SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

RAD: 9-3460- -17 **FECHA**: 2014-02-27 11:40:25
DEP: 2020 DIRECCION DE NUEVAS CREACIONES **EVE:** 378 FASE NACIONAL INCLUIDO CAPITULO I
TRA: 11 PCT-PATENTE DE INVENCION CAPITULOS I Y II **FOLIOS**: 10
ACT: 519 PRESENTACION

DIRECCIÓN DE NUEVAS CREACIONES

2020
Bogotá, D.C.,

Abogado(a)/Señor(a)
ERNESTO CAVELIER

Asunto: Radicación: 9-3460- -17
Trámite: 11
Evento: 378
Actuación: 519
Oficio: 2485
Folios: 10

Patente de Invención	**X**			Patente de Modelo de Utilidad	
Vía Nacional					
Vía PCT (Fase Nacional)	**X**	Capítulo I	**X**	Capítulo II	
No. Solicitud Internacional	PCT/IB2007/003074				
Fecha de Presentación Internacional	13/07/2007				

Como resultado del examen de fondo realizado, con fundamento en el Artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se comunica:

1. Documentos estudiados

Descripción:	Radicado No. 09-003460-00000-0000	16/Enero /2009
Reivindicaciones:	Radicado No. 09-003460-00000-0000	16/Enero /2009
	Radicado No. 09-003460-00010-0000	12/Marzo/2013
Dibujos:	Radicado No. 09-003460-00000-0000	16/Enero /2009
Oposiciones:	Radicado No. 09-003460-00003-0000	Procaps 19/Agosto/2010
Respuesta del solicitante:	Radicado No. 09-003460-00007-0000	10/Enero/2012
Prioridad:	EP 06291160.7	18/Julio/2006

2. Análisis de la Prioridad

Se acepta la reivindicación de prioridad porque ésta solicitud fue presentada dentro del plazo establecido (Art. 9 D 486) y su contenido técnico corresponde con el de la prioridad reivindicada.

3. Objeto de la invención

De acuerdo con lo establecido en el numeral 1.2.2.2 del capítulo primero del Título 10 de la Circular Única para efectos del examen de patentabilidad, se tendrá en cuenta la tasa pagada por el solicitante al momento de comenzar el examen para las reivindicaciones adicionales; esto es que cuando la solicitud contenga más de diez (10) reivindicaciones y no se haya pagado la tasa complementaria, sólo se estudiarán las cubiertas por la tasa pagada. En el presente caso, se tiene en cuenta la totalidad del nuevo capítulo reivindicatorio (Radicado No. 09-003460-00010-0000) para el examen de patentabilidad.

Título de la solicitud: “ANTICUERPOS ANTAGONISTAS DE EphA2 ANTICANCERÍGENOS”.

El problema planteado en esta solicitud consiste en la necesidad de diseñar anticuerpos antagonistas del receptor EphA2, inhibiendo su activación por el ligando efrina A1 e inhibiendo el crecimiento de las células tumorales dependiente de la quinasa de EphA2.

Para resolver este problema técnico la solicitud en estudio proporciona anticuerpos que se unen al receptor EphA2.

El objeto de la presente solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): C07K16/28.

4. De la solicitud de patente

Reivindicaciones (Art 30 D 486, Art 22 PCT)

Las reivindicaciones 1, 16, 17, 22, 23, 25, 26, 28, 29, 31, 32, 34, 35, 36, 38, 39, 41, 42, 44, 50, 51 carecen de concisión porque utilizan las expresiones “caracterizado porque dicho anticuerpo o fragmento de éste que se une al epítopo comprende uno o más regiones determinantes complementarias que tienen una secuencia de aminoácidos seleccionados de los grupos que consisten de las secuencias SEC ID No (…)”, “(…) donde dicho anticuerpo o fragmento de éste que se une al epítopo es un anticuerpo murino o un fragmento de éste que se une al epítopo y es producido por un hibridoma denominado 37.3D7”, “(…) en el que dicho hibridoma está depositado en la American Type Culture Collection con el número de registro PTA-7660”, “(…) un hibridoma denominado 37.1 F5, en el que dicho hibridoma está depositado en la American Type Culture Collection con el número de registro PTA-7661”, “(…) un hibridoma denominado 53.2H11, en el que dicho hibridoma está depositado en la American Type Culture Collection con el número de registro PTA-7662” “(…) hibridoma denominado EphA2-N1, en el que dicho hibridoma está depositado en la American Type Culture Collection con el número de registro PTM-8407”, “(…) o un hibridoma denominado EphA2-N2, en el que dicho hibridoma está depositado en la American Type Culture Collection con el número de registro PTM-8408.”, “(…) caracterizado porque dicho anticuerpo o fragmento de éste que se une al epítopo comprende una región variable de la cadena ligera que tiene una secuencia de aminoácidos seleccionada del grupo que consiste en las SEQ ID NO (…) ,“(…) caracterizado porque dicho anticuerpo o fragmento de éste que se une al epítopo comprende una región variable de la cadena pesada

que tiene una secuencia de aminoácidos seleccionada del grupo que consiste en las SEQ ID NO (…)", "(…) que comprende una cadena ligera variable que tiene una homología de secuencia de al menos aproximadamente 95% con (…)", lo cual dificulta determinar la extensión del objeto que se busca proteger. No se encuentra soporte en la descripción que un anticuerpo que comprenda solamente una secuencia de aminoácidos como entre las reclamadas, o que comprenda solamente un domino variable de cadena ligera o de cadena pesada, o una cadena pesada o una cadena ligera, o que presente una homología de secuencia de al menos 95%, comparta la actividad del anticuerpo reclamado. Es bien conocido en el estado del arte que la formación de un sitio de unión a antígeno intacto requiere la asociación de las regiones variables de las cadenas pesada y ligera, cada una de las cuales consiste en tres regiones determinantes de complementariedad (CDRs) y que incluso pequeños cambios en la secuencia de aminoácido de estas regiones, y particularmente de los CDRs, puede afectar dramáticamente la actividad de unión a antígeno. Se requiere al solicitante definir los anticuerpos producidos por los hibridomas a los que se hace referencia por la secuencia de aminoácidos de las cadenas pesada y ligera, la secuencia de aminoácidos de las regiones variables de las cadenas pesada y ligera o la secuencia de aminoácidos de las seis regiones determinantes de complementariedad (CDRs), en su conjunto.

Las reivindicaciones 2 – 15 utilizan las expresiones "(…) caracterizado porque dicho anticuerpo o fragmento de éste que se une al epítopo es capaz de inhibir el crecimiento de una célula cancerosa", "(…) caracterizado porque dicho anticuerpo o fragmento de éste que se une al epítopo es capaz de inhibir la migración de una célula cancerosa", "(…) caracterizado porque dicha célula cancerosa es una célula de un cáncer seleccionado del grupo que consiste en un cáncer de mama, cáncer de colon, cáncer de endometrio, carcinoma de ovario, osteosarcoma, cáncer cervical (…)", "(…) caracterizado porque dicho anticuerpo o fragmento de éste que se une al epítopo es capaz de inhibir la angiogénesis", "(…) caracterizado porque dicho anticuerpo o fragmento de éste que se une al epítopo es capaz de inhibir la fosforilación en tirosina de EphA2 en presencia de efrinaA1", que son resultados a alcanzar y no definen a la proteína de unión, puesto que la reivindicación incluiría no sólo la solución propuesta por el solicitante, sino todas las alternativas presentes o futuras que lleguen a ese resultado. Se requiere reemplazarlo por las características esenciales o estructurales que contribuyen a la obtención de ese efecto, como la secuencia de aminoácidos de las cadenas pesada y ligera, la secuencia de aminoácidos de las regiones variables de las cadenas pesada y ligera o la secuencia de aminoácidos de las seis regiones determinantes de complementariedad (CDRs), en su conjunto.

Las reivindicaciones 49 – 52 carecen de concisión porque utilizan las expresiones "un agente citotóxico", "un maitansinoide", "un fármaco pequeño", "un derivado de tomaimicina", "un derivado de leptomicina", "un profármaco", "un taxoide", "un análogo de CC-1065", (…)", lo cual dificulta determinar la extensión del objeto que se busca proteger. Se sugiere definir estos agentes por sus características técnicas esenciales o estructurales.

La reivindicación 58 hace referencia a una composición farmacéutica. Se recuerda al solicitante que una composición se caracteriza por sus componentes y proporciones dentro de la misma.

Las reivindicaciones 59 – 62 carecen de concisión porque utilizan las expresiones "un polinucleótido", "(…) caracterizado porque dicho polinucleótido tiene una secuencia que presenta una homología de al menos el 80% con un polinucleótido seleccionado del grupo que consiste en las SEQ ID Nos (…)", "un vector recombinante", "una célula anfitriona". Se recuerda al solicitante que un ácido

nucleico se caracteriza por su secuencia de polinucleótidos. Igualmente se sugiere definir el tipo de célula y el hibridoma por su número de depósito. Se requiere igualmente aclarar la dependencia de las reivindicaciones 60 y 61.

