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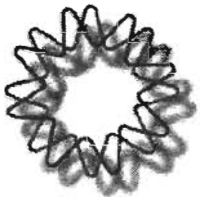

INSTITUTO DE BIOTECNOLOGIA

DEPARTAMENTO DE MEDICINA MOLECULAR Y BIOPROCESOS

AISLAMIENTO Y CARACTERIZACION DE ANTICUERPOS HUMANOS CONTRA LA TOXINA Cn2 DEL VENENO DEL ALACRAN *Centruroides noxius* A PARTIR DE UNA BIBLIOTECA DE DESPLIEGUE DE scFvs

TESIS PARA OBTENER EL GRADO DE
DOCTOR EN CIENCIAS BIOQUIMICAS

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A María del Carmen, Martín, Humberto, Susana y Elizabeth
A Colombia, "tierra querida"

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1. SUMMARY

Scorpion stings, a serious public health problem in Mexico and other regions of the world, are presently treated by passive immunization. Bivalent $F(ab')_2$ purified from hyper-immunized horses are used as antidote. Recombinant antibody fragments from human origin, specially the single-chain antibody fragment (scFv), should constitute the next generation of antisera. Toxin Cn2 is the major toxic component from venom of scorpion *Centruroides noxius*, the most dangerous Mexican species. In order to obtain neutralizing antibodies for Cn2 toxin, we constructed a human non-immune phage display library of $1.1X10^8$ members. Two specific scFvs (3F and C1), were isolated. The scFv 3F was affinity matured by directed evolution. After three cycles of maturation, the neutralizing scFv 6009F was isolated. It showed a 446 fold increment in the K_D [from 183 nM (3F) to 410 pM (6009F)], as determined by Biacore. The variant 6009F showed to be monomeric and capable of neutralizing 2 LD_{50} of Cn2 toxin or 2 LD_{50} of whole venom, when a molar ratio of 1:10 toxin:antibody fragment, was used. The mutant recognizes a different epitope than that of BCF2, a murine monoclonal antibody raised against scorpion toxin Cn2, which is capable of neutralizing both toxin Cn2 and the whole venom when tested in mice. The scFv 6009F is the first reported recombinant human antibody fragment capable of neutralizing *C. noxius* venom. These results pave the way for the generation of safer autologous recombinant neutralizing antisera against scorpion stings.

1. RESUMEN

Las picaduras de alacrán son un problema de salud pública en México y en otras regiones del mundo, las cuales son tratadas actualmente por inmunización pasiva. Los fragmentos $F(ab')_2$ obtenidos de caballos hiper-inmunizados, son utilizados como antídoto. Los fragmentos recombinantes de anticuerpos de origen humano, especialmente el fragmento variable de cadena sencilla (scFv), podría ser la próxima generación de antivenenos. La toxina Cn2 es el principal componente del veneno del alacrán *Centruroides noxius*, la especie más peligrosa de México. Con el fin de obtener anticuerpos neutralizantes de la toxina Cn2, construimos un banco no inmune de anticuerpos humanos de $1.1X10^8$ transformantes. Dos clonas específicas (3F y C1) fueron aisladas del banco. La afinidad de la clona scFv 3F fue mejorada por medio de evolución dirigida. Después de tres ciclos de maduración se seleccionó el scFv neutralizante 6009F. Este anticuerpo mostró un incremento de 446 veces en la K_D [de 183 nM (3F) a 410 pM (6009F)], determinación realizada en BIACORE. La proteína del scFv 6009F es principalmente monomérica y capaz de neutralizar 2 DL_{50} de la toxina Cn2 y 2 DL_{50} del veneno completo, cuando se emplea en una relación molecular 1:10 (toxina:anticuerpo). El anticuerpo humano 6009F reconoce a un epitopo diferente que el anticuerpo de origen murino BCF2, el cual tiene la capacidad de reconocer y neutralizar a la toxina Cn2 así como al veneno completo. Este es el primer reporte de un fragmento de anticuerpo humano capaz de neutralizar el veneno de *C. noxius*. Estos resultados son la base para la generación de antivenenos neutralizantes de anticuerpos autólogos recombinantes contra la picadura de alacranes.

2. INTRODUCCION

2.1 ALACRANISMO

Las picaduras de alacrán constituyen un problema de salud donde conviven humanos y alacranes, principalmente en las regiones tropicales y subtropicales como el norte y sur de Africa, América Latina, India y el Este Medio [1, 2].

Actualmente hay descritas 1500 especies de alacranes, y solo 25 de estas presentan riesgo para los humanos [3]. En México existen 221 especies, de las cuales 8 que pertenecen al género *Centruroides,* revisten peligro para la salud humana, estas son: *C. elegans, C. infamatus infamatus, C. limpidus limpidus, C. limpidus tecomanus,* C. *noxius, C. sculpturatus, C. pallidiceps y C. Suffusus suffusus* [4]. Los accidentes por picaduras de alacrán en México suman 50,000 casos al año aproximadamente. La mortalidad durante las décadas de los setenta y ochentas fue de 700 personas al año, para la década de los noventa esta cifra disminuyó a 300. En 1998 fue de 136 personas y en el año 2002 la mortalidad fue de 70 personas (Boletín epidemiológico semanal Secretaría de Salud de México). Esta disminución de la mortalidad se debe a la campaña nacional para el empleo del antiveneno realizada por el Instituto Mexicano del Seguro Social. El uso del antiveneno fue adoptado por el resto de los sistemas de seguridad social.

2.1.1 Alacranes

Los alacranes pertenecen al *phylum* Artropoda, clase Arácnida y orden Scorpionida. Estos animales se caracterizan por tener el cuerpo dividido en dos partes (Figura 1): cefalotorax (prosoma) y abdomen (opistosoma). El prosoma se encuentra cubierto por un caparazón

que aloja dos ojos situados a cada lado sobre un tubérculo ocular y de dos a seis ojos laterales organizados en uno o dos grupos. En el prosoma se encuentran seis pares de apéndices que son: un par de quelíceros, un par de pedipalpos con los cuales captura a la presa y cuatro pares de patas. El opistosoma esta dividido en dos regiones, un mesosoma amplio conformado por los siete primeros segmentos abdominales, y un metasoma angosto o cola, de cinco segmentos que termina en un aguijón, el cual está conectado a las glándulas venenosas [3]

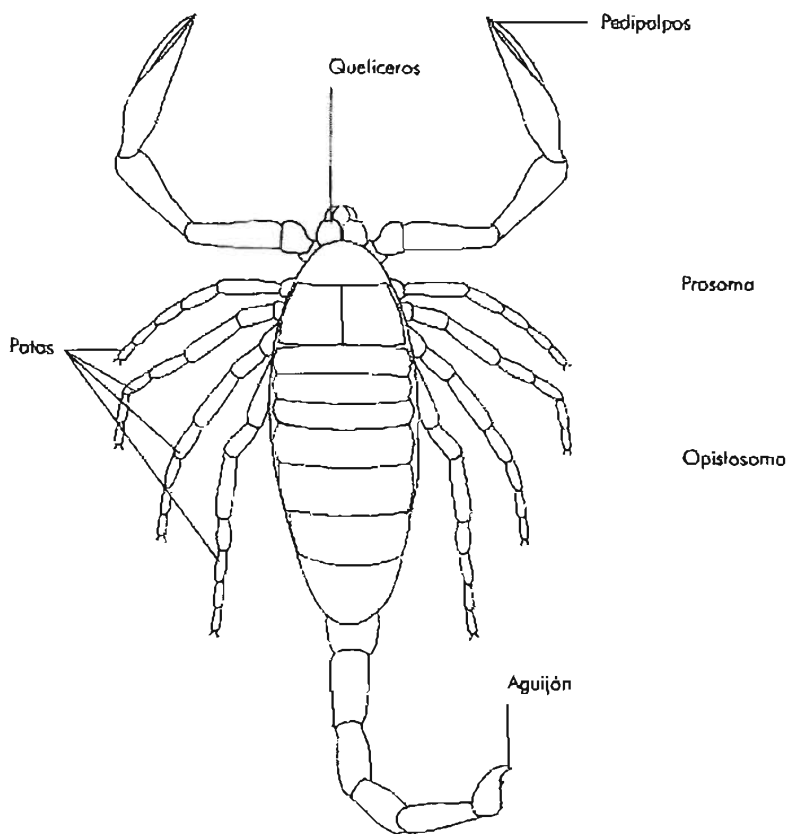

Figura 1. Morfología del alacrán (Tomado de Pineda 2002)

2.1.2 Veneno del alacrán

El veneno del alacrán es producido por un par de glándulas que se encuentran en la base del aguijón. El alacrán inocula el veneno mediante la contracción de los músculos que rodean a las glándulas. Generalmente la picadura es de tipo subcutánea y el veneno se distribuye en los tejidos de manera rápida [5]. Las principales características del envenenamiento son reacciones localizadas que ocurren en el 97% de las personas afectadas, las manifestaciones sistémicas (por ejemplo fiebre, transpiración, vómitos) no son comunes. Las manifestaciones cardio-respiratorias, principalmente shock cardiogénico y edema pulmonar son las causas principales de muerte. Los efectos farmacológicos y la letalidad del veneno de los alacranes pueden variar entre especies, debido a que cada uno presenta una composición diferente. Los venenos de diferentes especies de alacrán han sido analizados y se han logrado identificar una variedad de compuestos como son: enzimas, toxinas peptídicas, aminas, y otras sustancias que aún continúan siendo aisladas y caracterizadas [6]. Las toxinas son los componentes mas abundantes del veneno, estas son cadenas polipeptídicas que varían en peso molecular entre 4 y 9 KDa [7]. La estructura de las toxinas está estabilizada por 3 ó 4 puentes disulfuros [6]. Se ha determinado que estos péptidos presentan diversos niveles de interacción con canales iónicos como son los de sodio, potasio, cloro y calcio [8]. Estas toxinas se clasifican en varios grupos de acuerdo al sitio donde ejercen su acción (tipo de canal) y a la especie afectada (mamíferos, crustáceos e insectos). La sintomatología observada en los casos de alacranismo está asociada principalmente a los péptidos que modifican la actividad de canales de sodio [7].

2.2 INMUNIZACION PASIVA

La terapia más efectiva para tratar el envenenamiento por picadura de alacrán es a través de la inmunización pasiva o seroterapia [4, 9, 10]. La inmunización pasiva consiste en la neutralización de las toxinas circulantes, por medio de anticuerpos que atrapan o retienen a las toxinas, impidiendo que lleguen a su sitio blanco. El antiveneno actual es producido por la inmunización de caballos con extractos acuosos del telson de varias especies de alacranes. Después de varias inmunizaciones, el suero total se purifica por fraccionamiento y precipitación de las proteínas, para recuperar las inmunoglobulinas (Igs) [11]. Posteriormente las Igs son sometidas a digestión proteolítica con pepsina para generar el fragmento $F(ab')_2$, región divalente que retiene la especificidad al antígeno sin la región Fc, que es la causante principal de las reacciones inmunogénicas adversas en humanos. Este producto faboterápico (Alacramyn®, United States Patent No. 6,709,655 López de Silanes, et al. March 23, 2004) es efectivo, pero continúa siendo de origen heterólogo y va dirigido contra los diversos componentes de los venenos, muchos de los cuales no constituyen un problema en humanos. La producción presenta algunas desventajas como son: la diferencia de la respuesta inmune de los caballos empleados (variación de la respuesta inmunológica entre individuos), y el riesgo del empleo de un organismo heterólogo por la posibilidad de la transmisión de retrovirus y priones [12].

Los anticuerpos de origen humano son preferidos para la terapia en humanos, sin embargo es sumamente difícil obtener anticuerpos por diferentes razones que van desde el compromiso ético, hasta el poco éxito en la obtención de hibridomas humanos [13]. En la actualidad, se cuenta con estrategias alternativas para la obtención de anticuerpos de diversas fuentes incluyendo la humana: La tecnología de ADN recombinante que permite la

recuperación del repertorio de inmunoglobulinas presentes en un individuo y la selección de variantes de interés por medio del sistema de despliegue en fagos [14].

2.3 REPERTORIO DE ANTICUERPOS HUMANOS

2.3.1 Anticuerpos

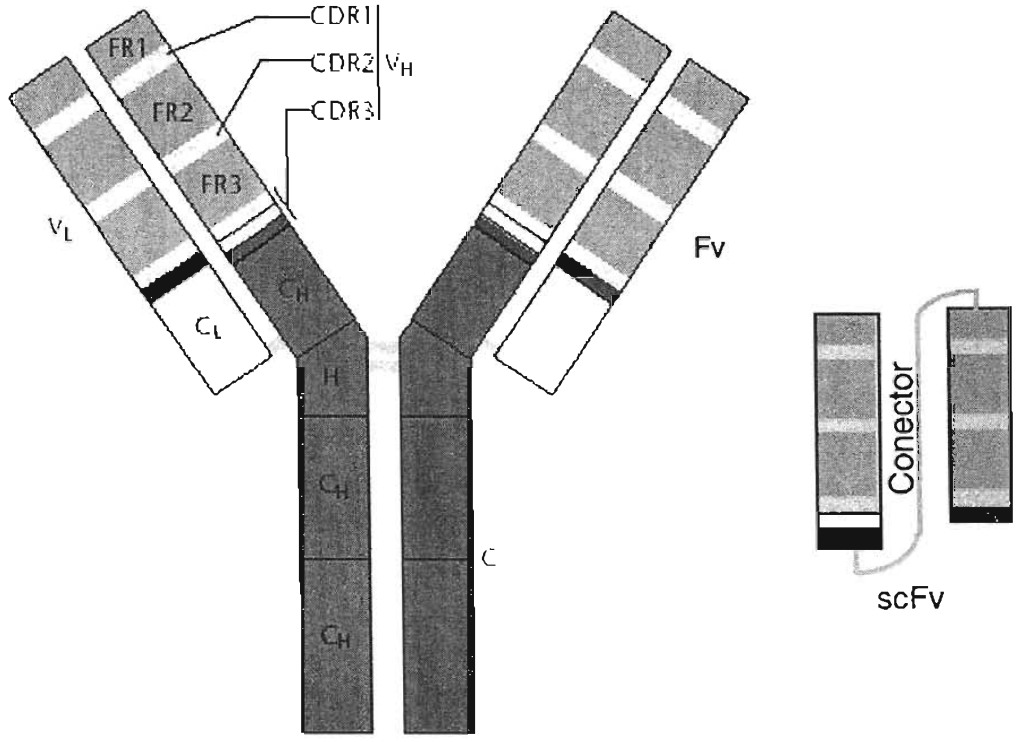

Figura 2. Descripción de las regiones de las inmunoglobulinas IgG: CH dominios constantes las cadenas pesadas, CL dominio constante ligera, VL región variable de la cadena ligera, VH región variable de la cadena pesada, FR *Frame work* (andamiaje), CDR región de determinante de complementariedad, H visagra, C sitio de glicosilación, Fv fragmento variable, scFv fragmento variable de cadena sencilla.

La inmunidad humoral conferida por las inmunoglobulinas o anticuerpos (Figura 2) se basa en la capacidad de reconocer elementos extraños al organismo. Los anticuerpos son proteínas producidas por los linfocitos B maduros, los cuales están formados por dos cadenas pesadas (VH-CH1-CH2-CH3) y dos cadenas ligeras (VL-CL). Las regiones

variables (VH y VL) son las responsables de la función de reconocimiento del antígeno. Los dominios de estas regiones adoptan una conformación de 2 láminas plegas β (Sandwich β), en los cuales se destacan los CDRs (regiones determinantes de complementariedad) (Figura 2). Estas regiones están ubicadas en 3 de las asas de cada dominio (VH y VL) formando el sitio de unión al antígeno. Los CDRs presentan una gran variabilidad en cuanto a la secuencia y al número de aminoácidos, esta plasticidad no sólo determina la capacidad de reconocimiento a diferentes antígenos, sino también la afinidad y especificidad de los anticuerpos.

El ser humano cuenta con un grupo de genes de línea germinal que contribuyen a la estructura de las regiones variables del anticuerpo. En los linfocitos B se dan los rearreglos entre estos genes para construir la región variable de la cadena pesada conformada por los genes VH-D-JH y la región variable ligera conformada por los genes VL-JL. El repertorio de la línea germinal tiene 50 genes funcionales localizados en el cromosoma 14, que codifican para los genes de VH [15], los cuales pueden ser agrupados en 6 familias de acuerdo a su identidad. También hay 27 genes de diversidad (D) y 6 genes de unión (JH). Los genes funcionales que codifican para las variables ligeras K son 40 comprendiendo 7 familias localizadas en el cromosoma 2 [16, 17] y 5 genes JK. Para las Vλ hay 30 genes funcionales agrupados en 10 subfamilias ubicadas en el cromosoma 22 [18, 19], con sus correspondientes 4 genes Jλ. Los procesos de recombinación entre dichos genes y entre las regiones variables permiten generar una diversidad de 10^9 variantes de anticuerpos [20]. Esto garantiza que el organismo pueda reconocer una gran cantidad de antígenos presentes en la naturaleza. En el momento del reconocimiento del anticuerpo acarreado por el linfocito B a un antígeno, se desencadena la selección clonal. El proceso ocurre durante la división celular del linfocito productor del anticuerpo [21] y mediante la mutación

de los genes que codifican para la región variable, se obtiene anticuerpos con mayor afinidad por el antígeno. Las modificaciones genéticas son almacenadas en las células B de memoria, las cuales en un segundo encuentro con el antígeno pueden responder rápidamente mediante nuevos procesos de división y mutación somática (respuesta secundaria), generando anticuerpos solubles con afinidad incrementada [22].

2.3.2 Fragmento variable de cadena sencilla (scFv)

Los dominios variables VH y VL del anticuerpo (fragmento variable o Fv) pueden ser obtenidos y expresados de forma individual. VH y VL tienen la capacidad de asociarse de manera natural para formar el Fv y mantener la afinidad por el antígeno, sin embargo no siempre se da una buena asociación entre los dominios [23]. El método más popular y eficiente para obtener un Fv estable es mediante la unión covalente entre los genes de VH y VL, a esta construcción se le denomina fragmento variable de cadena sencilla (scFv) (Figura 2). Para conectar genéticamente los dominios se emplea la secuencia de un péptido conector, la más ampliamente utilizada es $(Gly_4Ser)_3$ [24], la cual da flexibilidad a los dominios para establecer la interfase natural, sin modificar su estructura. La orientación de los dominios VH-conector-VL y VL-conector–VH son igualmente posibles.

2.3.3 Bancos de anticuerpos

2.3.3.1 Recuperación de la información genética

La variabilidad de los anticuerpos de un organismo puede ser recuperada de células donadoras (linfocitos B) provenientes de órganos linfáticos como timo, bazo y médula o de sangre periférica [25]. A partir de los linfocitos se recupera el ARN total que contiene los

mensajeros maduros de las secuencias de los anticuerpos (Figura 3). Por medio de RT-PCR se genera el ADNc y de éste se recuperan las regiones variables de las cadenas pesadas y ligeras. Se han diseñado oligos a partir de las secuencias reportadas en las bases de datos de secuencias y mediante técnicas de PCR se amplifican las regiones variables de las cadenas pesadas (VH) y ligeras (VK o Vλ) [25, 26]. La información genética que codifica para las regiones variables pesadas y ligeras, se unen por el segmento de ADN que codifica para el péptido conector. Posteriormente, esta información se clona en vectores de despliegue (fago o fagémido), los cuales permiten la fusión de la secuencia del scFv al gen 3 que codifica para una proteína de cubierta del bacteriófago M13 [14]. La información ligada al vector se transfiere a una cepa de *E. coli* por medio de una electrotransformación, y puede ser recuperada como fago anticuerpos (Figura 3). El tamaño de los bancos que se han generado es variado, los mejores bancos construidos de fuente natural presentan un tamaño de 10^{10} o 10^{11} genes fusionados [27, 28].

2.3.3.2 Fuente de inmunoglobulinas

Los bancos de anticuerpos pueden ser de tres clases:

Banco no inmune: los linfocitos son recuperados de un organismo sano que no ha sido expuesto con anterioridad a un determinado antígeno [26-29]. Estos bancos pueden ser fuente de anticuerpos contra cualquier antígeno. Los anticuerpos aislados de un banco no inmune, generalmente son de baja afinidad. Sin embargo es posible obtener anticuerpos de mayores afinidades (orden subnanomolar) cuando los bancos empleados son grandes, esto se debe a que al existir una mayor variabilidad se incrementa la probabilidad de seleccionar mejores anticuerpos [27].

