



No. 09-149371- -00005-0000

Fecha: 2011-07-19 09:25:56 Dep. 2020 DIR.NUEVASCR
Tra. 11 PATENTEIYII Eje: 378 FASENACIONALI
Act. 400 OPOSIOBSERVTER Folios: 35

FORMULARIO ÚNICO DE OPOSICIÓN

①

OPOSICIÓN A :

- | | |
|--|--|
| ☒ Patente de Invención. | ☐ Marca Colectiva. |
| ☐ Patente de Modelo de Utilidad. | ☐ Marca de Certificación. |
| ☐ Diseño Industrial. | ☐ Lema Comercial. |
| ☐ Esquema de Trazado para Circuitos Integrados. | |
| ☐ Marcas de Productos o Servicios. | |

②

SOLICITUD
PUBLICADA

Número del expediente: 09 - 149371
Solicitante: GLAXOSMITHKLINE LLC
Apoderado: CARLOS REYNALDO OLARTE GARCIA
Título de la nueva creación o denominación del signo: "FORMULACIONES DE
ANTICUERPOS ANTI-CD20 ESTABLES A LA TEMPERATURA Y CIZALLADURA"
Gaceta: 627

③ OPOSICIÓN PRESENTADA POR:

SOLICITANTE	Nombre: PROCAPS S.A.	IDENTIFICACIÓN C.C. ☐ NIT ☒ C.E. ☐ Otro ☐ Cual _____ Número <u>890.106.527-5</u>
	Dirección: CALLE 80 No. 78b-201 Nacionalidad o Domicilio: COLOMBIA Lugar de Constitución: BARRANQUILLA Teléfono: 371 99 00 Fax: 373 0815 E-mail: _____	
REPRESENTANTE O APODERADO	Nombre: ELIANA ISaura MORENO BOHORQUEZ Dirección: CARRERA 104 N°119-95 OF 104 Teléfono: 2131213 Fax: 6121979 E-mail: negocios@optimum-co.com	IDENTIFICACIÓN C.C. ☒ NIT ☐ C.E. ☐ Otro ☐ Cual _____ Número <u>51.975.664</u> Tp <u>83823</u>

④ FUNDAMENTOS DE LA OPOSICIÓN:

Art.14, 16, 18, 20 y 30 de la Decisión 486 de la CAN

Causal: No cumple con los requisitos de patentabilidad.

Signo opositor: _____

Justificación: _____

La presente solicitud no cumple con los requisitos de patentabilidad dado que lo
revelado, ya se encontraba en el estado de la técnica o es posible derivarlo de la
misma tal y como se demostrará en el capítulo "Fundamento de Hechos".

⑤ Comprobante de pago No.

11-0069665

Fecha

19/07/2011

⑥ ANEXOS

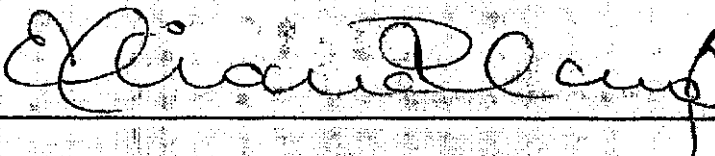
- ☒ Comprobante de pago de la tasa.
- ☒ Poderes, si fuere el caso.
- ☒ Pruebas de los fundamentos de la oposición.
- ☐ Documentos que acrediten el legítimo interés, si fuere el caso.
- ☒ Documento que acredita la existencia y representación legal de las partes, cuando sean personas jurídicas.

⑦

NOMBRE:

Francisco Morano B

FIRMA:



C.C. 51.975.664

TP. 83823



Derecho de la Competencia, Propiedad
Industrial y Comercio Exterior

Señor Jefe,

DIRECCION DE NUEVAS CREACIONES

Superintendencia de Industria y Comercio

E. S. D.

Referencia : Expediente N° 09 149371

Asunto : Oposición a la solicitud de patente de Invención titulada
"FORMULACIONES DE ANTICUERPOS ANTI-CD20
ESTABLES A LA TEMPERATURA Y CIZALLADURA"

Solicitante : GLAXOSMITHKLINE LLC.

Opositora : PROCAPS S.A.

Publicada en la Gaceta de la Propiedad Industrial

N° 627 del 20 de abril de 2011

Yo, *ELIANA ISAURA MORENO BOHORQUEZ*, abogada en ejercicio, identificada como aparece al pie de su firma, con tarjeta profesional No. *83823* del Consejo Superior de la Judicatura, en mi condición de apoderada de la sociedad **PROCAPS S.A.** Conformada bajo las leyes de la República de Colombia y domiciliada en la ciudad de Barranquilla, Colombia, atentamente manifiesto que estando dentro del término de ley y por orden expresa de mi representada, presento oposición a la concesión del registro de la Patente de Invención titulada "FORMULACIONES DE ANTICUERPOS ANTI-CD20 ESTABLES A LA TEMPERATURA Y CIZALLADURA", cuyos titulares son **GLAXOSMITHKLINE LLC**, y pido a Usted declarar fundada esta oposición y en consecuencia negar el registro de la patente solicitada.