Se requiere al solicitante hacer las aclaraciones necesarias.

5. Unidad de invención (Art 25 D 486)

La Solicitud no cumple con el Requisito de Unidad de Invención porque se refiere a 5 Grupos Inventivos.

Los Grupos Inventivos están definidos por las siguientes características esenciales:

1° Anticuerpo 37.3D7 que comprende 6 CDRs de SEQ ID Nos: 1-6. Reivindicaciones 1 – 63, parcialmente.
2° Anticuerpo 37.1F5 que comprende 6 CDRs de SEQ ID Nos: 7-12. Reivindicaciones 1 – 63, parcialmente.
3° Anticuerpo 53.2H11 que comprende 6 CDRs de SEQ ID Nos: 13-18. Reivindicaciones 1 – 63, parcialmente.
4° Anticuerpo EphA2-N1 que comprende 6 CDRs de SEQ ID Nos: 61-66. Reivindicaciones 1 – 63, parcialmente.
5° Anticuerpo EphA2-N2 que comprende 6 CDRs de SEQ ID Nos: 67-72. Reivindicaciones 1 – 63, parcialmente.

No se guarda un concepto común inventivo porque los anticuerpos 37.3D7, 37.1F5, 53.2H11, EphA2-N1 y EphA2-N2 comprenden diferentes conjuntos de CDRs, de manera que no comparten una estructura esencial para la actividad común.

Se sugiere presentar las Solicitudes Divisionales que considere necesarias, ó limitar la solicitud inicial a una sola invención, eliminando de ella las otras invenciones.

6. Análisis de la Oposición

Argumentaciones de Procaps

La sociedad opositora afirma que las reivindicaciones de la solicitud presentada no sólo están referidas a un anticuerpo, sino también hacen alusión al uso y a un método de tratamiento, y por lo tanto no son patentables según la legislación. Igualmente, menciona que la solicitud no es novedosa a la luz del documento WO 2004092343 que revela métodos y composiciones diseñadas para el tratamiento o la prevención de un trastorno de las células hipoproliferativas, especialmente los trastornos derivados de la destrucción, derramamiento, o la proliferación de los linfocitos intraepiteliales que comprende la administración de uno o más agentes antagonistas de EphA2 solos o en combinación con uno o varios otros agentes útiles para la terapia para un trastorno de las células hipoproliferativas.

Respuestas del Solicitante

Frente a tales argumentos, la solicitante afirma que el documento D1 da a conocer agentes antagonistas de EphA2, incluyendo anticuerpos, para el tratamiento de los trastornos de las células hipoproliferativas como la cistitis intersticial o enfermedad inflamatoria intestinal, mientras que los anticuerpos de acuerdo con la presente solicitud tienen por objeto impedir el crecimiento de células cancerosas, es decir

trastornos hiperproliferativos celulares y que por lo tanto, el documento D1 no revela el objeto de la solicitud.

Respuesta del Examinador Técnico

De acuerdo con lo expuesto, frente al nuevo capítulo reivindicatorio aportado, los argumentos presentados por la sociedad opositora no prosperan como elementos que permitan desvirtuar la patentabilidad de la solicitud que aquí se analiza.

7. Determinación del Estado de la Técnica

El estado de la técnica se determinó con base en la fecha de presentación de la solicitud de prioridad: 18 de julio de 2006, y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

Ítem	Documento	Título	Fecha de Publicación
D3	EP 0368684	"Single Domain Ligands, Receptors Comprising Said Ligands, Methods for Their Production, and Use of Said Ligands and Receptors"	17/Enero/1974
D4	Cancer Research, Vol. 54, Págs. 6160-6166	"Chimeric (Mouse/Human) Anti-Colon Cancer Antibody c30.6 Inhibits the Growth of Human Colorectal Cancer Xenografts in scid/scid Mice"	1/Diciembre/1994

D3[1] divulga un fragmento de anticuerpo que comprende la secuencia de aminoácidos SYWMH (Fig. 10A, Kabat IIB, A2).

D4[2] enseña un fragmento de anticuerpo que comprende la secuencia de aminoácidos DYNMH (Fig. 1A).

8. Examen de Patentabilidad (Art 14 D486)

Novedad (Art 16 D486)

La reivindicación 1 consiste en un anticuerpo o un fragmento de éste que se une al epítopo que se une específicamente a un receptor EphA2 y que es un antagonista de dicho receptor, caracterizado porque dicho anticuerpo o fragmento de éste que se une al epítopo comprende uno o más regiones determinantes complementarias que tienen una secuencia de aminoácidos seleccionados de los grupos que consisten de las secuencias SEC ID No 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71 y 72; donde dicho anticuerpo o fragmento de éste que se une al epítopo es un anticuerpo murino o un fragmento de éste que se une al epítopo y es producido por un hibridoma denominado 37.3D7, en el que dicho hibridoma está depositado en la American Type Culture Collection con el número de registro PTA-7660; un hibridoma denominado 37.1 F5, en el que dicho hibridoma está depositado en la American Type Culture Collection con el número de registro PTA-7661; un hibridoma denominado 53.2H11, en el que dicho hibridoma está depositado en la American Type Culture Collection con el número de registro PTA-7662; un hibridoma denominado EphA2-N1, en el que dicho hibridoma está depositado en la American Type Culture Collection con el número de registro PTM-8407; o un hibridoma denominado EphA2-N2, en el que dicho hibridoma está depositado en la American Type Culture Collection con el número de registro PTM-8408.

1 http://worldwide.espacenet.com/publicationDetails/biblio?DB=EPODOC&II=0&ND=3&adjacent=true&locale=en_EP&FT=D&date=19900516&CC=EP&NR=0368684A1&KC=A1

2 http://www.ncbi.nlm.nih.gov/pubmed/7954462

Características esenciales	D3	D4
Un anticuerpo o un fragmento de éste que se une al epítopo que se une específicamente a un receptor EphA2 y que es un antagonista de dicho receptor, caracterizado porque dicho anticuerpo o fragmento de éste que se une al epítopo comprende uno o más regiones determinantes complementarias que tienen una secuencia de aminoácidos seleccionados de los grupos que consisten de las secuencias SEC ID No 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71 y 72; donde dicho anticuerpo o fragmento de éste que se une al epítopo es un anticuerpo murino o un fragmento de éste que se une al epítopo y es producido por un hibridoma denominado 37.3D7, en el que dicho hibridoma está depositado en la American Type Culture Collection con el número de registro PTA-7660; un hibridoma denominado 37.1 F5, en el que dicho hibridoma está depositado en la American Type Culture Collection con el número de registro PTA-7661; un hibridoma denominado 53.2H11, en el que dicho hibridoma está depositado en la American Type Culture Collection con el número de registro PTA-7662; un hibridoma denominado EphA2-N1, en el que dicho hibridoma está depositado en la American Type Culture Collection con el número de registro PTM-8407; o un hibridoma denominado EphA2-N2, en el que dicho hibridoma está depositado en la American Type Culture Collection con el número de registro PTM-8408.	Un fragmento de anticuerpo que comprende la secuencia de aminoácidos SYWMH (Fig. 10A, Kabat IIB, A2)	Un fragmento de anticuerpo que comprende la secuencia de aminoácidos DYNMH (Fig. 1A)

Las características esenciales del anticuerpo de la reivindicación 1 habían sido divulgadas previamente en los documentos D3 y D4 porque:

El documento D3 enseña un fragmento de anticuerpo que comprende la secuencia de aminoácidos SYWMH (Fig. 10A, Kabat IIB, A2), que corresponde a la SEQ ID NO: 1 (SYWMH) de la presente solicitud.

El documento D4 divulga un fragmento de anticuerpo que comprende la secuencia de aminoácidos DYNMH (Fig. 1A), que corresponde a la SEQ ID NO: 67 (DYNMH) de la presente solicitud. El documento D4 también enseña un inmunocongujado del anticuerpo con metotrexato (Pág. 6160, Col. derecha, líneas 28 y 29).

En consecuencia, la reivindicación 1 y las reivindicaciones 2 – 29, 48, 49, 52 y 58 carecen de novedad, según lo divulgado en los documentos D3 o D4.

La reivindicación 59 se refiere a un polinucleótido que codifica un polipéptido seleccionado del grupo que consiste en las SEQ ID Nos: 20, 22, 24, 26, 28, 30, 32, 34, 36, 37, 38, 40, 42, 43, 45, 47, 48, 49, 50, 52, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 74, 76, 78 y 80.

La siguiente tabla presenta la comparación entre las características técnicas esenciales mencionadas en la reivindicación 59, y las reveladas en el documento D4.

