

Doctor

José Luis Salazar

Director de División de Nuevas Creaciones

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

E. _____ S. _____

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



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Solicitud de Patente de Invención titulada: "Anticuerpos humanizados que reconocen la alfa-sinucleína"

Solicitante: Neotope Biosciences Limited

RESPUESTA AL AUTO NOTIFICADO POR FIJACIÓN EN LISTA NO. 25
DEL 23 DE JUNIO DE 2015 OFICIO NO.6779 (EXAMEN DE FONDO)

ÁLVARO CORREA ORDÓÑEZ, mayor de edad y vecino de Bogotá D.C., identificado tal como aparece al pie de mi firma, en mi carácter de apoderado de la sociedad en referencia, doy respuesta al auto emanado por ese Despacho dentro del término de prórroga solicitado en mi memorial 14-113.186-00006-0000 de fecha 17 de septiembre de 2015:

1. Respuesta a las observaciones del Examinador

1.1 Claridad de la solicitud

- Reivindicación 1

Su Despacho establece que la reivindicación 1 no es clara, ya que hace referencia a porcentajes de similitud. El examinador sugiere que se caracterice las posibles variantes estructurales de las secuencias citadas en estas reivindicaciones por sus secuencias completas o por las sustituciones específicas a realizar.

- Reivindicaciones 1, 15 y 17

Su Despacho establece que las reivindicaciones 1, 15 y 17 no son claras porque la expresión "excepto que siempre y cuando" es imprecisa.

- Reivindicación 22

Su Despacho menciona que la reivindicación 22 no es clara porque no se especifica la respectiva secuencia de ácido nucleico que codifica la región variable de cadena pesada y de cadena ligera del anticuerpo aislado.

- Reivindicación 24

Su Despacho establece que la reivindicación 24 carece de sustento ya que no describe ni identifica el proceso para producir células transformadas como una línea celular y no define el vector especificando el tipo de vector y la secuencia recombinante que contiene.

- Reivindicación 25

Su Despacho establece que la reivindicación 25 no es clara porque no define la composición farmacéutica en términos de sus características técnicas esenciales, especificando sus ingredientes, excipientes en las cantidades o proporciones específicas características de dicha composición.

1.2 Modificación a una solicitud en trámite

Con el objeto de atender a las anteriores observaciones y de conformidad con el artículo 34 de la Decisión 486, presento un nuevo pliego reivindicatorio conformado por 10 reivindicaciones.

El nuevo pliego reivindicatorio se encuentra totalmente sustentado en la memoria descriptiva originalmente presentada.

El examinador podrá notar que la reivindicación 1 se modificó, las reivindicaciones 3 a 13 fueron canceladas.

El término "siempre y cuando" se cambió por "siempre que".

La reivindicación 22 fue modificada y las reivindicaciones 24 y 25 fueron canceladas.

De esta forma se da por superada las observaciones de claridad. Es decir, el nuevo pliego reivindicatorio cumple a cabalidad con el artículo 30 de la Decisión 486.

1.3 Novedad

Su Despacho reconoce la novedad de la solicitud en estudio. Por lo tanto, el requisito a sustentar es el nivel inventivo. Tal como se demuestra a continuación.

1.4 Nivel inventivo

El Examinador afirma que los documentos **D1**¹ (WO2010069603) y **D2**² (Masliah E. et al., 2011. Plos One. Vol. 6, page 1-17) afectan el nivel inventivo de las reivindicaciones 1 a 25 de la solicitud en estudio.

En forma resumida su Despacho argumenta que la diferencia entre la invención definida en la reivindicación 1 y el anticuerpo monoclonal humano anti-alfa-sinucleína divulgado en el documento D1, consiste en las secuencias de aminoácidos que comprenden la región variable de cadena pesada (VH) y ligera (VL); en las cuales las posiciones de aminoácidos L36 y L83, pueden ocuparse por Y o F y F o L respectivamente en VL, y las posiciones H73 y H93 pueden ocuparse por N o D y A o S respectivamente en VH.

Argumenta su Despacho que no hay evidencia de avances o ventajas asociadas al diseño de un anticuerpo que se una específicamente a la alfa sinucleína humana, debido a que el documento D1 evidencia resultados conductuales similares a los obtenidos por la solicitante

¹ "Human anti-alpha-synuclein autoantibodies

² "Passive Immunization Reduces Behavioral and Neuropathological Deficits in an Alpha-Synuclein Transgenic Model of Lewy Body Disease"

en el ejemplo II, de manera que el documento D1 divulga métodos terapéuticos para el tratamiento o prevención de una enfermedad generada por cuerpos de Lewy, o alfa sinucleinopatías, que resuelven el mismo problema técnico planteado por la presente solicitud.

Por lo tanto, el problema técnico objetivo que pretende resolver esta invención se puede formular así: "Cómo proveer un anticuerpo monoclonal humano alternativo que se una de manera específica a la proteína alfa-sinucleína humana, que sea capaz de disminuir la acumulación de la alfa-sinucleína, disminuyendo el riesgo de desarrollar una enfermedad de cuerpos Lewy".

Sin embargo, el documento D2 divulga que la inmunización pasiva a partir de anticuerpos dirigidos específicamente hacia el extremo C-terminal de la proteína alfa-sinucleína en el sistema nervioso central (SNC), podría tener un potencial terapéutico para el tratamiento de enfermedades asociadas con la acumulación de dicha proteína, tales como la enfermedad de Parkinson.

En consecuencia, la persona del oficio normalmente versada en la materia incluiría la sugerencia de realizar inmunización pasiva a partir de anticuerpos dirigidos específicamente hacia el extremo C-terminal de la proteína alfa-sinucleína como un potencial método terapéutico para el tratamiento de enfermedades asociadas con la acumulación de dicha proteína, divulgadas en el documento D2, en el procedimiento para la producción de anticuerpos anti-alfa-sinucleína divulgado en el documento D1.

Concluye su Despacho que la reivindicación 1 cumple con el requisito de novedad pero no cumple con el requisito de nivel inventivo.

1.4.1 Argumentos en favor del nivel inventivo de la solicitud en estudio

El nivel inventivo de una solicitud se encuentra definido en el Artículo 18 de la Decisión 486 y se establece cuando *"si para una persona del oficio normalmente versada en la materia técnica correspondiente, esa invención no hubiese resultado obvia ni se hubiese derivado de manera evidente del estado de la técnica"*.

Contrario a lo que plantea el Examinador, los documentos del arte previo citado no afectan el nivel inventivo de la presente solicitud por las siguientes razones:

Como se mencionó anteriormente se presenta un nuevo pliego reivindicatorio.

Las reivindicaciones como se encuentran ahora modificadas se dirigen al anticuerpo humanizado denominado anticuerpo V3H3 en las reivindicaciones. Este anticuerpo está ahora en ensayos clínicos en humanos para el tratamiento de la enfermedad de Parkinson.

Este anticuerpo tiene una retromutación de estructura principal de región variable en la cadena pesada en la posición H73 y dos retromutaciones de estructura principal de región variable de cadena ligera en las posiciones L36 y L83 (Tablas 2 y 3, párrafo 131). La estructura principal de región variable de cadena ligera es del anticuerpo humano AAY3335 y la estructura principal de región variable de cadena pesada es del anticuerpo AAC50998 (párrafo 12). La descripción proporciona datos de inmunoprecipitación (Fig. 5) que sugieren que el anticuerpo reivindicado tiene una unión más fuerte a alfa-sinucleína que el anticuerpo de ratón del que se deriva (la banda que corresponde a alfa-sinucleína aparece más fuerte). Las afinidades relativas han sido cuantificadas mediante análisis de afinidad SPR (véase más adelante) que proporciona evidencia adicional de que el anticuerpo H3L3 se une con más fuerza que el anticuerpo de ratón subyacente.

Un anticuerpo humanizado que combina las propiedades de unión más fuertes que el anticuerpo de ratón subyacente y que tiene sólo tres retromutaciones es un resultado notable más allá de lo que se espera razonablemente de la experiencia pasada en la que los anticuerpos más humanizados han tenido más retromutaciones y / o afinidad reducida.

En más detalle, la humanización de un anticuerpo a menudo implica un compromiso de consideraciones que compiten para lograr una alta afinidad sin inmunogenicidad. El trasplante de CDR de ratón en las estructuras principales de la región variable humana reduce la inmunogenicidad relativa a un anticuerpo de ratón, pero por lo general conduce a una gran pérdida de afinidad, debida a determinados residuos de estructuras principales de regiones variables de ratón (que varían de un anticuerpo a otro) contribuyen a la afinidad. La afinidad se puede aumentar al retromutar determinados residuos de estructura principal de región variable humana de nuevo a los residuos de ratón correspondientes, pero las

retromutaciones resultan en un carácter más similar a ratón y por consiguiente más potencial para inmunogenicidad. Por consiguiente, el objetivo en la humanización de un anticuerpo de ratón es lograr una afinidad de unión aceptable sin un número excesivo de retromutaciones y el consiguiente potencial de inmunogenicidad.

La mayoría de anticuerpos humanizados producidos hasta la fecha han incluido un número sustancial de retromutaciones. Las anteriores se ilustran por la Queen, US 5.585.089, que en las columnas, 43-44 proporciona la Tabla 1 que muestra las posiciones sustituidas en cinco anticuerpos humanizados que tienen de 11-23 variables sustituciones de estructura principal de región variable por anticuerpo.

El valor de un anticuerpo con números de retromutaciones más pequeños se ilustra mediante el siguiente extracto de un artículo sobre el diseño del anticuerpo comercial conocido Tysabri® (natalizumab).

Originalmente, se construyen y prueban dos versiones de cada una de las regiones AN100226FVL y VH. Todas las versiones probadas dieron buena unión al antígeno. Sin embargo, se obtuvo la mejor unión al antígeno, con el número mínimo de alteraciones en las FR humanas con la versión La de la región VL y la versión Ha de la región de VH. Combinadas representan un anticuerpo humano reformado que tuvo un total de cuatro cambios de aminoácidos para los FR de REI humanos en la región VL y cinco cambios de aminoácidos para los FR humanos 21/28'CL en la región VH.

