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Respuesta Requerimiento Art. 45
EXP: 15-80428



Señor
José Luis Salazar López
Director de Nuevas Creaciones
Superintendencia de Industria y Comercio
Dirección de Nuevas Creaciones
E. S. D.

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



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Act: 523 RESPUESTA Folios: 25

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Asunto	:	No. de Radicación	15-080428
Trámite	:	Patente de Invención	011
Evento	:	Registro	379
Actuación	:	Respuesta a	519
		Requerimiento	
		Artículo 45.	
Oficio	:		15665
Folios	:		15
Anexos	:		2

Juan Carlos Cuesta Quintero, mayor de edad, identificado como aparece al pie de mi firma, obrando como apoderado de **The Trustees of The University of Pennsylvania**, para la creación denominada "*Uso de las Células T Modificadas por Receptor de Antígeno Quimérico para Tratamiento de Cáncer*", por medio de este documento doy **respuesta** en tiempo oportuno al Oficio No. 15665, notificado por fijación en lista de fecha 29 de Diciembre de 2015, de conformidad con lo establecido en el Artículo 45 de la Decisión 486 de la Comunidad Andina de Naciones, en los siguientes términos.

Modificación a las reivindicaciones

En primer lugar se solicita al Examinador tener en consideración el nuevo capítulo reivindicatorio compuesto por 4 reivindicaciones, el cual se modificó de la siguiente manera:

1. Se han incluido las diferentes secuencias de ácidos nucleicos que codifican para cada una de las secuencias del receptor de antígeno quimérico en la reivindicación 1 y adicionalmente se han incluido las características funcionales que las relacionan para hacer más clara la materia reivindicada.



2. De igual manera se ha procedido en las reivindicaciones 2, 3 y 4 incluyendo las secuencias propias de CAR o aquellas que las codifican y su funcionalidad para que sea más clara su interrelación.

Presentación de solicitudes Fraccionarias

El Examinador menciona: *“... la solicitud fraccionaria no está bien presentada, porque reivindica la secuencia de ácido nucleico de SEQ ID NO: 8, el CAR aislado que comprende la secuencia de aminoácidos de SEQ ID NO: 12 y la célula ex vivo que comprende una secuencia de ácido nucleico aislada de SEQ ID NO: 8, que son materia que ya estaba reivindicada en la solicitud parental ”*

Frente a lo anterior se desea poner de presente lo que indica el Artículo 36 de la Decisión 486 de 2000, sobre solicitudes fraccionarias:

Artículo 36.- El solicitante podrá, en cualquier momento del trámite, dividir su solicitud en dos o más fraccionarias, pero ninguna de éstas podrá implicar una ampliación de la protección que corresponda a la divulgación contenida en la solicitud inicial.

En este caso, es deseo del solicitante argumentar que la solicitud divisional no amplía de ninguna forma la materia de la solicitud inicial, y que si bien, existe materia que se superpone, esta materia comprende modalidades particulares de la invención que no incurren en violación alguna a la norma al comprender una materia menor a la reclamada inicialmente.

Sin embargo, se ha tenido en cuenta esta observación y se ha modificado el capítulo reivindicatorio para que se observe más claramente la materia reivindicada.

Excepciones a la Patentabilidad

El Oficio 15665 menciona que *“El objeto de la reivindicación 12 no es patentable de acuerdo con el Artículo 20 lit. c) de la Decisión 486, puesto que hace referencia a células ex vivo que no se encuentran definidas en términos de una línea celular.”*

Frente a esta objeción me permito señalar de forma textual lo que cita la Decisión 486 en el Artículo 20, literal c:



Artículo 20.- No serán patentables:

“...c) las plantas, los animales y los procedimientos esencialmente biológicos para la producción de plantas o animales que no sean procedimientos no biológicos o microbiológicos;”

De acuerdo a lo anterior, el literal indica claramente que no serán patentable 3 items (2 productos y 1 procedimiento):

1. las plantas,
2. los animales,
3. los procedimientos esencialmente biológicos para la producción de plantas o animales que no sean procedimientos no biológicos o microbiológicos

En este caso, el solicitante no comprende porque se cita esta “no patentabilidad” dado que no es su pretensión reivindicar, una planta, un animal, o un procedimiento para producir una planta o un animal. Adicionalmente la legislación vigente no obliga la definición de este tipo de células mediante los términos de una línea celular, como lo menciona el Oficio 15665.

Es evidente que el Sr. Examinador no ha comprendido la esencia de lo reivindicado ya que en ningún momento de la descripción o las reivindicaciones se ha indicado la necesidad de obtener una planta o un animal, sino que la invención se refiere a una célula (capaz de replicarse) o un conjunto de células, que guardan una información genética en ellas, que corresponde a un Receptor de Antígeno Quimérico (en adelante CAR), las cuales son útiles para su posterior aplicación en tratamiento o diagnóstico de enfermedades como la leucemia linfocítica crónica.

Si bien, es importante mencionar que las células pueden provenir de un humano, la presente invención no se refiere a la producción de un humano o animal, o planta, sino para el tratamiento de un paciente mediante la identificación de las Células T por medio del CAR desarrollado, lo cual es una innovación mayor en el campo de la detección y prevención de este tipo de enfermedades.

Sin embargo, entendiendo que el tema debe ser comprendido por el técnico medio con conocimientos en biología molecular, se ha redactado nuevamente la reivindicación objetada que corresponde con la nueva reivindicación 3 que menciona una población de



Células T expandidas ex vivo y modificadas genéticamente para tener una secuencia aislada de un ácido nucleico con las secuencias reivindicadas previamente.¹

Mi representada desea explicar al Examinador que la característica novedosa de las células reclamadas es lo que es insertado dentro de ellas, que es, el ácido nucleico presentado en la parte caracterizante de la nueva reivindicación, NO el método de expansión NI el método de mutación de las mismas.

De acuerdo a lo anterior se entiende por superada la objeción por cuanto a "no patentabilidad" se refiere y se ruega analizar el nuevo capítulo reivindicatorio modificado.

1. DE LA SOLICITUD DE PATENTE

El Oficio menciona que capítulo reivindicatorio carece de claridad y concisión:

- *"Las reivindicaciones 1 a 6 no son claras porque reclaman secuencias que corresponden a elementos aislados del receptor CAR de la invención, se sugiere a la solicitante caracterizar en una sola reivindicación el receptor CAR incluyendo todos los elementos que comprende, es decir, un dominio de unión con el antígeno, un dominio transmembrana CD8alfa, una región de señalización co-estimuladora y un dominio de señalización CD3zeta."*
- *"Las reivindicaciones 10 y 11 no son claras porque reclaman el receptor CAR por referencia a las secuencias de ácidos nucleicos de SEQ ID No: 22 y 23. Se recuerda a la solicitante que una proteína no puede definirse por la molécula de ácido nucleico que la codifica y que debe indicarse claramente la secuencia de aminoácidos del receptor completo, no únicamente de algunos dominios o elementos del mismo."*

Las nuevas reivindicaciones han sido modificadas para atender las faltas mencionadas.

De acuerdo a lo acá presentado, se considera que las reivindicaciones modificadas superan las objeciones por claridad elevadas en el Oficio y se entienden por superadas.

¹ El sustento para esta modificación puede encontrarse en la publicación PCT:

- Páginas 44 a 48, donde se describen métodos de expansión de células T.
- Página 47, línea 29, hasta la página 48, se describe un método para expandir las células.
- Página 52, línea 28, hasta la página 53, se describen los métodos de expansión de las células T.
- Página 58, línea 32, hasta la página 59, línea 2 (ejemplos).
- Página 34, líneas 19 a 25 y en la página 82, se describe la modificación genética de las células T.

2. EXAMEN DE PATENTABILIDAD

El señor Examinador concluye que la materia reclamada carece de nivel inventivo (Art18 D486) en vista de los documentos

D1 – Kochenderfer et al., (2009) I. Immunother. 32(7):689-702

D2 – Kochenderfer et al., (2010) Blood 116(20): 4099-4102

D3 – WO2002/2077029 Jensen, City of Hope

D4 – WO2010/025177 Jensen, City of Hope

Es deseo de mi representada mencionar que ninguna de las referencias citadas revelan información que haga a las presentes reivindicaciones carentes de novedad o nivel inventivo.

Según el Oficio, es evidente que el Sr. Examinador considera que todos los CAR son idénticos entre ellos, sin importar si contienen o no los mismos constituyentes, lo cual no es cierto.

En este caso, los CAR reclamados comprenden:

- un anticuerpo CD19 scFv,
- un dominio bisagra CD8,
- un dominio transmembrana,
- un dominio 4-1BB co-estimulador; y
- un dominio señalizador CD3zeta.

Como se puede observar en las reivindicaciones, el solicitante revela las secuencias de amino ácidos que caracterizan a cada uno de los componentes del CAR tal como se ha sugerido siempre en la oficina de patentes colombiana.

Combinación de las enseñanzas de D1 y D2

Se observa que D1 revela dos (2) CAR diferentes: FMC63-28Z y FMC63-CD828BBZ.

FMC63-28Z comprende un anticuerpo anti-CD19 scFv que no es codificado por la secuencia SEQ ID No. 14 del solicitante. Tal secuencia del solicitante codifica una secuencia amino ácida de unión diferente entre las cadenas VL y VH del anticuerpo anti-CD19 scFv del solicitante.

Así las cosas, la secuencia aminoacídica de unión del solicitante es (G₄S)₃ (ver fragmento demarcado de la SEQ ID NO: 20 a continuación, la cual es codificada por la SEQ ID NO: 14 de la solicitud) mientras que D1 y D2 revelan una secuencia de unión GSTSGSGKPGSGEGSTKG que de ninguna forma puede ser igual o similar a la secuencia de unión señalada.

<210>	20														
<211>	242														
<212>	PRT														
<213>	Artificial Sequence														
<220>															
<223>	Chemically Synthesized														
<400>	20														
Asp	Ile	Gln	Met	Thr	Gln	Thr	Thr	Ser	Ser	Leu	Ser	Ala	Ser	Leu	Gly
1			5					10						15	
Asp	Arg	Val	Thr	Ile	Ser	Cys	Arg	Ala	Ser	Gln	Asp	Ile	Ser	Lys	Tyr
		20					25					30			
Leu	Asn	Trp	Tyr	Gln	Gln	Lys	Pro	Asp	Gly	Thr	Val	Lys	Leu	Leu	Ile
	35					40					45				
Tyr	His	Thr	Ser	Arg	Leu	His	Ser	Gly	Val	Pro	Ser	Arg	Phe	Ser	Gly
	50					55				60					
Ser	Gly	Ser	Gly	Thr	Asp	Tyr	Ser	Leu	Thr	Ile	Ser	Asn	Leu	Glu	Gln
	65				70					75				80	
Glu	Asp	Ile	Ala	Thr	Tyr	Phe	Cys	Gln	Gln	Gly	Asn	Thr	Leu	Pro	Tyr
		85						90						95	
Thr	Phe	Gly	Gly	Gly	Thr	Lys	Leu	Glu	Ile	Thr	Gly	Gly	Gly	Gly	Ser
	100						105							110	
Gly	Gly	Gly	Gly	Ser	Gly	Gly	Gly	Gly	Ser	Glu	Val	Lys	Leu	Gln	Glu
	115					120							125		
Ser	Gly	Pro	Gly	Leu	Val	Ala	Pro	Ser	Gln	Ser	Leu	Ser	Val	Thr	Cys
	130					135					140				
Thr	Val	Ser	Gly	Val	Ser	Leu	Pro	Asp	Tyr	Gly	Val	Ser	Trp	Ile	Arg
	145				150					155					160
Gln	Pro	Pro	Arg	Lys	Gly	Leu	Glu	Trp	Leu	Gly	Val	Ile	Trp	Gly	Ser
			165					170						175	
Glu	Thr	Thr	Tyr	Tyr	Asn	Ser	Ala	Leu	Lys	Ser	Arg	Leu	Thr	Ile	Ile
	180							185						190	
Lys	Asp	Asn	Ser	Lys	Ser	Gln	Val	Phe	Leu	Lys	Met	Asn	Ser	Leu	Gln
	195					200					205				
Thr	Asp	Asp	Thr	Ala	Ile	Tyr	Tyr	Cys	Ala	Lys	His	Tyr	Tyr	Tyr	Gly
	210					215					220				
Gly	Ser	Tyr	Ala	Met	Asp	Tyr	Trp	Gly	Gln	Gly	Thr	Ser	Val	Thr	Val
	225				230					235					240
Ser	Ser														

Adicional a lo anterior, la secuencia VL del solicitante es 16 aminoácidos más corta que aquella revelada en D1, por lo tanto, la secuencia VL del solicitante no solo es diferente en su tamaño sino en su estructura conformacional a la revelada por D1.

