Enzyme Research Laboratories). The FXa generation reaction was performed in the presence of 1 nM human FIXa, 140 nM human FX, 20 μ M synthetic phospholipid (10% phosphatidylserine, 60% phosphatidylcholine and 30% phosphatidylethanolamine; Avanti Polar Lipids) prepared as previously described [48], and antibodies at room temperature for 2 min in TBS containing 5 mM CaCl₂, 1 mM MgCl₂ and 0.1% (wt/vol) BSA (pH 7.6). The reaction was stopped by the addition of EDTA at appropriate time points. The activity of the FXa generated was determined by absorbance at 405 nm after the addition of chromogenic substrate S-2222 (Chromogenix). Data were collected in triplicate.

FR/CDR Shuffling of the Light Chains

CDRs of three light chains (c1L, c2L and c3L) were shuffled among each other then grafted onto the FRs of either c1L or c3L to generate light chain variants (supplementary Fig. S2A). Since c2L and c3L had identical CDR1 and CDR2 sequences, we generated twenty four light chains (twelve CDR combinations in two FRs) including parental c1L and c3L. Light chain variant genes were generated by assembly PCR, and the twenty four bispecific antibodies with these light chains were prepared as described above. FVIII-mimetic activity of each antibody was evaluated in APTT assay with standard techniques using APTT reagent (Sysmex) and FVIII deficient plasma (Sysmex).

Pharmacokinetic Study of Bispecific Antibodies in Mice

1 mg/kg doses of each bispecific antibody were administered to C57BL/6J normal mice (Charles River) by single subcutaneous injection ($n = 3$ for each group). Blood samples were collected at an appropriate time after each administration. Plasma concentration of bispecific antibodies was determined by human IgG-specific ELISA. Pharmacokinetic parameters were calculated by WinNonlin Professional software (Pharsight).

Determination of Isoelectric Point (pI) by Capillary Isoelectric Focusing (cIEF)

cIEF analyses of antibodies were performed with a PA800 plus Pharmaceutical Analysis System and 32 Karat software (Beckman Coulter) as described in the cIEF application guide (PN A78788AA). Briefly, antibody solutions (approximately 1 mg/mL in PBS) were diluted 1:25 with cIEF master mix solution containing cIEF gel, urea, Pharmalyte 3–10, arginine, iminodiacetic acid, and pI markers. A neutral capillary was preconditioned by rinsing with urea solution, and samples were injected and focused. The pI of each of the antibodies was determined from the pI markers.

Separation of Bispecific Antibodies by Cation Exchange Chromatography

Using an AKTAexplorer 10S (GE Healthcare), two HiTrap SP FF 1 mL columns (GE Healthcare) were connected in tandem and equilibrated by 20 mM sodium phosphate, pH 6.0. The elution buffer contained an appropriate concentration of NaCl in this equilibration buffer. The load sample was first prepared from culture supernatant using MabSelect Sure (GE Healthcare), and was then dialyzed with equilibration buffer. The sample antibody solution was applied to the HiTrap SP FF column at 1.5 mg/mL resin. After washing with equilibration buffer, antibodies were eluted with 10 column volumes (CV) of equilibration buffer containing 150 mM NaCl and then with 15 CV of 220 mM NaCl buffer in a stepwise manner, and finally with 15 CV of NaCl buffer in a linear gradient to 450 mM.

Solubility Analysis of Bispecific Antibodies

Stock solutions of bispecific antibodies were prepared by dialysis against water or 50 mM NaCl solutions, followed by ultrafiltration concentration. Samples were prepared by adding formulation stock solution to antibody stock solution using a Hydra II Plus One liquid-handling robot (Matrix). After centrifugation, 1 μ L of each sample was stored in an Intelli-Plate 96-2 (Art Robbins) at 20°C for 1 day, and then images of each well were taken by Rock Imager 54 (Formulatrix). The state of each antibody solution was determined as either a clear solution, precipitation, or liquid-liquid phase separation.

Accelerated Stability Study of Bispecific Antibodies

Solutions of bispecific antibodies were dialyzed against PBS, pH 7.4 (Sigma). 1 mg/mL of each antibody solution was stored at 40°C for 2 weeks and then analyzed by cation exchange chromatography (IEC) using BioPro SP columns (YMC) at room temperature. The mobile phase (A) was 20 mM sodium phosphate, pH 5.8, and the mobile phase (B) was 20 mM sodium phosphate and 500 mM NaCl, pH 5.8.

In silico Evaluation of Immunogenicity of the Variable Region of Bispecific Antibodies

T-cell epitope prediction of the bispecific antibody variants and immunogenicity risk scores of antibodies were provided by

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Epibase (Lonza). Immunogenicity scales of antibodies were provided by EpiMatrix (EpiVax).

Thrombin Generation Assay (TGA)

Calibrated automated thrombography [33] was employed using a 96-well plate fluorometer (Thermo Fisher Scientific) equipped with a 390/460 filter set, a dispenser, and the analyzing software (Thrombinoscope software version 3.0.0.29; Thrombinoscope) to measure thrombograms. Briefly, each concentration of bispecific antibody or rhFVIII (Bayer Healthcare) was added to FVIII-deficient plasma (<1% FVIII activity) either without inhibitors or with inhibitors against FVIII (George King Bio-Medical). Each concentration of bispecific antibody or rhFVIII was also added to plasma containing polyclonal rabbit anti-idiotypic antibodies against anti-FIXa Fab or anti-FX Fab (300 µg/mL each). Into each well was dispensed 80 µL of the plasma, to which was then added 20 µL of the triggering solution containing 0.47 nM human FIXa (Enzyme Research Laboratories) and 20 µM synthetic phospholipid but no Ca^{2+} . For calibration, 20 µL of Thrombin Calibrator (Thrombinoscope) was added instead of the triggering solution. 20 µL of FluCa-reagent prepared from FluCa-kit (Thrombinoscope) was dispensed to initiate the reaction. The thrombograms and peak height were analyzed by the software. Data were collected in triplicate.

Pharmacokinetic Study of hBS910 in Cynomolgus Monkeys

A single dose of 0.3 mg/kg of hBS910 was intravenously or subcutaneously administered to male cynomolgus monkeys ($n=3$ for each group). Blood samples were collected at an appropriate time after each administration. Plasma concentration of bispecific antibodies was determined by human IgG-specific ELISA. Pharmacokinetic parameters were calculated by WinNonlin Professional software (Pharsight).