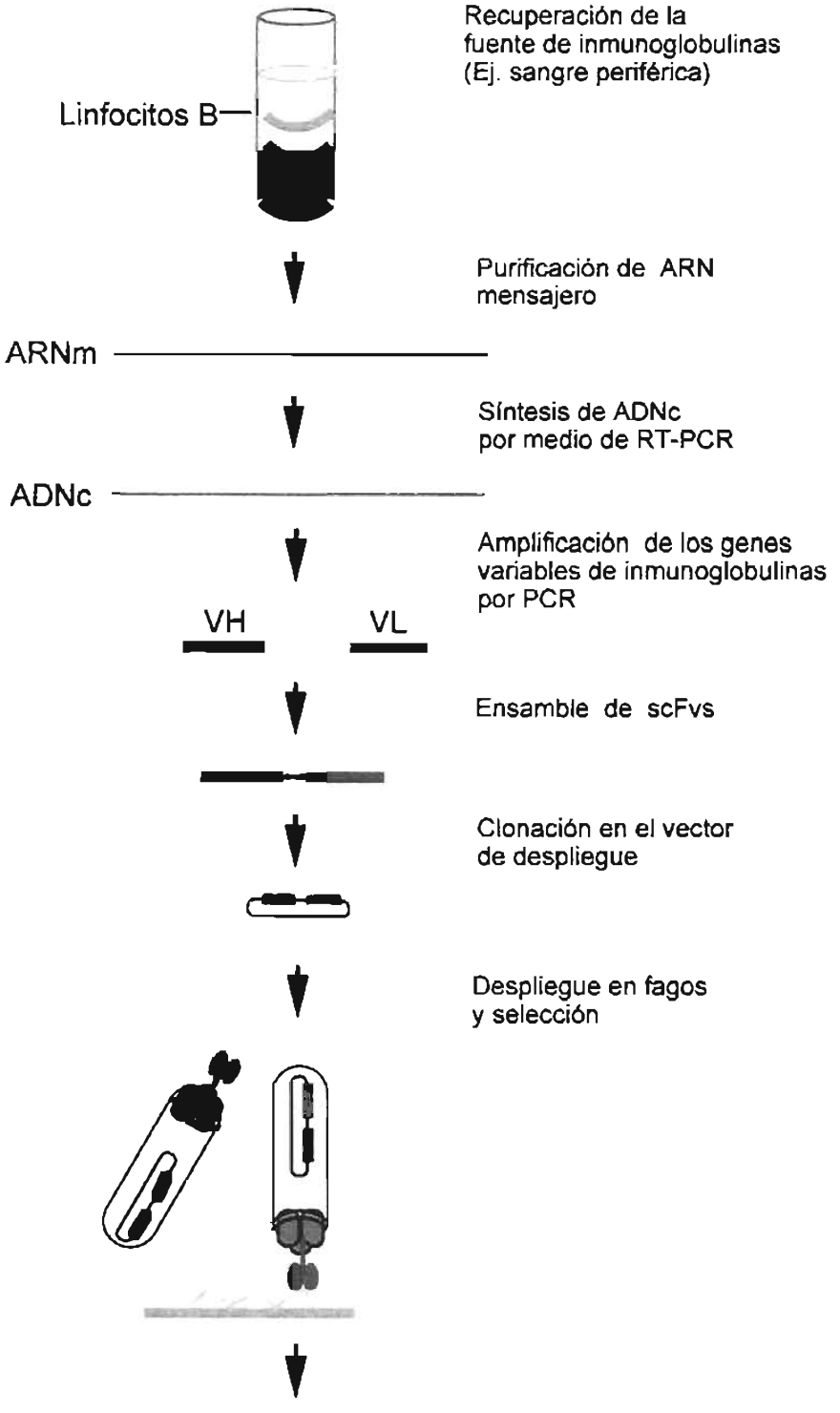

Figura 3. Descripción general de la obtención del repertorio de inmunoglobulinas y despliegue en fagos.

Banco inmune: los anticuerpos son recuperados de un individuo expuesto con anterioridad a un determinado antígeno, o pacientes con enfermedades como por ejemplo las autoinmunes [30]. La desventaja de este tipo de banco es que está restringido a un solo antígeno y las ventajas son:

- no se requiere que los bancos sean grandes (el óptimo puede ser $1X10^6$ variantes)
- los anticuerpos recuperados son de buena afinidad,

Bancos sintéticos o semi-sintéticos: estos se pueden generar por la clonación de CDRs sintéticos en los genes variables de línea germinal [31, 32]. Estos bancos presentan una gran variabilidad y también permiten seleccionar anticuerpos contra cualquier antígeno.

2.4 TECNOLOGIA DE DESPLIEGUE EN FAGOS

Esta técnica fue introducida por Smith [33] y permite la evaluación de grandes bancos de proteínas o péptidos, contra un ligando inmovilizado para caracterizar o descubrir nuevas interacciones. Esto es posible porque las proteínas de interés a ser evaluadas (fenotipo) están unidas con la información genética que codifica para ellas (genotipo). Para ello se emplean fagos filamentosos que portan la información genética en su interior (ADN de fago o fagémido) y la proteína anclada sobre la cubierta del fago. Esto se logra porque las secuencias de los scFvs se fusionan a la secuencia de una proteína de la cubierta del fago. Tiene la ventaja de ser un método rápido para el aislamiento de proteínas de interés, el cual puede ser empleado en:

- Descubrimiento de nuevos ligandos
- Caracterización de interacciones proteína-proteína
- Maduración de la afinidad de las interacciones ligando proteína

2.4.1 Fagos filamentosos

Son fagos de tipo no lítico como el M13, los cuales tienen forma de filamentos flexibles con un diámetro de aproximadamente 6.5 nm y 900 nm de largo. El genoma viral de M13 está constituido por una cadena de ADN circular (cadena+) de 6407 pb que codifica para 10 proteínas que le permiten la síntesis del ADN y las proteínas virales. El virus infecta solo a cepas de *Escherichia coli* que contienen al episoma F´; esto es a través de la unión y adsorción del extremo del fago al pilus de la bacteria. Posteriormente la bacteria retrae el pilus y el genoma viral penetra en la bacteria. Dentro de la célula, el genoma viral se replica, transcribe y traduce para generar las proteínas necesarias para el ensamble de nuevas partículas virales. Las proteínas que conforman la cubierta del fago son: PIII, PVI ubicadas en la región proximal del fago; PVII y PIX en la región distal y PVIII que constituye la mayor parte de la cubierta. El ensamble de los virus se lleva acabo en el citoplasma de las células infectadas, donde las nuevas proteínas de cubierta son almacenadas. Aproximadamente se producen entre 100 y 300 fagos durante el ciclo de vida de una bacteria infectada. Las proteínas PVIII y PIII (con 2710 proteínas y 3 a 5 proteínas por virión respectivamente) han sido las proteínas que inicialmente se utilizaron para desplegar diversos tipos de péptidos y proteínas [34], aunque recientemente se han realizados otros despliegues sobre PVII y PIX [35]. La proteína PIII se encuentra en uno de los extremos del virión y es la encargada del reconocimiento del pilus de *E. coli* para el proceso de infección [36, 37]. Esta es la proteína más utilizada para el despliegue de proteínas en la superficie del fago [14].

2.4.2 Tamizado de bancos de anticuerpos – Despliegue en fagos (*Phage Display*)

Existen dos tipos de sistemas para ser utilizados en el despliegue en fagos [38]: Uno es el sistema de fago, en donde la secuencia de interés es clonada como fusión traduccional en el genoma viral. El sitio de clonación se ubica entre la secuencia líder y la región que codifica para la proteína PIII, este sistema asegura el despliegue multivalente ya que los fagos generados tendrán todas las proteínas de PIII fusionadas a las proteínas de estudio.
El segundo sistema es el de fagémido, este vector contiene únicamente un gen viral (*pIII* o *pVIII*) bajo un promotor (generalmente Lac) y dos orígenes de replicación, uno viral y otro de replicación plasmídica, además de la resistencia a algún antibiótico. Las secuencias de estudio (bancos) son clonadas como fusión traduccional al gen de *pIII* o *pVIII*. La producción de los fago-anticuerpos se realiza cuando las células que contienen el fagémido son infectadas con el fago ayudador. Este contiene la información genética para expresar las proteínas necesarias para la construcción del fago. La replicación del ADN del fago ayudador es menos eficiente que la del fagémido, esto permite que el ADN del fagémido sea más abundante y empacado preferencialmente en la partícula viral. Debido a que el fago ayudador también genera proteínas PIII silvestres, el despliegue es monovalente ya que los fagos se ensamblan con proteínas silvestres y fusionadas. Este sistema presenta ventajas con respecto al sistema de fago como: la fácil manipulación, la estabilidad genética, la obtención de anticuerpos solubles y la selección de anticuerpos con mejores afinidades [38].

Las proteínas de los scFvs una vez anclados sobre las partículas virales (fago-anticuerpos) son sometidos a un tamizado de afinidad. En este proceso, el antígeno se adsorbe en la superficie de una columna, el cual será reconocido y unido por aquellos fago-anticuerpos

que presenten afinidad al mismo. Después de varios pasos de lavado que eliminan a las uniones no específicas, los fagos se eluyen y se amplifican por infección celular (Figura 3). Los fagos recuperados son sometidos a ciclos adicionales de selección para enriquecer la población de fagos que reconocen mejor al antígeno. Este proceso es análogo a la respuesta primaria de anticuerpos [39, 40] y también puede ser utilizado para incrementar la afinidad de un anticuerpo por medio de técnicas de evolución dirigida.

2.5 EVOLUCION DIRIGIDA

La evolución dirigida es una técnica empleada en el área de la ingeniería de proteínas, a través de la cual se modifica la secuencia de una proteína al azar. Se han creado diferentes estrategias para generar variabilidad, las cuales han mostrado ser cada día mas eficientes (Revisado en [41, 42]). El conjunto de variantes sintetizadas se evalúan con respecto a una propiedad de interés, como por ejemplo termoestabilidad, afinidad o actividad catalítica. Para la evaluación se emplean sistemas de selección y/o tamizado para identificar a las variantes ganadoras. De esta manera se han obtenido anticuerpos contra antígenos con afinidades incrementadas [43, 44]. Hay varios ejemplos de anticuerpos madurados por evolución dirigida que pueden llegar a tener constantes de afinidad del orden picomolar y femtomolar [45-48].

2.5.1 PCR mutagénica

Los errores en el proceso de incorporación de las bases durante la síntesis de ADN en la técnica de la reacción en cadena de la polimerasa (PCR), es la base de éste método. La relativa baja fidelidad de la enzima Taq polimerasa, ha sido explotada para la generación

de variabilidad *in vitro*, dando lugar a la construcción de bancos mutagénicos (revisado en [41]). El incremento de la tasa de error de la enzima puede ser logrado por la introducción de Mn^{2+} (en lugar del cofactor natural Mg^{2+}) y del empleo de dNTPs (deoxinucleótidos) en diferentes concentraciones. La presencia de Mn^{2+} y el desbalanceo de bases en las reacciones de amplificación, permiten alcanzar una taza de error de aproximadamente 0.6 % [49]. Estos valores pueden incrementarse con modificaciones en la síntesis ADN, logrando tasas de mutación alrededor del 1% [50] o mayores. La mutagénesis al azar es la técnica más ampliamente utilizada para la creación de diversidad en los experimentos de evolución dirigida.

2.6 ANTECEDENTES

2.6.1 Anticuerpos neutralizantes

Los anticuerpos neutralizantes son proteínas cuya función es impedir una interacción molecular que desencadena un efecto no deseado en un organismo. Para que un anticuerpo neutralizante sea empleado a nivel terapéutico debe:

- ser específico, para evitar interacciones con otras moléculas del organismo;
- ser estable para soportar las condiciones de producción y purificación de la proteína;
- tener buenas propiedades de difusión para penetrar eficientemente en los tejidos y unirse a sus sitios blanco.
- tener alta afinidad para competir por el antígeno y retenerlo hasta su eliminación.
- No ser inmunogénicos.

Estas propiedades se pueden encontrar en los Fab o scFvs de origen humano o humanizados.

Se han obtenido anticuerpos neutralizantes contra diferentes antígenos, a través de diferentes metodologías: hibridomas, bancos inmunes o nativos de anticuerpos seleccionados por despliegue en fagos y también anticuerpos madurados *in vitro*. La característica predominante en ellos es que tienen una afinidad alta por su respectivo antígeno. En la siguiente tabla se pueden observar algunos ejemplos de anticuerpos que han mostrado ser neutralizantes o inhibidores en diferentes formatos (anticuerpos completos, Fab, scfv monómero o dímero) y sus constantes de afinidad (K_D).

Antígeno	K_D nM	Tipo	Fuente
SARS	1.590	scFv-AbH	[51]
Toxina del Antrax	0.87 0.13	fabHI fabHI	[52]
Toxina del Antrax	0.25	scAbR	[53]
Toxina del Antrax	0.082	mAbHI	[54]
Toxina Botulínica	73.00 1.10	scFv scFv	[55]
Tox. B (Clostridium)	10.00	scFv-mAb	[56]
Factor de crecimiento vascular endotelial (VEGF)	0.10	FabH	[57]
α-Sinucleina	25.00	scFvH	[58]
Antigeno del virus de la Hepatitis B	0.98	scFvR	[59]
Factor TGFβ	2.32	scFvHE	[60]
Toxina Cn2	2.00	BCF2R	Grupo Dr. Possani *
Toxina Cn2	0.07	scFvDRE	[61]
Toxina Cn2	0.00001	scFvDPI	Bernardo Cortina *
Toxina AahI	0.20 1.50	mAbR scFvR	[62]
Toxina AahI	0.08	scFvDR	[63]
Toxina AahI	0.082	FabR	[64]
Toxina AahII	0.40	mAbR	[65]

Tabla 1. Ejemplos de Anticuerpos Neutralizantes. **H** humano; **I** inmune; **E** evolucionado; **Ab** anticuerpo completo; **mAb** Anticuerpo monoclonal; **scFv** Anticuerpo de cadena sencilla; **scAb** región variable unida a una región constante; **D** dímero; **R** Ratón, **P** pollo, I fuente inmune. * Datos sin publicar.

Geisow [66] afirma que el requerimiento para que un anticuerpo sea aplicado a nivel terapéutico es que tenga una afinidad menor a 1 nM, que es semejante a la de los anticuerpos obtenidos en la respuesta secundaria. Para el caso de las toxinas de alacrán, los anticuerpos que las neutralizan, presentan las afinidades más altas entre los anticuerpos reportados (ver Tabla 1). Lo anterior sugiere que los anticuerpos neutralizantes de este tipo de péptidos, deben presentar como principal característica el ser muy afines por sus antígenos (nM o menor).

2.6.2 Toxina Cn2

Dentro de los alacranes mexicanos, el veneno de la especie *Centruroides noxius* es el más tóxico, con una dosis letal cincuenta (DL_{50}) de 2.5µg/20g de ratón (cepa CD1) [67]. El componente principal de este veneno es la toxina Cn2, que constituye el 6.8% de la proteína total [68]. La toxina es un péptido de 66 aa, cuya estructura tridimensional está compuesta por una α-hélice y 3 hojas β, estabilizadas por 4 puentes disulfuros [69] (Figura 4).

La toxina ejerce su efecto sobre canales de sodio de mamíferos, la DL_{50} de Cn2 es de 0.25 µg /20g de ratón (cepa CD1) [67, 70]. Se han realizado varias determinaciones de la afinidad de la toxina por los canales de sodio de células de sinaptosoma de cerebro de rata y los valores son del orden nM [71, 72], otras determinaciones en preparaciones celulares de un solo tipo de canal muestran un valor de 1 nM (datos no publicados).

A

```
      BB                    HHHHHHH          BBBB        BBBB
   1   5     10    15    20    25    30    35   40         45    50    55    60    65
Cn2   KEGYLVDKNTGCKYECLKLGDNDYCLRECKQQYGKGAGGYCYA-----FACWCTHLYEQAIVWPLPNKRCS
```

B

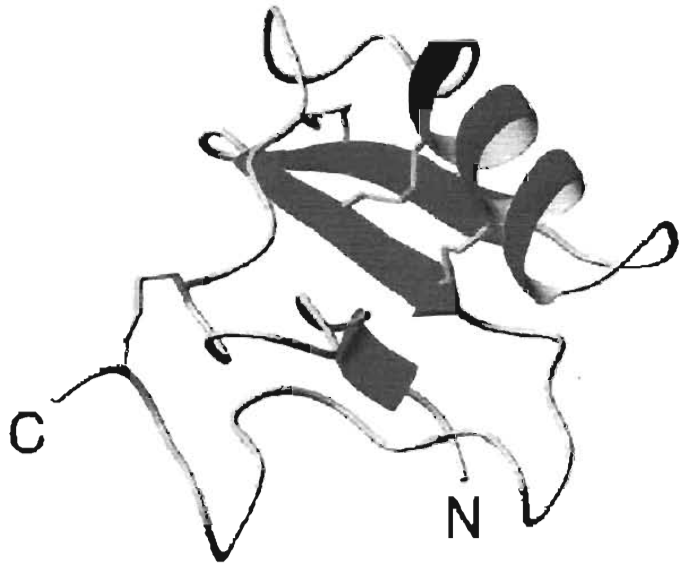

Figura 4. Toxina Cn2. A. Identificación de estructura secundaria en la secuencia primaria de la toxina Cn2. B. Estructura tridimensional de la molécula [69].

2.6.3 Anticuerpo monoclonal de ratón BCF2

Contra la toxina Cn2 se han generado anticuerpos monoclonales de ratón, de los cuales el BCF2 tuvo la capacidad de neutralizar el efecto de la toxina [70]. Este monoclonal murino tiene una constante de afinidad (K_D) por la toxina de 2nM (dato no publicado). El BCF2 también fue utilizado en pruebas de neutralización del veneno completo, donde se determinó que tiene la capacidad de inhibir su efecto al neutralizar a la toxina Cn2 [67]. Estos resultados muestran que aunque el veneno presenta diversas toxinas, es posible

identificar y bloquear a las toxinas más nocivas (en cuanto a abundancia y letalidad). Lo anterior podría ser aplicado para todos los venenos de especies peligrosas, ya que es posible identificar las moléculas toxicas a humanos y generar anticuerpos neutralizantes. Esta es una alternativa a la producción tradicional de antivenenos, ya que se podría generar un antiveneno altamente específico, con un menor contenido de anticuerpos diferentes y efectivo en concentraciones menores a las utilizadas en la actualidad.

En el marco del proyecto dirigido al desarrollo de anticuerpos terapéuticos (Anticuerpos Recombinantes Humanos) se ha visto viable la generación de fragmentos de anticuerpos humanos neutralizantes contra las toxinas del veneno de alacranes. Esto es posible mediante las técnicas ya descritas, empleando el formato de anticuerpo scFv, ya que presenta varias ventajas como son: la facilidad de expresión de las proteínas scFv en bacterias; el menor tamaño del fragmento scFv (28KDa) mejora las propiedades de difusión y penetrabilidad en los tejidos comparada con el anticuerpo completo (150 KDa). Asimismo los scFvs pueden ser modificados por métodos de evolución dirigida para incrementar las propiedades de afinidad y estabilidad. Esto permite que los scFvs sean fácilmente manipulados para el desarrollo de agentes de diagnóstico o terapia, seguros para la aplicación en humanos. En la actualidad hay numerosos trabajos de scFvs con potenciales aplicaciones terapéuticas, varios se encuentran en estudios clínicos, los cuales servirán como terapias alternativas.

3. HIPOTESIS

La construcción de una biblioteca humana a partir de genes que codifican para las regiones variables de anticuerpos, permitirá el aislamiento de scFv específicos contra la toxina Cn2. De los anticuerpos seleccionados será posible generar anticuerpos que neutralicen el efecto de Cn2, los cuales podrían ser empleados como uno de los componentes de un antiveneno recombinante de origen humano.

4. OBJETIVOS

4.1 OBJETIVO GENERAL

Obtener scFvs de origen humano con capacidad de neutralizar los efectos de la toxina Cn2 del alacrán *Centruroides noxius*, mediante la construcción de una biblioteca humana, el despliegue en fagos filamentosos y la evolución dirigida.

4.2 OBJETIVOS ESPECIFICOS

- Recuperar el repertorio de inmunoglobulinas humanas IgGs e IgMs mediante la construcción de un banco no inmune de scFvs a partir de una fuente de ARNm de linfocitos B.

- Seleccionar del banco humano, clonas específicas contra la toxina Cn2 de *C. noxius* mediante el sistema de despliegue en fagos.

- Caracterizar las variantes seleccionadas en cuanto a especificidad, afinidad y capacidad de neutralización en la cepa de ratón CD1.

- Madurar los scFvs humanos que resulten seleccionados, en caso de que éstos no tengan la capacidad de proteger.

- Caracterizar las clonas ganadoras del proceso de maduración

- Comparar los epitopos de los anticuerpos neutralizantes

5. MATERIALES Y METODOS (No publicados)

5.1 RECUPERACION DE LINFOCITOS B

Se obtuvo una muestra de sangre de un individuo sano. Las células se centrifugaron para obtener el concentrado leucocitario. La muestra se diluyó en una proporción de 1 a 1 con PBS 1X estéril. En tubos falcon de 50 ml se colocaron 10 ml de la mezcla sangre-PBS y se adicionaron 3 ml de Ficoll en el fondo de cada tubo. Se centrifugó a 1300 rpm durante 25 minutos sin freno, a temperatura ambiente. El gradiente formado genera tres capas, la del fondo de color rojo corresponde a los glóbulos rojos, la del medio de color blanco corresponde a los linfocitos y una transparente que es el suero. La capa blanca se recuperó en tubos Falcon de 50 ml, y se llevó a un volumen de 50 ml con PBS para lavar la muestra. Se centrifugó a 1300 rpm por 10 min, se eliminó el sobrenadante y cada muestra se resuspendió en un volumen total de 2 ml con PBS 1X, la muestra se centrifugó en tubos de 2 ml a 2000 rpm por 5 min. A partir de estas células se recuperó el RNA mensajero y de esta información se logró amplificar cada uno de los dominios variables de cadenas pesadas y ligeras (Procedimiento descrito en el Anexo 1).