Características esenciales	D4
Un polinucleótido que codifica un polipéptido seleccionado del grupo que consiste en las SEQ ID Nos: 20, 22, 24, 26, 28, 30, 32, 34, 36, 37, 38, 40, 42, 43, 45, 47, 48, 49, 50, 52, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 74, 76, 78 y 80.	Un fragmento de anticuerpo que comprende la secuencia de aminoácidos DYNMH (Fig. 1A)

De acuerdo con la tabla anterior, las características esenciales del polinucleótido de la reivindicación 59 habían sido divulgadas previamente en el documento D4 porque enseña un fragmento de anticuerpo de secuencia DYNMH y el nucleótido que lo codifica que tiene la secuencia GACTACAACATGCAC (Fig. 1A), siendo el fragmento de anticuerpo idéntico a la secuencia de aminoácidos SEC ID NO: 67 (DYNMH) de la presente solicitud. El documento D4 también enseña los vectores y células anfitrionas para la obtención del anticuerpo (Pág. 6161, Col. izquierda, líneas 17-33).

En consecuencia, la reivindicación 59 y las reivindicaciones 60 – 62 no son nuevas, según lo divulgado en el documento D4.

Nivel inventivo (Art18 D486)

La reivindicación 30 consiste en un anticuerpo o fragmento de éste que se une al epítopo según cualquiera de las reivindicaciones 1-17, caracterizado porque dicho anticuerpo o fragmento de éste que se une al epítopo comprende al menos una cadena pesada y al menos una cadena ligera, en el que dicha cadena pesada comprende tres regiones determinantes de la complementariedad secuenciales que tienen las secuencias de aminoácidos representadas por las SEQ ID NOS: 67, 68, y 69, y en el que dicha cadena ligera comprende tres regiones determinantes de la complementariedad secuenciales que tienen las secuencias de aminoácidos representadas por las SEQ ID NOS: 70, 71, y 72.

Características esenciales	D4
Un anticuerpo o fragmento de éste que se une al epítopo según cualquiera de las reivindicaciones 1-17, caracterizado porque dicho anticuerpo o fragmento de éste que se une al epítopo comprende al menos una cadena pesada y al menos una cadena ligera, en el que dicha cadena pesada comprende tres regiones determinantes de la complementariedad secuenciales que tienen las secuencias de aminoácidos representadas por las SEQ ID NOS: 67, 68,	Un anticuerpo que comprende una cadena pesada que comprende las regiones de complementariedad: CDR1: DYNMH CDR2: FIYPYNAGTGYNQKFKN CDR3: NDPHWYFV Y una cadena ligera que comprende las regiones de complementariedad CDR1: KSSQSLFNSRTRKNYLA CDR2: WASTRES CDR3: QSYNLYT

y 69, y en el que dicha cadena ligera comprende tres regiones determinantes de la complementariedad secuenciales que tienen las secuencias de aminoácidos representadas por las SEQ ID NOS: 70, 71, y 72.	(Fig. 1B)