Carrera 13 N° 119-95, Of. 104 Tel. (571) 2 13 12 13 - 6 20 50 21 - Fax. (571) 6 12 19 79
E-mail: negocios@optimum-co.com - www.optimum-co.com
Bogotá Colombia

1. INTERÉS PARA ACTUAR

Conforme a lo dispuesto en el artículo 42 de la Decisión 486 de 2000 de la CAN, estando dentro del término estipulado por la ley, manifiesto el legítimo interés de nuestra representada para oponerse al registro de la patente de la referencia, atendiendo a que la solicitud pretendida no cumple con los requisitos dispuestos en la ley como se analizará más adelante y de ser concedida afectaría directamente la comercialización de algunos productos de mi representada, que contienen, en particular ANTICUERPOS ANTI-CD20. Por lo tanto, solicito respetuosamente a ese Despacho se sirva tener en cuenta la presente oposición dentro del trámite administrativo que se surte en esta Superintendencia.

2. FUNDAMENTOS DE HECHOS

2.1. La solicitud objeto de la presente fue presentada el 30 de diciembre de 2009 reivindicando prioridad bajo la solicitud US20070948220 del 2007-07-06; y fue publicada en Colombia, en la Gaceta de la Propiedad Industrial N° 627 del 20 de abril de 2011, cuya publicación internacional corresponde a la WO2009009407 del 2009-01-15.

2.2. La solicitud colombiana 09 149371 objeto de la presente oposición se refiere a una formulación de anticuerpo anti-CD20 que comprende una cantidad terapéuticamente eficaz de un anticuerpo anti-CD20, en la que la formulación comprende además acetato sódico 10 a 100 mM, cloruro

sódico 25 a 100 mM, base libre de arginina al 0,5 a 5%, EDTA 0,02 a 0,2 mM, polisorbato 80 al 0,01 a 0,2% y está ajustada a pH 5,0 a 7,0.

2.3. En primera instancia, se debe advertir que en el capítulo reivindicatorio de la solicitud objeto de la presente oposición, se hace referencia a los usos de la composición, no sólo a la composición como tal o a un método de fabricación mediante la cual se obtenga un producto tangible y según la legislación, decisión 486 de la Comunidad Andina de Naciones, Artículo 14.- *“Los Países Miembros otorgarán patentes para las invenciones, sean de producto o de procedimiento (...)”*, los usos no están contemplados como patentables; y, Artículo 20.- *“No serán patentables (...) d) los métodos terapéuticos o quirúrgicos para el tratamiento humano o animal, así como los métodos de diagnóstico aplicados a los seres humanos o a animales”*. Solo serán patentables los productos o procedimientos. Por lo tanto a luz de la norma, dicha materia referente al uso no sería patentable.

2.4. Con base en el artículo 14 de la Decisión 486 de la CAN que consagra:

“Los países miembros otorgarán patentes para las invenciones, sean de producto o procedimiento, en todos los campos de la tecnología, siempre que sean nuevas, tengan nivel inventivo y sean susceptibles de aplicación industrial”, tenemos que las invenciones objeto de patente deben ser nuevas y tener nivel inventivo. Bien, pues antes de la fecha de prioridad de esta solicitud se encuentran los siguientes documentos que afectan la patentabilidad de la presente solicitud:

- D1: US 2006246004 publicada el 02 de noviembre de 2006



- D1: US 4816401 publicada el 28 de marzo de 1989

2.5. Ahora bien, el documento D1, revela un anticuerpo humanizado CD20 vinculante para el tratamiento de tumores malignos de células B y las enfermedades autoinmunes, formulación de anticuerpos anti-CD20 que comprende una cantidad terapéuticamente efectiva de un anticuerpo anti-CD20, en donde la formulación comprende además 10 a 100 mM de acetato de sodio, cloruro de sodio, arginina, 0,02 a 0,2 mM de EDTA, polisorbato 80 y se ajusta a un pH de 5,0 a 7,0. Dicho documento D1 también revela una concentración de NaCl y polisorbato 180.

2.6. El documento D2 por su parte se refiere a un medio de cultivo celular libre de suero que contiene menos de 10 ug / ml de proteínas. El medio, según dicho documento D2 cuenta con un medio basal suplementado con niveles de 2-aminoetanol sustancialmente más altos a lo divulgado anteriormente y se complementa con 2-mercaptoetanol, transferrina, insulina y aminoácidos esenciales libres. Según el mismo documento D2, los medios descritos en este documento apoyan el crecimiento y cultivo a largo plazo de una gran variedad de tipos de células incluyendo hibridomas y líneas de células tumorales, mientras que el mantenimiento tanto de potencial de crecimiento y las características diferenciadas de la línea celular particular. El documento D2 describe además un medio que puede ser utilizado para detectar anticuerpos y revela una concentración de base libre de arginina.