5.1.2 Amplificación de las familias de anticuerpos

A partir del ADNc obtenido y con oligos específicos de los genes de inmunoglobulinas [26], se amplificaron los genes que codifican para los fragmentos variables de las cadenas pesadas y ligeras. Las cadenas variables se unieron para formar el scFv, a través de un conector [73]. Las estrategias empleadas están descritas en la Figura 5.

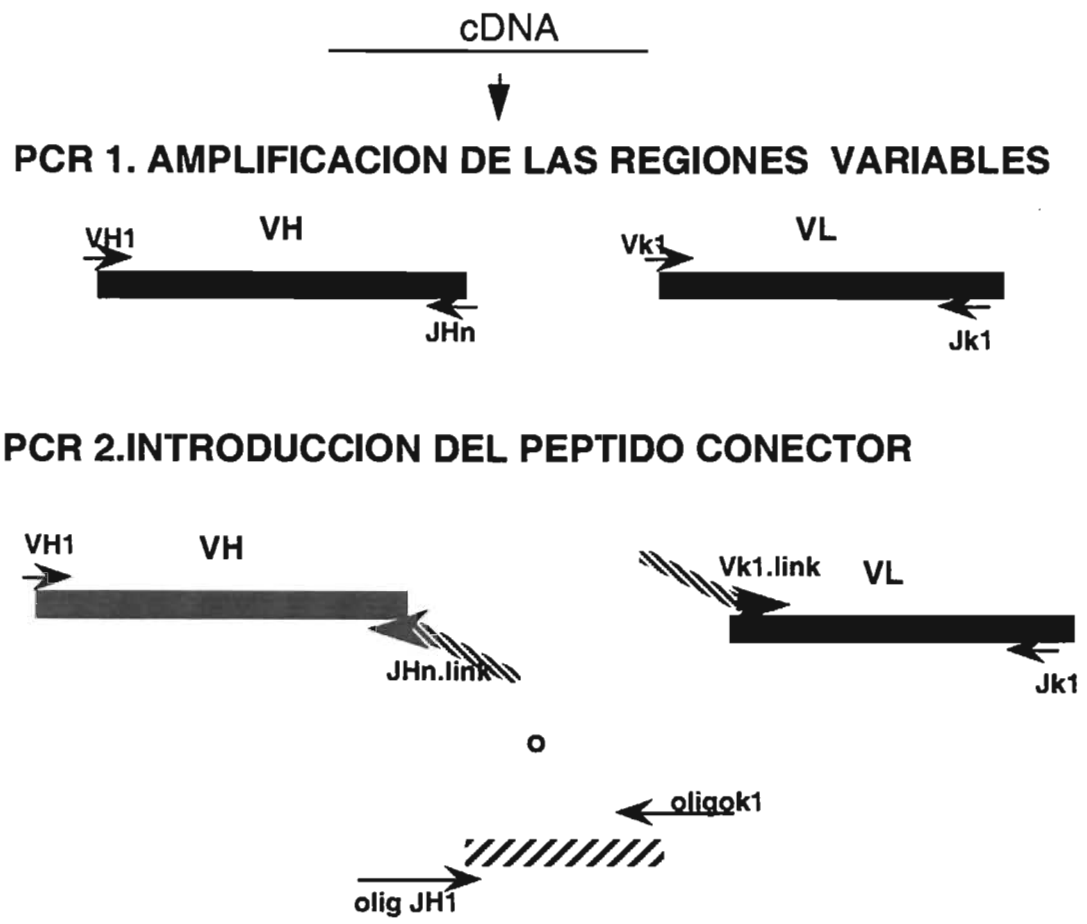

Figura 5. Esquema de las estrategias empleadas para realizar el empalme de VH y VL con introducción de la secuencia del péptido conector de 15 aa $(G_4S)_3$. Los genes de los segmentos de las variables VH y VL obtenidos por PCR son unidos por la secuencia que codifica para el conector. El cual puede ser generado por medio de oligos que incluyen la secuencia del conector y la secuencia del extremo de las cadenas pesadas y ligeras, luego se realiza el empalme de los dos fragmentos. La segunda alternativa es la generación de conectores a partir de una secuencia que posee el conector, éste se sintetiza en reacciones de PCR con los oligos que llevan las secuencias para unir las cadenas pesadas y ligeras en un empalme de tres fragmentos.

5.2 CARACTERIZACION DEL EPITOPO RECONOCIDO

5.2.1 Evaluación por ELISA

Para determinar si el anticuerpo humano reconoce o comparte el mismo epitopo que el anticuerpo monoclonal murino neutralizante BCF2, se realizó un ELISA en el cual se fijó a la placa (Corning) el anticuerpo humano a una concentración de 100ng/pozo, posteriormente se saturaron los sitios libres con BSA 0.5% en PBS1X. Después de la saturación se colocó la toxina Cn2, para que se una al anticuerpo humano. Después del lavado se colocó el anticuerpo monoclonal BCF2 (en diferentes concentraciones) y su unión al complejo scFv humano-Cn2 se evidenció al revelar con un anticuerpo de Cabra antiratón acoplado a peroxidasa.

5.2.2 Pruebas de competencias realizadas en BIACORE

Los siguientes experimentos se realizaron como se ha reportado [74], para determinar si los anticuerpos neutralizantes comparten el mismo epitopo. Una vez que el antígeno se fija al chip (Chip de Cn2 con 400 unidades de respuesta), se saturan los epitopos disponibles del antígeno con un anticuerpo que reconoce al antígeno. Posteriormente se inyecta un segundo anticuerpo, que también reconoce al antígeno. El sensograma generado permite detectar la unión del segundo anticuerpo (diferentes epitopos) o la competencia entre los anticuerpos (mismo epitopo).

5.2.2.1 Competencia entre los anticuerpos mAb BCF2 y scFv 6009F

En este caso se saturaron los sitios de reconocimiento de Cn2 con 6 inyecciones de 40 µl del anticuerpo BCF2 a una concentración de 200nM. Después se inyectaron 40 µl del scFv 6009F a una concentración de 20 nM. El flujo empleado durante el ensayo fue de 10µl/min y el sensograma obtenido fue analizado.

5.2.2.2 Competencia entre el antiveneno (Alacramyn®), mAbBCF2 y scFv 6009F

Se realizó el experimento para evaluar si la variante humana 6009F comparte algún sitio de reconocimiento en Cn2 con los anticuerpos presentes en el antiveneno (Alacramyn®) y el BCF2. Como se describió en el ensayo anterior, se realizaron 8 inyecciones de 40 µl de antiveneno (200nM) hasta alcanzar la saturación de los epitopos de la Cn2, posteriormente se inyectaron 40 µl de BCF2 (20nM) y finalmente 40 µl de 6009F (20 nM). El flujo fue de 10µl/min y el sensograma obtenido fue analizado.

6. RESULTADOS

La parte central de este trabajo de tesis fue publicada en FEBS Journal (Ver Anexo 1).

Con el título: A strategy for the generation of specific human antibodies by directed evolution and phage display: an example of a scFv that neutralizes a major component of scorpion venom.

Autores: Riaño-Umbarila, L., Juárez-González .V.R., Olamendi-Portugal, T., Ortiz-León, M., Possani, L.D. y Becerril, B.

También se contribuyó en el artículo publicado en JMB (Anexo 2)

Título: Directed Evolution, Phage Display and Combination of Evolved Mutants: A Strategy to Recover the Neutralization Properties of the scFv Version of BCF2 a Neutralizing Monoclonal Antibody Specific to Scorpion Toxin Cn2

Autores: Juárez-González, V. R., Riaño-Umbarila, L., Quintero-Hernández, V., Olamendi-Portugal, T., Ortiz-León, M., Ortiz, E., Possani, L. D. y Becerril, B.

6.1 CONSTRUCCION DE BANCOS (Datos no publicados)

Para la construcción de los bancos se procesó una muestra de 450 ml de sangre periférica de un individuo sano, después de procesar los linfocitos, se recuperó el RNA total que contiene el mensajero. El RNA mensajero se usó para generar el ADNc y de éste se

lograrón recuperar las secuencias que codifican para las regiones variables de las cadenas pesadas y ligeras (Materiales y métodos, Anexo 1). El ensamble de las regiones variables se realizó de dos maneras: empalme de 3 fragmentos VH-conector-VL o dos fragmentos donde las variables poseen la mitad de las secuencia del conector [26, 73] (Figura 5). La segunda alternativa se realizó porque, los genes de VH y Vλ no se ensamblaron, mientras que los VH y VK sí. Los productos ensamblados se digirieron con las enzimas *Sfi*I y *Not*I, posteriormente se fusionaron al gen *pIII* del fagémido pSyn2. Los bancos generados fueron evaluados en cuanto a tamaño (por PCR de colonia) y variabilidad (digestión con la enzima *Bst*NI de los productos de PCR y secuencia). Las características de los bancos se encuentran resumidas en la Tabla 2.

Banco	**VH3VK1**	**VHVK**	**HUMANO VH-VK-Vλ "COMPLETO"**
Estrategia empalme	Dos	Tres	Dos
ADN (inserto)	3.8 µg	7 µg	20 µg
Tamaño (variantes)	2.9×10^6	2.6×10^7	1.1×10^8
Digestión *Bst*NI	Positiva	Positiva	Positiva
Variabilidad Secuencia	Positiva	Positiva	Positiva

Tabla 2. Bancos generados de scFv humanos. La tabla incluye la información de la estrategia del empalme, tamaño y variabilidad de cada banco generado.

Banco VH3-VK1: Es el producto de la combinación de los genes de las familias de VH3 y VK1, ya que son los genes más utilizados en humanos para el reconocimiento de una gran variedad de antígenos, incluyendo moléculas pequeñas.

Banco VH-VK: En el proceso de construcción del banco completo de inmunoglobulinas humanas por medio del ensamble de 3 fragmentos, no se logró el empalme de los genes

VH-Vλ; mientras que VH-VK se empalmaron eficientemente. Este banco sesgado también es interesante ya que el organismo humano emplea las cadenas ligeras tipo K, en aproximadamente un 60% de los anticuerpos.

Banco VH-VK y VH-Vλ: El banco humano completo fue generado mediante el empalme de dos fragmentos. Este banco reúne la mayor combinación de cadenas pesadas y ligeras.

6.2 RESUMEN DE LOS RESULTADOS PUBLICADOS (Ver Anexo 1)

Se construyó un banco no inmune del repertorio de inmunoglobulinas de un donador sano. El tamaño del banco fue de $1.1X10^8$ variantes y su diversidad fue retada contra la toxina Cn2 del alacrán *C noxius* por medio del sistema de despliegue en fagos. En la cuarta ronda de selección se aislaron dos scFvs que reconocen a la toxina Cn2. Los scFv aislados fueron denominados 3F y C1, estos fueron caracterizados en cuanto a especificidad, afinidad y capacidad de neutralización. En las pruebas de ELISA los anticuerpos 3F y C1 mostraron ser especificos a la toxina Cn2. Los scFv fueron expresados y la afinidad fue determinada en BIACORE. Las constantes de afinidad de los anticuerpos mostraron valores característicos de anticuerpos generados en el reconocimiento primario de los anticuerpos (10^{-7}M), es decir característicos de fuentes no inmunes. Los anticuerpos no presentaron capacidad de neutralización en los ensayos de protección en ratones, por lo que se recurrió a la evolución dirigida para obtener variantes con mejores afinidades. El proceso resultó satisfactorio para la variante 3F, sobre la cual se realizaron tres ciclos de evolución dirigida: en el primero se obtuvo la variante 6F, en el segundo la variante 610A y en el último ciclo una de las variantes seleccionadas (6009F) mostró tener los niveles de afinidad y estabilidad necesarios para ser neutralizantes. Para

recuperar a la variante 6009F en el último ciclo de evolución dirigida, se modificó la metodología tradicional del tamizado y se emplearon condiciones astringentes. Esta mostró ser más eficiente que la metodología tradicional ya que se recuperaron un mayor número de clonas positivas. La proteína de la variante 6009F presentó la mejor afinidad de las clonas obtenidas y la capacidad de neutralizar el efecto de la toxina Cn2 (1 y 2 DL_{50}) y dos DL_{50} del veneno completo.

6.3 RESULTADOS NO PUBLICADOS

6.3.1 Competencia por el sitio de pegado con el anticuerpo monoclonal BCF2

El anticuerpo murino BCF2 tiene la capacidad de proteger a los ratones de los efectos de la toxina Cn2 y del veneno completo. Para saber si los anticuerpos neutralizantes BCF2 y 6009F reconocen el mismo epitopo en la toxina Cn2, se realizaron experimentos en ELISA de desplazamiento y BIACORE.

El experimento de ELISA mostró que la toxina Cn2 en complejo con el anticuerpo 6009F fue reconocida por el anticuerpo monoclonal BCF2 (Figura 6). Al aumentar la concentración del anticuerpo BCF2 la respuesta se incrementó gradualmente. Este resultado se explica si los dos anticuerpos permanecen unidos de forma simultánea a la toxina. El resultado sugiere que los anticuerpos reconocen sitios diferentes en la toxina, dado que los anticuerpos no se interfieren en el reconocimiento a Cn2, si fuera el mismo sitio, no se observaría ninguna señal en el ELISA.

Para corroborar que los anticuerpos BCF2 y scFv 6009F reconocen epitopos diferentes en la toxina, se realizaron pruebas de competencia en el BIACORE. El resultado obtenido muestra que la toxina fija en el chip, fue reconocida por el anticuerpo monoclonal BCF2

(Figura 7). Se saturaron todos los sitios disponible en Cn2, que son reconocidos por el BCF2 por medio de varias inyecciones del mismo (este efecto es evidente en el sensograma ya que no se observaron incrementos significativos en la señal después de varias inyecciones de BCF2). Finalmente se colocó el anticuerpo humano, el cual presentó una cinética de pegado similar al control, es decir el reconocimiento normal de 6009F a la toxina (sin competencia). Lo cual corrobora que BCF2 y 6009F reconocen epitopos diferentes de la toxina.

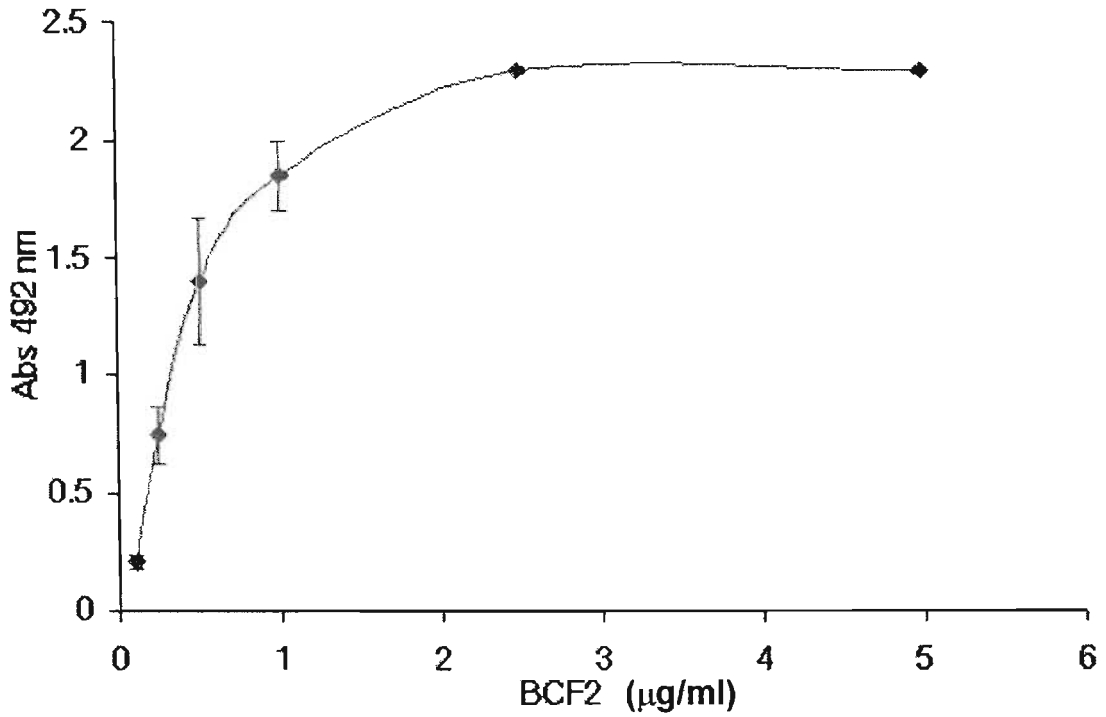

Figura 6. ELISA de competencia. En las placas se fijó el scFv 6009F a una concentración de 10 µg/ml toda la noche, posteriormente la placa se lavó y saturó con BSA (0.5% in PBS 1X) por 2 h. Se agregó la toxina Cn2 a una concentración de 3 µg/ml, se incubó por 1h y se lavó la placa. Se agregó el anticuerpo BCF2 a diferentes concentraciones (0.1, 0.25, 0.5, 1.0, 2.5 and 5 µg/ml). El pegado del BCF2 se observó con el anticuerpo de cabra antiratón acoplado a peroxidasa (HRP).

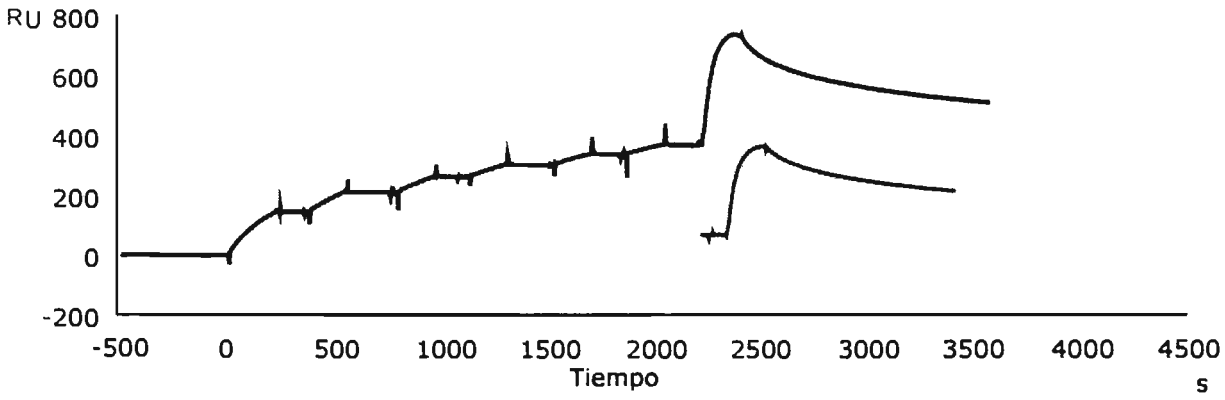

Figura 7. Análisis de competencia. La primera parte del sensograma hasta los 2300 seg, muestra la saturación de los sitios reconocidos por el BCF2 en Cn2 (6 inyecciones). Posteriormente se pasa una muestra de scFv 6009F a una concentración de 20 nM. Control: curva inferior: reconocimiento de 6009F a la toxina Cn2.

6.3.2 Competencia con el sitio de pegado con el antiveneno, BCF2 y 6009F

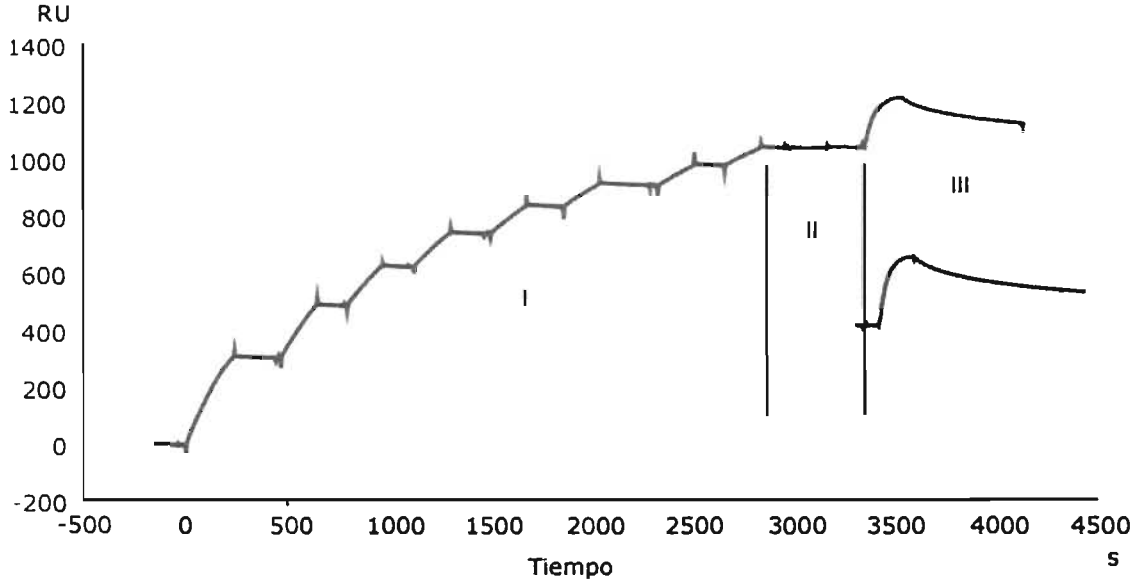

Figura 8. Análisis de competencia. La primera parte del sensograma hasta los 2900 seg, muestra la saturación de los sitios reconocidos por el antiveneno comercial (Alacramyn®) (8 inyecciones) (I). Posteriormente se ve que la inyección del anticuerpo BCF2 no produce cambio de la respuesta (entre 2900 y 3300 seg) (II). Finalmente la inyección del scFv 6009F (III). Control: curva inferior: reconocimiento de 6009F a la toxina Cn2.