El documento D4 es considerado el estado de la técnica cercano a la invención definida en la reivindicación 30 porque divulga un anticuerpo con actividad antitumoral que comprende las secuencias de cadena pesada y ligera que tienen los CDRs mostrados en la tabla 3 (Fig. 1A y 1B), donde la CDR1 de la cadena pesada es idéntica a la secuencia SEQ ID NO: 67 de la solicitud, y el CDR2 de la cadena pesada tiene un porcentaje de identidad del 88.24% con la secuencia SEQ ID NO: 68 de la presente solicitud:

```
  CLUSTAL 2.1 multiple sequence alignment

Sequence type explicitly set to Protein
Sequence format is Pearson
Sequence 1: D4 CDR2            17 aa
Sequence 2: SEQ ID NO:68       17 aa
Start of Pairwise alignments
Aligning...

D4 CDR2          FIYPYNAGTGYNQKFKN 17
SEQ ID NO:68     FIYPYNGGTGYNQRFKN 17
                 ******.******:***

Sequences (1:2) Aligned. Score: 88.24
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El documento D4 también enseña líneas celulares del anticuerpo (Pág. 6161, Col. derecha, líneas 33 a 50), así como inmunoconjugados del anticuerpo con ingredientes anticancerígenos (Pág. 6160, Col. derecha, líneas 28 y 29).

La diferencia entre la invención, definida en la reivindicación 30 y el anticuerpo del documento D4 consiste en las secuencias SEQ ID NOs: 69, 70, 71 y 72.

El efecto que logra el anticuerpo que tiene las secuencias SEQ ID Nos: 69, 70, 71 y 72 es la inhibición del crecimiento y supervivencia de células tumorales de colon HT-29 y LoVo; línea celular pancreática CFPAC-2 y BxPC3; y de melanoma UACC-257; así como la supresión del crecimiento del xenoinjerto de cáncer de colon humano HT-29 en ratones y la inhibición de metástasis mamarias tempranas MDA-MB-231 (Fls. 344 a 349).

El problema técnico objetivo que pretende resolver esta invención se puede formular así: cómo modificar el anticuerpo conocido en el documento D4 para proporcionar un anticuerpo alternativo para el tratamiento del cáncer.

Ahora bien, teniendo en cuenta que el documento D4 da a conocer anticuerpos con actividad antitumoral que producen lisis significativa de las líneas celulares tumorales COLO 205, HT29, LIM 1899 y LoVo (Pág. 6162, Col. derecha, líneas 54 a 5) y presenta una constante de afinidad de 1,5 X 10^8 (Pág. 6162, Col. derecha, líneas 39-43), el anticuerpo de la reivindicación 30 corresponde a una alternativa que no presenta ningún efecto técnico inesperado.

Por lo tanto la persona normalmente versada en la materia obtendría un anticuerpo alternativo para llegar al objeto de la reivindicación 30 con la expectativa razonable de que la actividad se conserve, lo cual es considerado obvio y por lo tanto, sin altura inventiva.

Puesto que las reivindicaciones dependientes 31 – 47, 50, 51, 53, 54 – 57 y 63 corresponden a otras realizaciones preferidas que tampoco presentan un efecto técnico inesperado, se considera que tales reivindicaciones tampoco tienen nivel inventivo.

9. Conclusión

Los requisitos de Patentabilidad de la presente solicitud están afectados así:

Ítem	Documento	Reivindicaciones afectadas	Requisito que afecta
D3	EP 0368684	1 – 29, 48, 49, 52 y 58	Novedad
D4	Cancer Research, Vol. 54, Págs. 6160-6166	1 – 63	Novedad y Nivel inventivo

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de sesenta (60) días contados a partir del día siguiente de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Se invita al solicitante se sirva indicar, si así lo considera, su renuncia al término faltante de sesenta (60) días para dar respuesta a este requerimiento y presentarla por escrito.

Finalmente, se le recuerda al solicitante que si como respuesta a este requerimiento llegara a modificar el capítulo reivindicatorio y éste contenga más de diez (10) reivindicaciones, no pagará la tasa de modificación voluntaria pero sí la correspondiente al pago por reivindicación adicional.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única No. 10 de 2001.

JOSÉ LUIS SALAZAR LÓPEZ
Director de Nuevas Creaciones (c)

El anterior requerimiento fue notificado por fijación en lista, de fecha 2014-03-04

Elaboró: Moncaleano Prado Juan David
Revisó: Consuelo Leguizamon Leguizamon

Chimeric (Mouse/Human) Anti-Colon Cancer Antibody c30.6 Inhibits the Growth of Human Colorectal Cancer Xenografts in *scid/scid* Mice

Peter F. Mount, Vivien R. Sutton, Wenjun Li, et al.

Cancer Res 1994;54:6160-6166.

[CANCER RESEARCH 54, 6160–6166, December 1, 1994]

Chimeric (Mouse/Human) Anti-Colon Cancer Antibody c30.6 Inhibits the Growth of Human Colorectal Cancer Xenografts in *scid/scid* Mice[1]

Peter F. Mount, Vivien R. Sutton, Wenjun Li, Jane Burgess, Ian F.C. McKenzie, Geoffrey A. Pietersz, and Joseph A. Trapani[2]

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ABSTRACT

The mouse monoclonal antibody, m30.6 (IgG2b), detects an antigenic determinant expressed predominantly on the surface of colorectal adenocarcinoma cells and has been shown previously to be a potentially useful therapeutic and diagnostic reagent for human colon cancer. We report the production and characterization of a mouse/human chimeric antibody, c30.6, with potent *in vitro* and *in vivo* antitumor activity. The genes encoding the variable domains for heavy and light chains were amplified by thermal cycling using degenerate oligonucleotide primers complementary to conserved immunoglobulin framework sequences. The gene segments were sequenced, subcloned into eukaryotic expression vectors containing human constant region genes (IgG1 and κ), and cotransfected into nonsecreting Sp2/0 mouse myeloma cells. There were significant differences in the biological activities of the murine and chimeric antibodies. The i.p. administration of c30.6 but not of m30.6 produced a marked growth inhibition of s.c. 30.6$^+$ COLO 205 tumors in *scid/scid* mice (~40% reduction in tumor size, measured 21 days after tumor inoculation). Reduced tumor growth was not due to altered binding characteristics of c30.6 because: (*a*) the chimeric antibody was shown by flow cytometry to bind exclusively to cell lines that expressed the 30.6 determinant; (*b*) c30.6 was able to completely inhibit the binding of m30.6 on 30.6$^+$ cells; and (*c*) the affinity of binding of the two antibodies was the same (K_a, ~1.50 × 10^8). Up to 15% of the total injected antibody dose/g tissue was localized in 30.6$^+$ tumors at 24 h, ~13% was present in the tumors at 48 h, and ~10% was present at 72 h. Furthermore, c30.6 demonstrated a shorter circulating half-life (53 h; m30.6, 72 h) when given i.p. to C57BL6 × BALB/c F_1 mice. Unlike m30.6, c30.6 was also strongly active in antibody-dependent cell-mediated cytotoxicity against a range of 30.6$^+$ tumor target cells *in vitro*. Up to 80% specific ^{51}Cr release was achieved using either freshly isolated human peripheral blood mononuclear cells or 2-day-old interleukin 2-stimulated human peripheral blood mononuclear cells as effectors. The enhanced antitumor activity of c30.6 suggests that it might be a useful immunotherapeutic reagent for colorectal carcinoma.

INTRODUCTION

CRC[3] is among the most common causes of cancer death in many Western countries. Presently available options for combating the high mortality and the morbidity associated with CRC are inadequate, and only those cases diagnosed at an early stage (tumor confined to the wall of the colon) are likely to be cured by surgical resection. Once a tumor has spread beyond the colonic wall, the prognosis is much poorer (1, 2), albeit that treatment with the cytotoxic agents 5-fluorouracil and levamisole in an adjuvant setting has been demonstrated to afford some prolongation of survival and some increase in the cure rate in this group (3). Unfortunately, there is no reliable screening test for early CRC.