Leger et al., Human antibodies 8, 1 hasta 18 (1997) p. 14, párrafo segundo.

La variante HaLa del anticuerpo humanizado fue llevada a ensayos clínicos y comercialización y ahora cuenta con aproximadamente dos mil millones de dólares en ventas anuales. Minimizar las retromutaciones fue un factor importante en su selección. El número de retromutaciones se redujo al mínimo incluso con más éxito en el anticuerpo reivindicado ahora (es decir, tres retromutaciones comparados con nueve en natalizumab).

A pesar de que incluye un número considerable de sustituciones de estructura principal de región variable, muchos anticuerpos humanizados producidos hasta la fecha han mostrado afinidad de unión inferior a sus homólogos murinos. Al resumir las afinidades de unión de

los anticuerpos humanizados en comparación con aquellos de los anticuerpos de roedores que son humanizados, Chiswell y McCafferty indican:

A pesar de nuestro conocimiento de la estructura de la molécula de anticuerpo ... anticuerpos humanizados no suelen tener la afinidad del mAb del roedor original. En la práctica, el éxito está generalmente reclamado por un anticuerpo con sólo el 33-50% de afinidad inicial.

TibTech 10, 80 (marzo de 1992).

Como se discutió anteriormente, los datos proporcionaron evidencia de que el anticuerpo reivindicado ha logrado una mejor afinidad que la reducción normal a nivel del 33-50%.

Hay una correlación general bien conocida entre la potencia y afinidad de anticuerpos (véase por ejemplo, Rathanaswami et al., BBR Comm. 334, 1004-1013 (2005), en el párrafo de puente páginas 1004-1005).

Además de la inmunogenicidad, las propiedades cinéticas del anticuerpo frecuentemente dictan su utilidad. Un anticuerpo de mayor afinidad o bien será capaz de unirse a su ligando más rápido (determinado por la constante de índice de asociación, k_{on}) permanecen unidos más tiempo (determinado por la constante de índice de disociación, K_{off}) o posee ambas propiedades. Dependiendo de la distribución en el tejido, formas expresadas (unidas a membrana o solubles), la actividad biológica y la concentración de una diana, puede haber una correlación directa entre afinidad y potencia de un anticuerpo. La administración de un anticuerpo de mayor afinidad y potencia también puede traducirse en una mejor eficacia in vivo. Los anticuerpos de mayor afinidad pueden ser capaces de ser utilizados a dosis más bajas para lograr los efectos clínicos deseados.

Por lo tanto, la mejora de la afinidad de un anticuerpo se considera como un resultado deseable.

Aunque la metodología general para humanizar anticuerpos ha existido durante muchos años, hay diferencias en la forma en que esta tecnología se aplica en la humanización de anticuerpos de ratón individuales que pueden hacer patentables determinados anticuerpos

humanizados. Se han emitido muchas patentes sobre anticuerpos humanizados particulares en todas las principales jurisdicciones de patentes a pesar de que la metodología general para producir dichos anticuerpos ha estado en la técnica durante muchos años.

Una primera decisión estratégica es qué anticuerpo humanizar. Hay muchos anticuerpos de ratón potenciales contra alfa-sinucleína como se explica en la referencia citada y en otros lugares.

Una decisión estratégica adicional es decidir cuántos y qué residuos retromutan. Como se discutió anteriormente la mayoría de anticuerpos más humanizados producidos hasta la fecha han incluido cifras considerables de sustituciones de estructura principal de región variable en la que un residuo humano se reemplaza con un residuo de ratón. El número de retromutaciones y sus posiciones varían dependiendo del anticuerpo de partida. Las consecuencias de retromutaciones en términos de su efecto sobre la afinidad no siempre son fáciles de predecir. Por esta razón, muchas permutaciones diferentes de retromutaciones se pueden ensayar en un intento por identificar una combinación que logra afinidad adecuada sin riesgo excesivo de inmunogenicidad.

Otra variable en la producción de un anticuerpo humanizado es la selección de secuencias aceptoras humanas para los que hay muchas posibilidades. Las opciones incluyen utilización de aceptores de secuencias de anticuerpos maduros o compuestos de dichas secuencias, secuencias consensus y secuencias de línea germinal. Secuencias aceptoras de cadena pesada y ligera pueden obtenerse de las mismas o de diferentes fuentes, como aquí. La selección de la secuencia aceptora humana es relevante para el número de sustituciones de estructura principal que deben llevarse a cabo, así como las características del anticuerpo humanizado resultante.

Visto desde una perspectiva de solución del problema, se sugiere que el documento D1 se considere como técnica anterior más próxima, ya que describe un anticuerpo 9E4 humanizado genérico. El documento D2 informa algunos de los mismos experimentos que el documento D1, pero se relaciona menos en no revelar los anticuerpos humanizados 9E4.

El problema puede ser visto como la producción de un anticuerpo 9E4 humanizado mejorado en relación con el anticuerpo humanizado genérico del documento D1. La

solución se encuentra en el anticuerpo con las secuencias de cadena pesada y ligera especificados en la reivindicación 1. Dicho anticuerpo combina una afinidad de unión por lo menos tan alta como la de ratón 9E4 con sólo tres retromutaciones.

Debido a las muchas variables en el diseño de anticuerpos humanizados, diferentes anticuerpos humanizados tienen distintos grados de éxito en términos de afinidad de unión y un bajo número de retromutaciones. La mayoría tienen menor afinidad con respecto al anticuerpo de ratón y más retromutaciones que el anticuerpo reivindicado, como se discutió anteriormente.

En vista de este estado de la técnica, la solución proporcionada por el anticuerpo reivindicado que combina una alta afinidad de unión con pocas retromutaciones no podría haberse esperado razonablemente.

En vista de lo anterior, la presente invención es inventiva con relación a los documentos D1 y D2.

Adicionalmente, se presentan datos experimentales (copia adjunta), que sustentan el nivel inventivo de la solicitud en estudio.

El análisis se realizó utilizando un Biacore T200 para comparar la afinidad de unión de 9E4 y PRX002 a la alfa-sinucleína humana recombinante. Cualquier anticuerpo Fc anti-ratón o anti-humano se inmovilizó sobre chip sensor CM5 a través de acoplamiento de aminas, y se capturó 9E4 o PRX002 (ligando) a un nivel para asegurar una máxima unión de analito de 50 RU (~ 250RU de unión del ligando). Varias concentraciones de alfa-sinucleína (que van desde ~ 1 nM a 500 nM) se pasó sobre el ligando capturado a 70 µl / min en regulador de serie (HBS + 0,05% P-20, 1 mg / ml de BSA) para asociación 180s / disociación 360s. Los datos fueron blancos sustraídos a un sensor irrelevante que no contiene anticuerpos y concentración de analitos de 0 nM. El análisis se realizó utilizando un ajuste global de 1: 1 con software de Evaluación Biacore.

En virtud de lo anterior, solicito que se acepten las razones expuestas en el presente documento y en consecuencia, se otorgue el privilegio de patente de invención para el invento que se tramita en este expediente.

Atentamente,



ÁLVARO CORREA ORDÓÑEZ
C.C.No.79.143.366 de Usaquén
T.P. 20.281

Anexos:

- Datos experimentales
- Capítulo reivindicatorio modificado conformado por 10 reivindicaciones
- Copia del documento "Phage antibodies: will new "coliclonal" antibodies replace monoclonal antibodies? (página 80 y 82)
- Copia del documento "Humanization of a mouse antibody against human alpha-4 integrin: A potential therapeutic for the treatment of multiple sclerosis" (página 1 y 14)
- Copia del documento "Demonstration of an in vivo generated sub-picolomar affinity fully human monoclonal antibody to interleukin-8 (1004-1005).

M

REIVINDICACIONES MODIFICADAS EN LA SOLICITUD DE PATENTE Núm.

14.113.186

Octubre 2015

1. Un anticuerpo que comprende una región variable de cadena pesada madura designada SEQ ID NO: 10 y una región variable de cadena ligera madura tiene una secuencia de aminoácidos designada SEQ ID NO: 5.

2. El anticuerpo de la reivindicación 1, en donde la región variable de cadena pesada madura se fusiona a una región constante de cadena pesada y la región constante de cadena ligera madura se fusiona a una región constante de cadena ligera.

3. El anticuerpo de la reivindicación 2, en donde la región constante de cadena pesada es una forma mutante de la región constante humana natural que tiene la unión reducida a un receptor Fcγ en relación a la región constante humana natural.

4. El anticuerpo de la reivindicación 2 o 3, en donde la región constante de cadena pesada es del isotipo IgG1 humano.

5. El anticuerpo de la reivindicación 2, en donde la región constante de cadena pesada tiene la secuencia de aminoácidos designada SEQ ID NO: 32 siempre que se pueda omitir el residuo de lisina C-terminal.

6. El anticuerpo de la reivindicación 2, en donde la región constante de cadena pesada tiene la secuencia de aminoácidos designada SEQ ID NO: 14 siempre que se pueda omitir el residuo de lisina C-terminal.

7. El anticuerpo de cualquiera de las reivindicaciones 2 a 6, en donde la región constante de cadena ligera tiene la secuencia de aminoácidos designada SEQ ID NO: 13.

8. El anticuerpo de cualquiera de las reivindicaciones 2 a 6, en donde la cadena ligera tiene la región constante designada SEQ ID NO: 28.

9. El anticuerpo de la reivindicación 5, en donde la región constante de cadena ligera tiene la secuencia de aminoácidos designada SEQ ID NO: 13.

10. Un ácido nucleico que codifica una región variable de cadena pesada madura y/o una región variable de cadena ligera madura que tiene una secuencia que comprende una cualquiera de la SEQ ID NO: 15, 17, 18, 19 y 20.