Para determinar si los anticuerpos policlonales del antiveneno Alacramyn® (obtenidos por inmunización con una mezcla venenos de alacranes mexicanos, que incluye el de *C. noxius*) pueden competir en el reconocimiento a la toxina Cn2 con al anticuerpo murino BCF2 y el humano 6009F, se realizó un análisis por BIACORE (Ver Materiales y Métodos). El sensograma muestra que después de 8 inyecciones de antiveneno se logró la saturación (anticuerpos policlonales que reconocen a la toxina Cn2) (Figura. 8). Posteriormente, la inyección de BCF2 no mostró un incremento en la señal de respuesta lo cual implica que el sitio reconocido por el BCF2 se encuentra ocupado por los anticuerpos provenientes del antiveneno. Luego, la inyección de la variante humana 6009F mostró una señal de respuesta que es comparable al reconocimiento de la 6009F a Cn2 sin competencia. Lo cual demuestra que la clona 6009F reconoce un sitio diferente en Cn2 que los sitios reconocidos por BCF2 y que los anticuerpos del antiveneno.

7. DISCUSION

En el desarrollo del proyecto se construyeron tres bancos empleando dos estrategias: empalme de 3 fragmentos VH-conector-VL y de dos fragmentos donde las regiones variables poseen la mitad de la secuencia del conector [26, 73] (Ver Figura 5). El empalme de dos fragmentos resultó ser más eficiente para la construcción del banco humano completo, debido a que se simplifica el proceso de formación del scFv a un sólo ensamble. Estos repertorios pueden ser utilizados de manera individual o conjunta (los tres repertorios) para aislar anticuerpos contra cualquier antígeno, incluso se puede estudiar la diferencia de respuesta inmunológica entre los anticuerpos que se seleccionen de cada banco.

El repertorio humano completo (1.1×10^8 variantes) se empleó para seleccionar anticuerpos contra la toxina Cn2. Las variantes 3F y C1 fueron aisladas en la cuarta ronda de selección por la capacidad de unirse a la toxina Cn2. Las secuencias de las dos variantes (Figura 1, Anexo1) fueron comparadas con las líneas germinales de la base de datos IMGT [75] y el análisis mostró que la clona 3F tiene como familias a VH3 y VK3 para 3F y la variante C1 a VH3 y Vλ1. Las clonas reconocen de manera específica a la toxina, esta es una característica importante teniendo en cuenta que el objetivo es encontrar anticuerpos para fines terapéuticos. Esto se confirmó en las pruebas de reconocimiento en ELISA, donde los scFv 3F y C1 no reconocieron a otras toxinas de alacrán (bloqueadoras de canales de sodios con alta identidad a Cn2) ni a otros antígenos (Figura 2, Anexo 1).

Los anticuerpos 3F y C1 fueron ensayados en cuanto a su capacidad de protección contra el efecto de la toxina Cn2 en ratones y se determinó que carecen de actividad protectora. Esto se debe a que no se observó un incremento en el número de ratones sobrevivientes comparado con el control ($1DL_{50}$ de Cn2), tampoco hubo retardo en la aparición de los síntomas de intoxicación. Al evaluar el reconocimiento de los scFvs 3F y C1 a Cn2 en el BIACORE, se determinó que la disociación de los anticuerpos a la toxina es muy rápida y que los valores de las constantes de afinidad son del orden de 10^{-7} M (Tabla 1, Anexo 1). Estos valores de afinidad son similares a los obtenidos de bancos no inmunes, típicos de una respuesta primaria de anticuerpos [76, 77]. Al comparar las K_D con los de la tabla 1, se observa que están alejados por dos órdenes de magnitud o más de las afinidades de los anticuerpos neutralizantes de toxinas, lo cual podría explicar la carencia del efecto protector.

Otros aspectos a considerar son:

La toxina Cn2 es muy afín por los canales de sodio (orden nM) [71, 72], y los anticuerpos 3F y C1 no tienen el nivel afinidad que les permita competir por la unión a la toxina, esto también explica porque los anticuerpos no tienen la capacidad de neutralizar el efecto de Cn2 en ratones. Además de la baja afinidad de los anticuerpos, el formato de anticuerpo scFv no es el natural por lo que pueden presentarse problemas de estabilidad funcional en algunos casos. Para lograr obtener mejores anticuerpos, se probaron estrategias alternativas como la construcción de dímeros y la evolución dirigida de los scFvs.

Las propiedades terapéuticas de los fragmentos scFvs pueden ser mejorados mediante la construcción de la forma dimérica [63, 78]. Se realizó la construcción de los dímeros de las variantes 3F y C1, por recorte del conector de 15aa a 7 aa. Estas construcciones fueron

expresadas y probadas en la capacidad de neutralización, pero no protegieron. Se recurrió a la evolución dirigida para lograr cambios graduales en las afinidades de los anticuerpos por la toxina.

En cuanto a los ensayos de evolución dirigida, se construyeron los bancos mutagénicos de las variantes 3F y C1 usando dos estrategias de mutagénesis [49, 50]. El banco del scFv C1 fue de $6X10^6$ transformantes (taza de mutación del 0.6%) y se realizaron tres rondas de selección, pero no se obtuvieron clonas mejores con respecto a la parental. En el caso del scFv 3F, el tamaño del banco mutagénico fue de $1X10^6$ transformantes (tasa de mutación del 0.9%) y se realizaron 4 rondas de tamizado contra la toxina Cn2. De este proceso se aisló la variante 6F, la cual tiene un cambio en el CDR2 de la cadena pesada (Ser54Gly) (Tabla 2, Anexo 1). La determinación de las constantes cinéticas por BIACORE mostró mejores constantes de asociación y disociación (7 y 1.5 veces respectivamente con respecto a la parental) y por lo tanto se incrementó la K_D en un orden de magnitud ($1.68X10^{-8}$ M). De esta variante (6F), se construyó un segundo banco mutagénico de $1.6X10^6$ variantes (tasa de mutación del 0.6%). El banco fue tamizado y se seleccionó en la tercera ronda de selección la clona 610A (Tabla 2, Anexo 1), la cual tiene un cambio en el CDR3 de la cadena pesada de Val101Phe. El cambio de un residuo alifático por uno aromático, mostró un incremento en la constante de afinidad (1nM) y el principal efecto se vió en una disminución de la constante de disociación. La variante 610A, fue sometida nuevamente a mutagénesis y selección. El banco de $1X10^7$ variantes del scFv 610A (tasa de mutación del 1%), fue tamizado utilizando 2 condiciones de selección: tamizado normal y tamizado en condiciones astringentes. Este último incluyó mayores tiempos de incubación de los fagos en presencia del antígeno (4 horas), la temperatura de incubación fue de 37°C, y después de los lavados normales se empleó un lavado adicional con

trietilamina (TEA) 100mM pH 13 por media hora para eliminar clonas con baja afinidad, que no resisten el cambio brusco de pH. Las clonas recuperadas de la tercera ronda de selección fueron evaluados por ELISA de proteína. En el tamizado astringente se obtuvo un mayor número de clonas positivas y de mejor intensidad en la señal (Abs a 492nm arriba de 2) con respecto al procedimiento normal. Se evaluaron las 6 mejores clonas obtenidas del tamizado astringente y 2 clonas del normal. A nivel de secuencia, las clonas del tamizado normal presentaron 1 ó 2 cambios, mientras que las clonas obtenidas por el método astringente presentaron entre 2 y 6 cambios. La TEA es una base débil ampliamente utilizada para la recuperación de fagos afines al antígeno en los pasos de tamizado; sin embargo, en este trabajo se empleó únicamente como agente astringente para seleccionar clonas con alta afinidad y estabilidad. Esto demostró que no todos los fago-anticuerpos son eluídos del inmunotubo con TEA, es decir que hay una considerable perdida de clonas de interés para quienes siguen la metodología tradicional. En este trabajo, la recuperación de las clonas que permanecieron unidas a la toxina después de los lavados (normal y astringente) se realizó con células de *E. coli*, las cuales son infectadas con los fagos que se mantienen adheridos al antígeno en el inmunotubo [79].

La mejor clona seleccionada por el método astringente fue la variante 6009F (Tabla 2, Anexo 1) que presentó 6 cambios con respecto a la clona 610A. Dos cambios fueron silenciosos y cuatro fueron de aminoácido: (Asp74Asn en el framework (FW) 3 de la cadena pesada, Thr152Ile en el FW1 de la cadena ligera, Tyr164Phe en el CDR1 de la cadena ligera y Ser197Gly FW3 de la cadena ligera) (Tabla 2, Anexo 1). El scFv 6009F fue expresado y purificado (Procedimiento experimental y Figura 1 Material suplementario, Anexo 1), en el último paso de purificación por exclusión molecular se observó que la mayor parte de la proteína es monomérica (Figura 2. Material suplementario, Anexo 1). La

proteína soluble del scFv 6009F al ser evaluada en BIACORE presentó una K_D de 410 pM, es decir que los tres ciclos evolución incrementaron 446 veces la constante de afinidad con respecto a la parental (clona 3F). Este valor es el resultado de una mejor asociación y menor disociación de la toxina (Tabla 2, Anexo1).

Las variantes seleccionadas del primer y segundo ciclo de evolución dirigida (6F y 610A), se construyeron en forma dimérica (por el recorte del conector a 7 aa). Se realizó la expresión de las proteínas de forma monómerica y dimérica y ninguna tuvo la capacidad de proteger. A diferencia de éstas, la proteína monomérica de la clona evolucionada 6009F, obtenida en el tercer ciclo de evolución sí lo hizo (Tabla 3, Anexo 1). Los ratones control (1 DL_{50} de la toxina Cn2) muestran un estado muy alterado entre 15 y 30 minutos después de la administración intraperitoneal de Cn2. Presentan acicalamiento excesivo, estado de alerta, agitación, chillidos, salivación, pérdida del control de miembros inferiores, dificultad respiratoria y la muerte ocurre entre 1 y 3 horas después de la inyección. La toxina administrada en presencia del scFv 6009F, no causa los síntomas de intoxicación. Esta variante fue capaz de proteger a los ratones contra una y dos dosis letales de la toxina Cn2, en una relación molecular de 1 a 10 (toxina: scFv), sin presentar síntomas de envenenamiento. Cuando se probó con 2 DL_{50} de veneno, encontramos que también protege a todos los ratones del ensayo, aunque tuvieron dificultad respiratoria causada por otras toxinas presentes en el veneno. Los ratones se recuperaron 7 horas después de la administración de la mezcla, mientras que todos los ratones control (con 2 DL_{50} de veneno) murieron en el transcurso de la primera hora. Estos resultados de protección indican que la variante humana 6009F alcanzó un buen nivel de afinidad y de estabilidad funcional para ser neutralizante.

La toxina Cn2 tiene varios epitopos inmunogénicos, esto fue demostrado por Zamudio et al [70], donde se generaron anticuerpos monoclonales obtenidos por la tecnología de hibridoma. Se determinó la presencia de 4 epitopos diferentes en la toxina Cn2, los cuales fueron reconocidos por los anticuerpos maduros, el único anticuerpo neutralizante fue el BCF2 y se estableció que éste reconoce a las regiones amino y carboxilo de la toxina [68, 80]. La existencia de dos anticuerpos neutralizantes (monoclonal BCF2 y scFv 6009F) de una toxina de solo 66 aminoácidos no resultó sorprendente. Lo interesante es que los dos anticuerpos neutralizantes reconocen sitios distintos. Las competencias (por ELISA Figura 6 y BIACORE Figura 7) mostraron que los epitopos reconocidos por el BCF2 y por el scFv 6009F son diferentes, es decir que aunque la toxina es una molécula pequeña, tiene más de un epitopo importante para la neutralización.

La competencia entre el Alacramyn®, el BCF2 y el scFv 6009F, mostró que dentro de los anticuerpos policlonales $F(ab')_2$ presentes en el antiveneno hay algunos que comparten el mismo epitopo con BCF2 (sensograma Figura 8). Esto es lógico ya que tanto el BCF2 como el Alacramyn® provienen de varios retos contra la toxina Cn2 en ratón y contra una mezcla de venenos (incluido el de *C. noxius*) en caballo; es decir, son anticuerpos obtenidos de la respuesta secundaria natural. En contraste, el scFv 6009F se une a un epitopo diferente que el reconocido por los anticuerpos policlonales. Sabemos que la respuesta inmune de anticuerpos puede ser imitada *in vitro* a partir de una fuente de anticuerpos y un sistema de selección que permite aislar anticuerpos que reconocen a un determinado antígeno. Sin embargo, la selección *in vitro* es independiente de la estimulación en la respuesta de anticuerpos [26, 27]. Cuando se realiza el proceso *in vitro* es posible establecer otras interacciones que probablemente nunca ocurran *in vivo*, es decir que se pueden obtener anticuerpos que reconocen diferentes epitopos a los

obtenidos por inmunización *in vivo.* Un trabajo similar, Amersdorfer [81] muestra que los anticuerpos obtenidos de fuentes inmunes reconocen sitios diferentes a los anticuerpos obtenidos de una fuente no inmune contra la toxina botulínica. Este resultado es muy interesante ya que uno de los problemas del sistema inmunológico es que existen moléculas que no son inmunogénicas, es decir no son detectadas como extrañas por el organismo. Por lo tanto no se desencadena el mecanismo de protección natural por anticuerpos. En caso del sistema *in vitro* es posible obtener anticuerpos contra cualquier antígeno, los cuales pueden ser empleados como agentes terapeúticos.

Otro aspecto interesante para comparar entre el scFv 6009F y el BCF2 es que el fragmento (scFv) derivado del monoclonal BCF2 no retiene la capacidad de neutralizar el efecto de la toxina Cn2. Por lo que fue necesario hacer un proceso de maduración y mutación dirigida para obtener una variante con una constante de afinidad de 75pM que sí protege (Anexo 2). Esta variante llamada triple mutante neutraliza 1 DL_{50} de Cn2, pero no elimina la manifestación de los síntomas de intoxicación causados por la toxina. Al compararla con la variante 6009F que es menos afín por la toxina (K_D 410pM), se observó que la clona humana neutraliza 1 DL_{50} y no presenta síntoma alguno de intoxicación. Resultado que es idéntico cuando se aumenta la toxina a 2 DL_{50}. Esto nos indica que el scFv 6009F presenta una mejor capacidad de protección debido probablemente a que el epitopo bloqueado por el anticuerpo humano es más importante que el reconocido por el anticuerpo de ratón, para la neutralización.

8. CONCLUSIONES Y PERSPECTIVAS

Se logró la construcción de un banco de fragmentos variables de cadena sencilla humanos que puede ser tamizado contra diversos antígenos. La construcción de los bancos no inmunes, permite tener una buena fuente de anticuerpos que puede ser explorada para cualquier antígeno. Lo cual ha sido comprobado ya que a partir de estos bancos se han logrado seleccionar anticuerpos en nuestro grupo contra otros antígenos como las proteínas de: la cadena ligera λ 6aJL2, Interleucina 6 y heveína.

Se aislaron dos scFv humanos con alta especificidad contra la toxina Cn2 por medio del sistema de despliegue en fagos.

El proceso de evolución dirigida de la clona 3F, permitió generar la variante 6009F con un nivel de afinidad adecuado para ser neutralizante. Esto gracias a las modificaciones del proceso de selección, ya que el empleo de condiciones astringentes mejoró la calidad de las variantes seleccionadas.

El scFv 6009F es el primer anticuerpo humano neutralizante de una toxina de alacrán. Este anticuerpo podría formar parte de un antiveneno recombinante humano, contra las picaduras de alacrán, el cual sería más seguro para ser empleado en humanos.

El scFv 6009F se podrá caracterizar en otros aspectos como: establecer el efecto de las mutaciones individuales (con respecto a la afinidad y capacidad de neutralización) y realizar estudios farmacocinéticos de la proteína.

Ya que la metodología del despliegue en fagos permite establecer nuevas interacciones, será interesante determinar por *docking* y/o cristalografía del complejo antígeno anticuerpo, el sitio de la interacción del anticuerpo 6009F que inhibe el efecto de la toxina.

Con las metodologías establecidas y la experiencia generada se puede pensar en obtener nuevos anticuerpos humanos contra otras toxinas. Esto puede ser mediante la búsqueda de nuevos anticuerpos a partir de los bancos humanos, o mediante un cambio de especificidad de la clona 3F, teniendo en cuenta la alta identidad entre las toxinas de canales de sodio. A largo plazo será posible generar un antiveneno de fragmentos humanos que reemplace al de caballo, el cual sería la siguiente generación de anticuerpos neutralizantes.

En la Oficina de Patentes y Marcas de Estados Unidos se inició el trámite para patentar los anticuerpos obtenidos en este trabajo.

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10. ANEXO 1

A strategy for the generation of specific human antibodies by directed evolution and phage display

An example of a single-chain antibody fragment that neutralizes a major component of scorpion venom

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Keywords
affinity maturation; directed evolution; human scFv library; phage display; scorpion toxin

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Note
The sequences reported have been deposited in the GenBank database under accession nos. AY781338, AY781339, AY781340, AY781341 and AY781342; corresponding to scFvs: 3F, 6F, 610 A, 6009F and C1.

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This study describes the construction of a library of single-chain antibody fragments (scFvs) from a single human donor by individual amplification of all heavy and light variable domains (1.1×10^8 recombinants). The library was panned using the phage display technique, which allowed selection of specific scFvs (3F and C1) capable of recognizing Cn2, the major toxic component of *Centruroides noxius* scorpion venom. The scFv 3F was matured *in vitro* by three cycles of directed evolution. The use of stringent conditions in the third cycle allowed the selection of several improved clones. The best scFv obtained (6009F) was improved in terms of its affinity by 446-fold, from 183 nM (3F) to 410 pM. This scFv 6009F was able to neutralize 2 LD_{50} of Cn2 toxin when a 1 : 10 molar ratio of toxin-to-antibody fragment was used. It was also able to neutralize 2 LD_{50} of the whole venom. These results pave the way for the future generation of recombinant human antivenoms.

In recent years, the demand for antibodies for therapeutic purposes has increased [1]. To cope with this demand, some technologies have been adapted to generate and improve these antibodies [2,3]. Two of these methods are phage display [4,5] and directed evolution [6,7]. These technologies have allowed the generation and improvement of different antibodies, which now reach affinities similar to those of a secondary immunological response [3]. Depending on the purpose for which the antibody fragments are intended, several expression formats have been developed [8]. The tendency to use smaller molecule formats [single-chain antibody fragment (scFv); 25 kDa], is due to their increased biodistribution, diminished immunogenic characteristics and clearance properties [9]. Display of antibody fragment libraries on the surface of filamentous phages has replaced hybridoma technology for the selection of human antibodies through the creation of large repertoires *in vitro* [10]. This process begins with the cloning and expression of cDNAs encoding the variable regions of the H and L chains of antibodies (V_H and V_L), allowing the *in vitro* generation of

Abbreviations
CDR, complementarity determining region; Cn2, toxin from *Centruroides noxius* scorpion; scFv, single-chain antibody fragment; TEA, triethylamine; V_H, heavy chain; V_L, light chain.

large antibody repertoires. From these libraries, specific antibodies can be selected by linking phenotype (binding affinity) to genotype, thereby allowing simultaneous recovery of the gene encoding the selected antibody. Selected antibody fragments that do not have the required affinity can be subjected to cycles of mutation and further selection (directed evolution) to enhance affinity [7]. Different selection strategies have been used to select variants with improvements in various properties, for example stability, affinity and expression level [6,7]. There has been little report of the use of these libraries to isolate antibody fragments against toxic components of animal venoms [11]. For therapeutic purposes, human antibody libraries would be the best source, because of their homologous character and their reduced allergenic or secondary reactions [12].

Here, we report the construction of a human nonimmune library in which all families of variable domains (H and L) were amplified independently and combined with each other, resulting in a repertoire of 1.1×10^8 different members. From this library, two specific clones (3F and C1) that recognize toxin Cn2 from the Mexican scorpion *Centruroides noxius* Hoffmann were isolated and functionally characterized. Cn2 is one of the most abundant and toxic components of *C. noxius* venom (6.8% of total venom; $LD_{50} = 0.25$ μg per 20 g of mouse weight) [13]. Clone 3F was matured by three cycles of directed evolution. The use of a set of stringent conditions in the third cycle allowed the selection of several improved clones. The best scFv obtained (6009F) had an affinity that was improved by 446-fold (from 183 nM to 410 pM). This scFv 6009F was able to neutralize 2 LD_{50} of Cn2 toxin when a toxin/antibody fragment molar ratio of 1 : 10, was used. It was also able to neutralize 2 LD_{50} of the whole venom. This is the first recombinant human antibody fragment that neutralizes *C. noxius* venom. To the best of our knowledge, this is the first report of the generation of a human recombinant antibody fragment capable of neutralizing the toxic effects of the whole venom from a deadly animal.