One strategy subject to much investigation has been the use of mAbs directed against CRC-associated antigens. Apart from their usefulness as the basis for screening tests and in diagnostic immunoscintigraphy, mAbs are potentially useful therapeutic reagents that are occasionally effective when used alone (4), or they can be used to deliver toxins (5), drugs (6), or radioactive isotopes (7) to tumors. Until recently, mAbs have almost invariably been of rodent origin, resulting in a HAMA response in patients, which accelerates the clearance of the mAb and precludes its repeated administration due to the risk of hypersensitivity reactions. To overcome this problem, recombinant DNA technology has been used to produce chimeric Abs combining the variable domains of the parental mouse antibodies and the human constant domain (8–11). Alternatively, CDR-grafted antibodies have used minimal antigen-binding domains to further reduce the mouse sequences in the recombinant product and to more faithfully mimic the original 3-dimensional antigen-binding domains (12). As well as reducing immunogenicity, these approaches enable the acquisition of improved effector functions, including antigen-dependent cell-mediated cytotoxicity and complement-mediated cytotoxicity.

The mouse mAb designated 30.6 (m30.6) (13) recognizes an antigen that is expressed on a high proportion of CRCs and has shown diagnostic and therapeutic potential. Immunoscintigraphy has shown that it is capable of localizing to primary and secondary tumor deposits in humans (14, 15). Furthermore, ^{131}I-radioimmunoconjugates (16) and methotrexate (17) immunoconjugates of 30.6 produced regression of human CRC xenografts in nude mice. However, in a phase I clinical trial, administration of m30.6 provoked a HAMA response that precluded the possibility of its repeated administration (18). We now present the production and characterization of a chimeric mAb, c30.6. The specificity and affinity of c30.6 are identical to those of m30.6, and its ability to inhibit the growth of 30.6$^+$ tumors in *scid/scid* (scid) mice demonstrates that this chimeric antibody is a good candidate for further development as a therapeutic reagent for CRC.

MATERIALS AND METHODS

Mice. Studies involving mice were carried out at the Biological Research Laboratory, Austin Research Institute. mAbs were purified from ascites fluid derived from CBA x BALB/c F_1 mice (m30.6) or BALB/c scid mice (c30.6) bearing i.p. tumors. m30.6 and c30.6 were purified by affinity chromatography on protein A-Sepharose (Pharmacia).

Cells and Cell Lines. The human CRC cell lines COLO 205, HT 29, LIM 1899, and LOVO and the human leukemia cell lines Sultan, Daudi, and K562 were maintained in RPMI supplemented with 10% (v/v) fetal calf serum (Flow Laboratories, Sydney, Australia), 2 mM glutamine (Commonwealth Serum Laboratories, Melbourne, Australia), 100 units/ml penicillin (Commonwealth Serum Laboratories), and 100 μg/ml streptomycin (Glaxo, Melbourne, Australia). PBMC were isolated from healthy donors by centrifugation through Ficoll-Paque (Pharmacia, Piscataway, NJ) and resuspensed in medium supplemented with 100 units/ml recombinant human IL-2, a kind gift of Dr. Mark Smyth, Biological Response Modifiers Program, National Cancer Institute, Frederick, MD.

Received 3/21/94; accepted 10/4/94.

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[1] This study was supported by the National Health and Medical Research Council of Australia Program Grant. J. A. T. currently holds a Wellcome Senior Research Fellowship in Medical Science. V. R. S. is supported by a R. Douglas Wright Fellowship from the National Health and Medical Research Council of Australia.

[2] To whom requests for reprints should be addressed.

[3] The abbreviations used are: CRC, colorectal carcinoma; ADCC, antibody-dependent cell-mediated cytotoxicity; V_H, immunoglobulin heavy chain variable domain; V_L/V_K, immunoglobulin lambda/kappa chain variable domain; HAMA, human anti-mouse antibody; CDR, complementarity-determining region; PBMC, peripheral blood mononuclear cells; E/T, effector/target; FITC, fluorescein isothiocyanate; scid, *scid/scid;* mAb, monoclonal antibody; PBS, phosphate-buffered saline; IL-2, interleukin 2.

Isolation of 30.6 Variable Region Genes. Thermal cycling was used to isolate mouse variable region gene segments. Cytoplasmic RNA was extracted from m30.6 hybridoma cells as described (19). Complementary DNA was synthesized from RNA by reverse transcription with mouse Moloney virus reverse transcriptase (BRL, Gaithersburg, MD) using random primers. The oligonucleotide primers used for thermal cycling were based on those published by Orlandi *et al.* (20) (Table 1). Reactions for the amplification of V_H genes used the primers VH1FOR2, VH1BACK1, VH1BACK2, VH1BACK3, and VH1BACK4. For amplification of V_K gene segments the primers used were VK1FOR and VK1BACK. The reaction products were analyzed by electrophoresis through 2% (w/v) agarose gels, purified on DEAE-cellulose (NA-45 paper; Schleicher and Schuell, Keene, NH), and cloned into *Sma*I-digested pBluescript (Stratagene, La Jolla, CA). Nucleotide sequencing was performed by the dideoxy method on double-stranded plasmid templates of four (V_H) or five (V_K) independent clones using Sequenase (United States Biochemical, Cleveland, OH).

Construction of Chimeric Antibody Expression Vectors. A modification of the method used by Orlandi *et al.* (20) was used. The vectors KS.M.V_H and KS.P.MV_K were constructed by substituting the pBluescript vector backbone for M13 in the original vectors, M13-VHPCR1 and M13-VKPCR1. Initially, V_H and V_K thermal cycling products were subcloned as blunt-ended fragments into pBluescript KS+. The presence of an internal *Pst*I site necessitated that the V_H segment be cloned into KS.M.V_H in two stages: (*a*) a 63-base pair 3′ *Pst*I-*Bst*EII fragment was force-cloned into the vector, and then the remaining 262-base pair *Pst*I fragment was inserted in the correct orientation into the *Pst*I site. The V_H expression cassette, comprising the immunoglobulin promoter, leader sequence, leader intron, and 3′ noncoding sequences, was recovered by digestion with *Hind*III and *Bam*HI and subcloned into the eukaryotic expression vector pSV.gpt, containing a human IgG1 constant-region gene. V_K gene sequences were removed from pBluescript by digestion with *Bgl*II and *Pvu*II and force-cloned into KS.P.MVK. The V_K expression cassette was then released with *Hind*III and *Bam*HI and subcloned into the expression vector pSV-hyg, which contains the human K-chain constant domain gene.

Transfection of Sp2/0 Cells. Plasmid DNA (20 μg) was linearized with *Pvu*I, reprecipitated, and introduced by electroporation into Sp2/0 cells. Cells were plated into 96-well tissue culture plates with 2.5×10^4 thymocyte feeder cells/well. Selection in medium supplemented with hygromycin B (400 μg/ml; Sigma Chemical Company, St. Louis, MO) was commenced on the next day. Wells containing transfectant colonies were screened for secretion of human immunoglobulin at 12–14 days using an enzyme-linked immunosorbent assay. Flexible polyvinyl chloride plates were coated with sheep anti-human immunoglobulin (50 μg/ml; Silenus, Hawthorn, Australia) and nonspecific binding sites were blocked with 2% bovine serum albumin in PBS. Hybridoma culture supernatant (100 μl) was added and incubated at 37°C for 1 h. Bound human immunoglobulin was detected with sheep anti-human immunoglobulin coupled to horseradish peroxidase (Amersham International, Sydney, Australia). The color reaction was developed using 50 μl of 0.03% [2,2′-azino-di-(3-ethylbenthiozoline)sulfonate] (Amersham) and read with an enzyme-linked immunosorbent assay plate reader at 405 nm. Wells expressing human immunoglobulin were cloned by limiting dilution, and the resultant clones were retested, expanded, and cryopreserved in liquid N_2.

Flow Cytometry. Cells (50 μl at 5×10^6 cells/ml) were washed three times in PBS and incubated with antibodies on ice for 60 min. After three washings in 0.5% PBS-0.1% bovine serum albumin-sodium azide, bound immunoglobulin was detected with rabbit anti-immunoglobulin (human or mouse) conjugated to FITC (ICN Immunobiologicals, Sydney, Australia). Fluorescence was measured by flow cytometry using a FACScan analyzer (Becton Dickinson, Mountain View, CA).