SPR affinity determinations

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Identity	k_a (M ⁻¹ s ⁻¹) (on-rate)	k_d (s ⁻¹) (off-rate)	k_D (M ⁻¹) (affinity)
m9E4	$4.29 \pm 0.23 \times 10^5$	$1.22 \pm 0.2 \times 10^{-2}$	$2.84 \pm 0.47 \times 10^{-8}$
h9E4 (PRX002)	$2.46 \pm 0.7 \times 10^6$	$3.37 \pm 0.45 \times 10^{-2}$	$1.41 \pm 0.2 \times 10^{-8}$

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Phage antibodies: will new 'coliclonal' antibodies replace monoclonal antibodies?

David J. Chiswell and John McCafferty

Antibodies can now be rapidly isolated from large and diverse recombinant libraries by displaying functional antibody fragments on the surface of bacteriophage particles and directly selecting with antigen. This method has been used to isolate antibodies, including human antibodies, with and without immunization, and to improve the affinity and specificity of antigen binding.

Monoclonal antibodies (mAbs) were arguably the most commercially successful of the new biotechnologies during the 1980s, being used mainly in sensitive *in vitro* diagnostic tests. Although the potential for therapeutic and *in vivo* diagnostic use for mAbs has still to be fully realized, more products are gaining regulatory approval, and antibodies could yet be the therapeutic molecules of the 1990s and beyond.

Most mAbs are still isolated using essentially the same technology as originally described by Köhler and Milstein¹: i.e. manipulation of the immune system to increase the number of B cells expressing antigen-specific antibodies, immortalization of these antibody-producing cells by cell fusion, and identification of stable cell lines secreting an antibody with the desired properties by extensive screening. The continued use of these techniques over 16 years of enormous technical change in the biosciences amply demonstrates the power and robustness of hybridoma technology. It now appears, however, that change is imminent.

'Coliclonal' antibodies

Recent advances have combined (1) the ability to produce functional antibody fragments in bacteria, (2) the isolation of antibody variable genes using polymerase chain reaction (PCR), and (3) powerful screening systems, to produce a radically more efficient methodology that enables isolation of antibodies more rapidly and more easily than has hitherto been the case with hybridoma-derived mAbs. We suggest that these new antibodies, isolated and manufactured in prokaryotic systems, be called 'coliclonal' antibodies. The new technology has the potential to extend the properties of coliclonal antibodies so that they are even more widely applicable than traditional mAbs have been.

D. J. Chiswell and J. McCafferty are at Cambridge Antibody Technology, Daly Research Laboratories, Babraham Hall, Cambridge CB2 4AT, UK.

This article illustrates the application of these new procedures, emphasizing particularly the recent advances made at Cambridge in the development of a powerful, bacteriophage-based screening system. For a recent comprehensive review dealing in depth with the earlier advances made in the field and the uses made of standard screening technology see Ref. 2.

Phage antibodies

McCafferty *et al.*³ demonstrated the feasibility of displaying fragments of antibodies on the surface of a bacteriophage and that these fragments folded correctly and bound antigen. Antibody variable-region genes were cloned into the gene encoding the minor coat protein (gene 3) of the filamentous bacteriophage fd. The fusion protein created, consisting of the antibody fragment at the N-terminus of the coat protein, is incorporated into the phage particle (a 'phage antibody'; see Fig. 1). Each recombinant phage genome contains the DNA encoding the specific antibody displayed on its surface, allowing the phage particle carrying antibody gene to be selected directly using the binding properties of the expressed protein.

This discovery opened a novel route for antibody isolation (Box 1). Repertoires of antibody genes² are amplified using PCR and cloned into phage, thus creating a large library of phage each displaying a specific antibody. Within these large libraries of phage antibodies (potentially representing between 10^6 and 10^9 different antigen-binding specificities) each phage expresses an individual heavy- and light-chain combination. To select an antibody of interest, the library of phage antibodies is passed over antigen bound to a solid surface such as a Sepharose column or a coated tube. Phage that display antibody fragments specific for the antigen are retained on the surface. These specific phage antibodies can be eluted and used to infect *E. coli* to give stable clones. The phage antibody can be both analysed and used directly from the culture supernatant as a reagent in techniques such as ELISA.

Box 1. Isolation of human/rodent antibodies using phage-antibody technology

(a) Antibody gene repertoire

The repertoire can be derived from the antibody genes expressed in a variety of B cells. A useful source of the genes is mRNA; IgG mRNA is preferred if using immunization, but more diverse libraries can be obtained by using IgM RNA⁶. Alternatively, repertoires could, in principle, be derived from foetal or germline antibody genes, or created artificially.

(b) Construction of phage-antibody library

The polymerase chain reaction (PCR) provides a convenient means of isolating whole repertoires of antibody variable genes². The genes are amplified using primers specific for conserved regions of the antibody gene families^{4,8} and cloned into phage vectors. It is important to use a cloning strategy that minimizes the use of restriction enzymes which are likely to cut within the coding regions of antibody genes, causing underrepresentation of whole antibody gene families in the library. We use only digests with very rare cutters (i.e. restriction endonucleases that have eight base-recognition sequences, or recognition sequences that are known to be rare in antibody genes). Heavy- and light-chain genes are amplified separately and linked using a PCR-based primer overlap strategy^{4,8}. The size of the library is directly influenced by the efficiency of transformation of the DNA into bacterial cells. For large libraries we use phagemid vectors that have a much higher transformation efficiency than phage vectors⁹.

(c) Antigen affinity selection

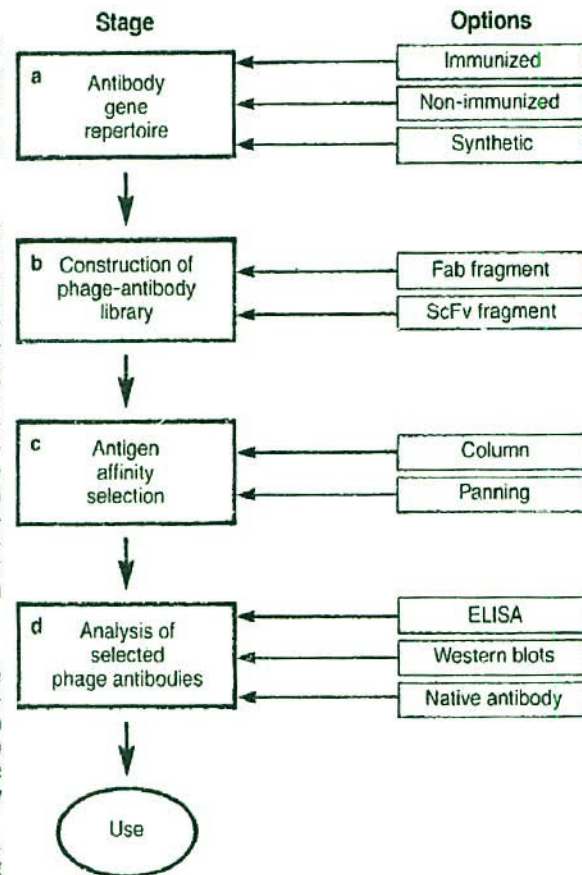
Antibodies are selected by subjecting the population of phage antibodies to antigen-affinity selection. Depending on the availability of the antigen, and the type of binding desired, this can be using columns of antigen immobilized on Sepharose³ or to 'pan' the library using plastic tubes or petri dishes which have been coated with antigen^{4,8}. This latter approach is generally simpler and uses less antigen.

After washing, binding phage can be specifically eluted, e.g. with an excess of antigen⁴, or nonspecifically eluted, e.g. using 100 mM triethylamine³.

Phage eluted from the affinity matrix are then used to infect *E. coli*. Since this phage genome carries a gene conferring drug resistance, infected cells can be selected on bacterial plates carrying antibiotic. Phage antibodies are prepared from individual clones by growth in liquid culture and harvesting the supernatant.

(d) Analysis of selected phage antibodies

Phage-antibody particles in the culture media can be used directly for subsequent analysis (e.g. by ELISA). Soluble fragments can easily be produced without the phage particle by subcloning, or (more easily) using vectors that contain an amber codon between the antibody and gene 3 (Ref. 5). These vectors produce soluble or phage antibodies depending on the bacterial strain infected. Single- or double-stranded DNA can easily be prepared for sequencing or mutagenesis.



shuffling' approach in which either the light or heavy chain of an antibody is retained and the partner replaced with a repertoire derived from another source. This has proven a fruitful approach to the improvement of both the specificity and affinity of antibodies. This approach has been used, for example, to remove unwanted crossreactivity from a steroid-hormone-specific mAb (A. Roberts *et al.*, submitted).