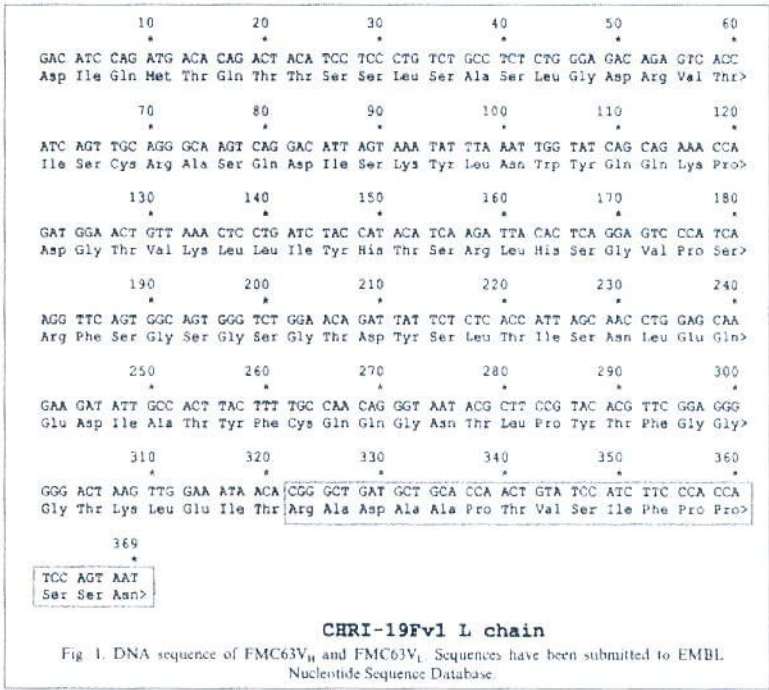
Lo anterior, se observa claramente al estudiar la secuencia de aminoácidos del anticuerpo anti-CD19 scFv de FMC63-28Z que es revelada en la referencia # 39 de D1²:

² "39. Nicholson IC, Lenton KA, Little DJ, et al. Construction and characterisation of a functional CD19 specific single chain Fv fragment for immunotherapy of B lineage leukaemia and lymphoma. Molecular Immunology. 1997;34:1157-1165."

Construction of the MSGV-FMC63-28Z and MSGV-FMC63-CD828BBZ
Recombinant Retroviral Vectors

The MSGV-FMC63-28Z recombinant retroviral vector encodes the MSGV (mouse stem cell virus-based splice-gag vector) retroviral backbone and the FMC63-28Z CAR. *The FMC63-28Z CAR consists of an anti-CD19 scFv that was derived from the FMC63 mouse hybridoma*³⁹, a portion of the human CD28 molecule, and the intracellular component of the human TCR- ζ molecule. The exact sequence of the CD28 molecule included in the FMC63-28Z CAR corresponds to Genbank identifier [NM_006139](#). The sequence includes all amino acids starting with the amino acid sequence IEVMYPPY and continuing all the way to the carboxy-terminus of the protein. To encode the anti-CD19 scFv component of the vector, we designed a DNA sequence which was based on a portion of a previously published CAR⁴⁰. This sequence encodes the following components in frame from the 5' end to the 3' end: an XhoI site, the human granulocyte-macrophage colony-stimulating factor (GM-CSF) receptor α -chain signal sequence, the FMC63 light chain variable region³⁹, a linker peptide⁴⁰, the FMC63 heavy chain variable region³⁹, and a NotI site ... (Párrafo 1, D1, página 3).

Al verificar tal secuencia VL de Nicholson, se encuentra que tiene 16 aminoácidos adicionales: RADAAPTVSIFPPSSN. Ver figura 1:



Por lo tanto, el solicitante llama la atención de ese Despacho en cuanto a que el alineamiento de la: **“Figura 3. Alineamiento BLAST entre la secuencia SEQ ID NO: 20 (WO_2012_079000_20) de la reivindicación 7 de la presente solicitud (Subject) y la SEQ ID NO: 2 (WO_2002_07729_2) (Query) divulgada en el documento D3”**, del Oficio 15665 es incorrecta. Como se aprecia en el alineamiento (figura 3, acción oficial) me permito demarcar con una línea el sitio donde este fragmento de la secuencia se encuentra ausente:

WO_2012_079000_20
Sequence ID: lc|Query_80837 Length: 242 Number of Matches: 1

Range 1: 1 to 242 [Graphics](#)

Score	Expect	Method	Identities	Positives	Gaps
454 bits(1168)	2e-161	Compositional matrix adjust.	230/245(94%)	230/245(93%)	3/245(1%)
Query 23	DIQHTQTSSLSASLGDRVTISCRASQDISKYLNNYQQKPDGTVKLLIYHTSRHSGVPS				82
Sbjct 1	DIQHTQTSSLSASLGDRVTISCRASQDISKYLNNYQQKPDGTVKLLIYHTSRHSGVPS				60
Query 83	RFSGSGSGTDYSLTISNLEQEDIATYFCQQGNTLPYTFGGGKLEITGSGSGKPGSGE				142
Sbjct 61	RFSGSGSGTDYSLTISNLEQEDIATYFCQQGNTLPYTFGGGKLEITGSGSGGSGG--S				117
Query 143	GSTRGEVKLQESGPGLVAPSQSLSVTCTVSGVSLPDYGVSWIRQPPRKGLEULGVINGSE				202
Sbjct 118	GGGGSEVKLQESGPGLVAPSQSLSVTCTVSGVSLPDYGVSWIRQPPRKGLEULGVINGSE				177
Query 203	TTYNSALKSRLTIINDNSKQVFLKMNLSLQDDTAIYYCAKHYYYGGSYANDYVGGQGS				262
Sbjct 178	TTYNSALKSRLTIINDNSKQVFLKMNLSLQDDTAIYYCAKHYYYGGSYANDYVGGQGS				237
Query 263	VTVSS	267			
Sbjct 238	VTVSS	242			

También se desea mencionar que el CAR en D1 **FMC63-28Z** contiene una porción de CD28 y el componente intracelular del TCRzeta humano, mientras que el CAR de la presente invención no contiene ninguna porción de CD28.

Por otro lado, en D1, el CAR **FMC63-CD828BBZ** contiene las mismas secuencias anti-CD19 scFv, CD28 y TCRzeta de FMC63-28Z ya analizadas y por lo tanto, el componente anti-CD19 scFv de este CAR es también diferente del anti-CD19 scFv de la invención.

En conclusión, el CAR reclamado contiene un anti-CD19 scFv codificado por un ácido nucleico de secuencia diferente a la secuencia codificante del anti-CD19 scFv tanto del FMC63-28Z como del FMC63-CD828BBZ.

Ninguno de estos dos CAR tienen actividad y function igual al reclamado en la presente invención.

El documento citado D1 revela un estudio experimental de las diferencias biológicas entre FMC63-28Z y FMC63-CD828BBZ, demostrando que FMC63-28Z tenía propiedades

biológicas superiores en comparación con FMC63-CD828BBZ, este último siendo más cercano estructuralmente al CAR de la invención.

Por su parte, en D2 - los mismos autores - utilizan sólo FMC63-28Z en un ensayo clínico del tratamiento en humanos y FMC63-CD828BBZ ni siquiera se menciona en D2 como un posible candidato de ensayo clínico.

En D1 los autores revelan que debido a los resultados superiores obtenidos por las células T transducidas por FMC63-28Z, se usó dicho receptor en los demás experimentos:

"Due to the superior recognition of primary CLL cells and the superior production of IL-2 by T cells that were transduced with FMC63-28Z, we used this receptor in the remainder of our experiments." (D1, página 9).

Entonces, se llama la atención del Examinador frente a que los CAR mencionados incluso en los mismos estudios de D1 y D2 son diferentes y que no se pueden utilizar indistintamente, como se entiende del Oficio 15665, teniendo en cuenta que:

- D1 revela la gran diferencia entre la función biológica de FMC63-28Z y FMC63-CD828BBZ
- FMC63-CD828BBZ es estructuralmente más cercano al CAR reclamado
- FMC63-28Z es estructuralmente más lejano del CAR reclamado
- FMC63-CD828BBZ es funcionalmente inferior a FMC63-28Z

Los documentos D3 y D4 no son suficientes para derivar la materia de la presente invención

Los documentos D3 y D4 también describen CAR que son diferentes al de la presente invención y de aquellos citados en D1 y D2.

D3 da a conocer un CAR que tiene una secuencia anti-CD19 scFv (similar a la de FMC63-28Z y FMC63-CD828BBZ), IgG4 Fc humana, dominio transmembrana de CD4 humana, y la cadena zeta citoplasmática humana. **Lo cual es diferente del CAR del solicitante.**

D4 divulga un CAR **diferente al CAR del solicitante,** que incluye una región CD4 transmembrana humana, una región transmembrana CD28 humana, o una región IgG4 Fc humana, así:



"[00011] The inventive CARs include those wherein the transmembrane region is a human CD4 transmembrane region, a human CD28 transmembrane region, or a human IgG₄ Fc region. Specific recognition elements of the CARs can be an IL13 molecule, an IL3 molecule or the extracellular binding domain of a single chain immunoglobulin that recognizes an antigen selected from the group consisting of Her/2Neu, α 3 integrin, CD20, CD19 and EGFRVIII and preferably is an IL13 molecule, most preferably an IL13 molecule that contains the E13Y mutation, such as IL13-CD28-41 BB ζ ."

Por estas razones un técnico medio versado en la materia no podría partiendo de las enseñanzas de ninguno de los documentos D1 – D4 obtener el CAR de la invención ya que en ninguno de los documentos se encuentran todas las características, tanto estructurales como funcionales, ni las partes exactas que componen el CAR de la invención. Si bien a grandes rasgos todos los CAR pueden ser moléculas funcionales similares, su actividad y su función específica no es la misma y el CAR de la presente invención no puede ser obtenida de forma obvia según los documentos citados.

Resultados inesperados logrados por la presente invención

La presente invención se trata de un CAR que es insertado en una célula T que se administra a un paciente humano que sufre de cáncer, dicho CAR tiene la propiedad sorprendente de aliviar el cáncer, tal y como se demuestra en los Ejemplos de la descripción de la presente invención.

Aunque en la descripción se describe el tratamiento en sólo tres pacientes humanos, los inventores han administrado el CAR de la invención a otros individuos, tal y como se revela Maude et al.³, en donde se menciona que 27 de 30 pacientes tratados con células que contienen el CAR de la presente invención presentaron una desaparición completa de todos los signos del cáncer en respuesta al tratamiento⁴ esos pacientes. Es decir, el 90% de los pacientes tratados.

Se observa que Maude et al., fue citado por el examinador de la solicitud original 13-137636, afirmando que el CAR en Maude no contiene una región bisagra CD8 alfa, lo cual **no es**

³ (2014) New England Journal of Medicine 37 ; 16 1507-1517 (Maude); página 1507. (Ver anexo).

⁴ Complete remission: The disappearance of all signs of cancer in response to treatment. This does not always mean the cancer has been cured. Also called complete response. (<http://www.cancer.gov/publications/dictionaries/cancer-terms?cdrid=45651>)



verdad, pues el CAR revelado por Maude et al., es el mismo CAR expuesto en la presente solicitud.

En resumen, la presente solicitud describe y reivindica un CAR que tiene resultados inesperados en el tratamiento de pacientes con cáncer y que no ha sido anticipado por ninguno de los documentos D1, D2, D3 o D4.

Por lo tanto, mi representada solicita a ese Despacho la concesión de la presente patente de invención en tanto cumple con los requisitos en materia de patentes en la región de Colombia.

Respuestas frente al análisis de nivel inventivo del Oficio 15665

Frente a D1 y D2 el Examinador menciona: *“... para una persona del oficio normalmente versada en la materia, resultaría evidente que al realizar mutaciones silentes o conservativas en la región entre los nucleótidos 67 y 801 de la secuencia de FMC63 (HM852952 Genbank), que codifica específicamente para el dominio de unión scFv, se pueden obtener secuencias que codifiquen proteínas con las mismas propiedades biológicas, por lo cual se considera que la secuencia de la reivindicación 1 no cumple con el requisito de nivel inventivo.”*

Respetuosamente, se pone de presente que ninguno de los documentos D1 o D2 sugiere al experto la forma de realizar supuesta mutación, así mismo, estas referencias revelan un CAR con una estructura muy diferente a la acá reclamada – como se mencionó previamente – y adicionalmente, la presente invención muestra un comportamiento superior en cuanto a la desaparición de signos de la enfermedad como se presenta en Maude et al.

El Examinador menciona: *“... la incorporación de dominios de estimulación y co-estimulación en receptores de antígenos quiméricos, ya había sido divulgada en el documento D4, el cual divulga un receptor quimérico de células T que comprende un elemento de reconocimiento, un dominio transmembrana, una región de señalización co-estimuladora y un dominio de señalización CD3 zeta de la secuencia SEQ ID No. 11 (Pág. 3, Párr. [007]), el cual presenta una identidad de 99% y una positividad del 100% frente a la secuencia SEQ ID NO: 24 del CAR reivindicado (Figura 4).*

Por lo tanto, para una persona medianamente versada en la materia, habría resultado evidente incorporar un dominio de estimulación CD3 y co-estimulación 4-BB tal como lo divulga el documento D4 (Pág. 3, Párr. [007]), en el CAR divulgado en el documento D3 que

comprende un dominio transmembrana de CD4 o CD8 (Pág. 16, Párr. [0050] a Pág. 17, Párr. [0050]) y un dominio extracelular específico para CD19 (Pág. 11, Párr. [0025] a Pág. 12, Párr. [0028]), para llegar así al objeto reclamado en la presente solicitud. Por ende, el receptor CAR divulgado en la presente solicitud se considera obvio y sin altura inventiva.”