2.7. Tomando los documentos D1 y D2 como el estado del arte más cercano a la solicitud objeto de la presente oposición, no es posible reconocer altura inventiva, ya que una persona experta en la materia, teniendo en cuenta que el uso de PH es un parámetro de control convencional y bien conocido en el estado del arte debido a la tendencia de una bio-molécula a ganar o perder protones en presencia de un ácido o una base y que la habilidad modificar el PH, es procedimiento de rutina para una persona experta en la materia, podría llegar de manera evidente a concebir una formulación de anticuerpo anti-CD20 que comprende una cantidad terapéuticamente eficaz de un anticuerpo anti-CD20, en la que la formulación comprende además acetato sódico 10 a 100 mM, cloruro sódico 25 a 100 mM, base libre de arginina al 0,5 a 5%, EDTA 0,02 a 0,2 mM, polisorbato 80 al 0,01 a 0,2% y está ajustada a pH 5,0 a 7,0.

2.8. En consecuencia y de acuerdo a lo descrito anteriormente, se deriva que la solicitud colombiana 09 149371 no puede pretender un monopolio por patente toda vez que lo que pretende no cumple con dos de los tres requisitos necesarios para su protección.

2.9. Que por consiguiente, respecto a la solicitud pretendida se tiene que:

2.9.1. CARENCIA DE ALTURA INVENTIVA. Según la Decisión 486 de la Comunidad Andina de Naciones, Artículo 18.- *“Se considerará que una invención tiene nivel inventivo, si para una persona del oficio normalmente versada en la materia técnica correspondiente, esa invención no hubiese resultado obvia ni se hubiese derivado de manera evidente del estado de la técnica.”*. A la fecha de prioridad, 06 de julio de 2007, a partir de los documentos D1 y D2, y teniendo en cuenta que el uso de PH es un

parámetro de control convencional y bien conocido en el estado del arte debido a la tendencia de una bio-molécula a ganar o perder protones en presencia de un ácido o una base y que la habilidad modificar el PH, es también conocida, un experto en la materia puede llegar de manera evidente a concebir una formulación de anticuerpo anti-CD20 que comprende una cantidad terapéuticamente eficaz de un anticuerpo anti-CD20, en la que la formulación comprende además acetato sódico 10 a 100 mM, cloruro sódico 25 a 100 mM, base libre de arginina al 0,5 a 5%, EDTA 0,02 a 0,2 mM, polisorbato 80 al 0,01 a 0,2% y está ajustada a pH 5,0 a 7,0; sobre lo cual busca protección en el capítulo reivindicatorio.

2.9.2. CARENCIA DE APLICACIÓN INDUSTRIAL La Decisión 486 de la CAN consagra en su artículo 14 lo siguiente: “*Los países miembros otorgarán patentes para las invenciones, sean de producto o procedimiento, en todos los campos de la tecnología, siempre que sean nuevas, tengan nivel inventivo y sean susceptibles de aplicación industrial*”. La presente solicitud carece de aplicación Industrial ya que como se demostró anteriormente, en el capítulo reivindicatorio se reclaman usos y estos no son aplicables industrialmente porque primero no se obtiene un producto o proceso; y segundo no es reproducible o repetible a escala industrial. Por lo tanto, el objeto de la presente invención referido a los usos, no cumple con el requisito de la aplicación industrial.

2.10. Dado lo anterior, es posible deducir que la solicitud y lo reivindicado no es objeto de patentabilidad pues es contrario a lo que dicta la Decisión 486 de la Comunidad Andina de Naciones, Artículo 14.- “*Los Países Miembros otorgarán patentes para las invenciones, sean de producto o de*

procedimiento, en todos los campos de la tecnología, siempre que sean nuevas, tengan nivel inventivo y sean susceptibles de aplicación industrial.”. La solicitud pretendida no cumple con dos de los tres requisitos de patentabilidad.

2.11. Por todo lo anterior, atentamente solicito a usted que se sirva declarar fundada la presente oposición y en consecuencia negar el registro a la solicitud de patente titulada “FORMULACIONES DE ANTICUERPOS ANTI-CD20 ESTABLES A LA TEMPERATURA Y CIZALLADURA”, cuyos titulares son GLAXOSMITHKLINE LLC, solicitud Colombiana que apareció publicada en la Gaceta de la Propiedad Industrial N° 627 del 20 de abril de 2011.