Affinity selection and CDR grafting

It is now possible to use the phage technology to isolate human antibodies directly for therapeutic use. However, if a suitable murine mAb has been isolated and many years spent in its characterization, it may still be more cost-effective to humanize this antibody by

reshaping⁹; phage technology can also help to achieve this. The binding properties of the rodent mAb are transferred to a human antibody framework by grafting the antigen-binding regions (complementarity determining regions; CDRs) onto a human framework, thereby creating a humanized antibody with almost the same binding characteristics as the rodent mAb. This process is not simply a matter of transferring only the CDRs; it also requires manipulation of the antibody backbone by identifying and altering key residues⁹⁻¹¹. However, despite our knowledge of the structure of the antibody molecule, these humanized antibodies do not usually have the affinity of the original rodent mAb. In practice, success is usually claimed for an antibody with only 33-50% of the original affinity^{9,10}. One possible consequence of this may be

Humanization of a mouse antibody against human alpha-4 integrin: A potential therapeutic for the treatment of multiple sclerosis

Olivier J.P. Léger, Ted A. Yednock*, Laura Tanner*, Heidi C. Horner*, Dawn K. Hines*, Simon Keen, José Saldanha, S. Tarran Jones, Lawrence C. Fritz*, and Mary M. Bendig

MRC Collaborative Centre, 1-3 Burtonhole Lane, Mill Hill, London UK; *Athena Neurosciences, 800F Gateway Boulevard, South San Francisco, CA USA

$\alpha 4 \beta 1$ integrin (VLA-4) is crucial for the adhesion of leukocytes to human vascular cell adhesion molecule-1 (VCAM-1) on inflamed endothelium. This cell adhesion event is the first step in leukocyte extravasation across the blood-brain barrier in inflammatory diseases of the central nervous system (CNS) such as experimental autoimmune encephalomyelitis (EAE). Prevention of leukocyte infiltration by antibodies against the $\alpha 4$ integrin, which block the $\alpha 4 \beta 1$ integrin/VCAM-1 interaction, have been shown to suppress clinical and pathological features of EAE. In this study, two mouse monoclonal antibodies (MAb) directed against human $\alpha 4$ integrin were analyzed in vitro for their ability to block the interaction of leukocytes with VCAM-1 under different assay conditions. The best blocking MAb, AN100226m, was humanized by complementarily-determining region grafting, associated with human C regions and expressed. We found that modification of two structural determinants (H27 and H29) for the heavy chain CDR1 loop in one hand, and modification of framework amino acid H38, H40 and H44 in the other hand, had no effect on antigen binding. In contrast, modification of a structural determinant (H71) for the heavy chain CDR2 loop resulted in loss of binding. The humanized antibody, AN100226, was equivalent to the murine antibody, AN100226m, in binding to $\alpha 4 \beta 1$ integrin and in blocking cell adhesion. More importantly, AN100226 was as effective as AN100226m in the reversal of active EAE in guinea pigs and thus may be useful in the treatment of autoimmune diseases such as multiple sclerosis. AN100226 is currently in phase II clinical trials in the UK for the treatment of multiple sclerosis exacerbations. [Hum Antibod 1997; 8: 3-16]

Keywords: Adhesion molecules; humanization; CDR-grafting; antibodies; $\alpha 4 \beta 1$ integrin; experimental allergic encephalomyelitis

Introduction

Inflammation is a response of vascularized tissues to infection or injury involving the adhesion of leukocytes to blood cells and subsequent leukocyte infiltration into the surrounding tissues. Under normal circumstances, inflammatory reactions are an essential part of host

defence, playing a critical role in the eradication of infectious agents. However, in pathologic inflammation, infiltrating leukocytes are over-responsive and can cause serious or fatal damage.

Adhesion of leukocytes to micro-vascular endothelium is essential for their migration into inflamed tissues. This response is mediated by the interaction of adhesion molecules expressed on the cell surface of leukocytes and venular endothelial cells¹. Very late antigen-4 (VLA-4, $\alpha 4 \beta 1$, CD49d/CD29) is a member of the $\beta 1$ integrin family of adhesion receptors. $\alpha 4 \beta 1$ mediates leukocyte adhesion to vascular cell adhesion molecule-1 (VCAM-

Correspondence and reprint requests to: Dr. Olivier Léger, MRC Collaborative Centre, 1-3 Burtonhole Lane, Mill Hill, London, NW7 1AD, UK. Accepted for publication September 1996.

those of AN100226m (i.e. 'best-fit' method). Thus the selected V_L and V_H were not derived from the same human Ab, but rather we selected human V regions that had the highest identity to the mouse V regions in an attempt to minimize the reintroduction of potentially immunogenic murine residues. In addition, a model of AN100226m V regions was built to help predict which amino acid residues in the FRs were likely to participate in some way in determining the specificity and/or affinity of binding to antigen. Care was taken to retain these critical amino acid residues in AN100226.

Originally, two versions each of AN100226 V_L and V_H regions were constructed and tested. All the versions gave good binding to antigen. Nevertheless, the best antigen binding, with the minimum number of alterations in the human FRs, was obtained with version La of the V_L region and version Ha of V_H region. Combined, they represented a reshaped human antibody which had a total of four amino acid changes to the human REI FRs in the V_L region and five amino acid changes to the 21/28'CL human FRs in the V_H region.

In the V_H region, four of the amino acid changes (Kabat positions 27 to 30) were adjacent to CDR1 and within an area defined by Chothia and Lesk⁴³ as part of the first loop structure. We have shown in the course of this humanization that amino acid residues at Kabat positions 27 and 29 can be kept human without altering the binding to antigen. The fifth amino acid change in the V_H region deals with the modification of amino acid at Kabat position 71 (H71). This amino acid comes in contact with both the CDR1 and CDR2 loops of the heavy chain⁴⁴. Based on crystallographic data, Chothia and colleagues^{44,45} have observed a systematic difference in position and conformation of a four amino acid loop sequence at the apex of some CDR2 loops depending on whether there is a small- to medium-sized amino acid (Ala or Val) or a large charged amino acid (Lys or Arg) at position H71. We observed a loss of binding to $\alpha 4\beta 1$ integrin when we substituted the murine residue Ala for the human residue Arg at position H71 in AN100226 (AN100226m has a four amino acid CDR2 structural loop). This difference in binding to $\alpha 4\beta 1$ integrin suggests that H2 is involved in antigen binding and that altering the residue at position H71 affects the conformation and position of AN100226 H2 loop. Better affinity is usually associated with maintaining the murine residue at position H71^{24,40,46} however Hsiao *et al.*⁴⁷ found that keeping the human residue at position H71 of the humanized form of 60.3 did not alter its binding to the CD18 target antigen.

Finally, in an attempt to better understand the process of humanization, we analyzed the amino acid residues at Kabat positions 38, 40 and 44 in the V_H region (H38, H40 and H44) of AN100226. These three residues often raise the dilemma of whether they should be kept human or reversed to the parental murine residues in the design of the reshaped human antibody. We found that all the combinations at these three positions, based on the residues from the selected human FR and the residues from the murine FR, gave similar binding to antigen. We conclude that amino acid residues at position H38, H40

and H44 are not critical for antigen binding and can therefore be kept as defined by the human template selected for CDR-grafting. This result constitutes useful information for the future design of humanization of Ab V regions.

In summary, with careful selection of human V regions to serve as the FR donors in CDR-grafting and with a few amino acid changes at carefully selected positions in these human FRs, a humanized form of AN100226m that displayed good binding to antigen was designed and constructed.

Further analysis to demonstrate that AN100226 (version Ha of the V_H region with version La of the V_L region) was fully functional were carried out with purified IgG4-antibody produced from stable cell lines. Because AN100226m MAb acts by blocking the interaction between $\alpha 4\beta 1$ integrin and VCAM-1, and not by mediating antibody-dependent cellular cytotoxicity, AN100226 (Ha/La) was produced as a human IgG4 isotype since this IgG subclass does not appear to bind the complement complex C1 and, therefore does not activate complement⁴⁸. Binding assays showed that AN100226m and AN100226 (Ha/La) exhibited almost identical affinity for human Jurkat T cells that express $\alpha 4\beta 1$ integrin on their surface. Both antibodies saturate the surface at a concentration of $0.2 \mu\text{g ml}^{-1}$ ($K_d=0.3 \text{ nM}$). Most importantly, AN100226 (Ha/La) was as efficacious as AN100226m in blocking the binding of Jurkat T cells to human VCAM-1, thus retaining the functional blocking characteristics of the mouse antibody.

Having shown that AN100226m and AN100226 behaved similarly *in vitro* in their ability to block $\alpha 4\beta 1$ integrin/VCAM-1 interactions, it was critical to establish that the humanized form of AN100226m was also effective in the treatment of EAE in animal studies. In EAE, circulating leukocytes enter the CNS producing inflammation, myelin damage and paralysis. The $\alpha 4$ integrin has been identified as an adhesion molecule critical for the entry of antigen-responsive cells from the blood into the brain, indicating that anti- $\alpha 4$ integrin inhibitors may afford a degree of specificity for treating mononuclear cell-mediated inflammation in the CNS. Antibodies to $\alpha 4$ integrin, such as AN100226m, can prevent mononuclear leukocyte infiltration into CNS and inhibit CNS inflammation in EAE⁹⁻¹¹. In this study, we demonstrate, as found, previously for AN100226m¹¹, that administration of AN100226 (Ha/La) after the onset of disease reversed the clinical symptoms of EAE and resulted in the clearance of leukocytes in the CNS. Thus the characteristics of AN100226m which make it a potentially useful therapeutic of autoimmune disorders of the CNS were fully retained by AN100226 (Ha/La). Because EAE is a disease with many similarities to multiple sclerosis (MS)⁴⁹, it is anticipated that inhibiting the action of $\alpha 4$ integrin by the use of antibodies to $\alpha 4\beta 1$ integrin, will provide an effective therapy for MS. Indeed, as discussed previously¹¹, anti- $\alpha 4$ integrin antibody has been shown to be effective in EAE in the three animal species where it has been examined; if this functional conservation extends to humans, this treat-



Demonstration of an in vivo generated sub-picomolar affinity fully human monoclonal antibody to interleukin-8

Palaniswami Rathanaswami^{a,*}, Shelly Roalstad^b, Lorin Roskos^c, Qiaojuan Jane Su^c, Steve Lackie^b, John Babcook^a

^a Abgenix Biopharma Inc., Burnaby, BC, Canada

^b Sapidyne Instruments Inc., Boise, ID, USA

^c Abgenix Inc., Fremont, CA, USA

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Abstract

The high specificity and affinity of monoclonal antibodies make them attractive as therapeutic agents. In general, the affinities of antibodies reported to be high affinity are in the high picomolar to low nanomolar range and have been affinity matured in vitro. It has been proposed that there is an in vivo affinity ceiling at 100 pM and that B cells producing antibodies with affinities for antigen above the estimated ceiling would have no selective advantage in antigen-induced affinity maturation during normal immune responses. Using a transgenic mouse producing fully human antibodies, we have routinely generated antibodies with sub-nanomolar affinities, have frequently rescued antibodies with less than 10 pM affinity, and now describe the existence of an in vivo generated anti-hIL-8 antibody with a sub-picomolar equilibrium dissociation constant. This confirms the prediction that antibodies with affinities beyond the proposed affinity ceiling can be generated in vivo. We also describe the technical challenges of determining such high affinities. To further understand the importance of affinity for therapy, we have constructed a mathematical model to predict the relationship between the affinity of an antibody and its in vivo potency using IL-8 as a model antigen.