Results

Human nonimmune library construction

The scFv library was generated by RT-PCR from total RNA purified from B lymphocytes of human peripheral blood. To avoid, as far as possible, a bias in antibody variable chain family representation, each V family of variable regions (V_H or V_L), was amplified by independent PCR. In a second PCR step, the sequence of the linker peptide was added to each individual V family. A PCR-overlapping process was performed in order to join both V domains (H and L). Every V_H family was overlapped to every V_κ or V_λ family (a total of 72 combinations). The DNA segments encoding the assembled products were fused to the *pIII* gene of the pSyn2 phagemid. The scFv library comprised 1.2×10^8 members. Twenty independent colonies were analyzed by PCR. Eighteen were of the right size and had different restriction patterns when digested with *Bst*NI (data not shown). Variability in the 18 different scFvs was confirmed by DNA sequence, which resulted in a library of 1.1×10^8 variants. We found different combinations of variable domains, which included the majority of V families.

Isolation and characterization of specific scFvs against Cn2 toxin

After four rounds of biopanning, the recognition capacity of scFvs was evaluated by means of phage-ELISA. Positive clones (15 of 88) were sequenced and analyzed individually. Two unique anti-Cn2 scFvs were identified and named scFv 3F and scFv C1 (Fig. 1). The

```
                                        CDR H1
C1  1   QVNLRESGGGLVQPGGSLRLSCAASGFSFGSYGMHWVRQA 40
3F  1   EVQLVESGGGLVQPGGSLRLSCAGSGFTFDNYAMHWIRQV 40

                    CDR H2
C1 41   PGKGLEWVAVISYDGSNKYYADSVKGRFTISRDNSKNTLY 80
3F 41   PGEGLEWVSGISRSSGDIGYADSVKGRFTISRDNAKKSLS 80

                                CDR H3
C1 81   LQMNSLRAEDTAVYYCAKDARDCLMCADWYFDLWGRGTLV 120
3F 81   LQMNSLRAEDTAVYYCAR-G-G----VGS-FDTWGQGTMV 113

          Linker
C1 121  TVSSGGGGSGGGGSGGGGSNFMLTQ-PHSASGTPGQRVTI 159
3F 114  TVSSGGGGSGGGGSGGGGSEIVLTQSPATLSVSPGERATL 153

                CDR L1                  CDR L2
C1 160  SCSGSSSNIGSNTVNWYRHLPGSAPELLIGSHNQRPSGVP 199
3F 154  SCRASQS--VRSYLAWYQQKPGQAPRLLISDASNRATGIP 191

                                        CDR L3
C1 200  DRFSASKSDTSASLAISGLQSEDEADYYCAAWDDSLIGYV 239
3F 192  ARFTGSGSGTDFTLTISSLEPEDFAIYYCQQY--RYSPRT 229

C1 240  FGTGTKLTVLGAAAEQKLISEEDLNGAAHHHHHH 273
3F 230  PGQGTKVEIKRAAAEQKLISEEDLNGAAHHHHHH 263
```

Fig. 1. Amino acid sequence alignment of scFvs selected from a human repertoire. These sequences include the C-myc C-terminal tag followed by a hexameric His tag. Complementarity determining regions (CDR) of V_H and V_L are delimited by a rectangle. The closest germ line, diversity and joining segments for the V_H domain of clone C1 were IGHV3-30*18, IGHD2-21*01 and IGHJ2*01, respectively. For the V_L domain, the germ line and the joining segments corresponded to IGVL1-44*01 and IGLJ1*01. The closest germ line, diversity and joining segments for the V_H domain of clone 3F were IGHV3-9*01; IGHD2-8*02; IGHJ3*02. For the VK, the germ line and the joining segments corresponded to IGVK3-11*01; IGKJ1*01.

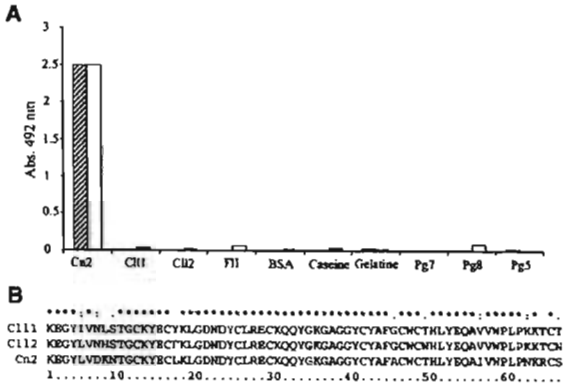

Fig. 2. Specificity of phage-antibodies 3F and C1. (A) Cross-reactivity: scFv 3F (hatched boxes) and scFv C1 (empty boxes). ELISA was used to determine binding to a variety of antigens. Cn2, CII1, CII2, Pg7, Pg8, specific toxins for sodium channels and Pg5, toxin specific for potassium channel, all at a concentration of 3 $\mu g \cdot mL^{-1}$; FII (toxic fraction II of *C. limpidus limpidus* venom) at 20 $\mu g \cdot mL^{-1}$. The titer of phage-antibodies was 1×10^{11} phages$\cdot mL^{-1}$. (B) Amino acid sequences of toxin Cn2 (*C. noxius*) and homologous toxins CII1 and CII2 (*C. limpidus limpidus*). Asterisks indicate identity, single dots indicate a 'weak' conserved group of residues and double dots indicate a 'strong' group of conserved residues as defined in CLUSTALX (v. 1.81).

Table 1. Kinetic rates and affinity constants of the soluble proteins corresponding to the scFvs 3F and C1. Kinetic rates and K_D were calculated using BIA-EVALUATION v. 3.2 software. SE, standard error.

scFv	K_{on} ($M^{-1} \cdot s^{-1}$)	SE/(K_{on})	K_{off} (s^{-1})	SE (K_{off})	K_D (M)
C1	2.0×10^4	2.3×10^2	1.40×10^{-2}	6.9×10^{-5}	5.40×10^{-7}
3F	7.0×10^4	1.7×10^3	1.28×10^{-2}	1.2×10^{-4}	1.83×10^{-7}

nucleotide sequences were compared with the databases using the BLAST algorithm. The best scores corresponded to human immunoglobulins. The nucleotide sequences were also compared with the IMGT databases [14] to determine the corresponding germ lines. For clone 3F, VH3-VK3 were the closest families for V_H and V_L domains, respectively. In the case of C1, VH3-Vλ1 were the families with highest scores. The specificity of these two scFvs was determined by phage-ELISA (Fig. 2A). These two clones were shown to be highly specific to Cn2 despite its high identity with control toxins CII1 and CII2 (Fig. 2B). The scFvs were recloned into the expression vector pSyn1 in order to characterize them as soluble proteins.

Characterization of clones 3F and C1

To discover whether the selected antibodies had the ability to protect mice against the toxic effects of Cn2, a neutralization assay was performed. The results showed that both antibody fragments were unable to protect the mice. The affinity constants were determined in a biosensor of molecular interactions in real time (BIACORE). Table 1 shows the values obtained for the binding kinetic constants. The affinity constants of both scFvs were similar, in the range of 10^{-7} M.

Affinity maturation

Clones 3F and C1 did not show the required affinity and/or functional stability to be neutralizing. Directed evolution and phage display were used to improve these properties. It has been shown that directed evolution allows a gradual increase in a particular property of the protein. Usually it is necessary to perform several evolution cycles in order to obtain the desired improvement. Three cycles of evolution were needed to obtain a variant of scFv 3F (6009F) with an adequate affinity level and that was capable of neutralization, whereas the directed evolution of scFv C1 was unsuccessful. In the first cycle, the library (1×10^6 variants; mutation rate 0.9%) obtained from scFv 3F was evaluated by phage display against Cn2 toxin. Variant 6F was selected (Table 2), which had a change (Ser54Gly) in CDR2 of the heavy chain. Determination of the kinetic constants (BIACORE) for this mutant showed a change in the K_D value from 1.83×10^{-7} M to 16.8 nM. Mutant 6F was subjected to a second maturation cycle (library size = 1.6×10^6 variants; mutation rate 0.6%), and clone 610A was selected. This variant showed a change at CDR3 of the heavy chain (Val101Phe). This mutation improved the K_D value from 16.8 to 1.04 nM (Table 2). A third cycle of evolution allowed us to select clone 6009F (library size = 1.0×10^7; mutation rate 1%). In this last maturation cycle, two alternative selection strategies were performed. The first was the standard procedure and the second included some stringent modifications intended to select variants improved in terms of their affinity and functional stability (see Experimental procedures). With the stringent selection, several clones were selected. The best clone was 6009F and their DNA sequence showed two silent mutations and four amino acid changes with respect to clone 610A (Table 2). One of these changes occurred at framework 3 of the heavy chain (Asp74Asn) and 3 of the light chain. Two of the changes (Thr152Ile and Ser197Gly) occurred at frameworks 1 and 3, respectively, and the third (Tyr164Phe), occurred at CDR1 (Table 2). Antibody 6009F was expressed in *Escherichia coli* and the presence of the protein was verified by SDS/PAGE (supplementary Fig. S1). The chromatographic elution profile of the antibody 6009F, showed a main peak corresponding to a monomer (supplementary Fig. S2).

Table 2. Characterization of scFvs selected by directed evolution and phage display. Results of sequence analyses allowing identification of the changes in amino acid residues that occurred during each cycle of evolution. For each selected variant, mutations with respect to clone 3F are indicated. The last five columns show the binding kinetic parameters of the scFvs to immobilized Cn2 determined by surface plasmon resonance (BIACORE). SE, standard error.

Evolution cycle	scFv selected	Change	Position	K_{on} ($M^{-1}\cdot s^{-1}$)	SE (K_{on})	K_{off} (s^{-1})	SE (K_{off})	K_D (M)
	3F			7.00×10^4	1.7×10^3	1.28×10^{-2}	1.2×10^{-4}	1.83×10^{-7}
1	6F	Ser54Gly	$CDR2V_H$	4.93×10^5	3.9×10^3	8.25×10^{-3}	9.0×10^{-5}	1.68×10^{-8}
2	610 A	Ser54Gly	$CDR2V_H$	6.35×10^5	8.3×10^3	6.63×10^{-4}	1.3×10^{-5}	1.04×10^{-9}
		Val101Phe	$CDR3V_H$					
3	6009F	Ser54Gly	$CDR2V_H$	7.4×10^5	3.7×10^3	3.00×10^{-4}	1.7×10^{-6}	4.1×10^{-10}
		Val101Phe	$CDR3V_H$					
		Asp74Asn	$FW3V_H$					
		Thr152Ile	$FW1V_x$					
		Tyr164Phe	$CDR1V_x$					
		Ser197Gly	$FW3V_x$					

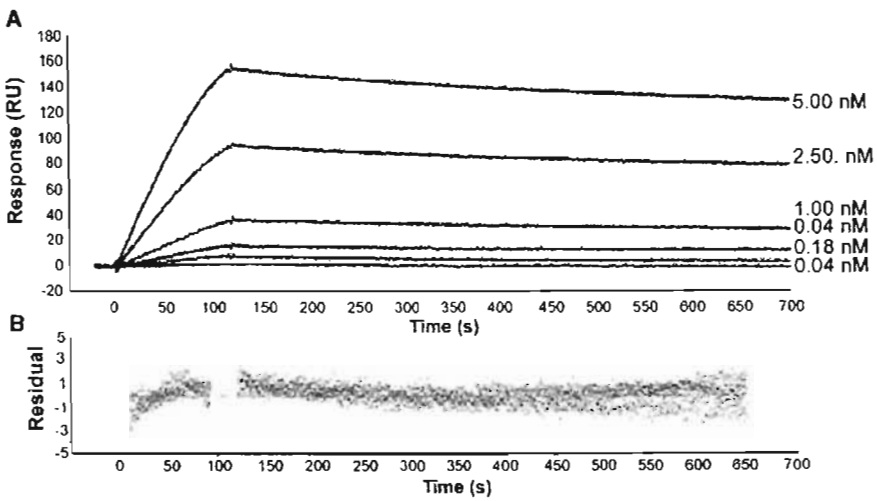

Fig. 3. Affinity determination of scFv 6009F. (A) BIACORE binding kinetics to Cn2 toxin. The Langmuir (1 : 1) binding model was used. (B) The variation between the theoretical and experimental data (residual values) shows the reliability of the fitting.

The total yield was typically 700 $\mu g \cdot L^{-1}$ of culture. To determine the neutralization capacity and binding kinetics, only the monomeric fraction was used. The BIACORE analysis (Fig. 3; Table 2) showed a K_D value of 410 pM, the best affinity value for the evolved variants.

Neutralization assays

The capacity of the soluble protein purified from clones 6F, 610A and 6009F to neutralize toxin Cn2 was evaluated in CD1 mice. Clone 6009F was the only one that had the capacity to neutralize the toxin. The protection showed by this antibody fragment was 100% (Table 3). No symptomatology was detected up to 24 h of observation, using 1 or 2 LD_{50} of toxin

Table 3. Neutralization assays. Results of mice groups challenged with Cn2 toxin or whole venom by intraperitoneal injection alone or in the presence of the indicated molar ratios of toxin/antibody. LD_{50}; Cn2 = 0.250 µg per 20 g of mouse weight and whole venom = 2.5 µg per 20 g of mouse weight.

Sample	LD_{50}	Molar ratio Cn2 : 6009F	Survival ratio (alive/total)
6009F			10/10
Cn2	1		6/10
Cn2	1	1 : 10	20/20
Cn2	2		6/18
Cn2	2	1 : 10	18/18
Whole venom	2		0/10
Whole venom	2	1 : 14 [a]	10/10

[a] Estimated assuming that Cn2 constitutes 6.8% of whole venom.

and a 1 : 10 molar ratio of toxin-to-antibody fragment. Two LD_{50} of whole venom were also tested using the same quantity of antibody as the one used to neutralize 2 LD_{50} of toxin. All the mice injected with the antibody/toxin mix survived. Slight symptoms of poisoning were observed up to 6 h after injection of the mix. One hour later the symptoms disappeared.

Discussion

Human scFv nonimmune library

The need to generate safer and more efficient antibodies to be used in human therapy has resulted in the development of recombinant antibodies from different sources. Ideally, the source itself should be human. In this study we constructed a scFv nonimmune library of 1.1×10^8 variants. Evaluation of the library in terms of variability revealed that it contained different combinations of variable domains.

From this library two anti-Cn2 clones (3F and C1) were selected. Although they were specific for Cn2 toxin (Fig. 2), they were not able to neutralize it. Analysis of the affinity constants showed values in the range 10^{-7} M (Table 1), which are typical affinity values for the primary immune response [15,16]. Clones 3F and C1 showed fast dissociation despite having good association, which suggests that the antibody fragments do not remain bound to the toxin for long enough to be neutralizing. It has been reported that the dimeric form of a scFv gives the molecule properties that are advantageous in therapeutic applications [17]. We constructed the dimeric form of our scFvs by shortening the linker from 15 to 7 amino acid residues. Neither of the diabodies, 3F or C1, was able to neutralize the toxin in the protection assay. They did not have the required affinity and/or functional stability to be neutralizing as shown for most examples of neutralizing antibodies, which have affinities in the nanomolar range and lower [18–20]. This result was expected, because the library is nonimmune, is of medium size and it is now known that higher affinity binders can be selected from bigger libraries [21–23]. The affinity of the toxin Cn2 for the sodium channels present in some cell preparations has been shown to be in the nM range [24,25]. These results suggest that an antibody with an affinity in this range at least is needed to neutralize the toxin. Taking this into consideration we matured the scFv 3F.

Affinity maturation

Three cycles of evolution were performed to obtain variant scFv 6009F to neutralize Cn2 toxin. The first cycle allowed selection of variant 6F (Table 2), with a change at CDR2 of the heavy chain. This mutant showed association and dissociation constants that were improved ≈7- and 1.5-fold, respectively, resulting in a change of one order of magnitude in the K_D value (from 183 to 16.8 nM; Table 2). These results show that scFv 6F binds more efficiently to the toxin, but it still detaches rapidly, suggesting that Gly at position 54 might play an important role in the interaction of the antibody with the toxin Cn2. Variant 6F was not able to neutralize the toxin despite having a better affinity constant than scFv 3F. The next cycle of evolution allowed selection of clone 610A. The change at CDR3 of the heavy chain improved both the association constant, and more importantly the dissociation constant. This result suggests that residue 101 in the CDR3 (Val101Phe) of the heavy chain might also be important for binding to the toxin. The change of Val to Phe may result in a better interaction in terms of an increased contact area. Changes at CDRs 2 and 3 in clone 610A had a synergistic effect on the affinity constant leading to a 176-fold change [183 nM (3F) to 1.04 nM (610A)] (Table 2). These improvements in affinity still did not confer a neutralizing capacity on this clone. For the third cycle, we used two alternative selection strategies: the standard and the stringent procedure to select variants improved in terms of their affinity and functional stability (see Experimental procedures). Drastic conditions were crucial for the selection of a variety of improved clones. Different strategies with the same purposes have been reported [26–29]. The standard procedure of phage selection gave a lower number of positive variants (including the first and second cycle) compared with the more stringent procedure. The number of nucleotide changes in the selected clones from the two procedures was different. Interestingly, clones selected from the standard procedure had fewer changes (usually one), whereas using the stringent strategy, the selected clones showed 2–6 changes. Clone 6009F was selected and showed four amino acid changes with respect to clone 610A (Table 2). Analysis of affinity measurements (Table 2 and Fig. 3), revealed that clone 6009F had a K_D of 410 pM, which is comparable with the affinities of other neutralizing antibodies of scorpion toxins [17,20,30,31]. The kinetic parameters showed that the additional changes present in clone 6009F improved the dissociation constant by approximately twofold compared with clone 610A, resulting in an affinity constant, as already mentioned, in the picomolar range, leading to a 446-fold change in K_D with respect to scFv 3F.

The evolution cycles of scFv 3F allowed the accumulation of changes in the sequence, which improved the affinity significantly. It has been suggested that changes at CDRs are the most important for improving the affinity of the antigen [32,33]. However, it has recently been shown that changes at frameworks improve not only affinity [34], but also expression level [7]. A similar phenomenon was seen during maturation of clone 3F, because scFv 6009F accumulated three changes at CDRs and three at the frameworks. We surmised that the changes at the frameworks contributed to the generation of a molecule with an improved affinity and an improved functional stability.

Neutralization capacity of variant 6009F

For the neutralization assays, two different doses of toxin Cn2 (1 and 2 LD_{50}) were used, whereas for the whole venom only 2 LD_{50} was assayed. When 1 LD_{50} of toxin and a 10 M excess of scFv 6009F were injected, all the mice survived compared with the controls (Table 3). Control animals showed typical symptoms of poisoning 30 min post injection. The first deadly effects of the toxin occurred 1.5 h after the injection. It is noteworthy that mice injected with the antibody/toxin mix did not present any symptoms associated with envenoming [35]. The next step consisted in using 2 LD_{50} of toxin. The mice did not show any signs of poisoning, demonstrating the effectiveness of our evolved human antibody (100% protection). When the mice were injected with 2 LD_{50} of toxin, the symptoms appeared 15 min after injection and the deadly effects started only 1 h after injection. In the case of whole venom, mice were protected but they presented some symptoms, such as respiratory distress, but they recovered 7 h later. This observation can be explained because the whole venom contains at least 70 different toxins (unpublished results), the majority affecting sodium channels. Despite Cn2 being the major toxic peptide, there are other toxins similar in toxicity but lower in concentration. This could imply that the toxicity of the whole venom is almost completely neutralized when toxin Cn2 is trapped by antibody 6009F but the remaining toxins exert an effect for some time until they are eliminated from the circulation. We would like to emphasize that antibody 6009F is capable of completely protecting against envenoming caused by two lethal doses of toxin Cn2 and confers reasonably good protection against two lethal doses of whole venom. The scFv 6009F is stable after 4 weeks stored in NaCl/P_i at 4 °C, as shown by a functional activity evaluation during 4 weeks (weekly; data not shown). The scFv 6009F showed protective activity during this period, indicating that it is functionally stable, as expected from the stringent selection strategy used. In the case of murine scFvs that recognize scorpion toxins, it has been shown that dimerization of scFv confers better affinity and stability [17]. We have also observed that dimerization, as a consequence of directed evolution [36] or shortening of the linker peptide (unpublished results), resulted in an improvement in the stability of the single chain. The diabodies of evolved clones 6F and 610A were constructed by shortening the linker. Despite showing better signals on ELISA, compared with their monomeric counterparts, none of these diabodies was capable of neutralizing toxin Cn2. The neutralization capacity of monomeric 6009F compared with clone 610A (monomer or dimer), indicates that the additional changes present in monomeric 6009F exerted a real positive effect on the affinity and functional stability.

We have obtained two scFvs highly specific to Cn2 toxin from a nonimmune human library (1.1×10^8 members). One of them (3F) was subjected to three cycles of directed evolution yielding a neutralizing variant named 6009F. It was able to neutralize 2 LD_{50} of toxin Cn2 and 2 LD_{50} of whole venom. Mutant 6009F was obtained after performing some modifications to the standard procedures of biopanning, specially the inclusion of a pre-elution step with 100 mM triethylamine (TEA) for 30 min to eliminate low stable and/or low affinity variants. The scFv 6009F blocked an epitope in Cn2 which seems to be very relevant for the interaction of the toxin with its target. These are the first recombinant human antibody fragments specific for toxin Cn2, which have been isolated from scFv libraries displayed on filamentous phages. The scFv 6009F could be used as a potential component of a recombinant antiserum against *Centruroides* stings. These results open new avenues for the generation of recombinant antisera against deadly animals.