3. PRUEBAS

De conformidad con lo dispuesto por las normas vigentes, solicito a usted tener como prueba en este asunto los siguientes:

- D1: US 2006246004 publicada el 02 de noviembre de 2006
- D1: US 4816401 publicada el 28 de marzo de 1989

4. FUNDAMENTO DE DERECHO

Fundamento esta observación en las normas legales siguientes:

4.1. Numeral 1 del artículo 586 del Código de Comercio.

4.2. Artículo 42 de la Decisión 486 de la Comisión del Acuerdo de Cartagena.

4.3. En las demás normas concordantes.

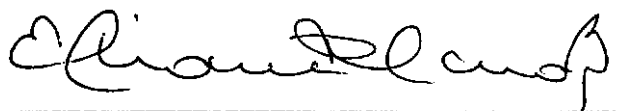
5. ANEXOS

Para los efectos correspondientes anexo al presente escrito los siguientes documentos:

1. Poder para actuar en el presente.
2. Documento que acredita al representante legal de **PROCAPS S.A.**
3. Recibo de caja del respectivo pago de tasa.
4. Copias de los documentos:
 - D1: US 2006246004 publicada el 02 de noviembre de 2006
 - D1: US 4816401 publicada el 28 de marzo de 1989

Para efectos de notificaciones que por disposición del artículo 50 del Decreto 01 de 1984 debe hacer su Despacho, informo que mi representada y la suscrita recibiremos notificaciones en la Secretaría de su Despacho o en mis oficinas situadas en la Carrera 13 N° 119-95, Of. 104 de esta ciudad de Bogotá.

Señor Superintendente,



ELIANA ISAURA MORENO BOHORQUEZ
C.C. 51'975.664 de Bogotá
T.P.A. 83823 del C.S. de la J.



PODER ESPECIAL

Yo, **WALTER TANAKA TATEKAWA**, mayor de edad y vecino de la ciudad de Barranquilla (Colombia), identificado como aparece al pie de mi firma, quien en el presente documento actúa en calidad de Representante Legal de la sociedad **PROCAPS S.A.** sociedad legalmente constituida y domiciliada en la ciudad de Barranquilla (Colombia), por el presente documento otorgo a la doctora **ELIANA ISAURA MORENO BOHORQUEZ**, abogada en ejercicio, identificada como aparece al pie de su firma, con tarjeta profesional No. 83823 del C.S. de la J., poder especial, amplio y suficiente, para formular oposiciones u observaciones a la solicitud de patente No. 09 149.371, titulada **"FORMULACIONES DE ANTICUERPOS ANTI-CD20 ESTABLES A LA TEMPERATURA Y CIZALLADURA"**, cuyo titular es **GLAXOSMITHKLINE LLC**, publicada en la gaceta No. 627 del 20 de abril de 2011.

Nuestro apoderado queda facultado, de manera expresa, para realizar todo tipo de actos necesarios para llevar a cabo el trámite de la oposición, incluyendo, sin limitación, preparar y presentar solicitudes, hacer declaraciones, pagar tasas, contribuciones e impuestos, recabar los títulos o certificados. Asimismo quedan autorizados para impugnar administrativamente todo lo que fuere resuelto en el expediente a que se hace expresa referencia en el presente poder, con todas las facultades generales y específicas que fuera requerido para ella.

De la misma manera, además de las facultades que le confiere la naturaleza del presente mandato, se encuentran facultados para recibir, desistir, transigir, sustituir, y reasumir el presente poder, incoar acciones, interponer recursos y demás facultades conferidas por la ley.

Dado y Firmado en Barranquilla (Colombia) a los 7 días del mes de Julio de 2011.


PROCAPS S.A. *-> adras.*
C.C. N° 16'251.923
NIT: 890.106.527-5
Representante Legal

Acepto:


ELIANA ISAURA MORENO BOHORQUEZ
C.C. 51'975.664 de Bogotá
T.P.A. 83823 del C.S. de la J.

NOTARIA
3023
NOTARIA TERCERA DEL CIRCULO DE BARRANQUILLA
DILIGENCIA DE RECONOCIMIENTO
Ante el Notario Tercero de Barranquilla se presento:
Walter Lora Lora
identificado con: 4116251923 Colombiano
Y declaro que el contenido del anterior Documento es cierto
y suya la firma que lo retendra.
Barranquilla, 11 JUL. 2011

[Handwritten signature]
[Handwritten signature]



SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
NIT : 800.176.089-2
- / -

Industria y Comercio SUPERINTENDENCIA	RECIBO DE CAJA	No. 11 - 0069665
		Bogotá D.C., Julio 19 de 2011 - 08:49:37

RECIBIDO DE : PROCAPSA S.A. NI 890.106.527

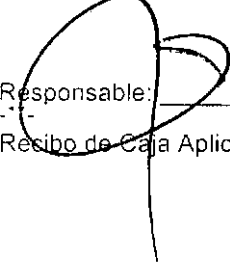
*** Soporte del Pago ***

TIPO PAGO	BANCO	CUENTA	No. PAGO	FECHA PAGO	VR. PAGO
CONSIGNACION	BANCO DE BOGOTA	062754387	407134965	07/07/2011	304.000.00