Radioiodination. c30.6 (100 μg) was radiolabeled with ^{125}I using chloramine-T (21) (2 min at 4°C). Free iodine was separated from radiolabeled protein by chromatography on Sephadex G-25 (PD-10 columns; Pharmacia Fine Chemicals, Uppsala, Sweden). Fractions of the initial (antibody) peak were easily distinguished from free ^{125}I denoted by a discrete second peak of radioactivity. These fractions were pooled and stored at 4°C until used. Binding affinity was determined by Scatchard analysis (22).

Competitive Binding Assay. c30.6 antibody was labeled with FITC (23). COLO 205 cells (3×10^5) were incubated with FITC-c30.6 (1.74 μg; molar incorporation of 1.5) and various concentrations of either c30.6 or m30.6 for 1 h in PBS-2% fetal calf serum. Cells were then washed three times and fluorescence was measured on a FACScan (Becton Dickinson).

ADCC. Target cells (2×10^6) were labeled with 100 μCi of sodium [^{51}Cr]chromate for 60 min at 37°C in 100 μl of RPMI supplemented as above. The target cells were washed and incubated with antibody (25 or 5 μg/ml) at 5×10^5 cells/ml. Effector cells were either freshly isolated human PBMC or PBMC-incubated for 36 h in medium supplemented with 100 units/ml recombinant human IL-2. Target and effector cells were mixed at various E/T ratios and incubated at 37°C for 4 h in U-bottomed wells. All assays were performed in triplicate. The percentage lysis was calculated as

$$\% \text{ of lysis} = 100 \times \frac{\text{test lysis} - \text{spontaneous lysis}}{\text{total lysis} - \text{spontaneous lysis}}$$

The spontaneous ^{51}Cr release was calculated from target cells incubated alone, and the maximum ^{51}Cr release was calculated from target cells incubated in medium containing 1.0% sodium dodecyl sulfate.

Biodistribution Studies. The biodistribution of ^{125}I-labeled mAb was analyzed in a mouse tumor model established by injecting the human COLO 205 cell line into BALB/c-scid mice. Mice were given 5×10^6 COLO 205 cells (in 0.2 ml PBS) s.c. on the anterior abdominal wall. Five days later, groups of four mice received c30.6 (5×10^5 cpm) i.v. via the tail vein. The specific activity of c30.6 was 2.99×10^5 cpm/μg and the immunoreactivity was 65–70%. Groups of mice were sacrificed at 24, 48, and 72 h. Samples of blood, tumor, heart, liver, spleen, lung, kidney, stomach, intestine, muscle, and skin were weighed and the radioactivity was measured in a gamma counter. Results were expressed as the percentage of injected dose/g tissue ± SE.

Inhibition of Tumor Growth *in Vivo*. 30.6$^+$ COLO 205 tumors were established as s.c. tumors in groups of six scid mice as described above. Five days later, when tumors measured 0.3–0.45 cm^2, mice were treated with either c30.6 or m30.6. Control groups received PBS. Four doses of 250 μg/dose were given by i.p. injection on days 5, 7, and 9 after tumor inoculation. Tumor size was measured daily on perpendicular axes and recorded as mean tumor size (cm^2) ± SE.

RESULTS

Isolation of 30.6 V_H and V_K Gene Segments. The gene segments encoding V_H and V_K regions were amplified by thermal cycling using complementary DNA derived from the m30.6 hybridoma and gene-

Table 1 *Nucleotide sequences of the synthetic oligonucleotide primers used in thermal cycling amplification*[a]

	5′ end	3′ end
V_H		
VH1BACK 1	5′ AGGT(C/G)AA(A/G)CTGCAG(C/G)AGTCAGG 3′	
VH1BACK2	5′ AGGT(C/G)CA(A/G)CTGCAG(C/G)AGTCAGG 3′	
VH1BACK3	5′ AGGT(C/G)CA(A/G)CTGCAG(C/G)AGTCTGG 3′	
VH1BACK4	5′ AGGT(C/G)AA(A/G)CTGCAG(C/G)ACTCTGG 3′	
VH1FOR2		5′ TGAGGAGACGGTGACCGTGGTCCCTTGGCCCC 3′
V_K		
	5′ GACATTCAGCTGACCCAGTCTCC 3′	5′ GTTAGATCTCCAGCTTGGTCCC 3′

[a] Restriction enzyme sites were introduced as indicated (underlined): 5′ CTGCAG 3′ (*Pst*I); GGTGACC (*Bst*EII); CAGCTG (*Pvu*II); GATC, (*Bgl*II).

specific primers. Agarose gel analysis of the amplified products indicated gene fragments of the predicted sizes, *i.e.*, ~350 base pairs for V_H and ~330 base pairs for V_K. Nucleotide sequencing of multiple clones confirmed the isolation of V_H and V_K sequences (Fig. 1). The predicted amino acid sequence of a representative V_H clone (Fig. 1*A*) was compared with sequences compiled by Kabat *et al.* (24), and it was demonstrated that the 30.6 V_H gene belonged to the subgroup IIA family. Invariant Cys residues at positions 22 and 92 that formed an intrachain disulfide bond were conserved. It was predicted that a single amino acid encoded by the consensus oligonucleotide primers would be different near the amino terminal of the wild-type 30.6 heavy chain. Glutamine, not glutamic acid, is an invariant residue at position 6 in this subclass of IgG (24). An additional amino acid, asparagine (position 94A in Fig. 1*A*), was also noted. The V_H clone was found to encode an internal *PstI* site, which necessitated subcloning into the subsequent expression plasmid in two stages (see "Materials and Methods"). Of five putative 30.6 V_K clones sequenced, one was identical to the defective NS-1 K chain (25) (data not shown). The remaining four clones encoded a novel sequence that was presumed to code for the 30.6 V_K region (Fig. 1*B*). Comparison with the other mouse V_K sequences demonstated that 30.6 is a member of the Group I V_K family (24). Once again, invariant Cys residues at positions 23 and 88 were conserved. Two amino acids encoded by the consensus oligonucleotide primers are likely to differ from wild-type 30.6. Valine, not glutamine, is virtually invariant at position 3 in class I V_K chains, and methionine is found in 95% of cases at position 4 (not leucine, which is present in only 4% of sequenced light chains). Additionally, proline at position 95 in CDR3 was absent in 30.6. This site is close to the site for *V-J* recombination, and the deletion may have arisen by in-frame truncation of the *V*-region gene. Importantly, this region was not encoded by the 3′ primer used for thermal cycling.

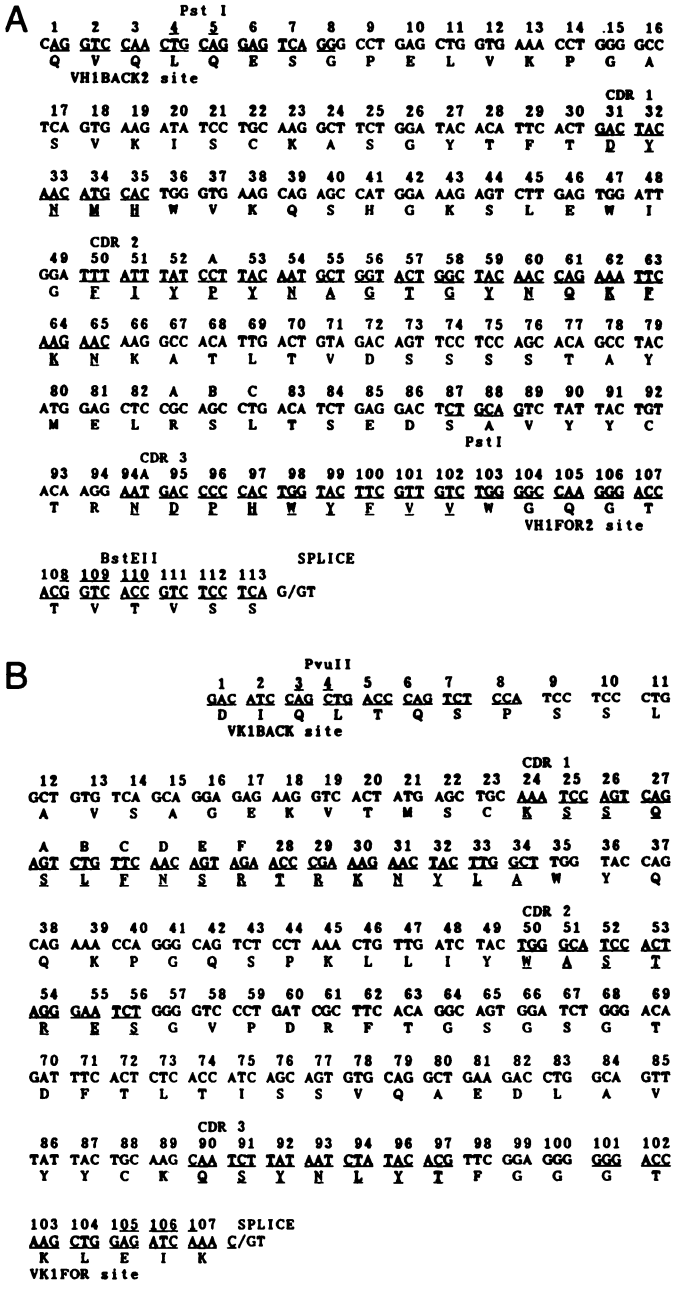

Fig. 1. Nucleotide and predicted amino acid sequences of V_H30.6 (*A*) and V_K30.6 (*B*). Oligonucleotide primers used in thermal cycling reactions (see also Table 1), restriction endonuclease sites used for cloning, and the predicted complementarity-determining regions of the putative heavy and light chains (underlined). Amino acid numbering and mapping of the complementarity-determining regions are predicted from the Kabat database (24). An additional *PstI* site which necessitated a two-step subcloning strategy for the VH clone is underlined at amino acid residues 87 to 89. "Splice" indicates the intron/exon boundary for both the heavy and light chain expression vectors.

Production of c30.6. The 30.6 V_H and V_K gene segments were used to construct chimeric antibody expression vectors, pSV.VH30.6 and pSV.VK30.6. Plasmid DNA was cotransfected into the non-immunoglobulin-producing mouse hybridoma cell line, Sp2/0, and clones incorporating plasmid DNA were selected in medium supplemented with hygromycin B. Eight clones were found to secrete human immunoglobulin into the culture supernatant on the basis of binding to an anti-human immunoglobulin reagent in an enzyme-linked immunosorbent assay (data not shown). Specific binding to 30.6 antigen was demonstrated by flow cytometry using 30.6^+ cell lines. The analysis of chimeric immunoglobulin from a representative clone is shown (Fig. 2). c30.6 bound strongly to each of the CRC cell lines COLO 205, HT29, LIM 1899, and LOVO, all of which express 30.6 antigen. The profiles observed were virtually superimposable with those obtained using m30.6. By comparison, neither c30.6 nor m30.6 bound the leukemic cell lines Daudi, Sultan, and K562 (data not shown).