Experimental procedures

Antigens

Toxin Cn2 (formerly II-9.2.2) was purified from venom obtained by electric stimulation of scorpions of the species *Centruroides noxius* Hoffmann. The venom was purified by Sephadex G-50 gel filtration and cation-exchange chromatography [37]. The other toxins used, CllI [38], CllI2 [39], Pg5, Pg7, Pg8 (T Olamendi-Portugal, BI García-Gomez, F Bosmans, J Tytgat, K Dyason, J van del Walt & LD Possani, unpublished data), and FII (toxic fraction II from

Table 4. Oligonucleotide primers used for PCR to append the sequence encoding the peptide linker [(Gly4-Ser)$_3$] to human V_H and V_L. The sequence corresponds to the 5′–3′ orientation.

VK1.link	GGCGGATCAGGAGGCGGAGGTTCTGGTGGAGGTGGGAGTGACATCCAGATGACCCAGTCTCC
VK2.link	GGCGGATCAGGAGGCGGAGGTTCTGGTGGAGGTGGGAGTGATGTTGTGATGACTCAGTCTCC
VK3.link	GGCGGATCAGGAGGCGGAGGTTCTGGTGGAGGTGGGAGTGAAATTGTGTTGACGCAGTCTCC
VK4.link	GGCGGATCAGGAGGCGGAGGTTCTGGTGGAGGTGGGAGTGACATCGTGATGACCCAGTCTCC
VK5.link	GGCGGATCAGGAGGCGGAGGTTCTGGTGGAGGTGGGAGTGAAACGACACTCACGCAGTCTCC
VK6.link	GGCGGATCAGGAGGCGGAGGTTCTGGTGGAGGTGGGAGTGAAATTGTGCTGACTCAGTCTCC
VL1.link	GGCGGATCAGGAGGCGGAGGTTCTGGTGGAGGTGGGAGTCAGCTCGTGTTGACGCAGCCGCC
VL2.link	GGCGGATCAGGAGGCGGAGGTTCTGGTGGAGGTGGGAGTCAGTCTGCCCTGACTCAGCCTGC
VL3b.link	GGCGGATCAGGAGGCGGAGGTTCTGGTGGAGGTGGGAGTTCTTCTGAGCTGACTCAGGACCC
VL3a.link	GGCGGATCAGGAGGCGGAGGTTCTGGTGGAGGTGGGAGTTCCTATGTGCTGACTCAGCCACC
VL4.link	GGCGGATCAGGAGGCGGAGGTTCTGGTGGAGGTGGGAGTCACGTTATACTGACTCAACCGCC
VL5.link	GGCGGATCAGGAGGCGGAGGTTCTGGTGGAGGTGGGAGTCAGGCTGTGCTCACTCAGCCGTC
VL6.link	GGCGGATCAGGAGGCGGAGGTTCTGGTGGAGGTGGGAGTAATTTTATGCTGACTCAGCCCCA
JH1-2.link	CCACCAGAACCTCCGCCTCCTGATCCGCCACCTCCTGAGGAGACGGTGACCAGGGTGCC
JH3.link	CCACCAGAACCTCCGCCTCCTGATCCGCCACCTCCTGAAGAGACGGTGACCATTGTCCC
JH4-5.link	CCACCAGAACCTCCGCCTCCTGATCCGCCACCTCCTGAGGAGACGGTGACCAGGGTTCC
JH6.link	CCACCAGAACCTCCGCCTCCTGATCCGCCACCTCCTGAGGAGACGGTGACCGTGGTCCC

Centruroides limpidus limpidus) [39], were obtained using the same procedure, from venoms of the species *C. limpidus limpidus* (Cll) and *Parabuthus granulatus* (Pg).

Construction of the library

A human nonimmune scFv library was prepared from a sample of 400 mL of peripheral blood provided by a healthy individual. cDNA was synthesized from total RNA isolated from B lymphocytes, using random hexamers (Roche RT-PCR Kit, AMV, Indianapolis, IN, USA). Variable domain repertoires of immunoglobulin heavy chains were amplified from the cDNA using Vent DNA polymerase (New England Biolabs, Beverly, MA, USA) in combination with each of the HuVHFOR primers and an equimolar mixture of HuJHBACK primers [40] in independent reactions for each family. For light chain variable domains, a similar procedure was performed using each HuVκFOR and a mixture of HuJκBACK for κ chains and each HuVλFOR with a mixture of HuJλBACK for λ chains. A GeneAmp PCR thermocycler (Perkin-Elmer 9600, Norwalk, CT, USA) was used for PCR. The conditions for the amplifications were: 3 min denaturation at 95 °C, followed by 30 cycles at 95 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min, with a final extension cycle at 72 °C for 10 min. PCR products were purified with a QIAquick PCR purification kit (Qiagen Inc., Valencia, CA, USA). These fragments were reamplified to append a DNA segment encoding half of the peptide linker [(Gly4-Ser)$_3$] in independent reactions. The connector primers were designed as described previously [41]. Their sequences are shown in Table 4. PCR products were gel-purified and overlapped by PCR. Each overlapped product (72 in total), was amplified in the same overlapping reaction mixture with primers that allowed the incorporation of *Sfi*I and *Not*I restriction sites. The following program was used: denaturation at 95 °C for 5 min followed by seven cycles of 1 min at 95 °C, 1.5 min at 64 °C, and 1 min at 72 °C without primers. Subsequently, external primers were added, followed by 30 cycles of 1 min at 95 °C, 1 min at 64 °C, and 1 min at 72 °C and a final extension at 72 °C for 10 min. Each PCR product was quantified and mixed in equimolar amounts to be digested. DNA segments were cut with restriction enzymes *Sfi*I and *Not*I and gel-purified. The resulting DNA fragments were ligated into the phagemid pSyn2 (kindly provided by J. D. Marks, UCSF, San Francisco, CA, USA) previously cut with the same restriction enzymes. Ligated DNA was electroporated into *E. coli* strain TG1. Twenty individual clones were analyzed by digestion with *BstN*I and sequenced. The sequences of the clones were determined with the primers forward (5′-ATACCTATTGCCTACGGC-3′) and reverse (5′-TTTC AACAGTCTATGCGG-3′) in the Applied BioSystems sequencer Model 3100 (Foster City, CA, USA).

Isolation of anti-Cn2 scFv by panning of phage-antibody repertories

The library of human scFv was displayed on filamentous phage and used for the selection of antibodies against Cn2 toxin. Biopanning was performed as described previously [40]. Some modifications to these procedures were as follows: 1 mL of the library (1×10^{13} phage antibodies) was incubated in the presence of different blocking agents (BSA or gelatin) before to biopanning in order to eliminate as many unspecific clones as possible. Pre-blocked library was poured into an immunotube (Maxisorp; Nunc, Roskilde, Denmark) previously coated overnight with 1 mL of Cn2 at 50 $\mu g \cdot mL^{-1}$ in $NaHCO_3$ buffer, pH 9.4 at 4 °C. Exten-

sive washings were performed to remove nonspecific phage. The bound phage-antibodies were recovered by the addition of 1 mL of TG1 cells of a mid-log phase ($A_{600} = 0.7$) culture [23,42]. After four rounds of panning, single phage-antibody clones were randomly picked and screened for specific binding to Cn2 by ELISA. High-binding polystyrene ELISA plates (Corning, NY, USA) were coated overnight with 0.3 μg of Cn2 (100 μL·well^{-1}) in bicarbonate buffer 50 mM pH 9.4 at 4 °C. Plates were washed three times with NaCl/P_i and 0.1% (v/v) Tween, then blocked with 0.5% (w/v) BSA in NaCl/P_i for 2 h at 37 °C. Phage-antibody supernatants were added to each well, incubated for 1 h at 37 °C and the plates washed. Bound phage-antibodies were detected with horseradish peroxidase (HRP)-conjugated anti-M13 serum (Amersham Pharmacia Biotech AB). HRP activity was detected by adding *O*-phenylenediamine. Plates were read at 492 nm in an ELISA reader (Bio-RAD Model 2550). Clones that bound to Cn2 with absorbance values > 2 were considered positive. Specific binding clones were sequenced.

Phage-antibody cross-reactivity

Selected phage-antibodies were tested for specificity with different antigens by ELISA. High-binding polystyrene immunoplates were coated with several proteins (Cn2, Cl1, Cl2, FII, Pg5, Pg7, Pg8, BSA, casein and gelatin) in bicarbonate buffer 50 mM pH 9.4 at 4 °C overnight. One hundred microliters of each selected variant containing 1×10^{11} phage-antibodies·mL^{-1} were added to the wells and detected as described.

Affinity maturation by error-prone PCR

Selected clones from the constructed library after four rounds of biopanning, were subjected to mutagenesis. Two standard techniques of error-prone PCR were used to construct random mutant scFv libraries with different mutation rates [43,44]. Both PCR products were mixed, digested with *Sfi*I and *Not*I, gel-purified and then ligated into the phagemid pSyn2. Ligated DNA was electroporated into electrocompetent *E. coli* TG1 cells. The library variability (mutation rate) was determined. The library was subjected to 3–4 rounds of biopanning as described previously [38]. Three cycles of evolution were performed.

For the last cycle of evolution, a second biopanning procedure was employed in order to obtain scFv clones with improved affinity and functional stability. It was performed according to the standard methods but with the following modifications: the immunotube was coated with 1 mL of Cn2 at 5 μg·mL^{-1}, the time of incubation was increased from 2 to 5 h and the temperature was increased from 25 to 37 °C. After the washing steps, 1 mL of 100 mM TEA (Pierce, Rockford, IL, USA), was added to remove the less stable or low-binding phage-antibodies. The incubation time was 30 min, after which the detached phages were eliminated. Immunotubes were rinsed with 1 mL of 1 M Tris/HCl, pH 7 to neutralize the TEA and then washed three times with NaCl/P_i. Phage-antibodies that remained bound to Cn2 were recovered with *E. coli* TG1 cells. The clones selected with this procedure were evaluated by ELISA as soluble proteins.

Expression of single-chain antibodies

The scFv inserts from the selected clones, were ligated into the expression vector pSyn1 [45,46]. This vector allows expression of the cloned segment under the control of *lac* promoter. The expressed product contains a C-myc tag and a hexa-His tag at the C-terminus. The constructs were transformed into *E. coli* strain TG1. Five hundred milliliters of recombinant cells were grown until an $A_{600} = 0.7$ was reached. Expression of the scFvs was induced with 1 mM isopropyl thio-β-D-galactoside. After 6 h the cells were harvested by centrifugation (6000 r.p.m., 10 min, to 4 °C). The pellet was resuspended in 12.5 mL of periplasmic buffer (PPB) extraction buffer (20% sucrose/1 mM EDTA/30 mM Tris HCl adjusted to pH 8). The mixture was incubated on ice for 20 min. Cells were centrifuged at 6440 *g* at 4 °C for 20 min. The supernatant containing the scFv protein was collected for further purification. The pellet was resuspended in 5 mM $MgSO_4$, kept on ice for 20 min and centrifuged at 6440 *g* at 4 °C for 20 min p.p.b. and $MgSO_4$ supernatants were mixed and dialyzed twice against 1× NaCl/P_i. The scFvs were purified by Ni^{2+}-NTA affinity chromatography (Qiagen, Hilden, Germany), and eluted with 1 mL of 250 mM imidazole. Finally, scFv preparations were purified by gel filtration chromatography on a Superdex™ 75 column (Phamacia Biotech AB, Uppsala, Sweden).

Neutralization assays

Purified scFv proteins were used to test their neutralization capacity against the toxic effects of Cn2 or the whole venom in mice. Groups of 10–20 female mice (CD1 strain) were injected with a mix of scFv and toxin Cn2 or venom. One or two LD_{50} (0.25–0.5 μg per 20 g of mouse weight) of Cn2 toxin or two LD_{50} (5 μg per 20 g of mouse weight) of whole venom, were mixed with each scFv at a final molecular ratio of 1 : 10 (toxin : scFv). The mix was incubated for 30 min a 37 °C and injected intraperitoneally. Three controls were used: venom (2 LD_{50}), Cn2 (1 LD_{50} and 2 LD_{50}) or scFv (8.7 μg per 20 g of mouse weight) were injected alone in independent assays. The amounts of antibody used to neutralize 1 or 2 LD_{50} of the toxin were 8.7 or 17.4 μg, which corresponded to a molar ratio of 1 : 10 in terms of Cn2 concentration. The number of animals was kept to a minimum, but was enough to validate the experiment. The protocols were approved by the ethical committee of animal

care at our institute, following the guidelines of the NIH (USA).

Surface plasmon resonance measurements

Kinetic constants for the interaction between scFv proteins and immobilized Cn2 toxin were determined in a BIACORE biosensor system (BIACORE X). Twenty-four micrograms of Cn2 toxin were bound onto a CM5 sensor chip using an equimolar mix of *N*-hydroxysuccinimide and *N*-ethyl-*N*-(dimethyl-aminopropil)carbodiimide) in 200 mM Mes buffer pH 4.7. Approximately 400 resonance units (RU) were coupled. The scFvs were diluted at various concentrations in HBS-EP buffer (BIACORE) and 60 μL were injected over immobilized Cn2 at a rate of 30 $\mu L \cdot min^{-1}$ with a delay in the injection of 700 s. Data were analyzed using BIA-EVALUATION (v. 3.2).

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Supplementary material

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Fig. S1. Expression and purification of scFv 6009F. (A) SDS/PAGE (12%). Lane 1, molecular mass markers; lane 2, antibody 6009F after affinity purification on Ni^{2+}-agarose; lane 3, periplasmic extract. (B) Lane 1, antibody 6009F after Superdex 75 column purification; lane 2, molecular mass markers.

Fig. S2. Purification by molecular exclusion. (A) Superdex 75 exclusion chromatography of antibody 6009F after affinity purification on Ni^{2+}-agarose. (B) Molecular mass standards: ovoalbumin (43 kDa), trypsinogen (23.9 kDa). The rate flux was 0.5 mL·min−1.

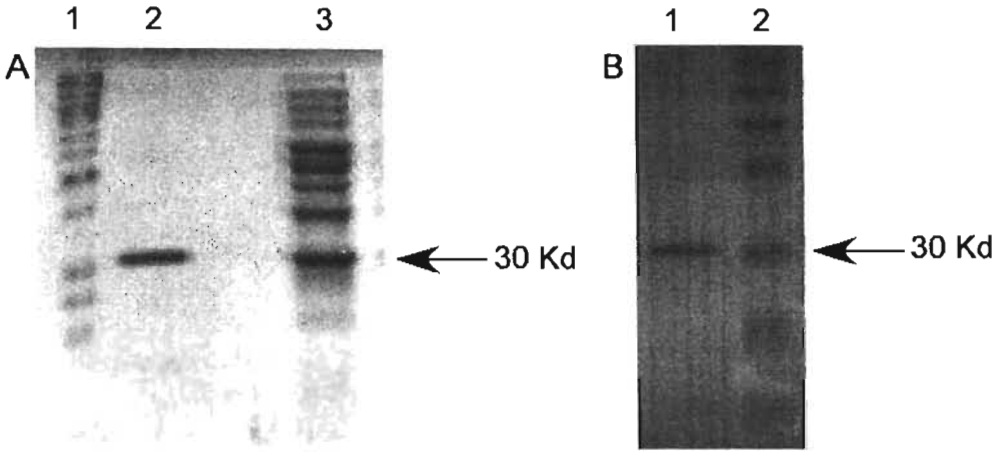

Fig. 1 (Supplementary Material). **Expression and purification of scFv 6009F.** A) SDS-PAGE (12%) Lane 1, MW markers; lane 2 antibody 6009F after affinity purification on Ni^{2+}-agarose; lane 3 periplasmic extract. B) Lane 1, antibody 6009F after Superdex 75 column purification; lane 2, MW markers.

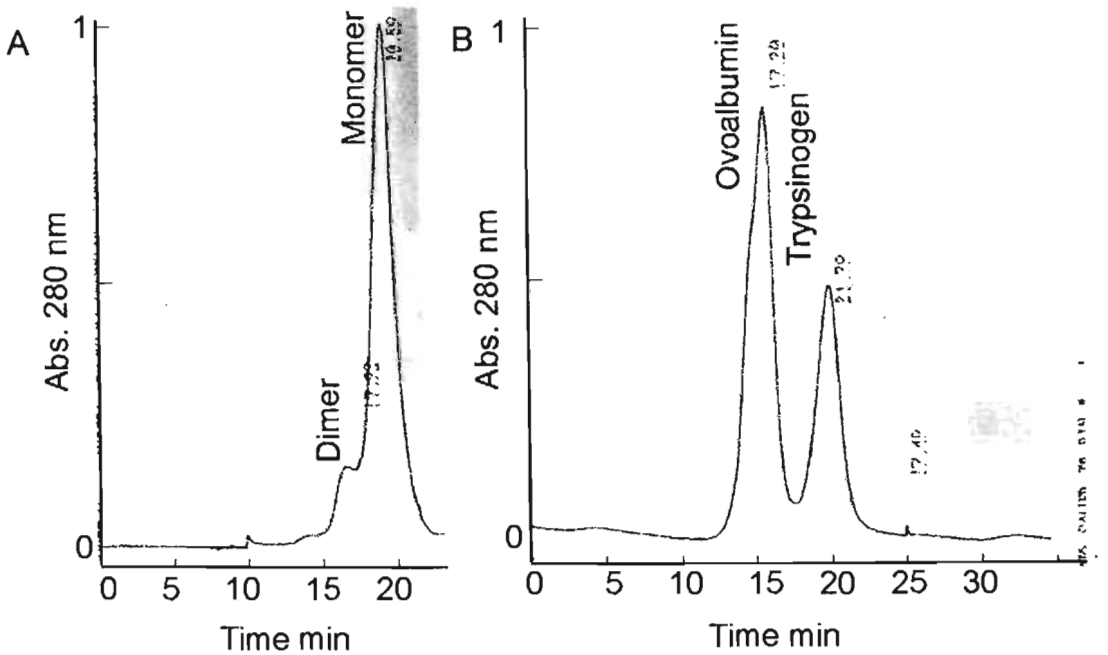

Fig. 2 (Supplementary Material). **Purification by molecular exclusion.** A) Superdex 75 exclusion chromatography of antibody 6009F after affinity purification on Ni^{2+}-agarose. B) Molecular weight standards: ovoalbumin (43 Kd), trypsinogen (23.9Kd). The rate flux was 0.5 ml/min.

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Directed Evolution, Phage Display and Combination of Evolved Mutants: A Strategy to Recover the Neutralization Properties of the scFv Version of BCF2 a Neutralizing Monoclonal Antibody Specific to Scorpion Toxin Cn2

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BCF2, a monoclonal antibody raised against scorpion toxin Cn2, is capable of neutralizing both, the toxin and the whole venom of the Mexican scorpion *Centruroides noxius* Hoffmann. The single chain antibody fragment (scFv) of BCF2 was constructed and expressed in *Escherichia coli*. Although its affinity for the Cn2 toxin was shown to be in the nanomolar range, it was non-neutralizing *in vivo* due to a low stability. In order to recover the neutralizing capacity, the scFv of BCF2 was evolved by error-prone PCR and the variants were panned by phage display. Seven improved mutants were isolated from three different libraries. One of these mutants, called G5 with one mutation at CDR1 and another at CDR2 of the light chain, showed an increased affinity to Cn2, as compared to the parental scFv. A second mutant, called B7 with a single change at framework 2 of heavy chain, also had a higher affinity. Mutants G5 and B7 were also improved in their stability but they were unable to neutralize the toxin. Finally, we constructed a variant containing the changes present in G5 and B7. The purpose of this construction was to combine the increments in affinity and stability borne by these mutants. The result was a triple mutant capable of neutralizing the Cn2 toxin. This variant showed the best affinity constant ($K_D = 7.5 \times 10^{-11}$ M), as determined by surface plasmon resonance (BIAcore). The k_{on} and k_{off} were improved threefold and fivefold, respectively, leading to 15-fold affinity improvement. Functional stability determinations by ELISA in the presence of different concentrations of guanidinium hydrochloride (Gdn-HCl) revealed that the triple mutant is significantly more stable than the parental scFv. These results suggest that not only improving the affinity but also the stability of our scFv were important for recovering its neutralization capacity. These findings pave the way for the generation of recombinant neutralizing antisera against scorpion stings based on scFvs.

Keywords: directed evolution; affinity maturation; functional stability; phage display; single chain antibody fragment (scFv)

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Introduction

Immune serum therapy (serotherapy), has been used during the last century for the treatment of poisoning caused by animal bites and stings in humans.[1] Scorpion stings in Mexico reach over 200,000 accidents per year with a mortality of

Abbreviations used: CDR, complementarity determining region; O/N, overnight; scFv, single chain antibody fragment; Gdn-HCl, guanidinium hydrochloride.