*** Conceptos Pagados ***

CANT.	RENTISTICO	CONCEPTO	Vr. UNIDITARIO	Vr. CONCEPTO
1	50005-01-04 PRESENTACION DE CERVACIONES	12 MARCAS Y LEMAS COMERCIALES	304.000.00	304.000.00
	A SOLICITUDES			\$304.000.00

SON: **TRESCIENTOS CUATRO MIL PESOS MONEDA CORRIENTE**

Responsable:  _____
Recibo de Caja Aplicado al Expediente No. _____

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

No. 09-149371- -00005-0000
Fecha: 2011-07-19 09:25:56 Dep. 2020 DIR.NUEVASCR
Tra. 11 PATENTEIYII Eva: 378 FASENACIONALI
Act. 400 OPOSIOBSERVTER Folios: 35



***** Pag. 1
 C A M A R A D E C O M E R C I O
 D E B A R R A N Q U I L L A
 09*****

CERTIFICADO DE EXISTENCIA Y REPRESENTACION LEGAL O DE INSCRIPCION DE DOCUMENTOS.

PROCAPS S.A.-----

NIT: 890.106.527-5.

EL SUSCRITO SECRETARIO DE LA CAMARA DE COMERCIO DE BARRANQUILLA, CON FUNDAMENTO EN LAS INSCRIPCIONES DEL REGISTRO MERCANTIL:

C E R T I F I C A

Que por Escritura Pública No. 1,190 del 22 de Julio de 1976, otorgada en la Notaria Tercera de Barranquilla, inscrito(as) en esta Cámara de Comercio, el 28 de Julio de 1976 bajo el No. 6,070 del libro respectivo, fue constituida la sociedad----- limitada denominada "PRODUCTORA DE CAPSULAS DE GELATINAS LIMITADA "PROCAPS LIMITADA".-----

C E R T I F I C A

Que por Escritura Pública No. 1,307 del 28 de Junio de 1979, otorgada en la Notaria Tercera de Barranquilla, inscrito(as) en esta Cámara de Comercio, el 23 de Julio de 1979 bajo el No. 10,039 del libro respectivo, la sociedad antes mencionada----- se transformo en sociedad anonima bajo la denominacion social de "PRODUCTORA DE CAPSULAS DE GELATINA S.A." PROCAPS S.A."-----

C E R T I F I C A

Que por Escritura Pública No. 3,810 del 31 de Dic/bre de 1980, otorgada en la Notaria Primera de Barranquilla, inscrito(as) en esta Cámara de Comercio, el 30 de Enero de 1981 bajo el No. 12,705 del libro respectivo, la sociedad antes mencionada----- se transformo en sociedad de responsabilidad limitada bajo al razon social de "PRODUCTORA DE CAPSULAS DE GELATINA LIMITADA "PROCAPS LI - MITADA".-----

C E R T I F I C A

Que por Escritura Pública No. 3,755 del 10 de Nov/bre de 1983, otorgada en la Notaria Unica de Soledad, inscrito(as) en esta Cámara de Comercio, el 22 de Nov/bre de 1983 bajo el No. 17,922 del libro respectivo, la sociedad antes mencionada----- se transformo en sociedad anonima bajo la denominacion social de "PRODUCTORA DE CAPSULAS DE GELATINA S.A. "PROCAPS S.A."-----

C E R T I F I C A

Que por Escritura Pública No. 3,615 del 06 de Dic/bre de 1996, otorgada en la Notaria 2a. de Barranquilla, inscrito(as) en esta Cámara de Comercio, el 20 de Dic/bre de 1996 bajo el No. 67,218 del

***** C O N T I N U A *****

CERTIFICADO DE EXISTENCIA Y REPRESENTACION LEGAL O DE INSCRIPCION DE DOCUMENTOS.

PROCAPS S.A.

NIT: 890.106.527-5.

libro respectivo, la sociedad antes mencionada-----
cambio de razon social, por la denominacion LABORATORIOS PROCAPS S.A

C E R T I F I C A

Que por Escritura Pública No. 3,775 del 18 de Dic/bre de 1996,
otorgada en la Notaria 2a. de Barranquilla, inscrito(as) en esta
Cámara de Comercio, el 23 de Enero de 1997 bajo el No. 67,704 del
libro respectivo, la sociedad antes mencionada-----
cambio de razon social, por la denominacion PROCAPS S.A. -----

C E R T I F I C A

Que dicha sociedad ha sido reformada por las siguientes escrituras
y/o documentos privados:

Numero	aaaa/mm/dd	Notaria	No. Insc o Reg	aaaa/mm/dd
39	1979/01/18	Notaria 3a. de Barranquilla.	9,452	1979/01/24
992	1981/04/30	Notaria 1a. de Barranquilla.	13,112	1981/05/06
1,464	1982/08/27	Notaria Unica de Soledad	15,533	1982/09/08
2,744	1983/08/18	Notaria Unica de Soledad	17,416	1983/08/26
3,826	1984/07/16	Notaria Unica de Soledad	19,554	1984/07/27
3,835	1985/08/26	Notaria Unica de Soledad	22,230	1985/09/04
2,260	1986/09/10	Notaria Unica de Soledad.	24,995	1986/09/15
1,849	1989/07/11	Notaria 2a. de Barranquilla.	33,909	1989/07/13
444	1990/02/21	Notaria 2a. de Barranquilla.	36,101	1990/03/16
3,090	1991/12/17	Notaria 2a. de Barranquilla	43,993	1992/01/28
269	1993/02/01	Notaria 2a. de Barranquilla	48,428	1993/02/16
2,342	1993/07/28	Notaria 2a. de Barranquilla	50,582	1993/08/10
3,652	1993/11/09	Notaria 2a. de Barranquilla	51,798	1993/11/22
3,615	1996/12/06	Notaria 2a. de Barranquilla	67,218	1996/12/20
89	1997/01/16	Notaria 2a. de Barranquilla	67,704	1997/01/23
2,070	1997/07/17	Notaria 2a. de Barranquilla	70,627	1997/07/31
2,281	1997/08/04	Notaria 2a. de Barranquilla	70,813	1997/08/14
765	1999/04/22	Notaria 3a. de Barranquilla	80,628	1999/04/23
459	2001/02/21	Notaria 2a. de Barranquilla	91,502	2001/02/23
2,608	2001/10/05	Notaria 2a. de Barranquilla	95,771	2001/11/08
1,730	2004/07/29	Notaria 2. de Barranquilla	112,999	2004/08/26
416	2005/03/03	Notaria 2. de Barranquilla	116,364	2005/03/10
1,613	2005/07/28	Notaria 2. de Barranquilla	119,322	2005/08/18
710	2009/05/05	Notaria 3 a. de Barranquilla	150,130	2009/06/30
1,054	2009/06/25	Notaria 3 a. de Barranquilla	150,130	2009/06/30

C E R T I F I C A

Que de acuerdo con la(s) escritura(s) arriba citada(s), la sociedad
se rige por las siguientes disposiciones:

DENOMINACION O RAZON SOCIAL:

PROCAPS S.A.-----

SIGLA: PROCAPS.

DOMICILIO PRINCIPAL: Barranquilla.

NIT No: 890.106.527-5.

MATRICULA MERCANTIL: 24,802.

***** C O N T I N U A *****



***** Pag. 2

CAMARA DE COMERCIO
DE BARRANQUILLA

09*****

CERTIFICADO DE EXISTENCIA Y REPRESENTACION LEGAL O DE INSCRIPCION DE DOCUMENTOS.

PROCAPS S.A.-----

NIT: 890.106.527-5.

C E R T I F I C A

Direccion para notificaciones judiciales:

CL 80 No 78 B - 201.

De la ciudad de Barranquilla.

Correo

electrónico:

mlozada@procaps.com.co

Pagina Web:

Telefono:

3719272

C E R T I F I C A

DURACION: El término de duración de la sociedad se fijó hasta el 18 de Agosto de 2073.

C E R T I F I C A

OBJETO SOCIAL: El objeto principal de la sociedad sera: a) La fabricacion, importacion y comercializacion de alimentos procesados y suplementos dietarios. b) La fabricacion de capsulas y otros productos similares a partir de materiales tales como gelatinas u otros de distinta naturaleza, que puedan ser utilizados como envases o recipientes de productos y sustancias quimicas, farmaceuticas, alimenticias y cosmeticas, para uso y consumo humano y veterinario. c) La investigacion, desarrollo y fabricacion de toda clase de productos y sustancias quimicas, farmaceuticas, alimenticias y cosmeticas para uso y consumo humano, animal y vegetal. d) La compra y venta de materias primas, insumos y productos intermedios necesarios para los procesos de fabricacion de sustancias quimicas, farmaceuticas, alimenticias y cosmeticas, asi como la comercializacion interna y externa de los productos terminados. e) La compra, venta, importacion, exportacion y distribucion de toda clase de elementos instrumentales y equipos medicos, quirurgicos, odontologicos y hospitalarios para la salud humana y animal. f) La compra, venta, exportacion, fabricacion, importacion, distribucion de dulces, confites, galletas y todo tipo de golosinas o alimentos para el consumo humano. g) Actuar como representante o agente de personas naturales y juridicas, nacionales y extranjeras en la realizacion de operaciones o transacciones relacionadas con la industria farmaceutica y en especial con cualquiera de las actividades descritas en los literales a), b), c), d) y e) antes descritos. h) Prestar los servicios de asesorias y consultoria en las areas administrativas, de mercadeo, analisis clinico, asistencia tecnica, investigacion, costos y produccion, exportaciones, importaciones, ventas,