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Ever since the development of hybridoma technology a quarter century ago, there has been a promise for monoclonal antibodies (mAbs) to be used as human therapeutic agents. Eighteen therapeutic mAbs are now on the market in the United States, with 16 of these having been approved in the last decade, and over 100 mAbs are currently in clinical development [1].

For the most part, the recent success of mAbs can be attributed to advances in antibody generation technologies. The first products of hybridoma technology were murine antibodies. The usefulness of these antibodies

as human therapeutics was limited by their immunogenicity; patients treated with murine antibodies rapidly mounted an immune response to these foreign proteins, a response known as a HAMA, for human anti-mouse antibody [2]. To avoid the HAMA response, we have developed XenoMouse strains that functionally recapitulate the human antibody response, including a vast repertoire of high affinity, somatically hypermutated human antibodies [3,4].

In addition to immunogenicity, the kinetic properties of the antibody often dictate their utility. A higher affinity antibody will either be able to bind its ligand faster (determined by the association rate constant, k_{on}), remain bound longer (determined by the dissociation rate constant, k_{off}) or possess both properties. Depend-

* Corresponding author. Fax: +1 604 676 8349.

E-mail address: swami.rathanaswami@abgenix.com (P. Rathanaswami).

ing on the tissue distribution, expressed forms (membrane-bound or soluble), concentration, and biological activity of a target, there can be a direct correlation between the affinity and potency of an antibody [5]. The administration of an antibody of higher affinity and potency may also translate into better in vivo efficacy [6,7]. Antibodies of higher affinity may be able to be used at lower doses to achieve the desired clinical effects. Lower dosing may allow for more convenient routes of administration and decreased injection volumes, which would translate into lower cost of goods.

Antibodies reported to be of high affinity are generally in the nanomolar range [8,9] and occasionally in the sub-nanomolar range [10]. Extensive in vitro modifications have been employed to increase the affinity of the antibodies to the picomolar range [7,11–13]. The use of XenoMouse mice, in conjunction with well-established antibody generation procedures, reproducibly results in high affinity fully human mAbs suitable for repeated administration to humans [14,15].

There are a number of technologies available to measure the affinity of an antigen–antibody interaction in the nanomolar range [16–20]. However, to measure the affinity of an interaction in the low picomolar range, a number of parameters may need to be modified [21]. It was recently shown that for two of these technologies, Biacore and KinExA, similar kinetic rate constants and affinities could be measured for a number of antibodies over a range of high affinity interactions [15]. Here, we describe the affinity characterization of very high affinity (low picomolar to sub-piccomolar) fully human antibodies generated from XenoMouse animals and the modifications required to measure these high affinities with precision using KinExA. Experiments were performed in two separate laboratories: Abgenix Biopharma, Burnaby, Canada and Sapidyn Instruments, Boise, USA.

Although it has been hypothesized that there could be an intrinsic affinity ceiling (100 pM) for the selection of antibodies generated in vivo [22,23], this report supports the existence of higher affinity antibodies derived from in vivo somatic hypermutation and affinity maturation, with affinities in the sub-piccomolar range. This implies that beyond the selection-driven affinity ceiling, higher affinities can still occur by chance and are not selected against. Alternatively, the assumptions underlying the limits of selection-driven affinity may need to be re-visited.

Materials and methods

Instrumentation and materials. Solution based kinetic exclusion assays were performed using the KinExA 3000 instrument (Sapidyn Instruments, Boise, ID). NHS-activated Sepharose 4 fast flow, CNBr-activated Sepharose 4B and protein A–Sepharose were obtained from Amersham Pharmacia (Amersham Biosciences, Piscataway, NJ). BSA was obtained from Sigma (St. Louis, MO). Cy5-conjugated (fluores-

cent dye based on indodicarbocyanine) affinity-purified goat anti-human IgG (Fc γ fragment-specific), goat anti-human IgG (H + L-specific) and mouse anti-human IgG [F(ab')₂ fragment-specific] Abs (all three have minimal cross-reactivity to bovine, horse and mouse serum proteins) were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). The recombinant human interleukin-8 (rhIL-8) (72 amino acid form) (CXCL8) (Cat. No. 200-08M) was obtained from PeproTech, Canada (Ottawa, ON). Titermax gold adjuvant was obtained from Sigma (Oakville, ON).

Generation of fully human anti-human interleukin-8 monoclonal antibodies. XenoMouse strains were produced as described previously [4]. Cohorts of XenoMouse mice were immunized with rhIL-8. The initial BIP (base of the tail by s.c. injection and i.p.) immunization was with 50 μ g of rhIL-8, mixed 1:1 v/v with complete Freund's adjuvant (CFA), per mouse. Subsequent boosts were made with 50 μ g of rhIL-8, mixed 1:1 v/v with incomplete Freund's adjuvant (IFA), per mouse. The animals were immunized on days 0, 14, 28, and 42. Then immunizations were continued for some mice with 50 μ g of rhIL-8 in Titermax gold adjuvant i.p. (on days 146, 160, and 181) and then with 10 μ g of rhIL-8 in PBS i.p. on day 205. A final boost was done with 10 μ g of rhIL-8 in PBS i.p. on day 226 (for two mice) and on day 234 (for two mice). Spleen and lymph nodes from XenoMouse animals were harvested 4 days after the last boost. The application of SLAM technology [24,25] resulted in the rescue of multiple high affinity, fully human anti-hIL-8 mAbs from the hyper-immunized XenoMouse animals. Briefly, B cells from the animals were harvested and cultured in plates [25]. Wells were screened by ELISA to identify B cells producing IL-8-specific antibodies. 1063 wells were identified as being positive for IL-8 binding. The single B cells secreting anti-IL-8 antibodies were identified using an antigen-specific hemolytic plaque assay, picked by micromanipulation, the heavy and light chain variable region cDNAs were amplified by RT-PCR and molecularly cloned into expression vectors as previously described [25]. The expressed recombinant antibodies were purified using protein A–Sepharose affinity chromatography and analyzed by non-reducing SDS–PAGE to assess purity and yield. Concentration was also confirmed by UV analysis at A_{280} and ELISA.

Preparation of antigen coated beads. Fifty micrograms of rhIL-8 was coupled to CNBr-activated Sepharose 4B. Alternatively, 25 μ g/ml or 100 μ g/ml of rhIL-8 was coupled to NHS-activated Sepharose. The remaining active groups on the beads were blocked as recommended by the manufacturer. The beads were then finally blocked with 10 mg/ml BSA in 1 M Tris and stored in the blocking solution.

KinExA equilibrium assays. The experiments with KinExA were performed using an automated flow immunoassay system, KinExA 3000 [26], in which rhIL-8-coupled beads served as the solid phase. Briefly, a constant amount of antibody between 0.67 and 5000 pM binding site concentration (two binding sites for an IgG) was incubated with titrating concentrations of rhIL-8 antigen starting at 100 nM in PBS with 0.1% BSA (sample buffer) to reduce non-specific binding. Antigen–antibody complexes were incubated at RT for 36–144 h to allow equilibrium to be reached. The mixture was drawn through the rhIL-8-coupled beads to accumulate unbound antibody. The captured anti-hIL-8 mAb is directly proportional to the remaining free binding sites [26], and was detected using solutions containing Cy5-conjugated anti-human secondary antibody in sample buffer. The concentrations, volumes, and flow rates of the secondary antibody solutions were varied to optimize the signal to noise ratio in each experiment. The schematics of the experiment and the measurement of free mAb have been discussed previously [20,26]. The bound signals were converted into relative values as a proportion of control in the absence of rhIL-8. Three replicates of each sample (in some experiments two replicates) were measured for all equilibrium experiments. The equilibrium dissociation constant (K_d) was obtained from non-linear regression analysis of the data using a one-site homogeneous binding model contained within the software [20,27]. The software calculates the K_d and determines the 95% confidence interval by fitting the data points to

a theoretical K_d curve. The 95% confidence interval is given as K_d low and K_d high.

KinExA kinetic assays. For measuring the association rate constant using KinExA, the same rhIL-8-coupled beads were used as the probe and the “Kinetics, Direct” method was used. The “Kinetics, Direct” experiments are identical to KinExA equilibrium assays with respect to bead column height, antibody capture, antibody concentration, and antibody detection. However, the mAb was mixed with an amount of antigen that bound approximately 80% of the mAb in the equilibrium experiments and the free antibody present in the sample was probed repeatedly, pre-equilibrium. Since the binding signals are proportional to the concentration of free antibody in the solution, the signals decrease over time until the solution has equilibrated. The volumes and flow rates of the antigen–mAb mixtures and the Cy5-labeled secondary antibody were varied depending upon the mAb tested. Data were analyzed utilizing the KinExA analysis software that comes with the KinExA 3000 instrument. This software graphically represents the decrease in binding signals over time, and fits the collected data points to an exact solution of the kinetic differential equations for binding. From this curve, an optimal solution for the k_{on} is determined. The k_{off} is indirectly calculated from solutions for the k_{on} and K_d .