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approximately 700 people during the seventies and eighties. For the nineties, the fatalities reported were 300 and by 1998, 136 (Weekly Epidemiological Bulletin, Mexican Health Ministry, week 52, 1998). During 2002 the number diminished to 70 due to an intensive serotherapy campaign. The antivenom currently used in Mexico consists of bivalent $F(ab')_2$ fragments obtained from hyper-immunized horses with a water extract from venomous glands of *Centruroides* scorpions.[2]

The monoclonal antibody BCF2 neutralizes the deadly effects of Cn2 toxin, which is present in the venom of one of the most dangerous species of Mexican scorpions (*Centruroides noxius* Hoffman).[3] Cn2 is one of the most abundant (6.8% of total venom), and toxic (LD_{50} = 0.25 μg/20 g mouse) peptides of this venom. BCF2 is able to neutralize also the whole venom.[4]

A new perspective for the treatment of scorpion poisoning by means of recombinant antibodies, has emerged recently. Two recombinant single chain antibody fragments (scFv) derived from their respective monoclonal antibodies have been expressed in bacteria.[5,6] Antibody 4C1 binds specifically to and neutralizes the most potent neurotoxin (AahII) from the scorpion *Androctonus australis* Hector.[5] Another antibody named 9C2 neutralizes the toxins AahI and AahIII from the same scorpion.[6,7] Both recombinant scFvs showed neutralizing activities similar to those of the original monoclonal antibodies. However, other examples of scFvs derived from their respective monoclonal antibodies have lost their recognition capacity or have shown a decreased affinity.[8–10] The scFv antibody format presents several advantages with respect to the whole antibody, like better diffusion, penetrability and faster elimination. However, their low stability makes them less attractive to be used as therapeutic molecules.[11–13] Directed evolution has been used to improve the affinity and/or the stability of scFvs.[14–16] Error-prone PCR (epPCR), allows us to introduce different rates of mutation in the DNA segments encoding the scFvs by means of the use of Taq polymerase under specific conditions.[17,18] The repertoires generated by epPCR can be displayed on the surface of filamentous phages (phage display) for the subsequent selection (bio-panning) of the clones improved in the desired properties like recognition capacity and/or stability.[19–26]

A new generation of anti-venoms could be based on a set of recombinant antibody fragments, which would be capable of neutralizing the most abundant and toxic components of the venom of a dangerous group of animals.

Here, we report the construction, expression and functional characterization of the scFv from the monoclonal antibody BCF2 (scFv BCF2). Since this construction was not neutralizing, two mutants (G5 and B7) were generated by directed evolution of the DNA segment encoding the scFv BCF2. Both mutants showed an improved stability. They also had an increased affinity to the Cn2 toxin as compared with the parental scFv, but were unable to neutralize the toxin *in vivo*. The combination of both variants resulted in a dimeric scFv (triple mutant) with the adequate affinity and stability to neutralize the Cn2 toxin.

Results

Construction and displaying of scFv BCF2

The gene coding for scFv BCF2 was cloned into the pSyn2 displaying vector and the construction was verified by DNA sequencing. Figure 1 shows the scFv BCF2 amino acid sequence translated from sequenced DNA. The segments involved in the conformation of the three loops corresponding to the complementarity determining regions (CDRs) of the V_H and V_L domains,[27] are indicated. The scFv BCF2 was evaluated by phage-antibody ELISA. It was able to recognize specifically the Cn2 toxin (Figure 2). Other proteins, including close homologous scorpion toxins, such as CII1, CII2, Pg7, Pg8 and toxic fraction II, and unrelated proteins, trypsinogen, casein and BSA were not recognized.