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relacionadas con iguales o similares actividades a las que constituyen el objeto de PROCAPS S.A., así como los de publicidad y promoción de productos químicos, alimenticios, cosméticos y farmaceuticos para uso humano, animal o industrial. i) Adquirir, operar, administrar y explotar, a cualquier titulo, empresa o establecimientos dedicados a la investigacion, desarrollo, fabricacion o comercializacion de productos químicos, farmaceuticos, alimenticios, hospitalarios o cosméticos. En desarrollo de su objeto principal, la sociedad podra tomar interes social como accionista, socio o fundador de empresas que tengan igual o similar objeto, comprar, vender, importar y exportar maquinarias, insumos y repuestos requeridos para el desarrollo de sus actividades; tomar dinero en prestamo, financiar y garantizar las operaciones comerciales que celebre con terceros relativas a su objeto; celebrar y ejecutar todo tipo de actos y contratos necesarios para desarrollar sus operaciones; girar, aceptar, otorgar y protestar todo tipo de titulos valores e instrumentos negociables, comprar, vender y constituir gravámenes sobre bienes muebles e inmuebles, designar representantes apoderados en el pais y en el exterior y, en general, realizar cualquier acto o negocio que sea necesario o conveniente para el desarrollo de su objeto social y para la mejor proteccion de sus intereses corporativos o la de sus socios. La sociedad podra garantizar el cumplimiento de sus obligaciones adquiridas por terceros o por sus socios, para lo cual podra inclusive constituir gravámenes reales sobre sus bienes de cualquier naturaleza.

C E R T I F I C A

DE BARBACILLA

CAPITAL

Nro Acciones

Valor Acción

Autorizado

\$*****2,000,000,000 *****2,000,000 *****1,000

Suscrito

\$*****1,298,000,000 *****1,298,000 *****1,000

Pagado

\$*****1,298,000,000 *****1,298,000 *****1,000

C E R T I F I C A

ADMINISTRACION: La direccion y administracion de la sociedad correspondera a la Asamblea General de Accionistas, a la Junta Directiva, al Presidente, al Vicepresidente y a un Gerente General. Corresponde a la Junta Directiva las siguientes funciones y atribuciones entre otras: Autorizar cualquier acto o contrato cuya cuantia sea superior al equivalente en pesos Colombianos a doscientos cincuenta mil dolares de los Estados Unidos de America (US\$250.000), liquidados a la tasa de cambio representativa del mercado vigente el dia en que se surta la transacion. Cuando se trate de operaciones en virtud de las cuales la sociedad garantice obligaciones de terceros o de los socios, se requerira que la operacion sea aprobada con el voto de por lo menos 2/3 partes de los integrantes de la Junta Directiva. La sociedad tendra un Presidente, quien ejercera la representacion legal de la sociedad y tendra las siguientes atribu-

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PROCAPS S.A.-----

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ciones entre otras: Ejecutar las decisiones de la Asamblea General de Accionistas y de la Junta Directiva. Ejercer la Representacion Legal de la sociedad en todos los actos y contratos que se requieran para el desarrollo del objeto social, de conformidad con lo previsto en las leyes y en los presentes estatutos. Constituir apoderados judiciales y extrajudiciales, recibir notificaciones, absolver interrogatorios de parte, confesar obligaciones de la sociedad, transigir, disistir y allanar a la sociedad en cualquier asunto judicial. Aprobar y celebrar en nombre de la socieda todo acto o contrato cuya cuantia sea igual o inferior al equivalente en pesos colombianos a doscientos cincuenta mil dolares de los Estados Unidos de America (US\$250.000), liquidados a la tasa de cambio representativa del mercado vigente el dia en que se surta la transacion o celebracion del acto. Las demas funciones que le correspondan como Representante Legal por disposicion de la ley, de estos estatutos o de la Junta Directiva. El Presidente tendra un suplente, designado por la Junta Directiva, quien reemplazara al Presidente en sus faltas temporales, absolutas o meramente accidentales, con entidades facultades y sin que sea necesario para ello acreditar la ausencia del principal. La sociedad tendra un Vicepresidente designado por la Junta Directiva, quien tambien ejercera la representacion legal de la sociedad, pudiendo ejercerla conjunta o separadamente con el Presidente. El vicepresidente tendra las siguientes facultades entre otras. Ejecutar dentro de su competencia las decisiones de la Asamblea General de Accionistas, de la Junta Directiva y del Presidente. Ejercer la representacion legal de la sociedad en todos los actos y contratos que se requieran para el desarrollo del objeto social, de conformidad con lo previsto en las leyes y en los presentes estatutos. Constituir apoderados judiciales y extrajudiciales, recibir notificaciones, absolver interrogatorios de parte, confesar obligaciones de la sociedad, transigir, desistir y allanar a la sociedad en cualquier asunto judicial. Aprobar y celebrar en nombre de la sociedad todo acto o contrato cuya cuantia sea igual o inferior al equivalente en pesos colombianos a ciento cincuenta mil dolares de los Estados Unidos de America (US\$150.000), liquidados a la tasa de cambio representativa del mercado vigente el dia en que se surta la aprobacion o celebracion del acto, siempre que no se trate de enajenar bienes inmuebles o cualquier otro activo fijo de la sociedad, inclusive de naturaleza muebles, acciones, cuotas o partes de interes inversiones y todo tipo de derechos de la sociedad, o de constituir gravámenes sobre cualquiera de los anteriores, facultades que radican exclusiva y privativamente en cabeza del Presidente. Las demas funciones que le corresponda como Representante legal por disposicion de la ley, de estos estatutos o de la Junta Directiva. El Vicepresidente tendra (1) suplente,