Kinetic Analysis of Bispecific Antibodies Binding to FIXa and FX Using Surface Plasmon Resonance

Kinetic analysis of bispecific antibodies was performed by surface plasmon resonance (SPR) using a Biacore T200 system (GE Healthcare). MabSelect SuRe Ligand (Recombinant Protein A; GE Healthcare) was immobilized onto a CM4 sensor chip (GE Healthcare). Then, anti-FIXa or anti-FX monospecific antibodies were injected into flow cell 2 to be captured. Natalizumab (Biogen-Idec Inc.) as a control human IgG₁ antibody was also injected into flow cell 1 to be captured. Then, 0, 80, 160, 320, 640, or 1,280 nM human FIXa or FX dissolved in running buffer (10 mM HEPES [pH 7.4], 150 mM NaCl, 0.05% surfactant P20, 2.5 mM CaCl_2) was injected at a flow rate of 30 µL/min to monitor the association phase for 120 s and the dissociation phase for 30 s.

Supporting Information

Figure S1 Generation of anti-FIXa/FX bispecific antibodies. Heavy chain variable regions (V_H) of anti-FIXa or anti-FX antibodies were fused with engineered human IgG₂ or IgG₄ constant region having mutations to facilitate Fc heterodimerization. Light chain variable regions (V_L) were fused with human κ or λ constant region. Bispecific antibodies were generated by expression with two pairs of genes, anti-FIXa and anti-FX heavy chain and light chain genes (or a common light chain gene). (PDF)

Figure S2 FR/CDR shuffling of the light chain. (A) CDRs of three light chains (c1L, c2L, and c3L) were shuffled among each

other and grafted onto the FRs of c1L and c3L. Each light chain variant was expressed with the selected anti-FIXa and anti-FX heavy chains. (B) Effects of bispecific antibodies (67 nM) with light chain variants on APTT assay in FVIII-deficient plasma are shown. The Y-axis indicates the APTT (s). All the data were collected in duplicate and are expressed as mean. (PDF)

Figure S3 Effect of humanization of the lead chimeric antibody (BS15) on FVIII-mimetic activity. Effect of chimeric antibody BS15 (circles) or humanized antibody hBS1 (squares) on FX activation in the presence of FIXa, FX, and synthetic phospholipid. The Y-axis indicates the 405 nm absorbance at 120 min of chromogenic development in the chromogenic substrate assay. All the data were collected in triplicate and are expressed as mean $\pm$ s.d. (in many cases, the bars depicting s.d. are shorter than the height of the symbols). (PDF)

Figure S4 Positive charge cluster and Tyr30Glu mutation on anti-FIXa Fv of hBS106. The positive charge cluster consists of arginine or lysine residues at Kabat position 60, 61, and 95 in the heavy chain and Kabat positions 24, 27, 31, 53, 54, 61, and 66 in the light chain of hBS106. Tyrosine located at Kabat position 30 in the light chain was mutated to glutamic acid to neutralize the positive charge cluster. Blue, red and gray colored surface indicates positively charged, negatively charged and neutral protein surface, respectively. Red and green line indicates heavy and light chain, respectively. (PDF)

Figure S5 Precipitation and liquid-liquid phase separation of bispecific antibody solution. Micro CCD camera images of states of bispecific antibody solution showing a clear solution, precipitation, and liquid-liquid phase separation. (PDF)

Figure S6 In silico prediction of immunogenicity of bispecific antibodies. (A) Immunogenicity risk score of BS15, hBS1, hBS910, trastuzumab, and palivizumab predicted by Epibase. (B) Immunogenicity scale of hBS910 and other marketed monoclonal antibodies by EpiMatrix. In both prediction systems, higher score indicates higher risk of immunogenicity in human. (PDF)

Figure S7 Necessity of bispecific binding to FIXa and FX for FVIII-mimetic activity. Effect of the bispecific antibody (hBS910) (circles), monospecific anti-FIXa antibody (squares), monospecific anti-FX antibody (triangles), or a mixture of the two monospecific antibodies (diamonds) on FX activation in the presence of FIXa, FX, and synthetic phospholipid. The Y-axis indicates the 405 nm absorbance at 30 min of chromogenic development in the chromogenic substrate assay. All the data were collected in triplicate and are expressed as mean $\pm$ s.d. (in many cases, the bars depicting s.d. are shorter than the height of the symbols). Monospecific antibodies against FIXa or FX antibodies or the mixture of them did not exhibit any detectable activity even at 120 min of chromogenic development. (PDF)

Figure S8 Surface plasmon resonance analysis of bispecific antibodies binding to FIXa and FX. Sensorgrams of hBS1, hBS106, and hBS910 binding to FIXa (A) and FX (B) at a concentration of 80 nM, 160 nM, 320 nM, 640 nM, and 1280 nM. (PDF)

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Isomerization of an Aspartic Acid Residue in the Complementarity-Determining Regions of a Recombinant Antibody to Human IgE: Identification and Effect on Binding Affinity

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ABSTRACT: This report describes the effect on antigen binding of an isomerized aspartate residue located in the complementarity-determining regions (CDRs) of a recombinant monoclonal antibody. The antibody, which binds human IgE, contains two Asp-Gly sequences within its CDRs, but only one site was found to be labile to isomerization. Isolation and characterization of antibody fragments differing in the labile sequence were facilitated by using a technique involving hydrophobic interaction chromatography (HIC) that separates aspartyl, isoaspartyl, and cyclic imide variants to the residue located in CDR-L1. The variants were isolated for structural characterization and for determination of their relative antigen binding affinities. Mutants were constructed with altered residues to obviate the effects of isomerization and were evaluated for their ability to bind to IgE. Inspection of published crystal structures of CDRs of antibodies indicated that hydrogen binding of the Asp side chain of the unreactive residue may be the constraint that prevents isomerization. The strategy outlined here may prove to be of general utility in the biochemical and immunochemical characterization of recombinant antibodies.