Affinity. Minimal difference was found between the mouse and chimeric antibodies in their ability to bind 30.6. The affinity of binding of c30.6 and m30.6 was measured by Scatchard analysis on 30.6^+ tumor cell lines and was identical for the two antibodies ($K_a = 1.5 \times 10^8$). In the course of these studies it was noted that a considerable fraction of m30.6 was inactivated as a result of iodination, resulting in an immunoreactive fraction of only ~10%. Therefore, the relative affinities of m30.6 and c30.6 were also compared using a competitive binding assay (Fig. 3). FITC-c30.6 was allowed to bind to 30.6 antigen in competition with varying concentrations of unlabeled c30.6 and m30.6. Fifty % inhibition of maximum binding of FITC-c30.6 was obtained with 0.59 μg c30.6 and 1.44 μg m30.6. Therefore, in this assay c30.6 had an apparent affinity ~2.4 times that of m30.6. This apparent mild increase in affinity of the chimeric antibody is unlikely to be functionally significant.

Antibody-dependent Cell-mediated Cytotoxicity. The c30.6 antibody was able to mediate significant lysis of ^{51}Cr-labeled target cells (COLO 205, HT29, LIM 1899, and LOVO) using human PBMC as effector cells in an ADCC assay (Fig. 4*A*). Lysis (28–42% specific ^{51}Cr release) of each of the cell lines was seen at an E/T ratio of 100/1 in the presence of 25 μg/ml c30.6, and reduced lysis was evident over a range of E/T ratios down to 10/1. Significant lysis was not seen when the antibody was deleted from the assay. The lysis of COLO 205 by PBMC in the presence of c30.6 (25 μg/ml) was then compared to that with m30.6 or with an isotype-matched control

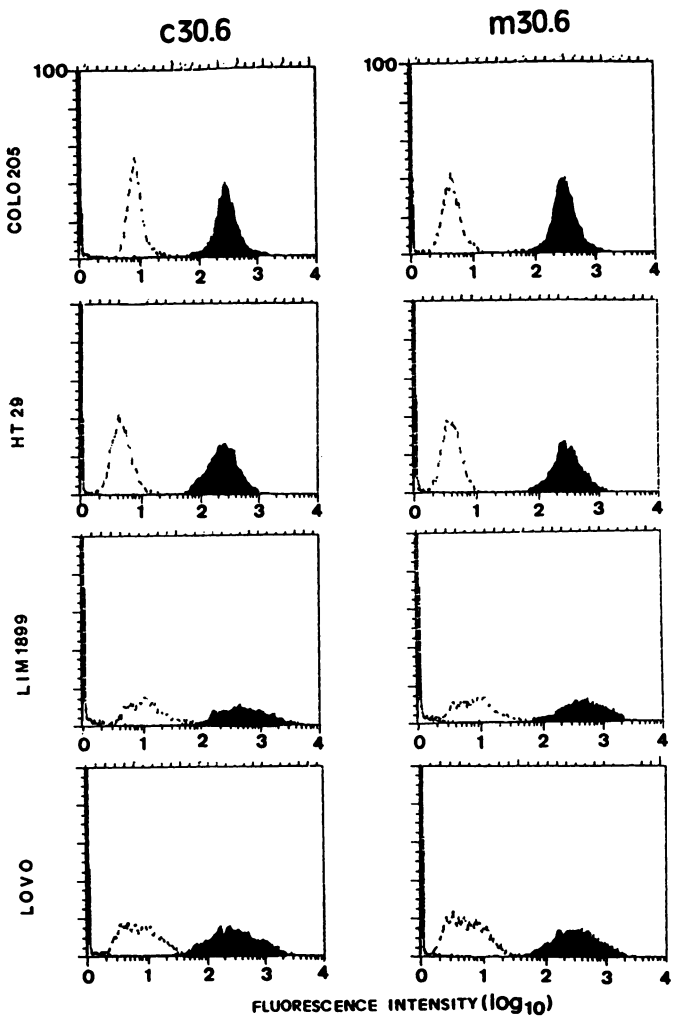

Fig. 2. Binding of c30.6 and m30.6 to human CRC cell lines. Analysis was performed on a FACScan cytofluorograph (Becton Dickinson) using purified antibodies at 25 μg/ml. *Shaded traces*, the binding of c30.6 and m30.6; *dashed traces*, negative controls: for c30.6, an isotype-matched chimeric mAb, cBC2, was used; and for m30.6, cells were preincubated in PBS instead of antibody prior to addition of fluoresceinated anti-mouse conjugate. *Ordinate*, relative cell numbers of each of the cell lines indicated. The human leukemic cell lines Daudi, Sultan, and K562 showed no binding of either c30.6 or m30.6 (data not shown).

chimeric antibody, cBC2 (Fig. 4*B*). Approximately 58% specific lysis was observed using c30.6, whereas <20% lysis was seen with m30.6 and <10% was seen with cBC2 at an E/T ratio of 100/1. This high level of lytic activity with c30.6 was maintained when lower E/T ratios were examined; however, m30.6 and cBC2 produced only background levels of lysis (<10%). When effector lymphocytes were prestimulated with IL-2 for 36 h prior to use, an increase in ADCC was noted with both 30.6 mAbs. However, this was accompanied by higher background lysis with cBC2 (Fig. 4*C*) and probably reflected the recruitment of antitumor lymphokine-activated killer cells.

Biodistribution of c30.6 in scid Mice Bearing COLO 205 Tumors. Initially, the circulating half-lives ($t_{½}$) of ^{125}I-labeled c30.6 and m30.6 were determined following i.p. administration to C57BL/6 × BALB/c F_1 mice. The $t_{½}\alpha$ of c30.6 was 1.01 h and that of m30.6 was 1.74 h. The $t_{½}\beta$ of c30.6 was 42.6 h and that of m30.6 was 135.3 h. The shorter circulating half-life of the chimeric antibody was in keeping with the findings for other mouse and chimeric antibodies (26).

^{125}I-labeled c30.6 was administered to groups of four COLO 205 tumor-bearing mice. Mice were sacrificed and tissues were counted for radioactivity after 24, 48, or 72 h (Fig. 5*A*). The accumulation of c30.6 in the tumors was 15%, 13%, and 10% injected dose/g at 24, 48, and 72 h, respectively. Significant accumulation of ^{125}I other than in the tumor was seen only in the spleen. Uptake in the spleen was 16%, 13%, and 6% injected dose/g, respectively, at the same time points. Splenic accumulation was therefore higher than it was in the tumor during the first 24 h after administration but fell to near background levels by 72 h in parallel with blood levels. Because this splenic accumulation may have been due to scavenging of denatured or aggregated antibody by reticuloendothelial cells or to micrometastases, biodistribution of ^{125}I-labeled 30.6 was studied in groups of four COLO 205 tumor-bearing scid, non-tumor-bearing scid, and C57BL6 × BALB/c F_1 mice 24 h after administration (Fig. 5*B*). The accumulation of ^{125}I-labeled c30.6 in scid and tumor-bearing scid was similar with 35% and 37% injected dose/g present at 24 h, respectively. This level of splenic accumulation was somewhat higher than that seen in the previous experiment (Fig. 5*A*). The scid mice used in the second experiment had, on the average, slightly larger spleens than those used in the first experiment, and this may have accounted for the increase in nonspecific accumulation. By contrast, the spleens of C57BL6 × BALB/c F_1 mice (much larger than those of scid mice) accumulated only a 4% injected dose/g over the same time, and ^{125}I-labeled c30.6 accumulation in the tumor was similar in the two experiments. Accumulation in the spleen was therefore not dependent on the presence of tumor cells or due to antibody denaturation and was related to the scid phenotype in general.

Inhibition of Tumor Growth *in Vivo* by c30.6. The antitumor activity of m30.6 and c30.6 was determined in scid mice bearing COLO 205 tumors. Groups of six scid mice were given c30.6 (250 μg i.p.), m30.6 (250 μg i.p.), or PBS on days 5, 7, and 9 after tumor implantation. The tumor size was measured daily, and at the conclusion of the experiment (day 21) the mean tumor size of the c30.6-treated group was reduced 40% by comparison with the control group tumor size ($P < 0.02$, by Student's t test; Fig. 6). In contrast to c30.6, administration of m30.6 had no effect on tumor size.

DISCUSSION

The antigenic determinant detected by mAb 30.6 is expressed on the cell surface of the vast majority of colorectal adenocarcinomas

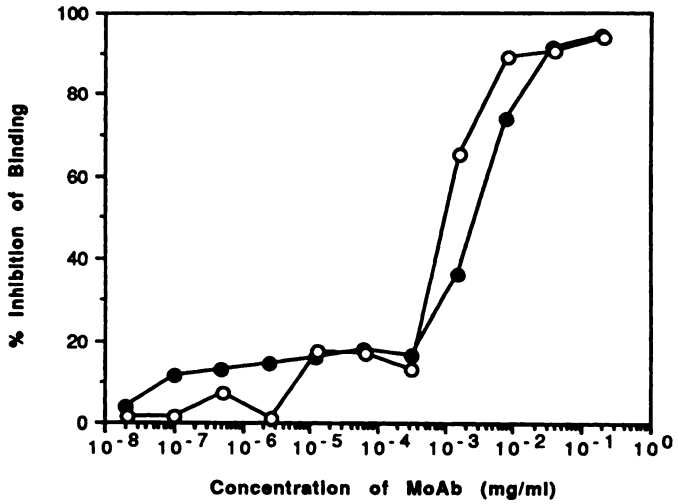

Fig. 3. Competitive binding assay of c30.6 and m30.6 on the 30.6$^+$ cell line COLO 205. COLO 205 cells (3×10^5) were incubated with FITC-c30.6 and various concentrations of either c30.6 (●) or m30.6 (○). The percentage inhibition of binding was calculated relative to the FITC binding observed in the absence of competing antibody. The experiment depicted was representative of three that were performed. *MoAb*, monoclonal antibody.

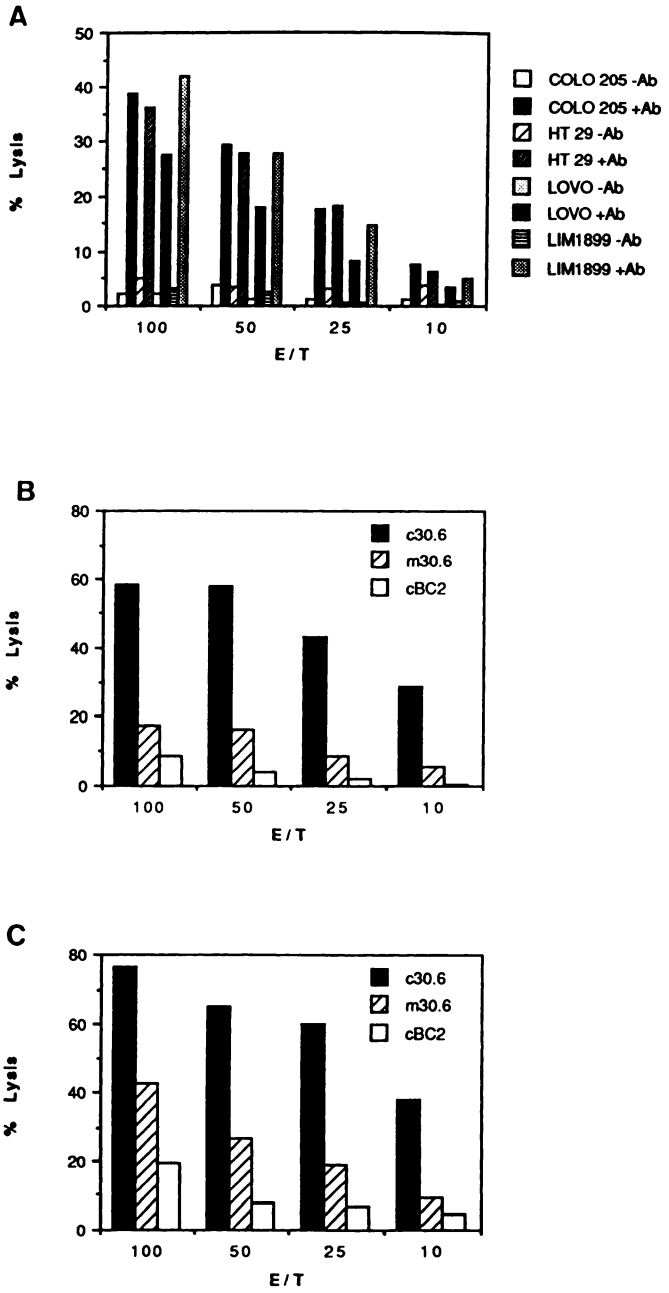

Fig. 4. Four-h ADCC assays using human CRC cell lines as targets. In *A*, assays were performed using freshly isolated human PBMC as effectors and four target cell lines in the presence or absence of c30.6 (25 μg/ml). SEs were <5% for each value. In *B*, PBMC were used as effectors and COLO 205 cells were used as targets in the presence of either c30.6, m30.6, or cBC2 at 25 μg/ml. SEs were <5% for each value. In *C*, assays were performed as in *B*, except that PBMC were incubated in medium supplemented with 100 units/ml recombinant human IL-2 for 36 h prior to use. In all experiments, E/T ratios varied from 100/1 to 10/1. All values were calculated as the mean of triplicate assays (see "Materials and Methods"). SEs were <5% for each value. These data are representative of four independent experiments performed using the PBMC of three unrelated donors. *Ab*, antibody.

(13). m30.6 was produced by conventional hybridoma technology from BALB/c mice immunized with fresh colon carcinoma cells and boosted with the colon carcinoma cell line, HT-29 (13). We have now produced a recombinant, chimeric mouse/human antibody, c30.6, that binds to the same epitope with equivalent affinity and, additionally, is able to mediate potent effector function against 30.6^+ tumors.