Theoretical effect of affinity on potency. A mathematical model was developed to simulate the effect of affinity on the dose of mAb required to suppress the serum IL-8 levels in vivo by at least 90% at steady-state following a multiple dose regimen. The model consisted of four differential equations describing the synthesis and elimination of endogenous IL-8, the dosing and elimination of mAb, and the monovalent and divalent binding of mAb to the IL-8. The half-life of the mAb was assumed to be 3 weeks, and dosing was conducted every 3 weeks for 15 weeks. The simulation was conducted at 3, 30, 300 pM, and 3 nM baseline levels of IL-8 to reflect concentrations that may be present in serum and at active sites of inflammation, respectively [28–30]. Simulations were conducted using SAAM II v. 1.2 (SAAM Institute, Seattle, WA). Numerical integration was conducted using the Rosenbrock method with an adjustable step size. The time course in serum of unbound IL-8 (IL8u), unbound mAb (ABu), mAb monovalently bound to IL8 (ABmv), and mAb bivalently bound to IL8 (ABbv) was described by the following equations:

$$\frac{dIL8u}{dt} = S_0 + \text{fluxunbindmv} + \text{fluxunbindbv} - \text{fluxbindmv} - \text{fluxbindbv} - \frac{CLag}{V}IL8u,$$

$$\frac{dABu}{dt} = \text{fluxunbindmv} - \text{fluxbindmv} - \frac{CLab}{V}ABu,$$

$$\frac{dABmv}{dt} = \text{fluxbindmv} + \text{fluxunbindbv} - \text{fluxunbindmv} - \text{fluxbindbv} - \frac{CLab}{V}ABmv,$$

$$\frac{dABbv}{dt} = \text{fluxbindbv} - \text{fluxunbindbv} - \frac{CLab}{V}ABbv,$$

where:

$$\text{fluxbindmv} = 2k_{on}\left(\frac{ABuIL8u}{V}\right),$$

$$\text{fluxbindbv} = k_{on}\left(\frac{ABmvIL8u}{V}\right),$$

$$\text{fluxunbindmv} = k_{off}ABmv,$$

$$\text{fluxunbindbv} = 2k_{off}ABbv.$$

The zero-order production rate of IL-8, S_0 , was varied to achieve different initial conditions for baseline levels of unbound IL-8. In the simulations, parameter values for CLab, CLag, and V were fixed at 0.0025, 0.493, and 0.064 L/kg, respectively. The clearance estimates were based on unpublished data, and the volume of distribution assumed limited distribution of mAb and IL-8 into extra vascular space. Parameter sensitivity analysis indicated that changes in assumptions regarding clearance and distribution of mAb and IL8 did not influence the estimate of optimum mAb affinity; however, the dose required to achieve 90% suppression of unbound IL-8 was influenced by these assumptions. The association rate constant, k_{on} , was fixed and changes in affinity were assumed to be related to changes in the dissociation rate constant, k_{off} . For any given affinity, changes in the values of k_{off} and k_{on} did not affect the simulated steady-state concentration of unbound IL-8.

Results

Affinity measurements of Anti-hIL-8 mAbs by KinExA

The affinity measurements of anti-hIL-8 mAbs from XenoMouse determined by KinExA are shown in Table 1. Generally, when the measured K_d of an antibody was in the range of high picomolar or above, the 95% confidence interval of the predicted K_d was within a narrow range. However, when the measured K_d was approaching low picomolar to sub-picomolar values, the 95% confidence interval tended to be broader, indicating the measured K_d was not as precise. For the low to sub-picomolar K_d measurements, the binding site concentration of mAb used in the equilibrium mixture was higher than the K_d , causing the precision of these

Table 1
 K_d measurements of anti-hIL-8 mAbs by KinExA using standard conditions

Anti-hIL-8 mAb	K_d (pM)	K_d range (pM)	mAb conc. (pM)
33	280	150–420	10
142	400	190–680	25
203	190	64–340	25
215	360	230–450	50
469	870	640–1010	200
809	2.2	0.36–4.8	23
837	11	0.054–31	90
861	2.9	0.010–8.2	25
928	0.057	<0.010–1.8	20
1064	54	29–72	50
1080	630	160–980	25
1093	200	76–300	100

Serial dilutions of rhIL-8 were mixed with the respective binding site concentration of anti-hIL-8 mAbs and allowed to reach equilibrium for 36 h. A proportionate amount of free mAb present in the equilibrium mixture was measured by flowing 0.25–3 ml of the sample on rhIL-8 coupled to CNBr-activated Sepharose beads and detected using 1.0 ml of 1.7 µg/ml of Cy5-labeled goat anti-human IgG Fcγ fragment-specific secondary antibody. Each sample was run in triplicate. The K_d was calculated by the KinExA software and the 95% confidence interval is given as the K_d range.

measurements to be less accurate, thus warranting KinExA signal optimization experiments.

Signal optimization

In order to make an accurate K_d measurement by KinExA, the active binding site concentration of the antibody must be near or below the actual K_d . To measure antibodies with very high affinities, as described in this paper, one needs to work at low picomolar binding site concentrations of antibody. At these low antibody concentrations the sensitivity of the assay needs to be further optimized. To increase the detection sensitivity of free antibody, bead coating concentrations and sampling parameters were optimized and different labels were evaluated.

Optimization of bead coating and labeled secondary antibody

Immobilization of certain antigens to beads may affect their ability to bind antibodies, due to steric hindrance by passive coating or destruction of epitopes by side chain reactive coupling. To test whether the signal in the bead pack could be increased, NHS-activated Sepharose beads were coupled with rhIL-8 and the bead saturation was tested, as previously described [26]. It was determined that there was no signal gain above a coupling concentration of 100 $\mu\text{g/ml}$ of antigen. At 25 $\mu\text{g/ml}$ of antigen, approximately 70% of the maximum signal was observed. Subsequently, the NHS-Sepharose beads were coupled with 100 $\mu\text{g/ml}$ of rhIL-8 for the low antibody binding site concentration (K_d controlled) experiments. The K_d measurements with high binding site concentration of mAb and on-rate experiments were performed with 25 $\mu\text{g/ml}$ of rhIL-8 to conserve antigen.

Different Cy5-labeled anti-human secondary antibodies were checked to identify one that would give the highest signal-to-noise ratio. When a 100 pM solution of anti-hIL-8 mAb was tested with various secondary labels (goat anti-human IgG (H + L)-specific, Fc γ fragment-specific or mouse anti-human IgG F(ab')₂ fragment-specific), the highest signal to noise ratio was generated using the Fc γ -specific label (9.9 times higher than background). This Fc γ fragment-specific goat anti-human secondary antibody was used for subsequent equilibrium and on-rate measurements.

Optimization of sample volume and flow rate

Since the initial equilibrium analysis of a few anti-hIL-8 mAbs showed low picomolar to sub-picomolar K_d s with broader 95% confidence intervals (Table 1; mAb 809, 837, 861, 928), the sample volume and flow rate were then optimized to obtain a higher signal. When a 100 pM of

anti-hIL-8 mAb 928 was tested either flowing 0.5 ml at 0.25 ml/min or 3.0 ml at 1.5 ml/min for 120 s, it was determined that increasing the sample volume and flow rate gave about 20% higher signal (data not shown).

The binding site concentration of the anti-hIL-8 mAb 928 was then lowered to 2 pM and a signal test was performed by flowing 30 ml at 1.5 ml/min for 1200 s. However, when the sample flow volume and the rate were raised, bubbles were forming in the bead pack, which affected the raw data traces. This problem was solved by degassing the samples and label immediately before running an experiment.

Time to reach equilibrium and sample viability

The antibody binding site concentration was subsequently lowered to 1 pM, to measure the K_d of low to sub-picomolar mAbs. At this very low binding site concentration of antibody, the samples require a longer time to reach equilibrium. Using the K_d curve obtained at a low mAb concentration and the measured k_{on} , it was estimated in the kinetic theory curve software from Sapidyne Instruments that the minimum time needed to reach equilibrium would be 6 days (data not shown). Otherwise, one could experimentally determine whether the antigen-antibody mixture reached equilibrium by measuring the free mAb left in solution, which should remain constant during triplicate measurements, if equilibrium has been achieved. The viability of 1 pM anti-hIL-8 mAb 928 was then checked at 0, 3, and 6 day incubation times and found that the antibody activity had not decreased during this period at room temperature. Subsequently, all equilibrium reactions were allowed to take place for 6 days.

Equilibrium and on-rate measurements of mAbs having low picomolar affinity

The K_d measurements were then repeated, for the mAbs (809, 837, 861, and 928) which had K_d s below 20 pM and broad 95% confidence intervals (Table 1), with optimized parameters for bead coupling, sample volume, flow rate, and secondary antibody-label. In KinExA, the percent of free mAb left in solution is plotted against each concentration of antigen, generating a sigmoidal curve, as shown in Fig 1. A theoretical curve is fit to the data and 95% confidence intervals are calculated by the KinExA software. It is known that when the binding site concentration of mAb is very close to, or below the K_d value, the shape of the curve is constant and is referred to as a K_d controlled curve (Fig. 1, curve 1). However, for mAb binding site concentrations above the K_d , the curve shifts towards the right and becomes steeper (Fig. 1, curve 2). These steeper curves are referred to as mAb concentration controlled curves, and will contain little information on the K_d . One rationale