Monoclonal antibodies (MAbs) are characterized by highly selective binding of a target antigen. The selectivity can be exploited for detection of the antigen in diagnostic applications, for purification in immunochromatographic techniques, to inhibit biochemical interactions *in vitro*, or as human therapeutics. The therapeutic usefulness of MAbs can be improved in many applications by employing recombinant DNA technology to produce humanized MAbs (rhMAbs). These molecules are produced by splicing the residues from, e.g., a murine MAb that confer antigen specificity into a human immunoglobulin framework (Jones *et al.*, 1986). This process yields a protein that is identical to human antibodies, save for the highly variable residues within the complementarity-determining regions (CDRs). The resulting molecule is less prone to eliciting an immunogenic response when administered to humans than are MAbs derived from other species, such as mice.

rhMAbs are subject to degradation reactions that affect all proteins, such as isomerization of aspartate residues. As shown in Figure 1, aspartate residues (I) in Asp-Gly sequences can isomerize to isoaspartate (II) through a cyclic imide intermediate (III) at an appreciable rate (Geiger & Clarke, 1987). The equilibrium, rate, and pI dependence of this reaction have been studied in model peptides separated by reversed-phase HPLC¹ (Oliyai & Borchardt, 1993). Since the reaction is reversible, and the intermediate is relatively

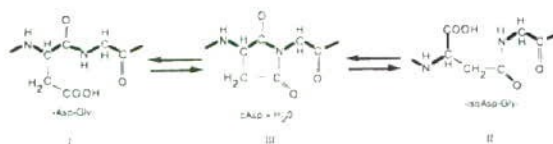


FIGURE 1: Chemical mechanism for isomerization of Asp-Gly (I) sequences. The reaction proceeds reversibly through a stable cyclic imide intermediate (III), resulting in an isoaspartic acid-containing sequence (II).

stable, peptides with labile Asp residues can be found in three forms containing either Asp, iso-Asp, or cyclic imide (cAsp) at the pertinent position. The tendency to undergo isomerization has been postulated to depend on the local flexibility of the portion of the molecule containing the Asp-Gly sequence (Geiger & Clarke, 1987). In this mechanism, sufficient local flexibility is required to accommodate the structural differences of the three species. The three forms interconvert and so approach an equilibrium that is temperature- and pH-dependent (J. Frenz and J. Cacia, in preparation).

As human therapeutics, monoclonal antibodies are subject to the same stringent oversight of their structural characteristics and consistency of manufacture as other protein pharmaceuticals. Sensitive analytical techniques are required to characterize a MAb intended for clinical uses to fully characterize its relevant features. Variants generated by spontaneous mutagenesis or degradative processes may alter the specificity and strength of antigen binding, particularly if the residue(s) involved are in the CDRs of the MAb. Variants that are silent with respect to antigen binding also present manufacturing and product consistency issues but may not directly impair the efficacy of the MAb as a drug. One example is the variable degree of post-translational processing of the C-terminal lysine in the Fc region of IgGs, which has been attributed to carboxypeptidase activity (McDonough *et al.*, 1989). A second example is the recent

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¹ Abbreviations: huFcγRI, human high-affinity IgE receptor; HIC, hydrophobic interaction chromatography; iso-Asp, isoaspartate; cAsp, cyclic imide (succinimide) intermediate; HPLC, high-performance liquid chromatography; EDTA, ethylenediaminetetraacetic acid; PTH, phenylthiohydantoin.

CDR-L1	RASQSVVDYDGD ³⁶ SYMN ³⁶
CDR-L2	AASYLES ⁶⁰
CDR-L3	QQSHCDPYT ¹⁰¹
CDR-H1	GYSWN ³⁶
CDR-H2	SITYDGGSTNYNPSVK ⁶⁶ G
CDR-H3	CARGSHYFGHW ¹¹⁰ HEAV ¹¹⁰

FIGURE 2: Primary structures of the complementarity-determining regions (CDRs) of the E25 rhMAb. The CDRs are defined as described in Presta *et al.* (1993).

identification of a genetic mutant of a recombinant monoclonal antibody, involving a point mutation in the Fc region of the molecule (Harris *et al.*, 1993).

E25 is a rhMAb that prevents binding of IgE antibodies to high-affinity receptors (Presta *et al.*, 1993) and thereby shows promise as a treatment for allergic diseases (Chang *et al.*, 1990). E25 (Figure 2) contains Asp-Gly sequences in CDR-L1 at 1.32–33 (i.e., residues 32–33 in the light chain) and in CDR-H2 at H55–56. This report describes the evidence for the occurrence of iso-Asp and cAsp residues in E25 samples incubated at 37 °C and pH 5.2.

EXPERIMENTAL PROCEDURES

Materials. Recombinant human monoclonal antibody E25 was cloned, expressed, and purified at Genentech, Inc. The antibody was prepared at 5 mg/mL in a buffer containing 10 mM acetate (pH 5) and incubated in a 37 °C water bath to generate the isomeric forms of the antibody characterized in this study. E25 mutant Fabs were prepared using site-directed mutagenesis and purified as described (Presta *et al.*, 1993).

Hydrophobic Interaction Chromatography. Analytical HIC was performed on a 4.6 × 30 mm TSK-Butyl NPR column (Tosoh Haas, Montgomeryville, PA). Buffer A consisted of 2 M ammonium sulfate in buffer B [20 mM Tris and 20% glycerol (pH 8.0)]. The column temperature was maintained at 40 °C. Intact E25 (5 µg) was injected and eluted with a 15 min linear gradient from 60 to 35% buffer A at a flow rate of 1 mL/min. Antibody fragments of E25 were separated with a 20 min linear gradient from 90 to 38% buffer A. The HIC column was washed with 100% buffer B for 10 min following each run and equilibrated at the initial gradient conditions for 10 min prior to injection. All chromatography was performed on a Hewlett-Packard (Palo Alto, CA) 1090M HPLC system with detection by UV absorbance at 214 nm.

Preparative collection was performed on E25 Fab and F(ab')₂ fragments for characterization using a TSK Phenyl 5PW column (7.5 × 75 mm). The column was run at 40 °C and 0.8 mL/min. Buffer A contained 2 M ammonium sulfate in buffer B [25 mM sodium phosphate and 20% glycerol (pH 7.0)]. A 70 min linear gradient was run from 70 to 20% buffer A. Fractions were collected manually and concentrated using Centricon-10 microconcentrators (Amicon, Beverly, MA).