Como se había presentado previamente, la información divulgada por D3 y D4 no habría permitido a un experto en la técnica llegar a la presente invención pues no revelan exactamente los mismos componentes del CAR, sino que mas bien revelan de forma general de los componentes de dichos receptores.

Y al igual que se mencionó previamente, el CAR de la presente invención muestra efectos sorprendentes e inesperados frente a lo que ya había sido revelado en la técnica.

Por consiguiente, la invención cumple además de novedad, el nivel inventivo y la aplicación industrial subsanando de esta forma las objeciones a la patentabilidad.

ANEXOS

- Nuevo capítulo reivindicatorio compuesto por 4 reivndicaciones
- Documento 39. “Nicholson IC, Lenton KA, Little DJ, et al. Construction and characterisation of a functional CD19 specific single chain Fv fragment for immunotherapy of B lineage leukaemia and lymphoma.” - Molecular Immunology - 1997;34:1157–1165.
- Documento “Chimeric Antigen Receptor T Cells for Sustained Remissions in Leukemia” - The New England Journal Of Medicine (2014) - 371;16: 1507- 1517.

NOTIFICACIONES

Mi mandante y yo recibiremos notificaciones en la Secretaría de la División de Signos Distintivos, y en mi oficina situada en la Calle 72 No. 10 – 07 oficina 603 de Bogotá D.C.. El solicitante y su apoderado las recibirán en la dirección indicada por este último en la solicitud inicial.

En vista de todo lo anterior, ruego a usted estudiar nuevamente la solicitud con base en las modificaciones realizadas y proceder de conformidad.

De Usted,

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Respuesta Requerimiento Art. 45

EXP: 15-80428



CUESTA & ASOCIADOS LTDA

13

Señor Superintendente,

Juan Carlos Cuesta Quintero.

C.C. 79.339.809 de Bogotá

T.P. 43.273 del C.S. de la J.



REIVINDICACIONES MODIFICADAS - OFICIO 11320

1. Una secuencia del ácido nucleico aislado que comprende las secuencias de ácido nucleico de SEQ ID NO: 14 que codifica un anticuerpo anti-CD19 scFv, SEQ ID NO: 15 que codifica el dominio de bisagra CD8, SEQ ID NO: 16 que codifica el dominio transmembrana CD8, SEQ ID NO: 17 que codifica el dominio de señal coestimuladora 4-1BB y SEQ ID NO: 18 que codifica el dominio de señalización CD3zeta.
2. Un receptor de antígeno quimérico aislado (CAR) que comprende las secuencias de aminoácidos de SEQ ID NO: 20 del anticuerpo anti-CD19 scFv, SEQ ID NO: 21 del dominio bisagra CD8, SEQ ID NO: 22 del dominio transmembrana CD8, SEQ ID NO: 23 del dominio de señal coestimuladora 4-1 BB y SEQ ID No. 24 del dominio de señalización CD3zeta.
3. Una población de células T expandidas exvivo que ha sido modificada genéticamente para tener insertada una secuencia aislada de ácido nucleico que comprende las secuencias de ácido nucleico de SEQ ID NO: 14 que codifica un anticuerpo anti-CD19 scFv, SEQ ID NO: 15 que codifica el dominio de bisagra CD8, SEQ ID NO: 16 que codifica el dominio transmembrana CD8, SEQ ID NO: 17 que codifica el dominio de señal coestimuladora 4-1BB y SEQ ID NO: 18 que codifica el dominio de señalización CD3zeta
4. Un vector que comprende las secuencias de ácidos nucleicos de SEQ ID NO: 14 que codifica un anticuerpo anti-CD19 scFv, SEQ ID NO: 15 que codifica el dominio de bisagra CD8, SEQ ID NO: 16 que codifica el dominio transmembrana CD8, SEQ ID NO: 17 que codifica el dominio de señal coestimuladora 4-1BB y SEQ ID NO: 18 que codifica el dominio de señalización CD3zeta.

ORIGINAL ARTICLE

Chimeric Antigen Receptor T Cells for Sustained Remissions in Leukemia

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ABSTRACT

BACKGROUND

Relapsed acute lymphoblastic leukemia (ALL) is difficult to treat despite the availability of aggressive therapies. Chimeric antigen receptor–modified T cells targeting CD19 may overcome many limitations of conventional therapies and induce remission in patients with refractory disease.

METHODS

We infused autologous T cells transduced with a CD19-directed chimeric antigen receptor (CTL019) lentiviral vector in patients with relapsed or refractory ALL at doses of 0.76×10^6 to 20.6×10^6 CTL019 cells per kilogram of body weight. Patients were monitored for a response, toxic effects, and the expansion and persistence of circulating CTL019 T cells.

RESULTS

A total of 30 children and adults received CTL019. Complete remission was achieved in 27 patients (90%), including 2 patients with blinatumomab-refractory disease and 15 who had undergone stem-cell transplantation. CTL019 cells proliferated in vivo and were detectable in the blood, bone marrow, and cerebrospinal fluid of patients who had a response. Sustained remission was achieved with a 6-month event-free survival rate of 67% (95% confidence interval [CI], 51 to 88) and an overall survival rate of 78% (95% CI, 65 to 95). At 6 months, the probability that a patient would have persistence of CTL019 was 68% (95% CI, 50 to 92) and the probability that a patient would have relapse-free B-cell aplasia was 73% (95% CI, 57 to 94). All the patients had the cytokine-release syndrome. Severe cytokine-release syndrome, which developed in 27% of the patients, was associated with a higher disease burden before infusion and was effectively treated with the anti-interleukin-6 receptor antibody tocilizumab.

CONCLUSIONS

Chimeric antigen receptor–modified T-cell therapy against CD19 was effective in treating relapsed and refractory ALL. CTL019 was associated with a high remission rate, even among patients for whom stem-cell transplantation had failed, and durable remissions up to 24 months were observed. (Funded by Novartis and others; CART19 ClinicalTrials.gov numbers, NCT01626495 and NCT01029366.)

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ENGINEERED T-CELL THERAPY IS A NEW strategy for the treatment of relapsed and refractory acute lymphoblastic leukemia (ALL), which is associated with an extremely poor prognosis in adults and remains a leading cause of death from childhood cancer.¹⁻³ In initial proof-of-principle clinical trials involving patients with chronic lymphocytic leukemia (CLL), chimeric antigen receptor–modified T cells that target CD19 produced a durable complete remission in a small number of patients.⁴⁻⁶ Our group and others then extended these findings to relapsed and refractory B-cell ALL, and we found profound responses in a small number of children and adults.^{7,8}

Chimeric antigen receptors are genetically engineered receptors that couple an anti-CD19 single-chain Fv domain to intracellular T-cell signaling domains of the T-cell receptor, thereby redirecting cytotoxic T lymphocytes to cells expressing this antigen. With the use of lentiviral-vector technology for gene transfer and permanent T-cell modification, CTL019 (formerly known as CART19)–engineered T cells express a chimeric antigen receptor in which the T-cell activation signal is provided by the CD3-zeta domain, and the costimulatory signal is provided by the CD137 (4-1BB) domain.⁴

We previously reported a high degree of *in vivo* expansion of CTL019 cells that resulted in complete remission in two children with relapsed and highly refractory B-cell ALL.⁸ However, the rate of complete remission in a larger cohort, long-term persistence of chimeric antigen receptor–modified T cells, and the durability of remission remained unknown. We now report the results of CART19 (A Phase I/IIA Study of Redirected Autologous T Cells Engineered to Contain Anti-CD19 Attached to TCRzeta and 4-1BB Signaling Domains in Patients with Chemotherapy Resistant or Refractory CD19+ Leukemia and Lymphoma) showing the efficacy of CTL019 and provide follow-up of up to 2 years in our expanded cohort of 30 patients with relapsed and refractory ALL. Robust expansion of CTL019 cells rapidly induced complete remission in this cohort of patients who were previously considered to have refractory and incurable disease.

METHODS

TRIAL DESIGN AND OVERSIGHT

We conducted pilot clinical trials at Children's Hospital of Philadelphia and the Hospital of the

University of Pennsylvania that were designed to assess the safety and feasibility of CTL019 T-cell therapy in patients with relapsed and refractory CD19+ cancers; the protocols were approved by the respective institutional review boards. All the authors discussed and interpreted the study results and vouch for the data and analyses. All the patients or their parents provided written informed consent. Enrolled patients received CTL019 infusions between April 2012 and February 2014. Additional details regarding the study design are provided in the Supplementary Appendix, available with the full text of this article at NEJM.org.

Leukapheresis products were stimulated with paramagnetic beads coated with antibodies to CD3 and CD28 and transduced with the CD19-BB-zeta transgene as described previously.^{4,9} After leukapheresis, patients received interim therapy at the discretion of their treating physician. Chemotherapy aimed at depletion of T lymphocytes (Table S1 in the Supplementary Appendix) was administered 1 week before infusion of CTL019 (Table S2 in the Supplementary Appendix), except in three patients who had persistent cytopenias.

PATIENT CHARACTERISTICS

A total of 25 patients, 5 to 22 years of age, were treated at Children's Hospital of Philadelphia (pediatric trial), and 5 older patients, 26 to 60 years of age, were treated at the Hospital of the University of Pennsylvania (adult trial) (Table 1). Of these patients, 26 had B-cell ALL in a first to fourth relapse, 3 had primary refractory E-cell ALL, and 1 had relapsed T-cell ALL that expressed CD19. Eighteen patients had relapsed disease after allogeneic stem-cell transplantation. Three patients (Patients 2, 3, and 20) had disease that was previously shown to be refractory to blinatumomab (a bispecific antibody with one domain that binds to CD3 on T cells and the other that binds to CD19).

RESULTS

OUTCOMES

Twenty-seven of 30 patients (90%) were in a morphologic complete remission at the first assessment 1 month after the infusion of CTL019. A test to detect minimal residual disease by means of multiparametric flow cytometry was negative in 22 patients, positive in 3 patients (the levels of minimal residual disease were 0.1% [and subsequently negative at 3 months], 0.09%, and 0.22%,

respectively), and not performed in 2 patients. A complete remission was achieved in 2 of 3 patients who had previously been exposed to blinatumomab. The 2 patients in whom blast cells were detected in the cerebrospinal fluid at the time of infusion subsequently had no detectable central nervous system (CNS) leukemia as of the most recent follow-up (6 months), and no CNS relapses were observed.

Seven patients who had a complete remission subsequently had a relapse between 6 weeks and 8.5 months after infusion of CTL019 cells. Three relapses developed after early loss of CTL019-modified T cells at 2 weeks to 3 months, and in these patients the relapsed ALL remained CD19-positive. After recovery of normal B cells at 2 to 3 months, one relapse occurred rapidly at 3 months and two relapses were delayed (they occurred at 6 and 8.5 months). Patient 9, who had minimal residual disease (0.22%) at 1 month, had a relapse with CD19-positive ALL at 6 weeks. The disease rapidly progressed, and the patient died from ALL. This patient had highly refractory disease that was in the fourth relapse at the time of infusion and was not eligible for stem-cell transplantation because of coexisting conditions. In three patients, the loss of the expression of CD19 in leukemia cells resulted in a relapse; one of these patients (Patient 2) had received prior blinatumomab therapy. In these patients, CTL019 cells were not lost at the time of relapse.

Of the 27 patients who had a complete remission, 19 remained in remission: 15 patients received no further therapy, and 4 patients withdrew from the study to receive other therapy. In Patient 11, the myelodysplastic syndrome developed during ALL remission, and the patient withdrew from the study to receive other therapy. The median follow-up was 7 months (range, 1 to 24). No deaths were related to the study treatment. Seven patients died after disease progression or relapse, including 1 patient who died from the myelodysplastic syndrome. At 6 months, the event-free survival rate was 67% (95% confidence interval [CI], 51 to 88) and the overall survival rate was 78% (95% CI, 65 to 95) (Fig. 1A and 1B).