Expression and purification of soluble scFv BCF2

The scFv BCF2 gene sub-cloned into the pSyn1 vector, was expressed by IPTG induction using the TG1 *Escherichia coli* strain. The protein was extracted from the periplasm and purified by ion-metal affinity chromatography (IMAC). The purified fraction containing the antibody fragment was separated into two peaks after flowing through a Superdex 75 column. The faster peak (18 minutes, elution time), corresponded to the dimeric form of the scFv and the second peak (20.5 minutes, elution time), to the monomeric form. The typical yield was

```
                                   CDR-H1
  1 EVQLQQSGPELVKPGASMKISCKV SGYSFTDHTMNWVKQS  40

              CDR-H2
 41 HGKNLELIG LINPFNGDAT YKQKFTGKATLTVDRSSSTAF  80

                        CDR-H3
 81 MELLSLTSEDSAVYYCAR YGNYAMDY WGQGTSVTVSS GGG 120

     Linker
121 GSGGGGSGGGGS DIVLTQSPVSLAVSVGQRATISC KASQS 160

     CDR-L1                    CDR-L2
161 VDFDGESYMN WYQQKPGQPPKLLIY VVSNLES GIPARFSG 200

                              CDR-L3
201 SGSGTDFTLNIHPVEEEDAATYYC QQSNEDPLT FGAGTNL 240

241 ELK 243
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Figure 1. Amino acid sequence of the scFv BCF2. Amino acid residues corresponding to the CDRs[27] are underlined. Linker peptide is shown in bold. The numbers shown correspond to a continuous numbering which includes the linker.

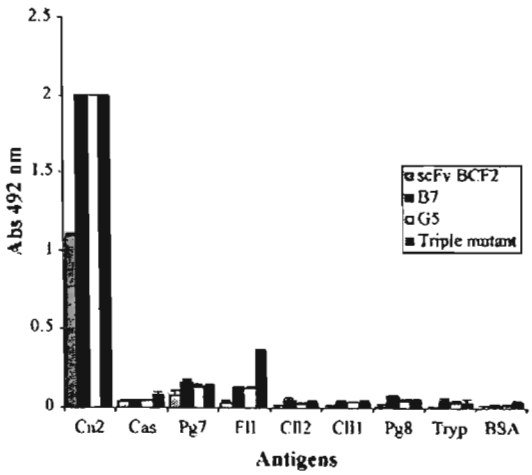

Figure 2. Specificity ELISA of phage-antibodies. Phage titers were normalized to 5×10^{11} phages/ml. Specificity of recognition was confirmed by using different antigens (Cn2, CII1, CII2, Pg7, Pg8, toxic fraction II, trypsinogen, casein and BSA). See Materials and Methods. Data are means of four experiments. Error bars show the standard deviation from the mean.

0.3 mg of soluble total protein per liter of culture. These two peaks represented an estimate of 30% and 70%, respectively, of the recovered protein (data not shown). Monomeric and dimeric fractions under SDS-PAGE conditions showed a M_r of approximately 30 kDa.

Neutralization of toxin Cn2 by scFv BCF2

The capacity of scFv BCF2 to inhibit the toxic effects of Cn2 was tested *in vivo* in CD1 female mice. In these assays the scFv BCF2 (monomeric or dimeric), despite being capable of recognizing specifically the Cn2 toxin in ELISA, was unable to inhibit the deadly effects of Cn2. We only show the data corresponding to the dimeric form (Table 1). We have observed that 20 minutes after the injection of Cn2, the first clinical symptoms of intoxication are present. The protective capacity of scFv BCF2 was practically non-existent as evidenced by the appearance of the poisoning symptoms almost at the same time as they occurred in the control animals (injected with Cn2 only). A subtle delay of five to ten minutes in the appearance of symptomatology was observed, but the mortality rate was consistently higher than in the control group injected with the toxin alone, suggesting that the scFv BCF2 caused a kind of potentiation of the Cn2 toxicity. This phenomenon was already reported by our group when a synthetic vaccine against the venom of *C. noxius* was assayed.[28]

Construction and characterization of the mutant libraries of scFv BCF2

The scFv BCF2 DNA segment was used as template to generate three random libraries: low, medium and high rate of mutation. The sizes were 7.8×10^7, 1.8×10^7 and 1.6×10^8 transformants, respectively. Ten independent plasmid clones from each library were sequenced to determine the diversity and the mutation rate. The experimental values of mutation rate (low 0.1%, medium 0.8% and high rate of mutation 4.7%) were very close to the theoretical reported values.[17,18] The analysis of the DNA sequences allowed us to conclude that the mutations were all different and randomly distributed throughout the frameworks, CDRs and linker.

Biopanning and characterization of the mutants of scFv BCF2

Mutagenic libraries were panned against the Cn2 toxin. From the fourth round of panning, we analyzed 88 colonies from each library by phage-antibody ELISA. About 90% of the positive clones showed higher signals than phage-scFv BCF2. We performed a second ELISA in which we tested the expressed soluble proteins (non-phage associated) from those variants. We found that only eight clones recognized Cn2 with higher signals than scFv BCF2 (Table 2). Two of them were from the low rate of mutagenesis library and six from the medium rate one. The high rate of mutagenesis library did not yield any Cn2 recognizing clone. Those eight clones were sequenced, and the analysis of the DNA sequences revealed that only seven were different. Two mutants named G5 and B7, giving the strongest signals in a quantitative ELISA, were further characterized. Mutant B7 was found twice among the eight clones tested as soluble scFvs (Table 2). The mutant G5 showed two mutations,

Table 1. *In vivo* neutralization of toxin Cn2 by dimeric scFv BCF2, G5, B7 and triple mutant

Test	Cn2 $1LD_{50}$ 0.25 μg of Cn2/20 g mouse	Molar ratio Cn2: scFv	Survival ratio protected/ injected
No antibody	$1LD_{50}$	-	5/10
scFv BCF2	$1LD_{50}$	1 : 10	1/10
G5	$1LD_{50}$	1 : 10	3/10
B7	$1LD_{50}$	1 : 10	2/10
Triple mutant	$1LD_{50}$	1 : 10	10/10

Negative control (no antibody): the mice were injected with the toxin alone.

Table 2. Description of the selected mutants

Clone	Library	Number of changes	Change[a]	Position[b]	Region
G5	L	2	Q27R	159	CDR1 V_L
			V51A	187	CDR2 V_L
E11	L	2	G7R	124	Linker
			Q27R	159	CDR1 V_L
A9	M	3	L82cH	86	Fw3 V_H
			N92K	228	CDR3 V_L
			E93V	229	Fw4 V_L
B3	M	4	F53Y	54	CDR2 V_H
			K60N	61	CDR2 V_H
			S113T	117	Fw4 V_H
			I58V	194	Fw3 V_L
B7	M	1	N35I	35	Fw2 V_H
D1	M	1	N35I	35	Fw2 V_H
D3	M	1	K60N	61	CDR2 V_H
D4	M	1	Y98H	102	CDR3 V_H

Source: L, scFvs isolated from the low mutagenesis library; M, scFvs isolated from the medium mutagenesis library.
The number, the type and the location of the changes are indicated. Two numbering systems were used.
[a] Individual numbering of variable chains according to Chothia.[27]
[b] Continuous numbering which includes the linker. CDR, complementarity determining region; Fw, framework, V_H, heavy chain; V_L, light chain.

one at CDR1 (Q27R) and the other at CDR2 (V51A) of the light chain (Figure 1; Table 2). Mutant B7 showed a single change (N35I) at framework 2 of the heavy chain (Figure 1; Table 2). Phage-antibodies of mutants G5 and B7 were assayed by ELISA to confirm that these variants conserved their specificity to Cn2 toxin (Figure 2). G5 and B7 were expressed as soluble proteins and purified. The Superdex 75 elution profile for G5 revealed the presence of two peaks corresponding to the monomeric and dimeric forms. In these cultures the yield of the scFv of G5 was 0.4 mg/l. In the case of B7, it showed a single peak corresponding to the dimeric form with a yield of 0.8 mg/l of culture. The dimeric form from both mutants were employed in the stability and neutralization assays.

Functional stability in guanidinium hydrochloride

The recognition capacity of the different scFv proteins (all dimeric) in the presence of increasing amounts of guanidinium hydrochloride (Gdn-HCl), as a measure of the functional stability, was evaluated by ELISA. These results revealed that 1 M Gdn-HCl was the critical concentration for functional stability, because the ability of scFv BCF2 to recognize Cn2 decreased significantly. The scFv BCF2 lost approximately 50% of its binding capacity, G5 still showed a significant high signal (lost only 10%) and B7 still had 100% binding (Figure 3). Similar results were obtained when the corresponding phage-antibodies were tested under the same conditions (data not shown).

Neutralization of toxin Cn2 by G5 and B7 mutants

In spite of showing an increased binding activity to Cn2 toxin and also having an improved stability, the mutants G5 and B7 (dimeric forms) were unable to neutralize the toxic effects of Cn2 (Table 1). However, some interesting observations were made: B7 mutant showed similar effects as compared to scFv BCF2 in terms of the time at which the poisoning symptoms appeared. Importantly, the mutant G5 did not show any symptoms up to three to four hours, after the typical envenoming symptoms appeared.

Construction and characterization of the triple mutant

The mutations present in the clones G5 and B7

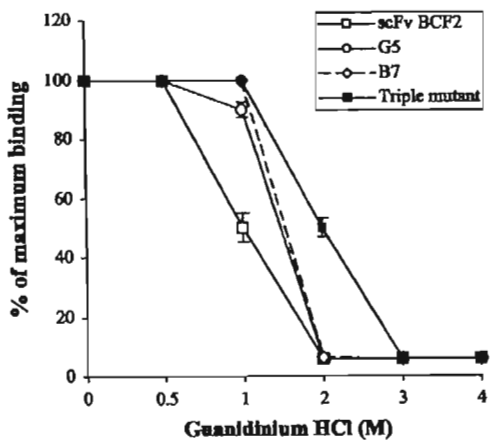

Figure 3. Functional stability determination of dimeric scFv antibody fragments. Cn2 binding by scFv BCF2, G5, B7 and triple mutant in the presence of different concentrations of guanidinium hydrochloride as determined by ELISA. The antibody concentration was normalized to 10 µg/ml. Data are means of four experiments. Error bars show the standard deviation from the mean.

were combined by means of PCR to obtain a variant with a significant improvement in affinity and stability, as a result of the synergistic contribution of both mutants. This combination resulted in a triple mutant whose identity was verified by DNA sequencing (Figure 1). This mutant was purified by gel filtration and its elution profile revealed the presence of a single peak with an elution time corresponding to a dimeric scFv (data not shown). This variant had the best expression level; the yield was 1 mg of soluble protein per liter of culture. The functional stability in Gdn-HCl of this protein was evaluated. It was shown to be significantly more stable than scFv BCF2 (Figure 3). At 2 M Gdn-HCl this variant still showed 50% binding while the other scFvs lost more than 90% of their binding activity.

Surface plasmon resonance (BIAcore) measurements

The affinity constants for all the scFvs were determined using soluble protein from their dimeric forms (Table 3). The K_D values of the scFvs were: scFv BCF2=1.1×10^{-9} M; G5=4.3×10^{-10} M and B7=7.1×10^{-10} M. The affinity constants of G5 and B7 were 2.5-fold and 1.5-fold higher than parental scFv BCF2, respectively. The triple mutant showed the best affinity constant ($K_D=7.5\times10^{-11}$ M). These results correlate with the functional stability assays (Figure 3). This increment in the affinity of the triple mutant, is another aspect that reflects the additive effect of the changes already present in mutants G5 and B7.

Neutralization test of the triple mutant against toxin Cn2

A molar ratio 1 : 10 (toxin:dimeric triple mutant), was used for protection assays *in vivo*. After eight to ten hours post-injection, the mice showed minor symptoms of poisoning but recovered rapidly. All of them survived after 36 hours of observation (Table 1).

Discussion

Characterization of scFv BCF2

In our laboratory, several monoclonal antibodies against Cn2 have been generated.[3] BCF2 was the only antibody that protected against toxin Cn2 and the whole venom of *C. noxius* Hoffmann.[4] This species is one of the most dangerous scorpions in Mexico and probably in the world.

The possibility to generate an antibody fragment like an scFv capable of neutralizing a toxin, is an important alternative in the therapeutic antibody area, because they have a reduced size and cause low immunological reactions in heterologous organisms. This new approach for the treatment of scorpion stings by means of recombinant antibodies has appeared recently. The scFv fragments derived from monoclonal antibodies 4C1 and 9C2 were able to neutralize their respective toxins.[5–7] The scFv of BCF2 generated here, although having a K_D in the nanomolar range (1.1×10^{-9} M; Table 3), did not neutralize toxin Cn2. Possible explanations to this fact can be given by: (a) a fast dissociation (4.5×10^{-4} s^{-1}), which facilitates the detachment of the toxin from the antibody-toxin complex, allowing the toxin to bind to its target; (b) the conformation adopted by V_H and V_L domains, which might not have the same orientation as in the monoclonal antibody, resulting in a decrease of the binding capacity, as it has been reported for other scFvs;[12,13] and (c) the low functional stability of the scFv BCF2, which in the presence of 1 M Gdn-HCl lost 50% of activity (Figure 3).

Characterization of evolved scFv BCF2 variants

The loss of neutralizing activity of the scFv BCF2 prompted the initiative to look for variants improved in their binding capacity and stability, aimed at recovering the neutralization capacity. For this purpose, we decided to generate three error-prone PCR libraries of different rates of mutagenesis (low, medium and high) in order to explore a wide range of combinatorial mutations. After four rounds of panning several phage-antibodies were isolated, which gave stronger signals in ELISA as compared to phage-scFv BCF2. When these phage-antibodies were tested as soluble proteins, only about 10% maintained their binding capacity, indicating that most of them had lost their recognition ability. This phenomenon has been well characterized and it has been explained in terms of the stabilizing effect of pIII on the surface of the phage.[29] Some scFvs, when they are displayed on the surface of filamentous phages recognize efficiently their respective antigens, but when they are expressed as free proteins, lose their recognition capacity due to a decrease in stability, indicating that only those conserving their stability will be functional. When we assayed the selected clones as soluble proteins, we chose two of them with the best affinities: G5 and B7, which maintained their specificity to Cn2 toxin (Figure 2). The variant G5, with one mutation at CDR1 (Q27R) and another at CDR2 (V51A) of the light chain, showed an improvement in affinity (Table 3), and stability (Figure 3). At 1 M Gdn-HCl it showed an activity

Table 3. Affinity and binding kinetics for the dimeric scFv of BCF2 and its mutants

Antibody	k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})	K_D (M)
scFv BCF2	4.0×10^{5}	4.5×10^{-4}	1.1×10^{-9}
G5	6.7×10^{5}	2.9×10^{-4}	4.3×10^{-10}
B7	2.3×10^{5}	1.6×10^{-4}	7.1×10^{-10}
Triple mutant	1.1×10^{6}	8.5×10^{-5}	7.5×10^{-11}

Association (k_{on}) and dissociation (k_{off}) rate constants were determined using surface plasmon resonance (BIAcore) and K_D was calculated as k_{off}/k_{on}.

higher than scFv BCF2 (approximately 40%) (Figure 3), however, it did not neutralize Cn2 toxin (Table 1).

Similar results have been obtained by other members of our laboratory, which indicate that not only a high affinity but also a good stability are necessary to reach the capacity to neutralize a toxin (L.R.-U., V.R.J.-G., T.O.-P., M.O.-L., L.D.P. & B.B., unpublished results). It has been shown that mutations at CDRs generally result in an improvement of the binding capacity due to the direct contact of loop residues with the antigen.[14] Our results are in agreement with these data. The changes at CDRs of G5 improved the affinity of this variant. An improvement of the stability was also observed. This level of improvement was still insufficient to confer to this mutant the capacity to neutralize the toxin. The most important observation was that mutant G5 delayed the onset of poisoning symptoms, indicating that the improvements in affinity and stability were characteristics that favor neutralization.

On the other hand, it has been reported that certain mutations at frameworks can improve the affinity of the antibody for its antigen.[14,16,30] The mutant B7 has a single change (N35I) at framework 2 of heavy chain. When it was purified by gel filtration, a single peak with an elution time corresponding to a dimeric scFv was recovered. An interesting effect of this mutation was an increase in the protein yield (2.6-fold) as compared to scFv BCF2. This mutation produced a dimeric variant that is more stable. It was demonstrated in the functional stability assays that this clone, at 1 M Gdn-HCl, was 50% more stable than scFv BCF2 and 10% more stable than mutant G5 (Figure 3). The increase in stability of mutant B7 can be explained based on its intrinsic dimeric properties. This mutant showed a similar K_D to G5 (Table 3). Noteworthy, this single mutation at the framework 2 of B7, improved both its affinity and stability, although not sufficient to confer a neutralizing capacity.

Combination of improved scFv BCF2 variants (triple mutant)

The properties shown by mutants G5 and B7 in terms of their increase in affinity and stability with respect to the parental scFv BCF2, suggested experiments aimed at combining the mutations carried by these variants in order to obtain an antibody fragment improved in these two parameters and hopefully producing a neutralizing antibody. The triple mutant was constructed and expressed, giving the best yield (1 mg/l of culture). The protein was tested in the neutralization assays and it was capable of neutralizing Cn2 toxin (Table 1). This variant protected 100% of mice. Its affinity constant (K_D), was 7.5×10^{-11} M (Table 3), in which the k_{on} and k_{off} were improved approximately threefold and fivefold, respectively, as compared to scFv BCF2, leading to 15-fold affinity improvement. With respect to the functional stability of the triple mutant (Figure 3), while the other variants showed only marginal values, at 2 M Gdn-HCl, this mutant still retained approximately 50% activity. These results indicate that in this mutant two determining characteristics were combined, which resulted in its neutralizing capacity: a significant improvement in affinity and stability as a result of the contribution of mutations at CDRs of the V_L and the mutation at the framework 2 of the V_H.

Dimeric scFvs with shortened linker, which showed a better affinity and higher stability than the monomeric form, have been reported. This is the case of a scFv specific for AahI scorpion toxin, whose dimeric form had a K_D in the sub-nanomolar range. It was neutralizing and very stable.[31] In another case, the dimeric format was required to neutralize the human cytomegalovirus.[32] We have also observed consistently in our laboratory that dimerization, both as a consequence of shortening the linker joining the variable domains or by directed evolution without shortening the linker, has resulted in an improvement of the stability of the single chain. These results suggest that dimerization was important for functional stabilization.

Putative effects of the mutations on the interaction antibody-toxin

The NMR structure of the Cn2 toxin,[33] a model of the Fv fragment of BCF2 and experimental data generated by epitope mapping,[34] have been used to propose a spatial model of the interaction between BCF2 and Cn2.[35] We made use of this model in order to obtain some insights into the nature of the changes that the mutations herein described could imply at the structural level (Figure 4). The light chain Q27R substitution, places a positively charged amino acid in a region where two acidic amino acids (light chain E93 and Cn2 E28), one belonging to the antibody and the other to the toxin, are expected to be located in close proximity. The R27 could be involved in the formation of new salt bridges with at least one of them, releasing in this way the tension generated by the natural repulsion between the two negatively charged residues. This could directly lead to an increase in the affinity of the interaction. On the other hand, the heavy chain position 35, although not directly located at the V_H–V_L interface, is a residue in close contact with the hydrophobic layer of amino acids that do conform this interface. We postulate that the replacement of a hydrophilic residue at this position (Asn) for a hydrophobic one (Ile) could lead to a change in the geometry and/or stability of the intramolecular V_H–V_L interaction, favoring the formation of the dimeric scFv, and consequently affecting in a positive way the recognition properties of the whole antibody fragment. We could not come to a plausible explanation for a putative effect of the V_L V51A mutation on the BCF2–Cn2 interaction in light of the structural model. There seems to be no

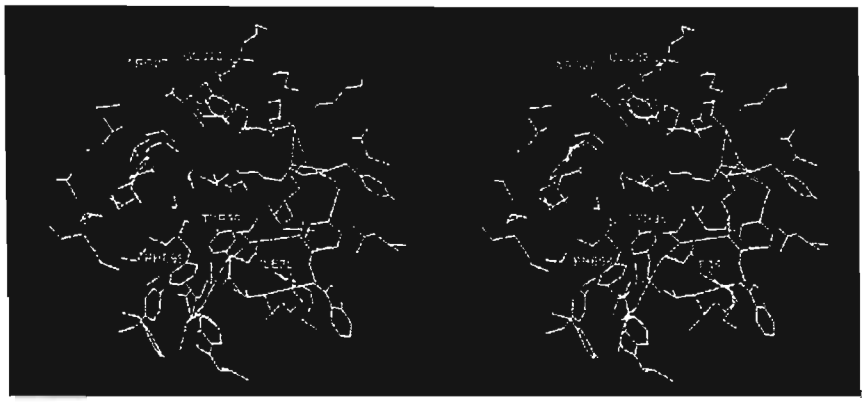

Figure 4. Stereo view of a segment of the BCF2–Cn2 complex model. Restricted part of the C^{α} trace and side-chains of the complex with the Q27R (V_L) and N35I (V_H) mutations included. The mutant amino acid residues are shown in magenta. The remaining side-chains are represented in RGB colors. The BCF2 light and heavy chain traces are shown in blue and yellow, respectively. The Cn2 toxin trace is displayed in green. Image created using the software package Swiss PDB Viewer (http://www.expasy.org/spdbv/).

significant change in the structure due to the replacement of Val by the similar Ala. However, we cannot rule out the possibility that this mutation could have caused a local change undetectable at the model level but contributing with the Q27R mutation to increase the affinity and/or stability of G5 mutant.

In conclusion, we constructed the single chain Fv (scFv) of the monoclonal antibody BCF2 raised against toxin Cn2 from the venom of the Mexican scorpion *Centruroides noxius* Hoffmann, and realized that this single chain was unable to neutralize Cn2 toxin. Two mutants (G5 and B7) were isolated from three different libraries with a wide range of variability generated by random mutagenesis. None of these mutants was able to neutralize the Cn2 toxin. The combination of the mutations from G5 and B7 resulted in a triple mutant, which was capable of neutralizing Cn2 toxin *in vivo*. The analysis of the regions in which the mutations occurred, based on the reported model for the structure of the complex antibody-toxin, provided a working hypothesis to explain the effect of these mutations on the stability and affinity of the triple mutant.

Using the approaches here described, low affinity and low stability antibody fragments (scFvs), can be modified in order to improve such properties. There seems to be a relationship between the improvement in those parameters (stability and affinity) and the protein yield.

A new generation of anti-venoms containing a limited number of highly specific antibody fragments capable of neutralizing the most abundant and toxic components of a venom can be obtained by means of the procedures reported here.

Materials and Methods

Toxins

All the scorpion toxins used here are specific for sodium channels and were purified and characterized in our laboratory. Toxin Cn2 was isolated from the venom of *C. noxius* Hoffmann.[3] Pg7 and Pg8, were isolated from *Parabutus granulatus* (unpublished data). Cll1[36] and Cll2, were isolated from *Centruroides limpidus limpidus*.[37]

Toxic fraction II was isolated from the venom of the scorpion *Centruroides limpidus limpidus*. This fraction was obtained by molecular weight sieving in Sephadex G-50 column chromatography, and contains a mixture of all Na^+ channel specific toxins.[36]

Enzymes

NotI, SfiI, BstNI, Taq polymerase, high-fidelity Vent polymerase and T4 DNA ligase, were purchased from New England Biolabs (Berverly, MA, USA).

Escherichia coli strains

TG1: K12 Δ(lac-pro), supE, thi, hsdD5/F′traD36, $proA^+B^+$, $lacI^q$, lacZΔM15.

XL1-Blue: F′::Tn*10* $proA^+B^+$, $lacI^q$, Δ(lacZ)M15/ recA1, endA1, gyrA96 (Nal^r), thi, hsdR17 ($r_k^- m_k^+$) glnV44 relA1, lac.

Construction of scFv BCF2

The segments of DNA used for the assembly of the scFv BCF2 were amplified from the pMrec-chFab-BCF2 phagemid[38] by means of a three-step PCR using high-fidelity Vent polymerase. In the first step, the variable domains were amplified. For the heavy chain, two primers were used: V_HBCF2 5′, a forward oligonucleotide (5′ GAG GTT CAG CTG CAA CAG TCT 3′) and V_HBCF2 3′, a reverse oligonucleotide (5′ TGA GGA GAC GGT GAC TGA GGT 3′). In the case of the light chain, the combination of oligonucleotides was V_κBCF2 5′, forward (5′ GAC ATT GTG TTG ACC CAA TCT 3′) and V_κBCF2 3′, reverse (5′ TTT CAG CTC CAG GTT GGT CCC 3′). In the second PCR step, overlapping sequences corresponding to the scFv linker $((Gly_4Ser)_3)$ were introduced into each variable domain encoding fragment. The primers used were V_HBCF2 5′ and linkV_H (5′ AGA GCC ACC TCC GCC TGA ACC GCC TCC ACC TGA GGA GAC GGT

GAC 3′) for heavy chain, and linkV_κ (5′ GGC GGA GGT GGC TCT GGC GGT GGC GGA TCG GAC ATT GTG TTG ACC 3′) plus V_κBCF2 3′ for light chain. The conditions used for the first and second steps of PCR were: one hold at 94° C for three minutes, 30 cycles at 94 °C for one minute, 55 °C for one minute and 72 °C for one minute, with a final extension hold at 72 °C for ten minutes. In the third step, a combination of overlapping PCR and amplification of the overlapped product took place; $V_H$forSfiI (5′ GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC GAG GTT CAG CTG CAA CAG TCT 3′) and $V_k$revNotI (5′GAG TCA TTC TCG ACT TGC GGC CGC TTT CAG CTC CAG GTT GGT CCC AGC ACC 3′), were the primers used for the amplification of an approximately 800 bp DNA segment encoding the scFv BCF2. These primers include restriction sites for SfiI and NotI. For the overlapping reaction, the conditions were: eight cycles at 94 °C for 1.30 minutes, 60 °C for 1.30 minutes and 72 °C for 1.30 minutes, in the absence of primers and for the amplification, 30 cycles at 94 °C for one minute, 60 °C for one minute and 72 °C for one minute, with a final extension hold at 72 °C for ten minutes. A GeneAmp PCR thermo-cycler (PERKIN ELMER 2400, Norwalk, CT, USA), was used for all PCR reactions. PCR products were run in 1% (w/v) agarose gel electrophoresis and purified using a QIAquick gel extraction kit (QIAGEN GmbH, Hilden, Germany). The scFv BCF2 DNA was digested sequentially with SfiI and NotI, run in 1% agarose gel, purified as described and cloned into pSyn2 vector (SfiI/NotI digested), in order to display the scFv on the surface of a filamentous phage.

DNA sequencing

The identity of the scFv BCF2 and its variants was verified by DNA sequencing using the forward primer pSynDir (5′AAA TAC CTA TTG CCT ACG GCA 3′). As reverse primers we used RevpSyn2 (5′ACT TTC AAC AGT CTA TGC GGC 3′) for pSyn2 and RevpSyn1 (5′ ATG GTG ATG ATG GTG ATG TGC GGC CCC 3′) for pSyn1 in an Applied Biosystems 3100 Gene Analyzer (Foster City, CA, USA).

Expression and purification of soluble scFv BCF2 and its mutants

The scFv BCF2 DNA segment was subcloned into the pSyn1 which contains the same restriction sites as pSyn2. The construction was electroporated into *E. coli* TG1 cells. The cells were grown in 500 ml YT2X medium supplemented with 0.1% (w/v) glucose plus ampicillin (200 μg/ml) at 37 °C. When the culture reached an $A_{600\ nm}=0.7$, the scFv expression was induced with 1 mM IPTG at 30 °C for six hours. The culture was centrifuged at 4500 rpm (in a Beckman J2-21 centrifuge and the JA-20 rotor) at 4 °C for 15 minutes and the pellet resuspended in 12.5 ml of PPB extraction buffer (30 mM Tris–HCl (pH 8.0), containing 1 mM EDTA and 200 mg/l sucrose). The suspension was incubated on ice for 20 minutes and centrifuged at 5000 rpm for 15 minutes. Supernatant was collected and the pellet resuspended in 12.5 ml of 5 mM $MgSO_4$. The resupended pellet was incubated on ice for 20 minutes and centrifuged at 15,000 rpm (in a Beckman J2-21 centrifuge and the JA-20 rotor) for 15 minutes and the supernatant collected. Both supernatants (periplasmic extracts) were mixed and dialyzed against PBS1X at 4 °C overnight (O/N). After dialysis, the periplasmic extracts were purified by Ni^{2+}-NTA affinity chromatography (QIAGEN GmbH, Hilden, Germany). Samples were eluted with 1 ml of 250 mM imidazole/PBS1X. The scFvs were purified by gel filtration chromatography on a Superdex®75 column (10 mm×300 mm; Pharmacia Biotech AB, Uppsala, Sweden). Eluted fractions containing the dimeric scFv were used for analysis in ELISA tests and for toxin neutralization assays.

In vivo neutralization assays

CD1 female mice were used for the *in vivo* neutralization assays. The toxin dose was normalized for mouse weight to correspond to 1 LD_{50} (0.25 μg/20 g of mouse weight). One LD_{50} was incubated with 16 μg of scFv corresponding to a molar ratio of 1 : 10 (toxin:scFv dimeric). The antibody-toxin mixture was incubated for 30 minutes at room temperature and injected intraperitoneally into mice. As control, the toxin alone was used. The animals were observed for up to 36 hours after injection. Ten animals were used for each test condition and the survival ratio recorded. These conditions were considered sufficient for validation of the experimental results and were approved by the Committee for Animal Welfare of our Institute.

Mutant libraries

The scFv BCF2 DNA was used as template to generate three libraries with different rates of mutation (high, medium and low) by means of error prone-PCR. A single round of error prone-PCR, in the conditions required to obtain the different rates of mutation, was carried out by the methods described (medium rate[17] and high rate[18]). For low rate of mutagenesis, we used conventional PCR conditions but increasing the concentration of $MgCl_2$ to 70 mM. The primers used to amplify the mutant libraries were $V_H$forSfiI and $V_k$revNotI. One hold at 95 °C for five minutes, 30 cycles at 95 °C for one minute, 60 °C for one minute and 72 °C for 10 minute, with a final extension hold at 72 °C for 10 minutes were the conditions used. Eight μg of the mutagenized scFv DNA from each library and 8 μg of pSyn2 phagemid, both previously digested with SfiI and NotI enzymes, were purified as already described and ligated at a 1 : 5 molar ratio (vector:insert) at 16 °C for 18 hours in a final volume of 200 μl. The ligated products were electrotransformed into competent *E. coli* XL1-Blue cells. The size of the libraries was determined by plating serial dilutions of the transformed cells on YT2X plates supplemented with 2% glucose, ampicillin (200 μg/ml) and tetracycline (30 μg/ml). The phagemid DNA from ten isolated colonies was purified and sequenced to determine the diversity and the mutagenesis rate in each library.

Biopanning

Low, medium and high rate of mutagenesis libraries were separately panned against the toxin Cn2 of *Centruroides noxius*. Two hundred μl from the respective library were inoculated into 15 ml of YT2X supplemented with 2% glucose plus 200 μg/ml ampicillin and grown at 37 °C with shaking (250 rpm). When the absorbance at 600 nm reached 0.7, 20 μl of helper phage VCSM13 (7×10^{12} phages/ml) were added. The growing of the culture and harvesting of phages was performed as described.[21] Four rounds of panning were performed, using decreasing concentrations of the Cn2 toxin in order to enrich phage-antibodies with increased affinities for this toxin. MaxiSorp inmunotubes (Nunc-Immuno™

tubes Brand products, Roskilde, Denmark) were coated overnight with 50 µg/ml, 10 µg/ml, 5 µg/ml or 1 µg/ml of Cn2 in 50 mM sodium bicarbonate buffer (pH 9.4) at 4 °C. The bio-panning was performed as described.[21] After washing with PBS1X Tween-20, bound phages were detached by adding 1 ml of XL1-Blue cells ($A_{600\ nm}=0.7$), incubating at 37 °C for 30 minutes without shaking and 30 minutes with shaking as described.[39,40] The cells were then plated on two YT2X-agar plates supplemented as described and incubated at 37 °C O/N. Selected phagemid DNA molecules were once again rescued as infecting particles from the scraped cells with helper phage as described above. The phages were used to initiate a new round of panning. Resulting colonies from the fourth round were collected and characterized.

Phage-antibody ELISA

A total of 88 isolated colonies from the fourth round of panning of each library were grown in 100 µl of YT2X supplemented with 2% glucose plus ampicillin (200 µg/ml) in the wells of a cell culture plate with flat bottom (Costar, New York, USA). Culture plates were incubated at 37 °C with shaking at 250 rpm O/N (Master plate). Replica plates (round bottom) were prepared inoculating 2 µl of the previous culture in 125 µl/well of YT2X supplemented as described. Replica plates were incubated at 37 °C with shaking at 250 rpm for three hours. Aliquots of 25 µl of 1×10^9 helper phages/ml were added to each well. The plates were incubated at 37 °C for 30 minutes without shaking and 30 minutes with shaking at 250 rpm. The culture plates were centrifuged at 3500 rpm, 4 °C, ten minutes and the supernatant was remove and the cells resuspended in 125 µl/well of YT2X supplemented with 200 µg/ml ampicillin and 30 µg/ml kanamycin. A final incubation O/N at 30 °C with agitation was done. The culture plates were centrifuged at 3500 rpm, 4 °C, ten minutes and the supernatant collected for ELISA.

Duplicate ELISA plates were coated with 100 µl/well of Cn2 toxin at a concentration of 3 µg/ml in 50 mM sodium bicarbonate buffer (pH 9.4) and incubated at 4 °C O/N. Plates were washed thrice with PBS1X/0.1% Tween-20 and saturated with 0.5% (w/v) BSA (200 µl/well) for two hours at 37 °C. Plates were washed thrice with PBS1X/0.1% Tween-20. Fifty µl of phage supernatant were mixed with 50 µl of PBS1X and added to each well of the previously saturated ELISA plate and incubated at 37 °C for one hour. Plates were washed thrice with PBS1X/0.1% Tween-20. The presence of bound specific phage-antibodies was revealed by adding 100 µl of a 1 : 2000 dilution (PBS1X) of horseradish peroxidase (HRP)-conjugated anti-M13 antibody (Amersham Pharmacia Biotech, Buckinghamshire, UK). Plates were incubated in the dark one hour at 37 °C and washed thrice with PBS1X/0.1% Tween-20. HRP activity was detected by adding 100 µl/well of 0.1 M sodium phosphate buffer (pH 5), containing *O*-phenylenediamine and H_2O_2 as substrates. After ten minutes, the reaction was stopped by adding 100 µl/well of 6 M HCl and the absorbances read at 492 nm in a BIO-RAD ELISA autoreader, model 2550 EIA Reader (BIO-RAD, Richmond, CA, USA). Positive clones were named according to the position of their respective well in the master plate.

ELISA of soluble single-chain antibodies

Procedures were similar to phage ELISA except that the expression of soluble scFvs was initiated by growing of the colonies from master plate in YT2X/0.1% glucose supplemented with 200 µg/ml ampicillin, until the $A_{600\ nm}$ was 0.7. Plates were incubated for six hours at 37 °C with shaking at 250 rpm. Culture plates were induced by adding 25 µl/well of YT2X medium with IPTG (USB, Cleveland, OH, USA) and ampicillin to final concentrations of 1 mM and 200 µg/ml, respectively. Plates were incubated at 30 °C O/N and centrifuged at 3500 rpm for ten minutes at 4 °C. Fifty µl of these supernatants were added to their respective wells of an ELISA plate previously coated with 3 µg/ml of Cn2 toxin in 50 mM sodium bicarbonate buffer (pH 9.4). ELISA plates were incubated at 37 °C for one hour and washed. Aliquots of 100 µl/well of a 1 : 2000 dilution (PBS1X) of mouse anti-c-myc antibody (ZYMED, San Francisco, CA, USA) were added. Plates were incubated one hour at 37 °C and washed. Aliquots of 100 µl/well of a 1 : 2000 dilution (PBS1X) of goat anti-mouse-HRP antibody (ZYMED, San Francisco, CA, USA) were added. Plates were incubated one hour at 37 °C in the dark and washed. HRP activity detection and plate reading were done as already described.

Combination of mutations from G5 and B7

The mutations in G5 and B7, isolated from the panning procedures, were combined by means of PCR. In the first PCR the oligos $V_H$forSfiI and Iso35rev (5′ CTT CAC CCA GAT CAT GGT GTG 3′) were used in an equimolar ratio in order to amplify the segment containing a mutation at position 35 (N35I). The B7 DNA was used as template. The product of this reaction, whose size was 147 bp, was purified and used as a mega-primer in a second step of PCR. This second step was carried out using G5 DNA as template. The mega-primer generated in the first round of PCR and oligo $V_k$revNotI were used as primers. One hold at 94 °C for three minutes, 30 cycles at 94 °C for one minute, 60 °C for one minute and 72 °C for one minute, with a final extension hold at 72 °C for ten minutes were the conditions used in both steps. The PCR product was extracted, purified from an agarose gel and digested sequentially with SfiI and NotI. One hundred ng of the 800 bp fragment were ligated with 100 ng of pSyn2 and pSyn1 vectors. The constructions were transformed into *E. coli* TG1. The combination of mutations was verified by DNA sequencing.

Specificity test of phage-antibodies

High binding ELISA plates (polyestyrene; COSTAR 3366, New York 14831, NY, USA) were coated (100 µl/well) with different antigens at the indicated concentration, in 50 mM sodium bicarbonate buffer (pH 9.4). The toxins used as antigens were all specific for sodium channels, approximately 7 kDa M_r, and their concentration was 1 µg/ml (Cn2, Cll1, Cll2, Pg7 and Pg8). The proteins used at 20 µg/ml were: casein, BSA and trypsin. Toxic fraction II of scorpion *C. limpidus limpidus* at 20 µg/ml, was also employed. The phage-antibodies were added at a concentration of 5×10^{11}/ml in PBS 1X (100 µl/well). ELISA plates were processed as described.

Functional stability assays

High binding ELISA plates were coated as described with Cn2 toxin. After washing and blockade with BSA, 100 µl of 10 µg/ml of soluble scFvs were added in the presence of different concentrations (0.5–4 M) of guanidinium hydrochloride in PBSIX (100 µl/well) and

incubated one hour at 37 °C.[41] The remaining of the ELISA process was performed as described.

Surface plasmon resonance measurements

All the experiments were performed at 25 °C using a BIAcoreX instrument (Amersham Pharmacia Biotech). Toxin Cn2 was covalently immobilized onto a CM5 sensor chip (BIAcore AB) using the amino coupling procedure, at a concentration of 15 μg/ml in 10 mM Mes buffer (pH 6) as recommended by manufacturers. Approximately 480 resonance units (RU) of protein were immobilized. To determine the kinetic constants, different concentrations of dimeric antibody fragments were injected, flowing at a rate of 30 μl/minute in PBS1X (pH 7.4) containing 0.005% P20 (polyoxyethylenesorbitan). The injection times were: for association, 120 seconds and for dissociation, 600 seconds. For the triple mutant, the injection times for association and dissociation were 180 seconds and 1200 seconds, respectively. The surfaces were regenerated by injecting 10 μl of a 10–15 mM NaOH solution, flowing at a rate of 5 μl/minute. Binding data were fitted to 1 : 1 Langmuir binding model of BIA evaluation software ver 3.2 (BIAcore AB), using rate equations with the term for drifting base line. For the triple mutant, a clear mass transfer phenomenon was observed. In order to obtain more realistic data for the determination of the K_D of this mutant, the Langmuir binding model with mass transfer limitation was applied.

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