Pepsin Digestion of E25. Samples of E25 were diluted 5-fold with 0.1 M sodium citrate (pH 3.5). Pepsin (Worthington Biochemicals, Freehold, NJ) was freshly resuspended in citrate buffer and added to E25 samples in a final

pepsin:E25 ratio of 1:100 (w:w). The samples were then incubated at 37 °C for 2 h and injected into the HPLC.

Papain Digestion of E25. Samples of intact or F(ab')₂ E25 were exchanged into papain digest buffer containing 0.1 M sodium phosphate and 2 mM ethylenediaminetetraacetic acid (EDTA) (pH 7.0) using Bio-Spin 30 columns (Bio-Rad, Hercules, CA). Freshly prepared L-cysteine was added to the samples to achieve a concentration of 10 mM. Papain (Worthington Biochemicals) was added at a papain:E25 ratio of 1:100 (w:w) to the samples which were then incubated at 37 °C for 4 h. Samples were frozen at –20 °C until Fab fragments were preparatively collected or analyzed by HIC.

Tryptic Peptide Mapping. S-Carboxymethylation of E25 Fab fragments was performed prior to digestion with trypsin. Samples were concentrated and exchanged using Centricon-30 microconcentrators (Amicon) into a solution containing 8 M urea, 0.35 M Tris, and 1 mM EDTA at pH 8.5. Digestion and mapping conditions were essentially as described (Harris *et al.*, 1993). Peak fractions were collected manually.

Liquid Chromatography–Mass Spectrometry (LC–MS). Mass spectrometric analyses were performed using a Sciex (Thornhill, Ontario) API III instrument operating in the positive-ion mode. The HPLC solvents A and B were 0.1% trifluoroacetic acid (TFA) in water and 0.1% TFA in acetonitrile, respectively. The E25 Fab fragments were injected onto a TSK-Phenyl 5PW column equilibrated with 10% solvent A at 45 °C with a flow rate of 0.5 mL/min. A linear gradient was run to 60% solvent A in 40 min. The column eluate was split at a ratio of 1:100 and introduced into the mass spectrometer at a flow rate of 10 µL/min.

N-Terminal Sequence Analysis. The cyclic imide intermediate was identified and sequenced using a previously described procedure (Kwong & Harris, 1994). E25 Fab tryptic fragments were sequenced on an Applied Biosystems (Foster City, CA) Model 477A/120A system. Molar values for the PTH amino acids were generated by peak area comparisons to an external standard mixture.

IgE Receptor Binding Inhibition Assay. The biological activity of E25 variant fragments and site-directed mutants was assessed by measuring their respective abilities to inhibit the binding of human IgE to the human high-affinity IgE receptor, hFcεRI, as described previously (Presta *et al.*, 1993). An intact E25 standard was used to quantify samples.

RESULTS

HIC Separation of E25 Isoforms

The hydrophobic interaction chromatography conditions—including column choice, buffer composition, and pH and column temperature—were optimized for resolution of isoforms of intact E25, as described elsewhere (J. Frenz, J. Cacia, and R. Keck, in preparation). The chromatogram of E25 incubated at 37 °C for 21 days, shown in Figure 3, contains seven peaks that represent forms of the antibody containing combinations of heterogeneities at different sites on the bivalent antibody, as detailed below. One source of heterogeneity is the partial processing of a C-terminal lysine on the heavy chain. This can be shown by treatment of the antibody with carboxypeptidase B, which cleaves basic residues from the C-terminus of proteins and eliminates peaks

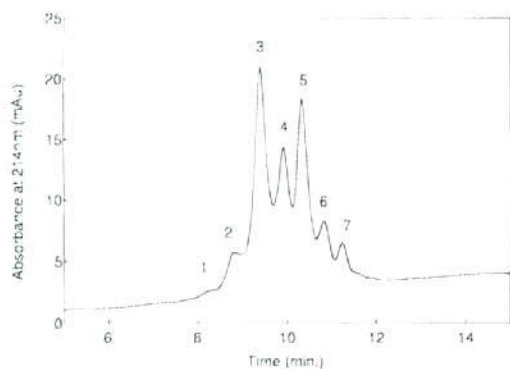


FIGURE 3: Separation by hydrophobic interaction chromatography of intact E25. Peaks labeled 1–7 correspond to the variants identified in Table 1. Separation conditions are described in the text.

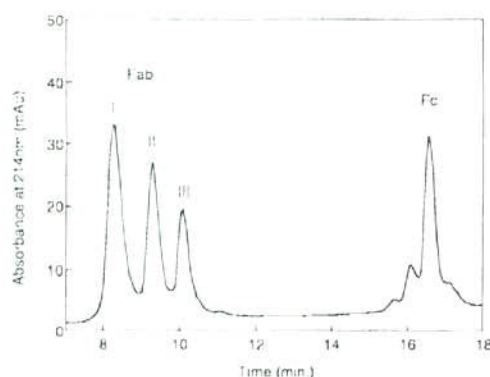


FIGURE 4: Separation by hydrophobic interaction chromatography of the mixture resulting from papain digestion of E25. The portion of the chromatogram labeled Fab includes the three heterodimeric fragments containing either Asp, iso-Asp, or cAsp at position 32. The portion labeled Fc includes the homodimeric Fc fragments that contain one or both of the unprocessed C-terminal lysine residues, as well as the major constituent, the fully processed fragment. The separation conditions are described in the text.

1 and 2 from the chromatogram, without affecting the positions of peaks 3–7 (data not shown). The remaining source of heterogeneity in these peaks was identified as described below.