IN VIVO EXPANSION AND PERSISTENCE OF CTL019

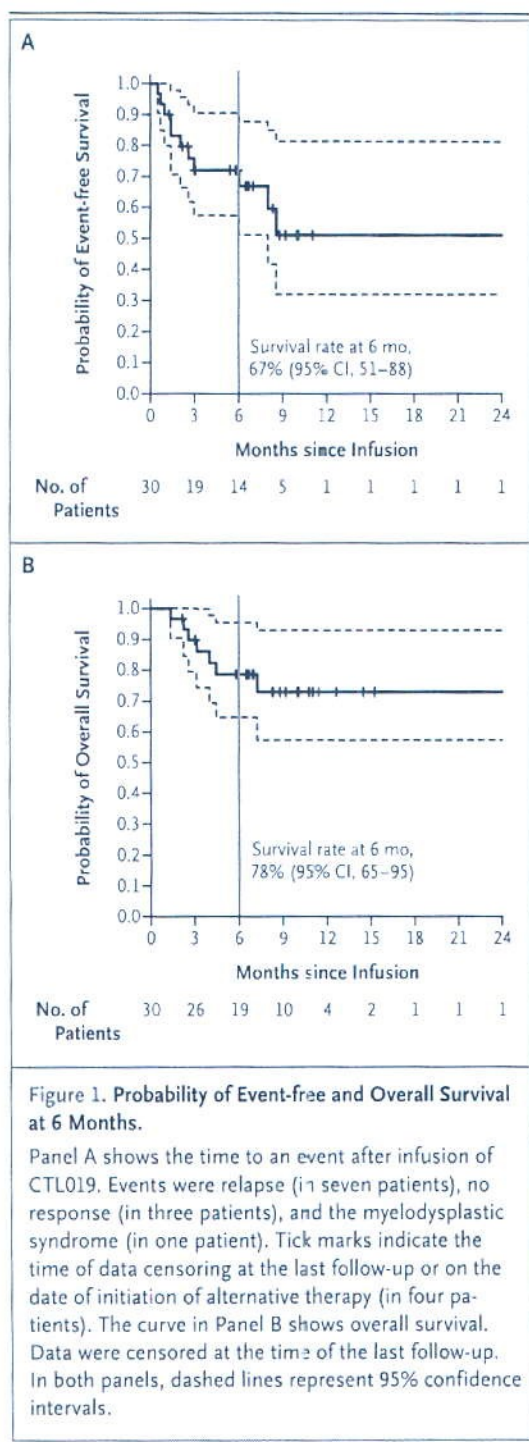
The CTL019 cells were easily detectable by means of flow cytometry, thus reflecting high in vivo proliferation. In 27 patients who had a response, high peak proportions of CTL019-modified T cells were detected by this method (median, 39.8% of

Table 1. Baseline Characteristics of the Patients.*			
Characteristic	Pediatric Cohort (N = 25)	Adult Cohort (N = 5)	Total (N = 30)
Sex — no. (%)			
Female	11 (44)	1 (20)	12 (40)
Male	14 (56)	4 (80)	18 (60)
Age at infusion — yr			
Median	11	47	14
Range	5–22	26–60	5–60
Allogeneic transplantation — no. (%)	18 (72)	0	18 (60)
Primary refractory disease — no. (%)	0	3 (60)	3 (10)
Relapse — no. (%)			
1	3 (12)	2 (40)	5 (17)
≥2	22 (88)		22 (73)
Baseline burden of acute lymphoblastic leukemia — no. (%)			
Presence of detectable disease†	20 (80)	4 (80)	24 (80)
Morphologic remission‡		1 (20)	1 (3)
Absence of minimal residual disease	5 (20)		5 (17)
High-risk cytogenetic factors — no.			
BCR–ABL1	2		
IKZF1 deletion	2		
iAMP21	1		
MLL translocation	1		
Hypodiploidy	2		
CNS status — no.§			
CNS-1	23		
CNS-2	2		

* CNS denotes central nervous system, and iAMP21 intrachromosomal amplification of chromosome 21.
† Disease was detected by assessment of bone marrow and cerebrospinal fluid morphologic features and measurement of minimal residual disease.
‡ Minimal residual disease was not measured.
§ CNS status was determined only in the pediatric trial. CNS status was defined as CNS-1 (no detectable blast cells in a sample of cerebrospinal fluid), CNS-2 (blast cells detected in a sample with <5 leukocytes per cubic millimeter and <10 erythrocytes per cubic millimeter), and CNS-3 (blast cells detected in a sample with ≥5 leukocytes per cubic millimeter and <10 erythrocytes per cubic millimeter). The presence of CNS-3 disease was an exclusion criterion.

CTL019-positive cells in CD3-positive cells; range, 4.4 to 69.3), whereas in the 3 patients who did not have a response, 0.2%, 0.6%, and 8.2% of CD3-positive cells, respectively, were CTL019-positive at peak levels. CTL019 cells were detectable in the blood by means of flow cytometry for up to 11 months (Fig. 2A and 2B).

The probability of persistence of CTL019 at 6 months was 68% (95% CI, 50 to 92). CTL019



sequences remained detectable by means of quantitative polymerase-chain-reaction (PCR) assay in patients with sustained remissions until 2 years (Fig. 2C and data not shown). This assay showed very high levels of proliferation of CTL019 cells; all patients had peak levels greater than 5000 copies per microgram of genomic DNA, and 26 patients had peak levels greater than 15,000 copies

per microgram of genomic DNA. One patient (Patient 17) received infusions again at 3 months and 6 months because of early loss of CTL019 cells with B-cell recovery, and this patient subsequently had persistence of CTL019. In the patient with the longest remission (2 years), B-cell aplasia (absence of CD19-positive cells) (Fig. 3) continued for a year after the loss of CTL019 cells detectable by flow cytometry, suggesting functional persistence of CTL019 cells below the limits of detection by flow cytometry, whereas CTL019 remained detectable by means of quantitative PCR. The probability of relapse-free B-cell aplasia at 6 months was 73% (95% CI, 57 to 94).

CTL019 FOR RELAPSE AFTER ALLOGENEIC STEM-CELL TRANSPLANTATION

In the 18 patients who were treated for relapse of disease after allogeneic stem-cell transplantation, the median donor chimerism at the time of leukapheresis was 100% (range, 68 to 100). No graft-versus-host disease was observed after infusion of CTL019. Event-free survival and overall survival did not differ significantly between the patients who had previously undergone stem-cell transplantation and those who had not undergone stem-cell transplantation ($P=0.21$ for event-free survival and $P=0.24$ for overall survival).

THERAPY AFTER ADMINISTRATION OF CTL019

Five patients withdrew from the study after the administration of CTL019 to receive other therapy; three of these patients underwent allogeneic stem-cell transplantation while their disease was in remission, and the disease remained in remission 7 to 12 months after the infusion of CTL019. Patient 12, who had undergone a previous stem-cell transplantation, had a post-transplantation relapse of T-cell ALL that aberrantly expressed CD19, was refractory to two intensive reinduction regimens, and entered a morphologic remission after the infusion of CTL019, but the patient had minimal residual disease (0.09%). She subsequently received bortezomib and an infusion of donor lymphocytes, and the disease remained in remission without minimal residual disease at 11 months. In Patient 11, the myelodysplastic syndrome developed and led to overt acute myeloid leukemia with a monosomy 8 clone that also shared cytogenetic features with the original B-cell ALL.

CYTOKINE-RELEASE SYNDROME

A major toxic effect associated with CTL019 is the cytokine-release syndrome, a systemic inflammatory response that is produced by elevated levels of cytokines; these elevations are associated with T-cell activation and proliferation. The cytokine-release syndrome ranges from mild and self-limiting, with high temperatures and myalgias, to severe and life-threatening, with a clinical course that also includes vascular leak, hypotension, respiratory and renal insufficiency, cytopenias, and coagulopathy. Several aspects of the cytokine-release syndrome mirror those of the macrophage activation syndrome.⁸ All the patients in our studies had the cytokine-release syndrome, which was mild to moderate in 22 of the 30 patients. These patients required hospitalization for febrile neutropenia and received broad-spectrum antibiotics and pain medications. Severe cytokine-release syndrome, which required intensive care with varying degrees of respiratory support (from placement of a nasal cannula to mechanical ventilation), developed in 8 patients (27%), and all these patients required vasopressor support for hypotension. Coagulopathy, with elevated prothrombin and partial-thromboplastin times as well as severe hypofibrinogenemia, was observed in patients who had severe cytokine-release syndrome, although clinical bleeding was rare (observed in 3 patients).

Severe cytokine-release syndrome started a median of 1 day after infusion, whereas cytokine-release syndrome that was not severe started a median of 4 days after infusion ($P=0.005$). Laboratory markers of systemic inflammation, including C-reactive protein and ferritin levels, were elevated in all the patients. Patients who had severe cytokine-release syndrome had higher peak levels of interleukin-6 than did patients who did not have severe cytokine-release syndrome ($P<0.001$) (Fig. 4A); they also had higher peak levels of C-reactive protein ($P=0.02$), ferritin ($P=0.005$), interferon- γ ($P<0.001$), and soluble interleukin-2 receptor ($P<0.001$) (Fig. S2 in the Supplementary Appendix). The baseline disease burden (the percentage of blast cells in bone marrow before infusion) correlated with the severity of the cytokine-release syndrome; a higher disease burden was significantly associated with severe cytokine-release syndrome ($P=0.002$) (Fig. 4B). Patients with severe cytokine-release syndrome also had higher levels of CTL019-positive

CD8 cells ($P=0.012$) and CTL019-positive CD3 cells ($P=0.026$).

We previously found marked elevation of interleukin-6 levels after CTL019 therapy and rapid reversal of severe cytokine-release syndrome with the interleukin-6–receptor blocking antibody tocilizumab⁸; therefore, tocilizumab was incorporated into the management of severe cytokine-release syndrome in this study. Nine patients received tocilizumab, which resulted in rapid defervescence and stabilization of blood pressure, with improvement (weaning from vasopressor support) over a period of 1 to 3 days. Six patients also received short courses of glucocorticoids, and four patients received a second dose of tocilizumab for recrudescence of the cytokine-release syndrome after transient improvement with the first dose. All the patients recovered fully, and there was a complete reversal of symptoms and a normalization of laboratory results. Relapses occurred in two of the nine patients who received immunosuppressive therapy for the cytokine-release syndrome.

ENCEPHALOPATHY

Thirteen patients had neurologic toxic effects, which ranged from delirium during the period of high temperatures to global encephalopathy with one or more of the following: aphasia, confusion, delirium, and hallucinations. Six patients had delayed encephalopathy that occurred after high temperatures had resolved and was independent of the severity of the cytokine-release syndrome and whether the patient had received prior tocilizumab therapy. Symptoms were self-limiting (lasting 2 to 3 days and resolving over 2 to 3 days), and they resolved fully without further intervention or apparent long-term sequelae. One patient with encephalopathy had two seizures that may have been caused by concomitant electrolyte abnormalities. Several patients had normal computed tomographic or magnetic resonance imaging of the head and lumbar puncture that was negative for infection or leukemia.

B-CELL APLASIA

We performed flow cytometry to detect CD19-positive B cells in order to monitor patients for the development of B-cell aplasia, which can be used as a pharmacodynamic measure of CTL019 function (Fig. 3). B-cell aplasia occurred in all

the patients who had a response and persisted for up to 1 year after CTL019 cells were no longer detectable by means of flow cytometry. Patients with B-cell aplasia received immunoglobulin replacement to maintain IgG levels greater than 500 mg per deciliter.

DISCUSSION

The engineering of T lymphocytes to express chimeric antibodies that target tumor antigens has been studied for more than 20 years.^{10,11} Clinical progress had been limited by poor in vivo expansion of engineered T cells and failure of these cells to persist after infusion.¹²⁻¹⁶ We and others have documented a high level of proliferation, activity against bulk disease, and long-term persistence of chimeric antigen receptor T cells.^{4-8,17,18}

In this study, we found a 90% rate of complete remission among 30 children and adults who received CTL019 for ALL that was relapsed or refractory. With a follow-up period of 2 to 24 months, sustained remissions were observed in 19 patients (15 of whom received no further therapy) and were associated with the persistence of CTL019 and B-cell aplasia that continued beyond 2 to 3 months, suggesting continued effector function. Three patients who were in remission subsequently underwent allogeneic stem-cell transplantation. However, for several reasons (e.g., the lack of a suitable donor, prior stem-cell transplantation, or family choice), most patients did not undergo stem-cell transplantation; this allowed for longer follow-up. The rate of event-free survival at the median follow-up of 6 months was 67% in this heavily pretreated population. Several patients with relapse received salvage therapy, and the rate of overall survival at 6 months was 78%. In comparison, with the use of clofarabine, nelarabine, and liposomal-encapsulated vincristine — the most recently approved drugs for relapsed ALL¹⁹⁻²¹ — the Food and Drug Administration label indicates rates of complete remission of less than 25%, with a median documented duration of response of 4 to 9 weeks.