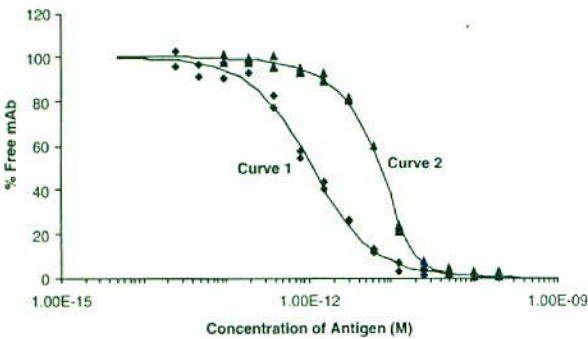


Fig. 1. K_d measurement of mAb 928 by KinExA. Twofold dilutions (50 pM to 24 fM) of rhIL-8 were mixed with 1 pM binding site concentration of anti-hIL-8 mAb 928 and allowed to reach equilibrium for 144 h. A proportionate amount of free mAb present in the equilibrium mixture was measured by flowing 22.5 ml of the sample at 0.25 ml/min on rhIL-8 coupled to NHS-activated Sepharose beads. The bead bound mAb was detected by flowing 2.0 ml of 2.0 μ g/ml of Cy5-labeled goat anti-human IgG Fc γ fragment-specific secondary antibody at 0.25 ml/min. Each sample was run in duplicate. The K_d calculated by the KinExA software by single curve analysis for a representative experiment (curve 1) was 870 fM and the 95% confidence interval calculated as the K_d high and K_d low was 1.3 pM and 500 fM, respectively. A representative mAb controlled experiment (curve 2) was performed using 20 pM binding site concentration of anti-hIL-8 mAb 928, mixed with two fold dilutions (200 pM–97 fM) of rhIL-8 and allowed to reach equilibrium for 48 h. A proportionate amount of free mAb present in the equilibrium mixture was measured by flowing 5 ml of the sample at 0.5 ml/min on rhIL-8 coupled to NHS-activated Sepharose beads. The bead bound mAb was detected by flowing 1.0 ml of 2.0 μ g/ml of Cy5-labeled goat anti-human IgG Fc γ fragment-specific secondary antibody at 0.25 ml/min. Each sample was run in triplicate. The K_d was calculated to be 790 fM by using n -curve analysis, by the KinExA software and the 95% confidence interval was calculated to be between 1.1 pM (K_d high) and 570 fM (K_d low). Analysis of curve 2 also determined the active binding site concentration of antibody present in each reaction to be equal to 67%.

for performing a mAb concentration controlled experiment is that the KinExA software will be able to calculate precisely the active binding site concentration of mAb present in solution during the equilibrium reaction.

Additionally, one can further increase the accuracy of very high affinity measurements by performing one or more experiments using a mAb binding site concentration near or slightly higher than its K_d value and at least one or more experiments with a 10- to 100-fold higher binding site concentration of mAb than the first experiment [15]. All these experiments can then be analyzed simultaneously with the KinExA software by using the “ n -curve analysis” selection. This “ n -curve analysis” selection allows one to obtain a precise K_d value (Table 2) by fitting all of the given curves to a single K_d value simultaneously. We performed a number of experiments for n -curve analysis of mAb 928. In these experiments, we used mAb 928 either near the K_d concentration or at a concentration of about 15- to 20-fold higher than the K_d . By n -curve analysis, the K_d of mAb 928 was

Table 2
 K_d measurements of the highest affinity anti-hIL-8 mAbs by KinExA using optimized conditions

Anti-hIL-8 mAb	K_d (pM)	K_d range (pM)	mAb conc. (pM)
809	3.3	1.9–5.2	4.6, 27
837	16	9.3–25	18, 120
861	3.0	2.0–4.2	1.3, 13
928	0.61	0.38–0.94	0.68, 2.0, 14

Serial dilutions of rhIL-8 were mixed with the respective anti-hIL-8 mAbs in the binding site concentrations shown above and allowed to reach equilibrium for 36 h for the high binding site concentration of each mAb, or for 144 h for the low binding site concentration of each mAb. A proportionate amount of free mAb present in the equilibrium mixture was measured in KinExA under optimized conditions. For example, for the mixtures containing the low binding site concentration of mAb, 18–36 ml of the sample was flowed through rhIL-8 coupled to NHS-activated Sepharose beads and detected using 1–2 ml of 2.0 μ g/ml of Cy5-labeled goat anti-human IgG Fc γ fragment-specific secondary antibody. The K_d was calculated using KinExA software by n -curve analysis and the 95% confidence interval is given as the K_d range. Six experiments were performed for mAb 928 and two were performed for mAbs 809, 837, and 861.

determined to be 610 fM (K_d high = 940 fM and K_d low = 380 fM), which is very close to the K_d determined by low mAb binding site concentration experiments analyzed in single curves (590 $\pm$ 220 fM). The K_d distributions of individual measurements of mAb 928 are given in Fig. 2.

The on-rate measurement by KinExA is not limited by the mAb binding site concentration used in the experiment. Therefore, the measurements of k_{on} for the very high affinity antibodies were straightforward and did

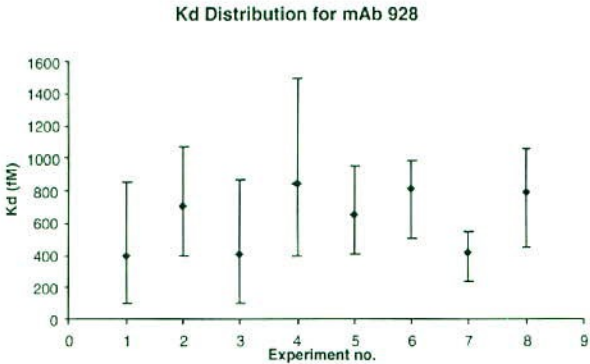


Fig. 2. Distribution of K_d measurements for the sub-picomolar affinity anti-hIL-8 mAb 928 by KinExA. Two fold dilutions of rhIL-8 (50 pM–24 fM) were mixed with anti-hIL-8 mAb 928 at an active binding site concentration of either 2 pM (Experiment No.1) or 670 fM (Experiments 2–4). Experiments 5–8 represent n -curve analysis, done either with 2 pM and 13.5 pM mAb (Experiment No. 5) or with 670 fM and 13.5 pM mAb (Experiments 6–8). The diamonds represent the optimal K_d determined by KinExA data analysis for each experiment. The error bars represent the 95% confidence intervals from KinExA data analysis. The error bars are not centered on the optimal K_d value because of the non-linear fit of the theoretical curve to the equilibrium.

Table 3
On-rate measurements for the highest affinity anti-hIL-8 mAbs in KinExA

Anti-hIL-8 mAb	k_{on} ($M^{-1} s^{-1}$)	k_{on} range ($M^{-1} s^{-1}$)	k_{off} (s^{-1})
809	4.8E+6	4.5E+6–5.5E+6	1.6E–5
837	1.1E+6	1.1E+6–1.2E+6	1.9E–5
861	4.4E+6	4.0E+6–5.0E+6	1.3E–5
928	6.0E+6	5.6E+6–6.4E+6	3.7E–6

The binding site concentration of mAb used for on-rate experiments was the same as those used in equilibrium binding experiments. An amount of IL8 that bound 80% of the mAb in equilibrium binding experiments was mixed with the mAb. The amount of free mAb left in the mixture was measured in KinExA by flowing 1 ml of the mixture repeatedly through rhIL-8 coupled to NHS-activated Sepharose beads and detected using 1 ml of 2.0 µg/ml of Cy5-labeled goat anti-human IgG Fcγ fragment-specific secondary antibody. The k_{on} was measured using the KinExA software by the kinetic direct method and the 95% confidence intervals are given as k_{on} range. The k_{off} for the mAb was calculated from the measured k_{on} and K_d of the respective mAb.

not require further optimizations. The measured k_{on} and the calculated k_{off} are given in Table 3.

Simulated effect of affinity on potency

The theoretical dose required to suppress IL-8 in vivo by at least 90% at steady-state is shown in Fig. 3 as a function of affinity for four baseline levels of endogenous IL-8 in serum. Improvements in mAb affinity are predicted to provide proportional improvements in potency until the K_d falls to 1/10th the endogenous IL-8 level. When the antigen level is at least 10 times greater than the K_d , the dose of mAb required to suppress IL-8 by 90% is determined by antigen level and not by affinity; thus, further improvements in affinity

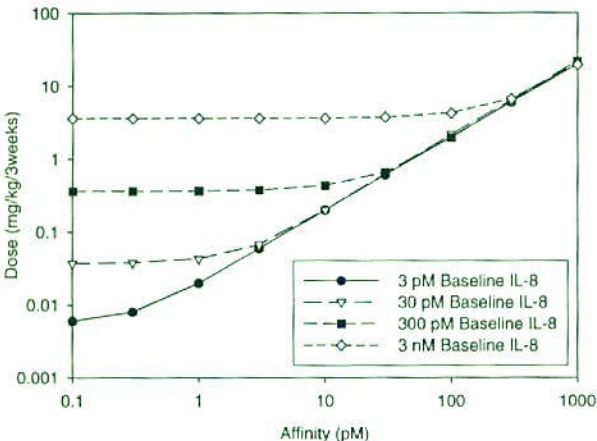


Fig. 3. Theoretical effect of mAb affinity on potency. The dose (mg/kg/3 weeks) of mAb required to suppress IL-8 levels in vivo by at least 90% at steady-state was simulated as a function of mAb affinity (K_d) at four baseline levels of endogenous IL-8 (3 pM, circles; 30 pM, triangles; 300 pM, squares; 3 nM, diamonds). When the K_d of the mAb is less than 1/10th the baseline IL-8 levels, further improvements in affinity will not provide any improvements in potency.

are not predicted to yield a further improvement in potency. For example, the mean concentration of IL-8 in exudates from psoriatic plaques was reported to be 250 pM [30]. Improvement of mAb affinity to approximately 25 pM is predicted to decrease the dose required to suppress IL-8 concentrations in the psoriatic plaque; a further increase in affinity is not predicted to improve potency.