Characterization of E25 Isoforms

Papain Digestion and HIC Separation of C-Terminal Heterogeneity. A convenient way to localize the sources of heterogeneities on an antibody molecule is by selective protease digestion followed by separation of the resulting fragments. Both papain and pepsin digestion followed by HIC separation were employed to characterize the heterogeneity observed in the E25 antibody. Papain cleaves the E25 molecule on the C-terminal side of His-H228 to yield two Fab fragments and an Fc fragment. The digest mixture can be resolved on an HIC column as shown in Figure 4. Two peaks in the chromatogram yielded masses that correspond to the expected mass of the Fab fragment, 48 194, by LC-MS, as shown in Table 1. The third peak yielded a mass 16 amu smaller. The Fc fragment was resolved into three peaks, but treatment of the antibody with carboxypeptidase B prior to papain digestion (data not shown) results in disappearance of the two peaks eluting slightly ahead of

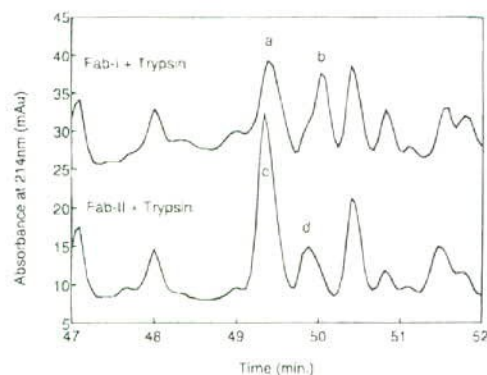


FIGURE 5: Portions of the chromatograms of mixtures resulting from trypsin digestion of purified Fab-I and Fab-II E25 variants. This portion of the chromatogram contains the four peaks labeled a–d that were collected and subjected to N-terminal sequence analysis. The reversed-phase HPLC separation of the digest mixture was carried out as described in the text.

Table 1: Mass Spectrometric Analyses of E25 Fab Variants

Fab sample	expected mass	observed mass	identity
Fab-I	48 194	48 194	L1–217, H1–228 (Asp @ LC-32)
Fab-II	48 194	48 194	L1–217, H1–228 (iso-Asp @ LC-32)
Fab-III	48 176	48 178	L1–217, H1–228 (cAsp @ LC-32)

Table 2: N-Terminal Sequence Results of E25 Fab Tryptic Peptides

peak	sequences observed	identity
a	40 pmol of GPSVFPLAPSSK	H128–139
	2.5 pmol of DYFPEP	H154–159
b	5.0 pmol of ASQSDVDYDGDSYMNW	L25–46 (Asp @ 32)
	5.0 pmol of NTFYLMQNSLR	H177–87
	1.5 pmol of LLIY	L50–53
c	6.0 pmol of ASQSDVY ^a	L25–46 (iso-Asp @ 32)
	75 pmol of GPSVFPLAPSSK	H128–139
	3.0 pmol of DYFPEP	H154–159
d	10 pmol of NTFYLMQNSLR	L50–53
	2.0 pmol of LLIY	H177–87

^a Sequence reaction terminated.

the main peak. Hence, these peaks represent forms of the antibody that retain either or both of the C-terminal lysines. This procedure thus provides a means for quantification of the extent of processing of these residues; in this case, about 3.5% of the E25 molecules contains both lysine residues, 16.5% contains a single lysine, and 80% lacks both residues.

Characterization of Fab Fragments. The identities of the Fab fragments were established as follows. The peaks identified in Figure 4 as Fab-I and Fab-II, which have the mass expected of the correct Fab fragment (Table 1), were individually collected and digested with trypsin and the resulting peptides separated by reversed-phase HPLC. The tryptic maps for the two forms of Fab were essentially identical except for the portions compared in Figure 5. The four peaks labeled a–d were collected and subjected to mass spectrometry and 15 cycles of N-terminal sequencing. Mass spectrometric analysis of the collected fractions indicated that peaks b and c both contained a peptide with the mass expected (2568 amu) for the full length L25–46 peptide that contains the labile Asp-L32 residue. The sequences obtained for the four peaks are shown in Table 2 and reveal that peak b from the map of the Fab-I fragment contains the full length unmodified L25–46 peptide (Table 2). Peak c, in the map

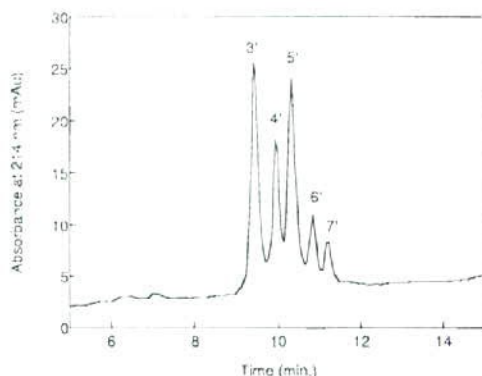


FIGURE 6: Separation by hydrophobic interaction chromatography of the mixture resulting from pepsin digestion of E25. The peaks labeled 3'–7' correspond to the variant identities summarized in Table 3. The separation conditions are described in the text.

of the Fab-II fragment, contains a peptide beginning with Ala-L25 along with the two peptides found in peak a. Termination of the sequencing reaction at cycle 6, just before an expected Asp residue in a peptide that mass spectrometry indicates is the full length L25–46 tryptic peptide, suggests that the Asp-L32 residue has isomerized to isoaspartate (Allen, 1981). Isomerization of the Asp residue does not change the mass of the peptide. Hence, Fab-I is the fragment containing the expected Asp-L32 residue, and Fab-II is the fragment with the isoAsp variant.

The Fab-III fragment was found by LC–MS to have a mass of 49 178 (Table 1). This mass is in close agreement with the value of 48 176 expected of the succinimide intermediate of the isomerization reaction, according to the scheme shown in Figure 1. The identity of this fragment as the cyclized aspartate (cAsp) form of the Fab was consistent with its observed rapid conversion to a mixture of Fab-I and Fab-II under mild conditions (data not shown). This assignment was further supported by a procedure that has been described for direct identification of cyclic imide intermediates in proteins (Harris & Kwong, 1994). HIC peaks Fab-I and Fab-III were collected and subjected to this procedure in which hydroxylamine is used to cleave the protein at succinimide residues. Fab-I yielded only the expected N-terminal sequences of the heavy and light chains after hydroxylamine treatment (data not shown). Fab-III, on the other hand, gave a sequence beginning with Gly-L33 in addition to the heavy and light chain sequences, consistent with the chain cleavage of the cyclic imide at Asp-L32 that occurs upon treatment with hydroxylamine.

Hence, these results demonstrate the occurrence of the isomerization products in CDR-L1 of the E25 antibody and the assignment of Fab-I as the Asp-containing fragment, Fab-II as the iso-Asp-containing fragment, and Fab-III as the cAsp form.