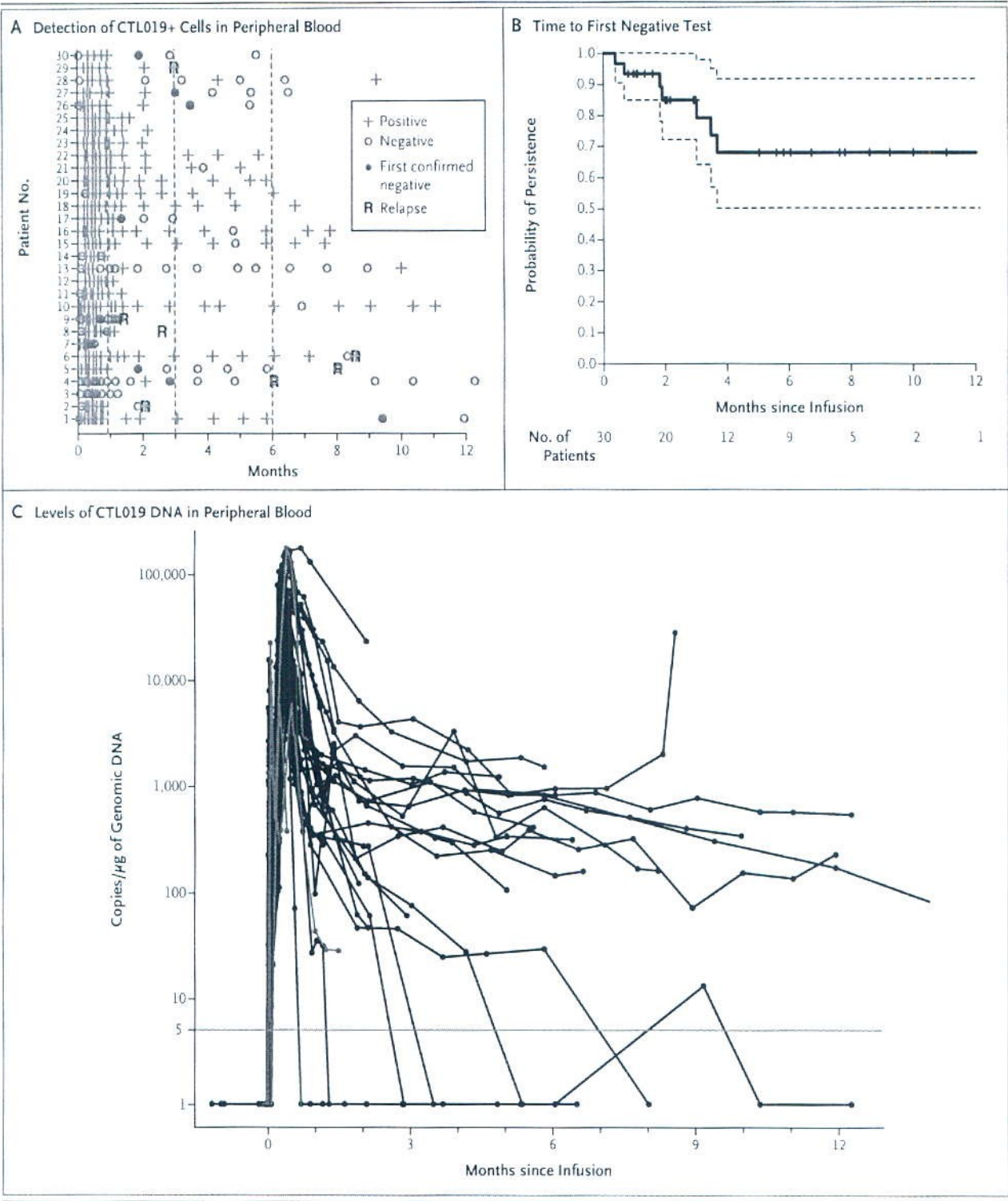
Davila et al. recently reported results from an expanded cohort of 16 adult patients with B-cell ALL.²² The short-term results were similar to those in our study; the overall complete remission rate was 88% with the use of a CD19 chimeric antigen receptor with the CD28 costimulatory domain. However, the only long-term remissions that

Figure 2 (facing page). Persistence of CTL019.

Panel A shows the results of detection of CTL019-positive T cells detected by means of flow cytometry in peripheral-blood samples. "Confirmed negative" was defined as the first of two consecutive negative measurements (<0.1% CTL019-positive cells in CD3-positive cells). Patients 1 through 25 were participants in the pediatric trial (which included children and young adults 5 to 22 years of age), and Patients 26 through 30 were participants in the adult trial (which included patients 26 to 60 years of age). CTL019-modified T cells were also detected in the cerebrospinal fluid of 17 of 19 patients with specimens that could be evaluated. Panel B shows the Kaplan–Meier curve of the time to the first confirmed negative measurement in peripheral blood and bone marrow. Data were censored at the time of the last follow-up. Dashed lines represent 95% confidence intervals. Panel C shows measurements of CTL019 gene-modified T cells in peripheral blood as assessed by means of quantitative real-time polymerase-chain-reaction (PCR) assay. Genomic DNA was isolated from samples of whole blood obtained at serial time points before and after infusion of CTL019. The horizontal line at 5 copies per microgram of DNA represents the lower limit of quantification of this assay. Data on patients who did not have a response are shown in red. In general, the levels of CTL019 detected by means of quantitative PCR correlated well with the level of CTL019-positive cells detected by means of flow cytometry, with the exception of the levels in 3 patients who did not have a response and whose peak levels measured by means of quantitative PCR (6066, 5982, and 178,481 copies per microgram of genomic DNA, respectively) did not correspond with detection of CTL019 cells by means of flow cytometry or the induction of B-cell aplasia. CTL019 sequences were detected (23 copies of CTL019 cells per microgram of DNA) at month 24 by means of quantitative PCR in the 1 patient who remained in complete remission at the 2-year follow-up. Data at the first time point were obtained before infusion of CTL019 cells. Doses of cells were determined according to the total amount of cells available after manufacturing. The manufacturing goal of 1.5×10^7 to 5×10^9 total cells (3×10^5 to 1×10^8 cells per kilogram of body weight) was achieved in all treated patients (see Table S2 in the Supplementary Appendix). A split-dose strategy was used to determine safety with 0.1×10^8 to 1×10^8 cells per kilogram infused over 1 to 3 days (5×10^8 to 50×10^8 cells in patients who weighed 50 kg or more). The transduction efficiency ranged from 5.5 to 45.3%; this yielded a dose of 0.76 to 20.6×10^6 CTL019 cells per kilogram.

were reported occurred in patients who crossed over to allogeneic stem-cell transplantation. The median duration of persistence of 19-28z chimeric antigen receptor T cells was 30 days (range, 0 to 120). As a bridge to transplantation, high

CHIMERIC ANTIGEN RECEPTOR T CELLS FOR LEUKEMIA



levels of proliferation with a short duration of persistence is sufficient to achieve remission and eligibility for stem-cell transplantation. However, in patients who are ineligible for stem-cell transplantation, short persistence of chimeric antigen receptor T cells is unlikely to produce long-term

remission. We observed prolonged persistence of CTL019 cells and B-cell aplasia for as long as 2 years in this cohort and for more than 3 years in patients with CLL.⁴ There was no discernible effect of the density of CD19 antigen or cell dose on either efficacy

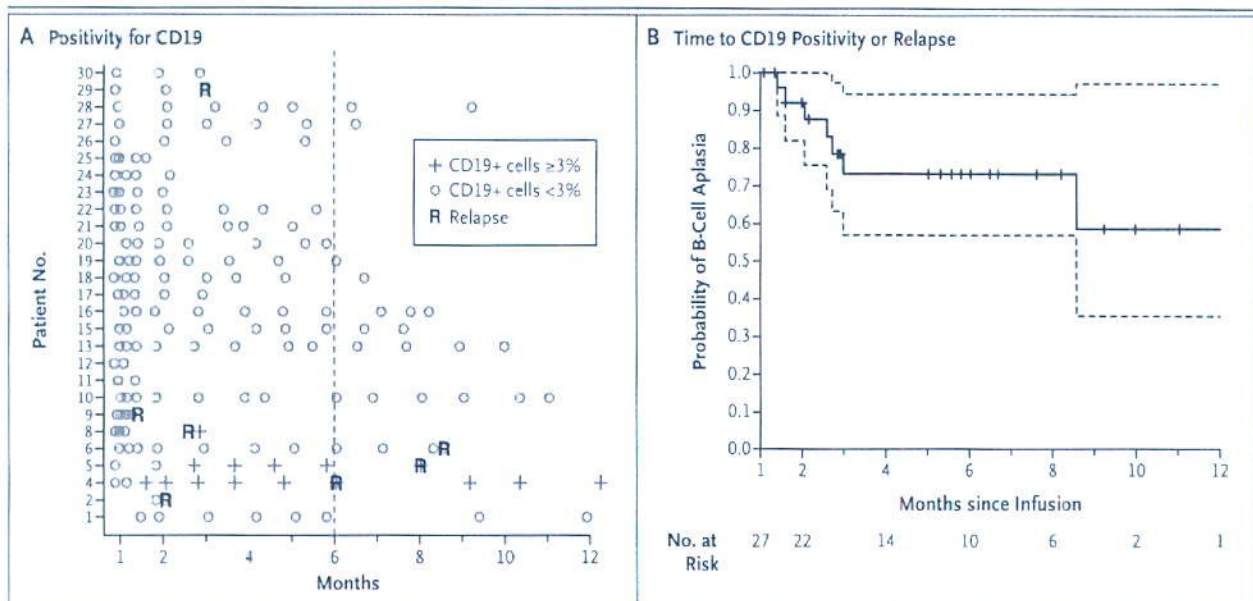


Figure 3. B-Cell Aplasia.

Panel A shows the results of testing to detect the percentage of CD19-positive lymphocytes in peripheral-blood samples by means of flow cytometry. Patients 1 through 25 were participants in the pediatric trial (which included children and young adults 5 to 22 years of age), and Patients 26 through 30 were participants in the adult trial (which included patients 26 to 60 years of age). Negative results were defined as less than 3% of lymphocytes that were positive for CD19. An outlier sample (Patient 16 at month 3) with 4% CD19-positive lymphocytes was discrepant with the measurements on clinical flow cytometry ($< 1\%$ CD19-positive cells), bone marrow measurements at the same time point, and four subsequent monthly evaluations and was, therefore, considered to be negative. Panel B shows a Kaplan-Meier curve of the time to either CD19 positivity or relapse. Data on patients in remission were censored at the time of the last follow-up (indicated by tick marks). Dashed lines represent 95% confidence intervals. All patients required intravenous immunoglobulin replacement, and no serious infectious complications were observed as a result of B-cell aplasia; however, bronchitis (in one patient), acute otitis media (in two patients), salmonella infection (in one patient), and recurrent urinary tract infections (in one patient) were observed.

or toxicity (Table S2 in the Supplementary Appendix), although this study was not powered to investigate these correlations. Both complete remissions and severe cytokine-release syndrome were observed at the lower doses of cells administered. Although there was generally good concordance of CTL019 proliferation as measured by means of flow cytometry and quantitative PCR, in the cases of the three patients who did not have a response, the degree of proliferation of CTL019 cells determined by means of quantitative PCR was matched neither by the detection of cells expressing surface chimeric antigen receptor nor by the induction of B-cell aplasia. The mechanisms underlying this observation, including possible gene silencing of CTL019, are unknown. B-cell aplasia can be used as a pharmacodynamic measure of the persistence of functional CD19-targeted T cells. Continued B-cell aplasia was seen in all patients who had a sustained remission, and none of the patients with B-cell aplasia had a CD19-positive relapse.

Relapses were associated with either lack of CTL019 persistence or CD19 escape variants. In two cases of loss of CTL019, relapse was heralded by recovery of normal B cells, suggesting that monitoring for the return of normal B cells will be important to identify patients at the highest risk for relapse and potentially provide a window for repeat treatment or stem-cell transplantation. Selection of CD19-negative clones or down-regulation of CD19 expression are concerns associated with CD19-directed therapies. Data are lacking to determine the mechanism of CD19-negative relapses.