The 30.6 determinant is not expressed exclusively on malignant cells, but it has been shown in immunoperoxidase and immunofluorescence studies to be expressed on benign and malignant lesions and on secondary tumor deposits. It is expressed weakly only on normal gastrointestinal epithelial cells and on some other secretory epithelia (13). Expression is greatest on well- differentiated tumors, is less pronounced on poorly differentiated tumors, but is usually absent from the most anaplastic lesions. The biochemical nature of the 30.6 antigen has not been fully elucidated, but its expression has been shown to be confined to the luminal surface of glandular cells and it is not secreted. The antigen has been shown not to be carcinoembryonic antigen or mucin on the basis of antibody-blocking studies. Immunoprecipitation experiments detected bands corresponding to molecules with molecular weights of 22,000, 25,000, 27,000, 31,000, and 42,000; however, the reason for these multiple species is uncertain. The selective expression of the 30.6 epitope on CRC provides a possible target for anticancer immunotherapy and the possibility of an improvement in therapy as compared with existing nonspecific modes of nonsurgical treatment. Zalcberg *et al.* (16) demonstrated by immunoscintigraphy that radiolabeled 30.6 localized to human CRC xenografts in nude mice and that ^{131}I-labeled antibody strongly inhibited the growth of these tumors. Notably, however, unlabeled m30.6 was no more effective than irrelevant antibodies at inhibiting tumor growth. In a phase I/II trial, *N*-acetylmelphalan that was coupled to

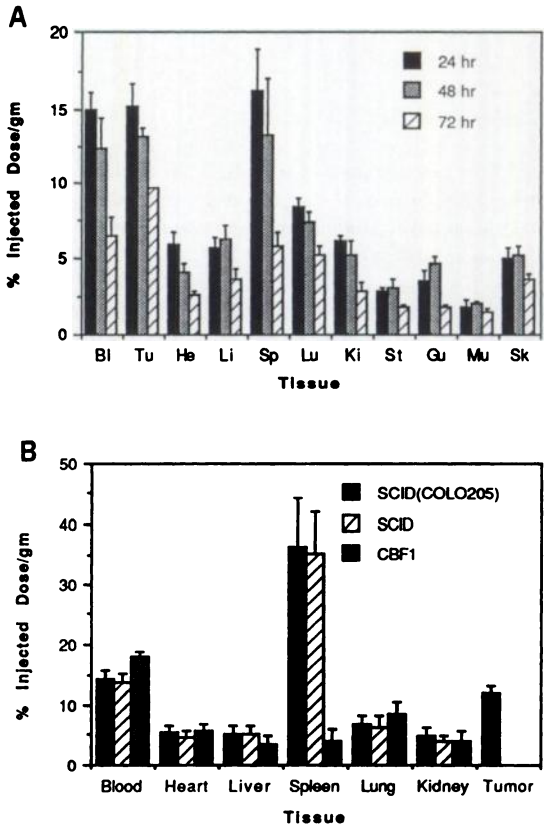

Fig. 5. Biodistribution of ^{125}I-c30.6 in human CRC tumor-bearing scid mice. In *A*, COLO 205 tumors were established s.c. on the anterior abdominal wall of scid mice, and ^{125}I-c30.6 administered by tail vein injection (see "Materials and Methods"). Mice were sacrificed at the times indicated and immediately dissected. Specimens were assayed for radioactivity in a gamma counter. The radioactivity detected is expressed as the mean of triplicate determinations ± SE (*bars*)/g tissue. *Bl*, blood; *Tu*, tumor; *He*, heart; *Li*, liver; *Sp*, spleen; *Lu*, lung; *Ki*, kidney; *St*, stomach; *Gu*, intestine; *Mu*, skeletal muscle; and *Sk*, skin. *B*, distribution of radioactivity in COLO 205-bearing scid mice, control scid mice, C57BL/6 x BALB/c F_1 (CBF_1)mice following administration of ^{125}I-c30.6. Mice were sacrificed at 24 h and organ specimens were assayed for radioactivity. The apparent increase in splenic accumulation in scid mice relative to *A* was due to a smaller average spleen size in the scid mice used in A. The radioactivity detected is expressed as the mean of triplicate determinations ± SE (*bars*).

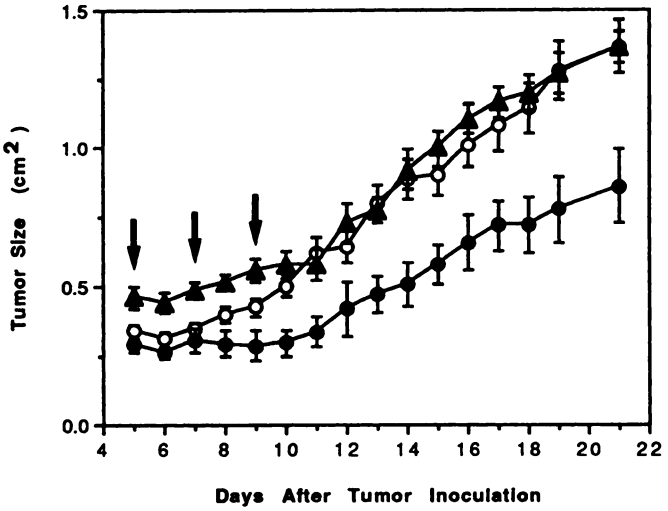

Fig. 6. Inhibition of tumor growth in COLO 205-bearing scid mice. Mice bearing s.c. tumors were treated with c30.6 (●), m30.6 (○), or PBS (▲) administered i.p. 5, 7, and 9 days after tumor inoculation (*arrows*). Tumor size was determined by measuring on two perpendicular axes and is expressed as the mean ± SE (*bars*) of six mice in each group.

30.6 was well tolerated at doses that normally produce myelosuppression; however, all patients receiving the mAb produced a HAMA response that precluded further dosage escalation (18).

The current study attempts to address the problem of HAMA by producing an antibody in which the Fc portion (~70% of the entire antibody) is replaced with a human Fc piece. The immunogenicity of chimeric antibodies compared with totally xenogeneic antibodies appears to be variable and dependent on the individual antibody. The first chimeric antibody studied for immunogenicity and pharmacokinetics was chimeric 17-1A (27), which detects a CRC-associated antigen. The results were encouraging in that the mean half-life was 6 times that of the mouse antibody and only 1 of 10 patients developed a mild HAMA response. By contrast, another antibody cB72.3 was as immunogenic as its mouse counterpart (28). Further studies are required before generalizations can be made about immunogenicity of chimeric antibodies. Indeed, CDR-grafted antibodies may not be free of immunogenicity because anti-idiotype and anti-allotype responses may be evoked when large doses are given. Furthermore, CDR-grafted mAbs frequently require framework remodeling to restore or increase affinity, which may lead to unexpected immunogenicity (29). Our initial results of administration of c30.6 to patients indicated that it was as well tolerated as the mouse antibody, and it evoked only weak anti-idiotype responses.[4]

Apart from reducing the likelihood of HAMA, substitution of the mouse Fc piece (IgG2b) by the human Fc of choice (in this case human IgG1) may allow the acquisition of novel effector functions *in vitro* and *in vivo*. This was clearly the case with c30.6, which was able to mediate markedly enhanced lysis of 30.6^+ target cells in comparison with m30.6 in *in vitro* antibody-dependent cell-mediated cytotoxicity. The same cells could not be lysed by either c30.6 or m30.6 in the presence of human or rabbit complement. Similar results have been found with a number of chimeric antibodies (30). Some such antibodies were able to bind C1q, and an inability to lyse cells was probably related to low antigen density on the target cells (31). It remains to be established whether the acquisition of effector functions such as ADCC and complement-mediated cytotoxicity *in vitro* will be of therapeutic significance *in vivo*.

A further significant difference between the biological activities of m30.6 and c30.6 was the ability of c30.6 to significantly inhibit the growth of human CRC tumors in scid mice. While a significant overall inhibition of tumor size was noted in mice given the chimeric antibody, close examination of the data suggested that the majority of this effect occurred between days 5 and 10, *i.e.*, early in the course of tumor growth and while antibody was being administered (Fig. 6). Providing that this effect is also observed in humans, this suggests that c30.6 might be of use in an adjuvant setting, particularly in the treatment of minimal residual disease following excision of a primary lesion and mesenteric nodal metastases (Duke's Stage 3 CRC). In this regard, it is encouraging that the therapeutic effect in scid mice was observed with unconjugated c30.6. If c30.6 proves to be weakly immunogenic in humans its anticancer properties may be enhanced by conjugation with cytotoxic drugs or radioisotopes because ^{131}I and methotrexate have been shown to confer antitumor activity on m30.6, which otherwise shows no intrinsic therapeutic benefit (16, 17).

The mechanism to explain the therapeutic effect of c30.6 in scid mice remains to be elucidated. Given the similarity of binding affinities of the two reagents it seems likely that the therapeutic effect resides in the Fc portion of the molecule. Because scid mice lack significant T and B cell responses any effector response would have to reflect either a direct toxicity of antibody for the tumor cells or the recruitment of other cells with cytotoxic capacity, such as neutrophils, monocytes/macrophages, or natural killer cells. The first possibility seems most improbable because direct cytotoxicity has not been noted with 30.6 in several previous studies. If monocytes, neutrophils, or natural killer cells are involved this would suggest binding of these cells to c30.6 via FcγRI/FcγRII (monocytes and neutrophils) or FcγRIII (natural killer cells). It is noteworthy that mouse IgG2b antibodies are generally able to efficiently bind FcγRII and FcγRIII but not FcγRI (32). By contrast, human IgG1 antibodies can generally bind to all three classes of Fc receptors. These observations point to a possible role for FcγRI in the inhibition of CRC tumor growth in scid mice. These receptors are constitutively expressed on monocyte/macrophages and are potently up-regulated by γ-interferon (33). While activated T cells are absent from scid mice, a source of γ-interferon might be natural killer cells localized to the tumor via their FcγRIII receptors. We are currently assessing the localization of these two effector populations to CRC tumors in our scid model.

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