Pepsin Digestion Followed by HIC Separation. Two sources of heterogeneity were identified by papain mapping: partial processing of the C-terminal lysine on each of the heavy chains and isomerization of Asp-L32. To demonstrate that these two features account for the occurrence of seven peaks in the E25 chromatogram shown in Figure 3, digestion with pepsin was employed. This treatment cleaves the Fc portion of the antibody, removing C-terminal heterogeneity. The resulting mixture can be separated into five components by HIC, as shown in Figure 6, that

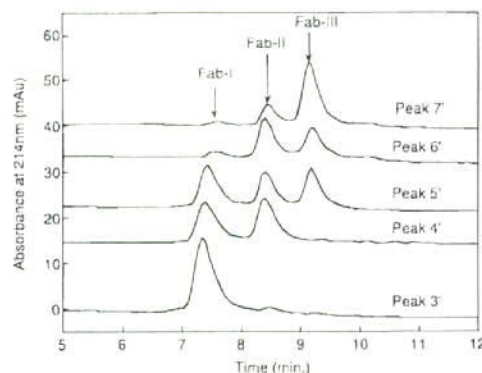


FIGURE 7: Separation of mixtures generated by papain digestion of $F(ab')_2$ variants of E25. Peaks 3'–7', shown in Figure 6, were individually collected, subjected to papain digestion and separated under the same conditions as in Figure 4, resulting in the three Fab fragments labeled Fab-I, Fab-II, and Fab-III.

correspond with peaks 3–7 in the chromatogram of intact E25 shown in Figure 3. The $F(ab')_2$ fragments are better resolved on the HIC column, and so these were used rather than the intact antibody for characterization of the combinations of variants found in E25. The $F(ab')_2$ peaks are labeled 3'–7' to emphasize their relationship to the separation of the components of intact E25.

Papain Digestion and CDR Mapping of $F(ab')_2$ Fragments. Each of the $F(ab')_2$ peaks in Figure 6, denoted 3'–7', was individually collected and subjected to digestion with papain in order to identify differences among the species. Papain digestion of each of the bivalent $F(ab')_2$ variants yields two antigen binding fragments that can be resolved by HIC. Figure 7 shows chromatograms identifying each of the Fab fragments released by digestion of each of the peaks collected from the chromatogram shown in Figure 6. The papain digest of peak 3' comprises only the Fab-I variant; that is, it contains the expected Asp-L32 residue in both antigen binding sites. Peak 4' yields roughly equal amounts of Fab-I and Fab-II and so has Asp-L32 in one binding site and iso-Asp-L32 in the other binding site of the antibody, while peak 5' shows all three forms of Fab in the papain map. Peak 6' yields mainly Fab-II and Fab-III in an approximately 2:1 ratio, with only trace amounts of Fab-I. Peak 7' yields mainly Fab-III, with much smaller amounts of the other two species. The assignments of identities to peaks 5'–7' are complicated by the relatively rapid hydrolysis of cAsp residues under the conditions employed for the papain digestion. Fab-III is the isoform containing cAsp-L32 and appears in the papain maps of peaks 5'–7'. Hydrolysis of this residue to Asp and iso-Asp must be taken into account in order to interpret the papain maps of these fragments. Hence, peak 5' represents the species of antibody with Asp-L32 in one antigen binding site and cAsp-L32 in the other. By this reasoning, the appearance of a relatively small amount of the iso-Asp form of the Fab following digestion is due to hydrolysis of the cAsp form, Fab-III. Peak 6' represents the coelution of antibody fragments containing iso-Asp in both binding sites and the isoform with iso-Asp in one site and cAsp in the other. Finally, peak 7' consists of the fragment with cAsp in both antigen binding domains. Hence, this analysis distinguishes those isoforms that are unmodified (peak 3') from those with one unmodified antigen binding site (peaks 4' and 5') and from those that are

Table 3: Identities and Binding Affinities of E25 Isoforms Resolved by HIC

peak ID ^a	F(ab') ₂ peak ID ^b	number of Lys residues at position H451 ^c	residues at L-32 sites in bivalent antibody ^d	relative F(ab') ₂ binding affinity ^e
1	NA	2	Asp-Asp	NA
2	NA	1	Asp-Asp	NA
3	3'	1	Asp-Asp	100 ± 3.8
4	4'	0	Asp-iso-Asp	42 ± 1.5
5	5'	0	Asp-cAsp	51 ± 0.6
6	6'	0	iso-Asp-iso-Asp	15 ± 0.5
			iso-Asp-cAsp	
7	7'	0	cAsp-cAsp	22 ± 0.7

^a HIC peak number of the intact antibody, as shown in Figure 3.

^b HIC peak number of the F(ab')₂ antibody fragment, as shown in Figure 5.

^c NA = not applicable owing to removal of Fc fragment by pepsin.

^d 2 indicates the presence of both Lys-H451 residues on the antibody, 1 indicates a single remaining Lys residue, and 0 denotes that both Lys residues have been removed from the molecule. ^e Entries refer to the principal variant occurring in residue 32 in each of the light chains.

The two entries indicate the two individual light chains in the bivalent antibody. Asp = aspartate, iso-Asp = isospartate, and cAsp = cyclic imide intermediate in the conversion between Asp and iso-Asp.

^e Activity measurements are normalized to the native Asp-Asp form of the antibody.

modified in both sites (peaks 6' and 7'). The assignments of the seven peaks in the chromatogram of E25 shown in Figure 3 can be made by analogy to the results obtained on pepsin-digested antibody and are summarized in Table 3 along with the identities of the F(ab')₂ variants. The assignments in Table 3 only show the principal components of each of the peaks of the intact antibody, since there are expected to be coelutions of minor components that sport different combinations of light chain Asp-L32 and heavy chain Lys-H451 heterogeneity.

In Vitro Activity of E25 F(ab')₂ Variants

The effect of isomerization of Asp-L32 on the affinity of E25 for IgE was assessed by measuring the ability of F(ab')₂ variants purified by HIC to inhibit binding of IgE to its high-affinity receptor. Peaks 3'–7' from a separation such as shown in Figure 6 were collected and tested for their relative ability to block binding of IgE to the receptor, huFcεRI, with results shown in Table 3. The singly modified forms of the antibody (peaks 4' and 5') have roughly half the binding affinity for IgE as the native form of the antibody (peak 3'). Furthermore, when the antibody is modified at both functional valences (peaks 6' and 7'), the relative binding affinity is again halved with respect to the singly modified species.