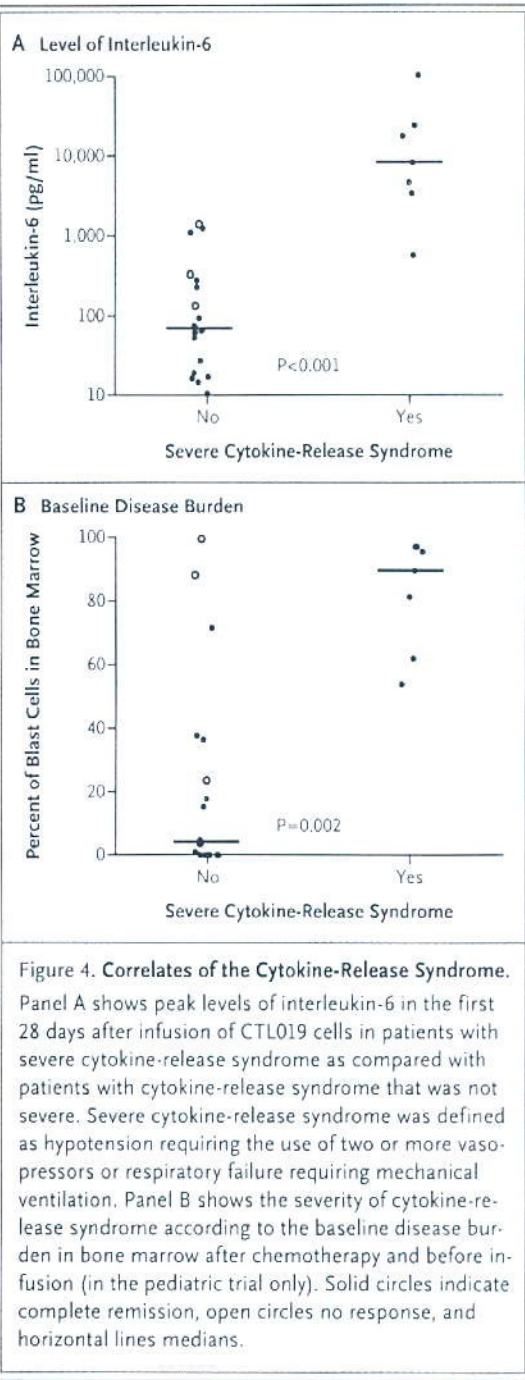
The most prominent and serious toxic effect of chimeric antigen receptor T-cell therapy is the cytokine-release syndrome, an inflammatory process marked by dramatic elevations of cytokine levels.²³ Because this cytokine release probably both results from and contributes to T-cell activation, some degree of cytokine-release syndrome is probably necessary for efficacy. In the major-

ity of our patients, the cytokine-release syndrome was self-limited, with high temperatures and myalgias that resolved spontaneously in a few days. However, in approximately 30% of the patients in our study, hypotension and respiratory insufficiency associated with this syndrome required treatment in the intensive care unit. The cytokine-release syndrome has been described by several groups in association with CD19 chimeric antigen receptor T-cell therapy,^{8,22} but until recently, management was nonspecific, glucocorticoid-based, and directed primarily toward supportive care. In severely affected patients, glucocorticoids may have a limited effect on toxicity but a substantial negative effect on T-cell proliferation and persistence.^{4,7,8,22}

We previously observed marked elevation of interleukin-6 levels in a patient with severe cytokine-release syndrome after infusion of CTL019, and our study showed the benefit of cytokine-directed therapy.⁸ Tocilizumab, an interleukin-6-receptor blocking antibody, produced a rapid and profound improvement in severe manifestations of the cytokine-release syndrome without an apparent impact on the efficacy of CTL019. We have now incorporated tocilizumab as a first-line agent in the management of severe cytokine-release syndrome. Tocilizumab could blunt maximal T-cell proliferation by interfering with a cytokine feedback loop; therefore, we have limited its use to patients with grade 4, or life-threatening, toxic effects. Despite this theoretical concern, in this study, all the patients who received tocilizumab had robust proliferation of CTL019 cells, a complete remission, and the continued persistence of CTL019 cells. Future studies are needed to determine whether tocilizumab can be used preemptively without compromising efficacy.

We observed that many findings in patients with the cytokine-release syndrome were similar to those in patients with the macrophage activation syndrome or hemophagocytic lymphohistiocytosis.^{8,24,25} Hyperferritinemia is a hallmark of these two conditions, and we observed marked elevations in ferritin levels during the period of maximal T-cell proliferation. The degree of elevation in ferritin and C-reactive protein levels was significantly higher in patients with severe cytokine-release syndrome.

Neurologic toxic effects were seen in a subgroup of patients. Encephalopathy typically occurred after symptoms of the cytokine-release



syndrome peaked and was self-limited. CNS toxic effects have been associated with other T-cell-activating therapies, including other chimeric antigen receptor-modified T-cell therapies and blinatumomab.^{22,26,27} The pathophysiology of encephalopathy after the use of CTL019 is not known. The mechanism could involve direct T-cell-mediated toxicity or it could be cytokine-mediated. We have observed no clear predictors of neurologic toxic effects. CTL019-modified

T cells were detected in the cerebrospinal fluid of 17 of 19 patients with specimens that could be evaluated. This may have implications for successful surveillance of CNS disease, yet only a fraction of the patients had encephalopathy. The 2 patients (of the 25 patients in the pediatric trial) in whom cerebrospinal fluid leukemic blasts were detected (CNS-2 disease) did not have encephalopathy. Neurologic toxic effects did not obviously correlate with the severity of the cytokine-release syndrome and was not prevented by tocilizumab. Further studies are needed to determine the potential predictors, mechanism, and treatment of neurologic toxicity.

Relapsed ALL is a considerable therapeutic challenge, particularly in patients who do not have a second complete remission or have a relapse after stem-cell transplantation.^{1-3,28,29} Salvage therapy induces remissions in only 40% of children who have had two or more bone marrow relapses, and long-term survival is quite poor.³ This study included patients with multiple relapses or with disease that was refractory to two or more attempts at reinduction, and 18 patients (60%) had had a relapse after allogeneic stem-cell transplantation. Two complete remissions were achieved in 3 patients with disease that was refractory to blinatumomab, a promising CD19-directed bispecific antibody for relapsed ALL,³⁰ suggesting that a lack of response to CD19-directed therapy may not preclude successful therapy with CTL019. The complete re-

mission rate of 90% and sustained remissions of up to 2 years that were seen in this study are encouraging.

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CHIMERIC ANTIGEN RECEPTOR T CELLS FOR LEUKEMIA

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CONSTRUCTION AND CHARACTERISATION OF A FUNCTIONAL CD19 SPECIFIC SINGLE CHAIN Fv FRAGMENT FOR IMMUNOTHERAPY OF B LINEAGE LEUKAEMIA AND LYMPHOMA

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Abstract—The B cell specific antigen CD19 is a target for the immunotherapy of B lineage leukaemias and lymphomas. We have engineered a single chain Fv (scFv) fragment from the mouse hybridoma cell line FMC63 which produces monoclonal antibody specific for CD19. The genes encoding the FMC63 heavy and light chain variable regions were amplified from cDNA and a scFv was constructed by splice overlap extension PCR. Analysis of staining of lymphoblastoid cell lines, peripheral blood lymphocytes and tonsil sections demonstrated that the monovalent scFv fragment has the same cellular specificity as the parent hybridoma antibody. Kinetic studies with radiolabelled material showed that the scFv binds target cells with a K_d of 2.3×10^{-8} , compared with 4.2×10^{-8} for the parent antibody. This CD19 scFv will be used in experimental models to test its therapeutic efficacy and immunogenicity, with a view to application in the diagnosis and treatment of human B cell cancers. © 1997 Elsevier Science Ltd. All rights reserved.

Key words: scFv, CD19, antibody therapy, leukaemia, lymphoma.

INTRODUCTION

Antibody directed imaging and immunotherapy of tumours relies on targeting tumour-associated antigens. CD19 is expressed on most B lineage malignancies, including acute lymphoblastic leukaemia, chronic lymphocytic leukaemia and non-Hodgkin's lymphoma. Because CD19 is absent from bone marrow progenitor cells it is a potential target for immunotherapy of these malignancies (Uckun *et al.*, 1988). Antibodies against CD19 inhibit the growth of tumour cells (Ghetie *et al.*, 1994). CD19 is not readily shed from cells (Uckun *et al.*, 1988) and is internalised with bound antibody, allowing delivery of anti-CD19-linked toxins (Uckun *et al.*, 1988). Animal models have indicated the potential value of antibodies to CD19 (Jansen *et al.*, 1992; Pietersz *et al.*, 1995). Antibody alone (Hekman *et al.*, 1991), with IL-2 (Vlas-

veld *et al.*, 1995), or conjugated to toxin (Grossbard *et al.*, 1993; Stone *et al.*, 1996) have been used in clinical trials for therapy of leukaemia and lymphoma and CD19 scFv have been described (Bejcek *et al.*, 1995).

Some of the limitations of therapeutic monoclonal antibodies can be overcome by engineering smaller and more effective antibody fragments (Winter *et al.*, 1994). scFv are single gene fusions of the antibody heavy and light chain variable regions joined by a peptide linker. Because they are smaller than whole antibodies, scFv show improved penetration into poorly vascularised tumours (Yokota *et al.*, 1992) and in clinical trials have shown negligible immunogenicity (Bengt *et al.*, 1996). Functional moieties such as toxins, enzymes, or sites for binding drugs or radioisotopes can be incorporated (Ghetie and Vitetta 1994; Pietersz *et al.*, 1992). Engineered antibody fragments can be produced on a large scale in bacterial or mammalian expression systems (Pack *et al.*, 1993; Bebbington, 1995).

We describe the production and characterisation of a CD19 scFv, CHRI-19Fv1. Staining of lymphoblastoid cell lines, peripheral blood lymphocytes and tonsil sections indicates that CHRI-19Fv1 has the same cellular specificity as the parent antibody and has retained a high level of binding albeit with a 2-fold reduction in affinity.

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MATERIALS AND METHODS

Cloning of antibody variable region genes

FMC63 (CD19; IgG2a/ κ ; Zola *et al.*, 1991) hybridoma cells were grown in RPMI1640 with 10% fetal calf serum. Total RNA was isolated using RNeasy (Biotech Laboratories, Houston, TX, U.S.A.). cDNA was synthesised using reverse transcriptase (Promega, Madison, WI, U.S.A.). Heavy chain variable region (V_H) cDNA was amplified using a degenerate primer to the first eight codons (MVH.FWR) (Orlandi *et al.*, 1989) and a primer to the γ constant region (MVH. γ) (Gavilondo *et al.*, 1990). Light chain variable region cDNA (V_L) was amplified using a mixture of primers to the first eight codons of framework 1 (MVK.B5-1-B5-5) (Leung *et al.*, 1993) and a primer to the κ constant region (MVK. κ) (Gavilondo *et al.*, 1990) (Table 1). PCR products were purified using the Wizard PCR Prep System (Promega), cloned into pGem-T (Promega) and transformed into *E. coli* TG1 cells. Recombinant colonies were selected by blue-white screening and insert size estimated using heavy chain primers MVH.FWR and MVH.JOIN, and light chain primers MVK.B5 and MVK.FOR2 (Table 1). Inserts from positive colonies were amplified using M13 sequencing primers and sequenced.

Assembly of scFv fragment

V_H and V_L DNA templates were extended at the 3' and 5' ends by PCR using primers encoding the linker sequence (Gly₄Ser)₃ (Huston *et al.*, 1988). V_H DNA was amplified with the primers Vector. V_H and H.SOE.anti; V_L with B5-2.SOE.Sense and V_L .Vector.2 (Table 1). Products were purified using the Wizard PCR Prep System and the extended V_H and V_L products were used for assembly of the scFv by SOE-PCR (Clackson *et al.*, 1992). Cycling began (in the absence of primers) with denaturation at 95°C for 5 min followed by five cycles of 74°C for 1 min, 95°C for 1 min then a final extension at 74°C for 10 min. For amplification of the scFv template, Tli DNA polymerase, dNTPs and the primers Vector. V_H and V_L .Vector.2 were added directly to the PCR mix. The pooled products was amplified for 10 cycles using Taq polymerase to add an overhanging "A" residue for ligation into pGem-T. The ligation was transformed into competent *E. coli* TG1 cells. Recombinant colonies were screened for scFv by PCR using the MVH.FWR and MVK.FOR2 primers. The construct was re-amplified from positive colonies using the Vector. V_H and V_L .Vector.2 primers, digested with NcoI and NotI and cloned into the pHEN-1 phagemid (Hoogenboom *et al.*, 1991). The ligation was digested with XhoI to reduce the

Table 1. Primers used for isolation of immunoglobulin heavy chain and light chain variable region genes and the assembly of the scFv construct

Primer Name	Sequence (5' to 3')	Region	Strand
MVH.FWR	TTCSAGGTSMARCTGCAGSAGTCT	V_H FWR1	sense
MVH. γ	ACACACAGGRRRCAGTGGATAGAC	CH1	antisense
MVH.Join	TGAGGAGACGGTGACCGTGGTCCCTTGGCCCCAG	V_H FWR4	antisense
MVK.B5-1	GAAATTGKTCTCACMCARTCTCC	VK FWR1	sense
MVK.B5-2	GACATCCAGATGACMCAGWCTMC		
MVK.B5-3	GATATTGTGATGACMCAGGMC		
MVK.B5-4	GATGTTGTGATGACCCAACTCC		
MVK.B5-5	ARYATTGTGATGACCCAGWCTC		
MVK. κ	ACTGGATGGTGGGAAGATGGA	CK1	antisense
MVK.For.2	GTTAGATCTCCAGCTTGGTCCC	VK FWR4	antisense
Vector. V_H	CCATGACTCGCGGCCAGCCGGCCATGGCCSAGGTS MARCTGCAGSAGTCT	pHEN1- V_H FWR1	sense
V_L .Vector.2	TGCGGCCGCGCGTTTGATCTCCAGCTTGGTCCC	pHEN1-VK FWR4	antisense
H.SOE.Anti	ACCCGACCCACCAACCGCCGAGCCACCGCCACCTGAGGA GACGGTGACCGTGGTCCCTTGGCCCCAG		antisense
B5-2.SOE.Sense	TCGGGCGGTGGTGGGTGCGGTGGCGGCGGATCTGACA TCCAGATGACACAGACTACA		sense
M13.USP	GTAAAACGACGGCCAGT		sense
M13.RSP	CACACAGGAAACAGCTATGACCATG		antisense
pHEN.USP	CAGTCATAATGAAATACCTATTGCCTAC		sense
pHEN.RSP	CGTTAGTAAATGAATTTTCTGTATGAGG		antisense

non-recombinant background and was transformed into competent *E. coli* HB2151 (Carter *et al.*, 1985) for soluble expression of the scFv protein. Ampicillin-resistant colonies were screened for scFv using the MVH.FWR and MVK.FOR2 primers and positive clones were tested for production of soluble scFv protein.