Mutational analysis

The role of somatic mutation in optimizing hIL-8 binding affinity was analyzed for mAbs 928, 809, 215, and 1064. These mAbs were derived from the same germline sequence and thus the backbones should be essentially identical. Table 4 lists the mutations located within the complementary determining regions (CDRs) and framework regions (FRs) of each of these antibodies in comparison with the germline residues. The residues are numbered according to the scheme of Kabat et al. [31]. Mutational analysis indicates that these four mAbs were derived from an ancestral clone and a Val^{H50} → Asp mutation occurred within mAbs 215, 809, and 928. The light chain of the mAb 215 independently diverged from the precursor Val^{H50} → Asp clone and generated a Ser^{L27A} → Asn mutation resulting in a high affinity K_d of 362 pM. Then, mAbs 809 and 928 diverged from the ancestral Val^{H50} → Asp clone with the addition of a His^{H35} → Leu mutation. Antibody 809 independently further diverged at Tyr^{H79} → Phe in FR3 finally resulting in a higher affinity K_d of 2.23 pM. The heavy chain of mAb 928 independently continued to diverge in FR3 at Tyr^{H90} → Phe. The light chain of the mAb 928 further mutated in the CDRs from

Table 4
Somatic mutations in the CDR and FR regions of mAb 928, 809, 215, and 1064

Kabat number	Germline	mAb 928	mAb 809	mAb 215	mAb 1064
<i>CDR regions</i>					
H50	Val	Asp	Asp	Asp	Leu
H35	His	Leu	Leu		
L92	Gly	Asp			
L32	Tyr	Phe			
L27A	Ser			Asn	
L91	Tyr				Asp
H96	Arg				His
H31	Ser				Thr
L52	Ser				Tyr
L53	Ser				Arg
<i>FR regions</i>					
L39	Lys				Arg
L72	Thr	Ile			
H79	Tyr		Phe		
H90	Tyr	Phe			

Residues are numbered according to the scheme of Kabat et al. [31].

Gly^{L92} → Asp and Tyr^{L32} → Phe, as well as in FR3 Thr^{L72} → Ile, resulting in a final product with a superior affinity in the sub-picomolar range ($K_d = 610$ fM). However, mAb 1064 appears to have evolved independently from a germline ancestral clone and resulted in mutations in the CDRs at Val^{H50} → Leu, Ser^{H31} → Thr, Arg^{H96} → His, Ser^{L52} → Tyr, Ser^{L53} → Arg, and Tyr^{L91} → Asp, along with a mutation in FR2 at Lys^{L39} → Arg, which ultimately resulted in a high affinity K_d of 54 pM.

Discussion

There are a number of reports describing monoclonal antibodies with sub-nanomolar affinities [10,11,15]. However, in vitro affinity maturation by phage, yeast or ribosome display is often required to increase the affinity of an antibody to the picomolar range [11,13,32–34]. Here, we describe the affinity characterization of fully human mAbs generated in vivo from XenoMouse that were directly rescued using the selected lymphocyte antibody method (SLAM) [24] and were not subjected to further in vitro affinity maturation. Rigorous affinity analyses of these antibodies were performed at two separate laboratories utilizing KinExA technology.

A number of technologies, such as surface plasmon resonance (SPR) (Biacore) [16], resonant mirror (IASys) [19], solution based kinetic exclusion assay (KinExA) [20], isothermal titration calorimetry [18], and fluorescence-polarization [17] are used to measure equilibrium constants. Commonly used Biacore technology utilizes surface-based biophysical methods to measure the k_{on} and k_{off} , from which one can calculate the K_d . Whereas, KinExA utilizes solution-based biophysical methods to directly measure the K_d and k_{on} . The k_{off} can then be calculated using the measured K_d and k_{on} . Both Biacore and KinExA have been successfully used to measure affinities in the picomolar range [15,21,26,35]. Recently, it has been shown that for some antigen–antibody affinity measurements, KinExA and Biacore correlate closely [15]. However, Biacore requires proper experimental design to avoid avidity, mass transport, steric hindrance, aggregation, and crowding effects, as reviewed extensively before [21,36]. Here we have used KinExA technology, where both binding constituents are present in solution without being modified, to precisely measure low picomolar affinities. Since the concentration of IL-8 used in our kinetic equilibrium measurements were 100 nM or below and IL-8 exists as a monomer at this concentration [37,38], we were able to measure the true affinity, rather than the avidity, of the anti-hIL-8 mAbs. We also describe modifications to standard equilibrium measurement techniques for KinExA that allowed the precise measurement of sub-picomolar affinities.

The challenge in KinExA for sub-picomolar affinity measurements is to precisely measure the proportionate amount of very low concentrations of free mAb left in solution after equilibrium is reached. To achieve this, we modified a number of parameters. As shown for anti-hIL-8 mAb 928, by coupling the NHS-activated Sepharose beads with a saturating concentration of IL-8, increasing the sample flow volume, and using the optimized Cy5-labeled detection antibody, we were able to increase significantly the signal-to-noise ratio. These modifications together allowed us to measure the proportionate amount of free antibody left in solution to a very low concentration range. In addition, we incubated the antibody–antigen mixture for a sufficient period of time to allow the reactants to reach equilibrium. By applying these modified parameters, we performed a number of K_d controlled experiments, which were also used in n -curve analysis along with mAb controlled experiments and measured the average K_d of mAb 928 to be in the sub-picomolar range. Using n -curve analysis, one may even be able to extend the system to determine equilibrium dissociation constants in the low femtomolar range.

To our knowledge there has only been one publication describing a sub-picomolar affinity antibody [34]. This murine-derived, in vitro mutated and yeast display affinity matured antibody targets the hapten fluorescein. However, we have not seen any reports describing a sub-picomolar affinity antibody targeting a protein antigen, generated in vivo from any species. The reason for this might be due to either the inherent difficulties in rescuing very high affinity mAbs by available antibody generation technologies or in measuring high affinity interactions with existing technologies. We have shown here that if the technologies that measure the affinity, such as solution-based KinExA, are used properly, high affinity measurements between a protein antigen and an antibody can be measured with precision. The very high affinity determined for mAb 928 was confirmed to be sub-picomolar by two independent laboratories using KinExA.

Additionally, we have demonstrated for the first time that fully human mAbs that exhibit an equilibrium dissociation constant in the sub-picomolar range can be generated in vivo. In addition, we are now regularly isolating low picomolar (less than 10 pM) fully human antibodies from XenoMouse [15]. Using SLAM technology, we have also generated a number of low picomolar affinity antibodies directly from human peripheral blood B cells targeting a variety of antigens from human pathogens. We have also generated many low picomolar affinity antibodies, as well as a sub-picomolar affinity mAb from rabbit peripheral blood B cells, using SLAM technology (unpublished results). These observations indicate that the generation of exceptionally high affinity antibodies occurs across species in vivo.

It was proposed by Foote and Eisen [22] that, under physiological conditions, there is a ceiling at 100 pM on antibody affinity maturation in vivo for any antigen. It was proposed that above this threshold there would be no selective in vivo advantage for somatic mutation and affinity maturation to happen. This argument was based on the limits on both on- and off-rate constants of an antibody. The on-rate constant of an antibody is controlled by the diffusion coefficients of the antigen and antibody [39,40] and the maximum on-rate constant proposed was approximately 10^5 – 10^6 $M^{-1} s^{-1}$. The proposed limit on the off-rate constant is based on the residence time of an antigen complexed to an antibody on a B cell surface before it is taken up and processed by the B cell [23,41]. These studies led to the conclusion that B cells cannot differentiate between antibodies that have off-rate constants less than $10^{-4} s^{-1}$. However, no study describes a disadvantage for in vivo affinity maturation beyond these affinity ceilings. In fact, Foote and Eisen [22] themselves suggested that affinities beyond the ceiling could happen randomly. We have generated antibodies with on-rates greater than $10^6 M^{-1} s^{-1}$ [15,42], with off-rates less than $10^{-4} s^{-1}$ [15] and the present study extends those observations. Others have also reported on-rates higher than $10^6 M^{-1} s^{-1}$ for antibodies [43] and for Fab fragments [44]. Increased on-rates beyond the proposed diffusion limit may be explained by additional electrostatic forces [45]. For the association of barnase with barstar, a basal association rate constant of $10^5 M^{-1} s^{-1}$ was increased to over $5 \times 10^9 M^{-1} s^{-1}$ by electrostatic forces [45]. For off-rates, it is possible that B cell receptor turn-over in vivo may be slower or more heterogeneous than that measured in vitro using cell lines [23]. In this case, B cells expressing antibodies with off-rates slower than $10^{-4} s^{-1}$ may have a selective advantage. Alternatively, somatic hypermutation could stochastically result in the generation of antibodies with higher affinities than the proposed in vivo ceiling, which are neither selected for nor selected against in vivo. Supporting this statement, it has even been predicted that a single amino acid substitution can alter binding kinetics in protein–protein interactions by up to six logs [46]. In addition, somatic hypermutations in the CDR and frame work regions of an anti-HEL mAb resulted in a three log increase in affinity [44].

Among the four high affinity mAbs (215, 809, 928, and 1064) that were derived from the same germline sequences, it appears that the affinity has evolved through a progressive somatic mutation process. The superior affinity of mAb 928 ($K_d = 610$ fM) seems to be the result of a combination of enhanced electrostatic interactions by introduction of multiple aspartic acid mutations, and enhanced hydrophobic interactions with the introduction of phenylalanine and leucine mutations. Alternatively, it is possible that the parental germ-

line sequence could have existed in multiple preexisting conformational isomers [47,48] and that somatic mutations in the antibodies resulted in the stabilization of the high affinity binding isomers.

Antibodies with affinities in the picomolar or femtomolar range may provide significant improvements in mAb potency, depending on the biological characteristics of the antigen, particularly the in vivo concentration (Fig. 3). By using SLAM, a more efficient antibody generation procedure, to screen a larger proportion of the available immune repertoire in hyperimmune animals, it was possible to identify a number of exceptionally high affinity antibodies including one of sub-picomolar affinity without the need for time consuming in vitro manipulations.

We have determined the affinities of a panel of fully human anti-hIL-8 antibodies generated in vivo from XenoMouse animals to be in the low picomolar range. Furthermore, one of these antibodies was found to be a sub-picomolar affinity antibody. As far as we are aware, this is the first such description of an in vivo generated sub-picomolar affinity antibody. This report not only confirms the prediction that there can be affinity maturation in vivo beyond the proposed affinity ceiling [22], but in fact provides precise affinity measurements of an antibody that does so by over two orders of magnitude.

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