Interaction of E25 Variants with IgE

The effect of variations in the Asp-L32 residue on the affinity of E25 for IgE was further assessed by quantification of the ability of Fab variants to inhibit binding of IgE to its receptor. The residues important in affecting the affinity of E25 for IgE were previously defined (Presta *et al.*, 1993). This study included Fab fragments that were prepared by site-directed mutagenesis of the labile Asp-L32 and nearby residues. The mutants were used to probe the importance of various residues, including Asp-L32, in IgE binding and to examine the feasibility of stabilizing the molecule with respect to isomerization while maintaining antigen recognition. The results, summarized in Table 4, indicate that changing Asp-L32 decreased the relative binding affinity for

Table 4: Identities and Relative Binding Affinities for rhuMAb-E25 Fab Variants and Mutants

Fab variant or mutant	identity (variation from sequence shown in Figure 2)	relative binding affinity ^a
E25 Fab-I	CDR sequences shown in Figure 2	100 ± 3.9
E25 Fab-II	Asp-L32 → iso-Asp	13 ± 0.7
E25 Fab-III	Asp-L32 → cAsp	16 ± 2.6
V10	Asp-L30 → Ala	
	Asp-L32 → Ala	<1.0
	Asp-L34 → Ala	
V100	Asp-L32 → Ala	27 ± 2.7
V103	Asp-L32 → Ala	
	Asp-H55 → Ala	56 ± 2.0
	His-H105 → Ala	
V104	Asp-L32 → Glu	33 ± 2.1

^a Activity measurements are normalized to the native Asp-Asp form of the antibody.

IgE significantly, whether the mutant residue was an alanine (V100), glutamate (V104), or the isomerization variants of aspartate (Fab-II and Fab-III). Previous studies (Presta *et al.*, 1993) had shown that the affinity of the antibody for IgE can be eliminated by mutating Asp-L30 and Asp-L34 along with Asp-L32, as shown by the result obtained for the V10 mutant. The loss of binding suffered by changing Asp-L32 to Ala can be partially recouped by changing two residues in the heavy chain to Ala, as in the V103 Fab.

DISCUSSION

Among the modifications undergone by proteins following expression are chemical changes such as the isomerization of an Asp-Gly sequence described here. The biological significance of isomerization of aspartate residues is unknown, but an enzyme has been identified, protein isospartyl methyltransferase, that methylates iso-Asp residues and that may be a repair enzyme for reconversion of these residues to Asp (Aswad, 1984; Ota & Clarke, 1989). Hence, isomerization of Asp residues, under certain conditions, likely occurs *in vivo*. The *in vivo* role of isomerization of Asp residues in the CDR of a natural antibody is probably negligible, since polyclonal antibodies consist of a diverse assemblage of sequences with varying affinities, so any secondary chemical effect on a particular sequence will be insignificant. Hence, there would be no evolutionary pressure to avoid labile sequences in the CDR, as there might in other proteins, where significant chemical modifications would have structural and functional consequences for the organism. In the special case of monoclonal recombinant antibodies, which have significant technological importance, chemical modification of CDR residues following expression may be consequential and should be taken into account in the engineering of a therapeutic antibody.

An Asp-Gly sequence in a recombinant human monoclonal antibody was shown here to undergo isomerization to a β-amino acid, through a cyclic intermediate, giving rise to variants of the antibody that differed at this site, containing either iso-Asp-Gly or succinimide in place of the expected sequence. Identification of the reaction products was facilitated by the use of hydrophobic interaction chromatography for separation of variants of the intact antibody and fragments generated by selective protease digestion. Following digestion with papain, the three variant Fab fragments could be purified by HIC (Figure 4). The residue at the labile position, where Asp was expected, was determined by a

combination of tryptic mapping, mass spectrometry, and N-terminal sequencing. Termination of the Edman sequencing reaction was used to identify the isoAsp residue in a peptide that had the same mass as the expected sequence. Electrospray mass spectrometry of the Fab fragment was sufficiently accurate to demonstrate the increase in mass (18 amu) expected for the cyclic imide-containing variant, even though the fragment itself has a mass near 50 kDa. Pepsin digestion was then employed to generate $F(ab')_2$ fragments, to permit identification of forms of the antibody that carried different combinations of the isomerization product in each of the antigen binding sites. The occurrence of isomerization at a single residue accounted for all of the heterogeneity found in the CDRs of the antibody.

Evidence for isomerization was found in the Asp-L32-Gly-I-33 sequence in CDR-L1, but not for the other potential sequence, Asp-I155-Gly-I156, that is in CDR-H2 of the antibody. The two sequences are both present in loops that interact with antigen and so are exposed to the same environmental conditions during the incubation that gave rise to isomerization in CDR-L1. Analysis by HIC separation, peptide mapping, sequencing, and mass spectrometry indicated only the presence of the expected H2 sequence and no evidence for isomerization at this site. The marked difference in reactivity of the two sequences confirms the role of higher-order structure in the rate of reactions, such as isomerization and deamidation, that proceed in proteins through a cyclic intermediate (Geiger & Clarke, 1987).

Monoclonal antibodies, such as E25, provide a useful tool for investigation of the influence of structure on the reactivity of a peptide sequence since the crystal structures of many Fab fragments have been solved and can be compared to elucidate the likely conformation of a given sequence. In order to investigate why the Asp-Gly sequence in E25 CDR-L1 forms isoaspartate but the same sequence in E25 CDR-H2 does not, we compared CDR loops from Fab crystal structures in which the size and sequence of the CDRs were similar to those in E25. CDR-L1 is a member of the murine κ -light chain subgroup III, and in this subgroup, a Tyr-Gly sequence is most common, though 8 of 89 sequences contain Asp(or Asn)-Gly (Kabat *et al.*, 1991). Only two such crystal structures are available in the Protein Data Bank (Bernstein *et al.*, 1977): 1ACY has Tyr-Gly (Ghiara *et al.*, 1994) and 1GGB has Asp-Gly (Rini *et al.*, 1993). The CDR-L1 loops from these two crystal structures were superimposed using backbone atoms of the first two and last two residues of the CDR (i.e., residues 24, 25, 33, and 34). For these two loops, much of the loop can be superimposed except for the residues near the Tyr-Gly and Asp-Gly sequences (Figure 8, left). For CDR-H2, five Fab crystal structures contain a CDR-H2 with the same size and similar sequence as E25; 1GGB (Rini *et al.*, 1993) and 1VFB (Bhat *et al.*, 1994) have an Asp-Gly sequence (Asp side chains shown in Figure 8, right), 3HFM (Padlan *et al.*, 1989) and 1BAF (Brunker *et al.*, 1991) have a Ser-Gly sequence, and 7FAB (Saul & Poljak, 1992) has a Thr-Gly sequence. The five CDRs were superimposed using backbone atoms of residues 49, 50, 59, and 60. In contrast to CDR-L1, the five CDR-H2 loops have similar conformation, with 3HFM being the most dissimilar (Figure 8, right). Four of the five have a type I reverse turn (with the Gly residue as the fourth residue of the turn; 3HFM does not have the turn), and the side chain at position 54 accepts a hydrogen bond from the backbone peptide $>NH$ of residue