Expression, purification and size exclusion chromatography

scFv was prepared in 2YT medium containing 100 µg/ml ampicillin, grown to log phase, induced with 1 mM IPTG and incubated with shaking for 24 h at 22°C. A monoclonal antibody against c-myc (encoded as a tag in pHEN), 9E10 (Evan *et al.*, 1985; ATCC, Rockville, MD, U.S.A.), was used to detect scFv expression by slot-blot analysis on nitrocellulose. scFv was purified from concentrated culture supernatant using an affinity column made with 9E10 antibody. The purified scFv was analysed on a Superose12 FPLC column (Pharmacia); scFv was detected by slot-blot using 9E10 antibody.

Flow cytometry and immunohistology

Binding of scFv to Mann, JVM13, HRIK, KM3 (all CD19⁺) and Jurkatt (CD19⁻) cell lines to peripheral blood and tonsil cells was detected using 9E10 antibody, biotinylated horse anti-mouse IgG (Vector Laboratories, Burlingame, CA, U.S.A.) and streptavidin-conjugated phycoerythrin (Caltag Laboratories, San Francisco, CA, U.S.A.). FMC63 antibody was used as a positive control, detected with biotinylated horse anti-mouse IgG and streptavidin-conjugated phycoerythrin. The ability of CHRI-19Fv1 and several CD19 antibodies to inhibit the binding of FMC63 and two other labelled CD19 antibodies was tested using JVM13 and PE labelled FMC63 (AMRAD, Melbourne, Australia), FITC labelled B43 and CyChrome-labelled HIB19 (Pharmingen, San Diego, U.S.A.). Cells (5×10^5 in 50 µl) were incubated with blocking antibody for 30 min at 4°C, washed, mixed with labelled antibody, incubated for 30 min at 4°C, washed and analysed. Samples were analysed using a FACScan (Becton Dickinson, San Jose, CA, U.S.A.) or an EPICS Elite cytometer (Coulter, Hialeah, FL, U.S.A.).

Frozen sections of human tonsil were examined using a high-sensitivity immunofluorescence staining method (Zola *et al.*, 1995).

Binding kinetics

Purified scFv (1.8 mg/ml) and intact FMC63 (3.0 mg/ml) in PBS were transferred to 0.1 M sodium borate buffer pH 8.6 by centrifugal desalting (Lee *et al.*, 1993) using Bio-gel P6Dg (Biorad, Australia). Aliquots (300 µCi) of ¹²⁵I-Bolton Hunter Reagent (¹²⁵I-BHR; Du Pont—AMRAD, Australia) (Bolton and Hunter, 1973) dissolved in dry benzene were dried down in V-bottom vials using a gentle stream of nitrogen. The vials were cooled on ice and the proteins added and incubated on ice for 2 h with occasional mixing. The labelled antibodies were purified by centrifugal buffer exchange to PBS. The

immunoreactivities and affinities of the labelled antibodies were determined as described by Lindmo *et al.* (1984). Briefly, 10 ng of labelled antibodies were mixed with concentrations of Daudi cells from 0.12×10^6 cells in 1.0 ml of medium at 4°C. After constant mixing by inversion for 45 min at 4°C the cells were washed three times with medium. Antibody binding was determined by comparison to standards. Immunoreactivity, expressed as percentage binding, was derived by double reciprocal plot of percentage binding against cell number. Affinity was determined by Scatchard analysis (Lindmo *et al.*, 1984); aliquots of unlabelled antibody from 0–5 µg were mixed with 10 ng of labelled antibody and 6×10^6 cells in 1.0 ml medium.

Radiochemical purity

Radiochemical purity of labelled antibody was analysed using thin-layer chromatography developed with 10% trichloroacetic acid. Labelled antibody (1 ng) was spotted on the TLC strip and developed until the front reached the top of the strip. Strips were divided in two and counted separately. Over 90% of radioactivity remained at the origin, bound to the antibody.

RESULTS

Isolation of antibody variable region genes

DNA sequences of the heavy and light chain variable region genes used to construct the scFv are shown in Fig. 1. The sequences are in frame and contain no stop codons. Alignment to GeneBank confirmed that both sequences are unique mouse immunoglobulin genes. Two other light chain sequences were isolated; one derived from the fusion partner (Carroll *et al.*, 1988) while the second contained four termination codons.

Assembly of scFv fragment

The SOE-PCR generated one major band at 740 bp (the expected size for the scFv fragment) and two minor bands at about 500 bp and 350 bp. Following cloning into pGem-T recombinant colonies containing an insert of 740 bp were amplified and cloned into pHen-1. Clone CD19.105 was used in further studies. Nucleotide sequencing confirmed that the insert was a correctly assembled scFv construct.

Expression of scFv fragment

Slot blot analysis indicated that more scFv was secreted into the culture supernatant at lower temperatures and we routinely used a temperature of 22°C for 24 h after induction with IPTG. Yields were approximately 200 µg per litre in the supernatant. scFv derived from culture supernatant was used for the experiments described. Approximately 40 µg per litre of culture was recovered from the cell pellet by freeze-thaw extraction and approximately 1 mg/l was obtained from disrupted cells following 6 h cultures in media containing 10 mM MgSO₄.

10	20	30	40	50	60
*	*	*	*	*	*
GAG GTG AAA CTG CAG	GAG TCA GGA CCT GGC	CTG GTG GCG CCC TCA	CAG AGC CTG TCC	GTC	
Glu Val Lys Leu Gln	Glu Ser Gly Pro Gly	Leu Val Ala Pro Ser	Gln Ser Leu Ser	Val>	
70	80	90	100	110	120
*	*	*	*	*	*
ACA TGC ACT GTC TCA	GGG GTC TCA TTA CCC	GAC TAT GGT GTA AGC	TGG ATT CGC CAG CCT		
Thr Cys Thr Val Ser	Gly Val Ser Leu Pro	Asp Tyr Gly Val Ser	Trp Ile Arg Gln	Pro>	
130	140	150	160	170	180
*	*	*	*	*	*
CCA CGA AAG GGT CTG	GAG TGG CTG GGA GTA	ATA TGG GGT AGT GAA	ACC ACA TAC TAT AAT		
Pro Arg Lys Gly Leu	Glu Trp Leu Gly Val	Ile Trp Gly Ser Glu	Thr Thr Tyr Tyr	Asn>	
190	200	210	220	230	240
*	*	*	*	*	*
TCA GCT CTC AAA TCC	AGA CTG ACC ATC ATC	AAG GAC AAC TCC AAG	AGC CAA GTT TTC TTA		
Ser Ala Leu Lys Ser	Arg Leu Thr Ile Ile	Lys Asp Asn Ser Lys	Ser Gln Val Phe	Leu>	
250	260	270	280	290	300
*	*	*	*	*	*
AAA ATG AAC AGT CTG	CAA ACT GAT GAC ACA	GCC ATT TAC TAC TGT	GCC AAA CAT TAT TAC		
Lys Met Asn Ser Leu	Gln Thr Asp Asp Thr	Ala Ile Tyr Tyr Cys	Ala Lys His Tyr	Tyr>	
310	320	330	340	350	360
*	*	*	*	*	*
TAC GGT GGT AGC TAT	GCT ATG GAC TAC TGG	GGT CAA GGA ACC TCA	GTC ACC GTC TCC TCA		
Tyr Gly Gly Ser Tyr	Ala Met Asp Tyr Trp	Gly Gln Gly Thr Ser	Val Thr Val Ser	Ser>	

CHRI-19Fv1 H chain

10	20	30	40	50	60
*	*	*	*	*	*
GAC ATC CAG ATG ACA	CAG ACT ACA TCC TCC	CTG TCT GCC TCT CTG	GGA GAC AGA GTC ACC		
Asp Ile Gln Met Thr	Gln Thr Thr Ser Ser	Leu Ser Ala Ser Leu	Gly Asp Arg Val	Thr>	
70	80	90	100	110	120
*	*	*	*	*	*
ATC AGT TGC AGG GCA	AGT CAG GAC ATT AGT	AAA TAT TTA AAT TGG	TAT CAG CAG AAA CCA		
Ile Ser Cys Arg Ala	Ser Gln Asp Ile Ser	Lys Tyr Leu Asn Trp	Tyr Gln Gln Lys	Pro>	
130	140	150	160	170	180
*	*	*	*	*	*
GAT GGA ACT GTT AAA	CTC CTG ATC TAC CAT	ACA TCA AGA TTA CAC	TCA GGA GTC CCA TCA		
Asp Gly Thr Val Lys	Leu Leu Ile Tyr His	Thr Ser Arg Leu His	Ser Gly Val Pro	Ser>	
190	200	210	220	230	240
*	*	*	*	*	*
AGG TTC AGT GGC AGT	GGG TCT GGA ACA GAT	TAT TCT CTC ACC ATT	AGC AAC CTG GAG CAA		
Arg Phe Ser Gly Ser	Gly Ser Gly Thr Asp	Tyr Ser Leu Thr Ile	Ser Asn Leu Glu	Gln>	
250	260	270	280	290	300
*	*	*	*	*	*
GAA GAT ATT GCC ACT	TAC TTT TGC CAA CAG	GGT AAT ACG CTT CCG	TAC ACG TTC GGA GGG		
Glu Asp Ile Ala Thr	Tyr Phe Cys Gln Gln	Gly Asn Thr Leu Pro	Tyr Thr Phe Gly	Gly>	
310	320	330	340	350	360
*	*	*	*	*	*
GGG ACT AAG TTG GAA	ATA ACA CGG GCT GAT	GCT GCA CCA ACT GTA	TCC ATC TTC CCA CCA		
Gly Thr Lys Leu Glu	Ile Thr Arg Ala Asp	Ala Ala Pro Thr	Val Ser Ile Phe	Pro>	

369
*
TCC AGT AAT
Ser Ser Asn>

CHRI-19Fv1 L chain

Fig. 1. DNA sequence of FMC63V_H and FMC63V_L. Sequences have been submitted to EMBL Nucleotide Sequence Database.

Affinity purification and molecular size

The scFv was eluted from the 9E10 column as a single peak. Polyacrylamide gel analysis showed a single band of approximately 30 kDa. Size exclusion chromatography confirmed a molecular weight of 30 kDa under non-dissociating conditions; virtually no dimer was detected.

Target cell binding demonstrated by flow cytometry

The scFv and the parent FMC63 monoclonal antibody both stained CD19⁺ B and pre-B lymphoblastoid lines Mann, JVM13 (Fig. 2), KM3 and HRIK, but showed no reactivity with the Jurkatt T cell line (data not shown). About 10% of peripheral blood lymphocytes and 50% of tonsil lymphocytes reacted with the scFv (Fig. 2). The intensity of staining of the scFv was less than that of the parent monoclonal antibody. Pre-mixing the scFv with 9E10 anti-c-myc antibody to make a divalent complex increased the staining intensity, but not to the level obtained with parent FMC63 (Fig. 2).

Staining of lymphoid tissue

The pattern of staining observed with the CD19 scFv was similar to that seen with the monoclonal FMC63 antibody, with germinal centres stained strongly and scattered staining in the interfollicular area (data not shown). Staining intensities were lower with the scFv than with the parent antibody.

Competition with parent and other CD19 monoclonal antibodies

CHRI-19Fv1 was able to inhibit by 85% the binding of parent FMC63 monoclonal antibody (Table 2). The scFv also strongly inhibited binding of the HIB19 and B43 CD19 antibodies (Table 2).

Binding kinetics

The labelling efficiencies for the intact FMC 63 and scFv were 41% and 48.4% and specific activities were 2.5 and 1.8 mCi/mg respectively. Binding to Daudi cells was measured in the cold to minimise internalisation of the radioconjugates. The immunoreactivities, as determined through double reciprocal plots shown in Fig. 3, were 54.3% for ¹²⁵I-BHR-FMC 63 and 13.1% for ¹²⁵I-BHR-scFv. The *K_d* and number of antibody binding sites, derived from Scatchard plots (Fig. 4), were 4.17×10^9 M⁻¹ and 32,000 sites per cell for the whole antibody and 2.32×10^9 M⁻¹ and 30,000 sites per cell for CHRI-19Fv1.

Potential immunogenicity

The amino acids present at the solvent exposed residues in the FMC63 heavy chain and light chain proteins are indicated in Table 3. For the light chain, 13 of the 15 amino acids present in the FMC63 sequence were common in human sequences. For the heavy chain, 20 of the 26 residues were common in human sequences.

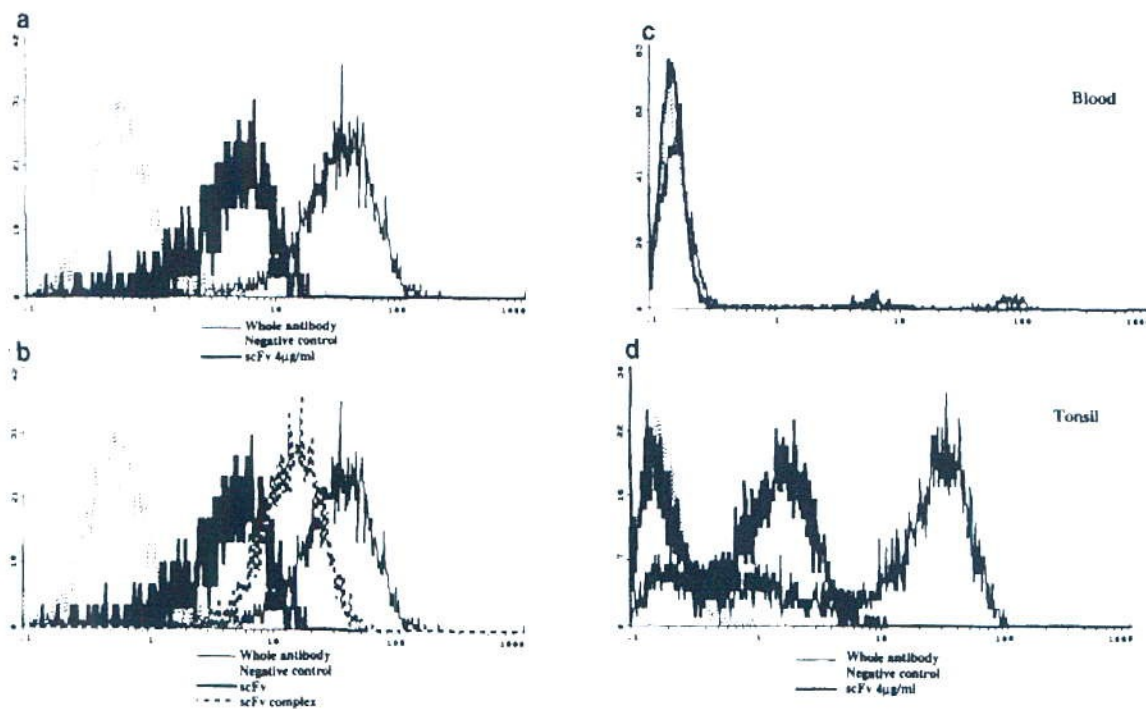


Fig. 2. **a:** Staining of the B lymphoblastoid cell line Mann with FMC63 scFv (bold trace) and parent antibody (normal trace). **b:** Staining of the B prolymphocytic leukaemia derived cell line JVM13 with FMC63 scFv (bold trace) and parent antibody (normal trace). The bold dotted trace shows scFv pre-complexed with anti-c-myc antibody. **c:** Staining of peripheral blood and tonsil **d:** cells with FMC63 scFv (bold trace) and parent antibody (normal trace). In each panel, the negative control X63 is shown as a dotted trace.

Table 2. Blocking of binding of directly-conjugated CD19 antibodies B43, FMC63 and HIB19 by CD19 antibodies and the CD19scFv

Blocking antibody	Reference	Staining antibody*		
		B43 (Pharmingen)	FMC63 (AMRAD)	HIB19 (Pharmingen)
X63	Negative control	100	100	100
FMC63	Zola <i>et al.</i> , 1991	16	3	8
CD19 scFv	This study	30	15	16
AB-1	Dorken <i>et al.</i> , 1989	73	30	55
B4	Nadler <i>et al.</i> , 1983	19	6	11
4G7 (Leu12)	Meeker <i>et al.</i> , 1984	21	2	8
HD37	Pezzutto <i>et al.</i> , 1987	31	10	10
HB15d	CD20 control	126	93	111

* Numbers represent mean fluorescence intensity, normalised to give a value of 100 when the blocking antibody is X63, a non-binding IgG1.

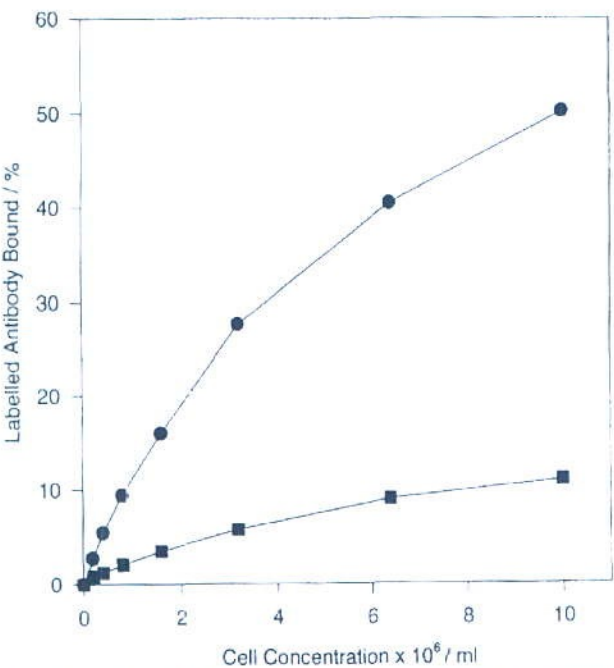


Fig. 3. Binding of ¹²⁵I-FMC 63 (circles) and ¹²⁵I-scFv (squares) to Daudi cells. Double inverse plots Lindmo *et al.* (1984) provided immunoreactive fraction values of 54.3% and 13.1% for the whole antibody and scFv respectively.

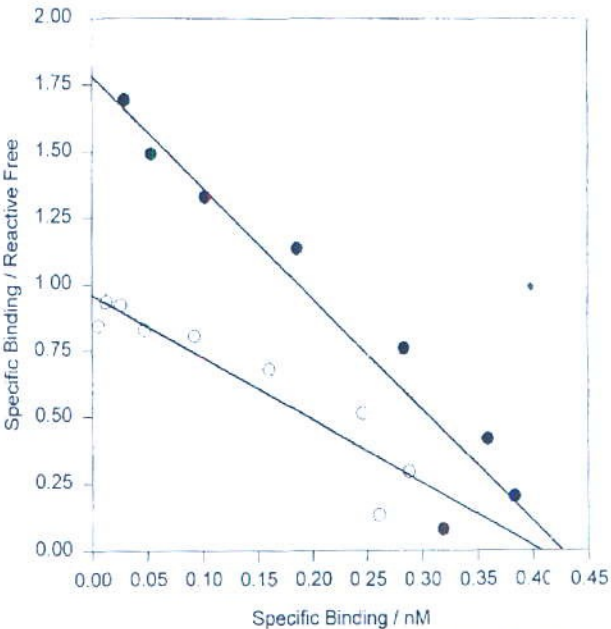


Fig. 4. Scatchard plots of binding of ¹²⁵I-FMC 63 (●) and ¹²⁵I-scFv (○) antibodies to CD19 expressing Daudi cells. From the slopes of the lines, the association constants for the two conjugates were $K_a = 4.2 \times 10^9 \text{ M}^{-1}$ and $2.3 \times 10^9 \text{ M}^{-1}$ respectively. From the intercept values at the abscissa, the binding capacity per cell were determined to be 32,000 and 30,000 molecules.

DISCUSSION

Functional immunoglobulin variable region gene sequences isolated from the CD19 hybridoma FMC63 were joined and used to encode a 30 kDa protein, CHRI-19Fv1. The scFv retained the specificity of the parent monoclonal antibody when tested against cell lines, blood and tonsil lymphocytes and frozen sections of human tonsil and was able to compete with parent antibody. Improved binding seen with a preformed divalent complex suggests that the reduction in binding affinity is at least in part due to the loss of valency. The linkage of the two V region domains using a peptide sequence, in

contrast to the natural antibody structure, may reduce affinity further. Scatchard analysis of the affinity of CHRI-19Fv1 and the parent antibody showed a 2-fold reduction in affinity. This reduction compares to 8–10-fold reductions reported for other scFvs (Milenic *et al.*, 1991; Wels *et al.*, 1992). It may be possible to increase the affinity by *in vitro* mutation and selection using phage display techniques (Winter *et al.*, 1994; Irving and Hudson, 1995) or by constructing a divalent form of the single chain antibody, which may be expected to show an

Table 3. Comparison of FMC63 surface exposed residues with accessible residues in human and mouse immunoglobulin variable regions. Numbering of residues is according to the Kabat system (Kabat *et al.*, 1990). np: FMC63 residue not listed as found at a frequency of 5% or greater.

Heavy Chain					Light Chain				
Residue Number	Mouse Amino Acids	Human Amino Acids	FMC63 V _H Amino Acid	Human Frequency	Residue Number	Mouse Amino Acids	Human Amino Acids	FMC63 V _L Amino Acids	Human Frequency
1	E, Q, D	E, Q	Q	46	1	D, Q, E	D, E, A, S	D	51
3	Q, K	Q, T	K	np	3	V, Q, L	V, Q, S, Y	Q	24
5	Q, V, L, K	V, L, Q	Q	13	5	T	T, L	T	61
9	G, P, A	G, A, P	P	18	9	S, A, L, P	P, S, G, A, L	S	26
10	E, G, S	G, A, E, D	G	53	15	L, P, V, A	P, V, L	L	12
11	L	L, V, F	L	61	18	R, K, S, Q, T	R, S, T, P	R	57
13	K, Q, R	K, Q, E	A	np	40	P, S	P	P	94
14	P, A	P	P	95	41	G, D	G	D	np
15	G, S	G, S, T	S	16	45	K, Q, R, T	K, R	K	43
19	K, S, R	R, K, S, T	S	17	57	G	G	G	91
26	G	G	G	96	60	D, A, S	D, S, A	S	25
28	T, S, N, A, D	T, S, N, I	S	32	67	S, I	S	S	96
41	P, H	P, S	P	84	70	D, S, A, K	D, T, S, E	D	43
42	G, E	G	R	np	80	A, P, T, E, Q	P, A, S, T	Q	np
43	K, Q, N, H	K, Q, R	K	76	81	E, D	E, D, G	F	71
61	E, P, D, A, Q	D, O, A, Q, T	S	np					
62	K, S, T	S, K, P	A	np					
64	K, Q	K, Q, R, N	K	53					
65	G, S, D	G, S, T	S	21					
73	T, K, N, D	T, D, N, K	N	19					
74	S, A	S	S	91					
75	S, K, Q	K, I, T, R	K	65					
83	T, R, K	R, T, K, D	Q	np					
84	S, A, T	A, P, S, T	T	8					
		E, A, D, S, Z,							
85	E, D	V	D	13					
87	T, S	T	T	91					

affinity similar to that of native divalent antibody (Pack *et al.*, 1993).

The major limit on therapeutic effectiveness of whole murine antibodies is the human anti-mouse antibody response (Grossbard *et al.*, 1993; Stone *et al.*, 1996). Murine antibodies lacking the murine *F_c* have exhibited reduced immunogenicity in humans (Hale *et al.*, 1988; LoBuglio *et al.*, 1989; Begent *et al.*, 1996). However, the variable regions of a murine antibody may retain some immunogenicity. Residual immunogenicity may be analysed by comparing the exposed amino acids present in mouse- and human-derived proteins (Pedersen *et al.*, 1994). For CHRI-19Fv1, 6 of 26 heavy chain amino acids and 2 of 15 light chain amino acids are very infrequent at the corresponding location in human antibody sequences (found in less than 5% of sequences). Analysis of other published CD19 antibody sequences showed a similar number of infrequent amino acids. Although the relationship between these differences and immunogenicity is indirect, the number of differences, coupled with the several other residues that are relatively infrequent (10–20%) suggests that immunogenicity may remain a clinical concern. This may be addressed by replacing potentially

immunogenic residues with more “human-like” residues (Roguska *et al.*, 1994; Rosok *et al.*, 1996), or by sequentially exchanging the V regions for sequences from human libraries (Jespers *et al.*, 1994).

FMC63 was selected in the present study because of its high affinity. The scFv retains significant binding activity. Radiolabelled scFv molecules may be useful for imaging (Begent *et al.*, 1996). The short *T_{1/2}* of scFv may limit their absolute tumour uptake, which has implications on therapy unless prolonged infusions are employed. Diabody/tribody scFv constructs may be suited to localisation studies and may also produce improved tumour/blood ratios for therapy (Casey *et al.*, 1996; Antoniwi *et al.*, 1996). Further genetic engineering of scFv may yield bispecific antibodies that activate NK or T cells at tumour sites (Renner *et al.*, 1996; Hartmann *et al.*, 1997).

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