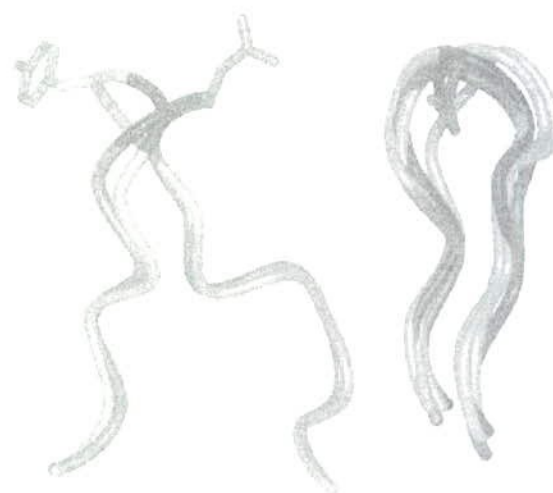


FIGURE 8: Crystal structures of (left) CDR-L1 and (right) CDR-H2 sequences similar to those of E25. The structures of the CDR loops were drawn from published coordinates and superimposed at the first and last two residues of the fragments illustrated as described in the text. The side chains of aspartic acid or asparagine residues in the position corresponding to Asp-L32 or Asp-I155 in the pertinent sequences are shown.

56. The presence of the reverse turn as well as the side chain-backbone hydrogen bonds may stabilize the conformation of CDR-H2 and thereby prevent formation of isoaspartate. The results suggest that the sequence is too constrained to undergo the isomerization reaction observed in CDR-L1. The relatively large differences in crystal structures of the CDR-L1 sequences, on the other hand, suggest that this part of the antibody is relatively unconstrained, accounting for its greater reactivity. Examination of the crystal structures of these sequences thus is a useful tool for the explanation of the large difference in reactivities of the two Asp-Gly sequences in the antibody.

No structural determination of the perturbation caused by isomerization has been reported for a protein, but the potential for alteration in structure is suggested by Figure 1. The products of isomerization differ from the Asp-Gly sequence, with the peptide backbone lengthened by a methylene group in the iso-Asp-Gly variant and incorporating a five-membered ring in the cAsp form. Small changes in sequence of the CDRs have been shown to cause significant losses of binding affinity in E25 (Presta *et al.*, 1993), so we examined the effect of isomerization on affinity of binding to IgE. $F(ab')_2$ fragments, which could be purified more easily than variants of intact E25, were tested in an assay for inhibition of binding of IgE to its receptor. The results (Table 3) demonstrate a reduced ability of the products of the isomerization reaction to bind IgE. The forms of E25 that are modified at one antigen binding site retain half the activity of the unmodified $F(ab')_2$ in this assay. Variants that contain isomerization products in both binding sites retain only about 20% of the activity of the fragment with the expected sequence. Hence, isomerization of the Asp-Gly sequence of CDR-L1 of E25 is associated with loss of affinity for antigen.

Further studies incorporating previously reported findings (Presta *et al.*, 1993) were undertaken to evaluate modifications to the CDRs that prevent isomerization in the antigen binding sites. A previous study (Presta *et al.*, 1993)

demonstrated the key importance of the three Asp residues found in CDR-L1 of E25 on antigen recognition. Mutation of the three residues to alanine eliminates affinity for IgE as shown by the result obtained for the V10 mutant. Mutation of only the labile Asp-L32 residue to alanine reduces the relative potency of the Fab by about 4-fold (Table 4). Substitution of Asp with Ala has the effect of removing the highly polar carboxylate side chain of the acidic residue, without, presumably, altering the protein's α -carbon structure. Hence, this side chain plays a role in affinity for IgE. The resulting mutant cannot undergo isomerization but exhibits an affinity that is only slightly better than that of the variant isoforms (Fab-II and Fab-III) and so is not an improvement over E25. The V104 mutant retains the acidic side chain of residue L32 by substituting a glutamate residue for aspartate, but this mutant also shows significantly reduced affinity for antigen, and also is no improvement over E25. Mutation of His-H105 to alanine has previously been shown (Presta *et al.*, 1993) to increase the affinity of E25 for IgE, so this change was incorporated into a mutant in which both potential sites of isomerization were mutated, with a partial improvement in binding affinity, as shown by the result obtained for the V103 mutant. This mutant cannot undergo isomerization, and so represents a chemically stable species, but does not exhibit the same potential as E25 for blocking the binding of IgE to its receptor. These studies demonstrate sensitivity of antigen recognition to subtle sequence changes in the CDRs and the tradeoffs incurred in engineering out a labile sequence.

The work reported here demonstrates the difficulty of predicting whether an Asp residue will undergo isomerization and the need for experimental techniques for making this determination. We have investigated the utility of hydrophobic interaction chromatography (HIC) in the characterization of the antigen binding domains of a monoclonal antibody that binds to IgE. Previous studies have shown that HIC is selective for gross differences in antibody structure and thus has been useful in the purification of bispecific antibodies (Neblock *et al.*, 1992), hybrid MAbs (Abe & Inouye, 1993), Fab' fragments (Morimoto & Inouye, 1991), and MAbs containing asymmetric Fab glycosylation (Grebenau *et al.*, 1992). Other modes of chromatography, such as chromatofocusing (Jungbauer *et al.*, 1990) and cation-exchange (McDonough *et al.*, 1989), have been used to identify and purify isoforms found in MAb preparations, generally reflecting much greater sequence and structural variations than expected for a recombinant protein. HIC under the conditions employed here shows high selectivity for the CDR of rMAbs and therefore may be useful for identification of structural differences that affect antigen binding affinities.

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