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designado por la Junta Directiva, quien lo reemplazara conjunta o separadamente en sus faltas temporales, absolutas o meramente accidentales, con identicas facultades y sin que sea necesario para ello acreditar la ausencia del principal. La sociedad tendra un (1) Gerente General, con su respectivo Suplente, designados por la Junta Directiva. El Suplente reemplazara al Gerente General, en sus faltas temporales, absolutas o meramente accidentales, quien junto con el gerente general, llevaran la representacion legal de la sociedad, con identicas facultades, pudiendolas ejercer conjunta o separadamente con el Presidente y Vicepresidente, con identicas facultades que el Gerente General y sin que sea necesario para ello acreditar la ausencia del titular. El Gerente General tendra las siguientes facultades entre otras: Ejercer la representacion legal de la sociedad, en todos los actos y contratos que se requieran para el desarrollo del objeto social, de conformidad con lo previsto en las leyes y en los presentes estatutos. Aprobar y celebrar en nombre de la sociedad todo acto o contrato cuya cuantia sea igual o inferior al equivalente en pesos Colombianos a Cincuenta Mil dolares de los Estados Unidos de America (US\$50.000), liquidados a la tasa de cambio representativa del mercado vigente el dia en que se surta la aprobacion o celebracion del acto, siempre que no se trate de enajenar a cualquier titulo bienes inmuebles o cualquier otro activo fijo de la sociedad, inclusive de naturaleza mueble, acciones, cuotas o partes de interes, inversiones y todo tipo de derechos de la sociedad o de constituir gravámenes sobre cualquiera de las anteriores facultades que radican exclusiva y privativamente en cabeza del Presidente.

C E R T I F I C A D O
DE BARRANQUILLA

Que según Acta No. 116 del 27 de Junio de 1997 correspondiente a la Asamblea de Accionistas en Barranquilla, de la sociedad:

PROCAPS S.A.-----

cuya parte pertinente se inscribió en esta Cámara de Comercio, el 15 de Agosto de 1997 bajo el No. 70,838 del libro respectivo, y según Acta No. 127 del 22 de Marzo de 2000 correspondiente a la Asamblea de Accionistas en Barranquilla, de la sociedad:

PROCAPS S.A.-----

cuya parte pertinente se inscribió en esta Cámara de Comercio, el 29 de Mayo de 2000 bajo el No. 87,250 del libro respectivo, fueron hechos los siguientes nombramientos:

CLASE: JUNTA DIRECTIVA

Principales

1. Minski Gontovnik Ruben	CC.*****7,463,982
2. Minski Gontovnik Meyer	CC.*****7,461,324
3. Minski Gontovnik Jose	CC.*****8,671,834

Suplentes

1. Minski Zirberblum Elliot	CC.*****72,219,541
2. Minski Deitel Daniel	*****
3. Minski Sharon	PA.*990,212,082,805

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C E R T I F I C A

Que según Acta No. 116 del 27 de Junio de 1997 correspondiente a la Asamblea de Accionistas en Barranquilla, de la sociedad: PROCAPS S.A. cuya parte pertinente se inscribió en esta Cámara de Comercio, el 15 de Agosto de 1997 bajo el No. 70,838 del libro respectivo, fueron hechos los siguientes nombramientos:

Cargo/Nombre	Identificación
Presidente.	
Minski Gontovnik Ruben	CC.*****7,463,982
Suplente del Presidente	
Minski Minski Abraham	CC.*****802,575
Vicepresidente	
Minski Gontovnik Meyer	CC.*****7,461,324
Suplente del vicepresidente	
Minski Gontovnik Jose	CC.*****8,671,834

C E R T I F I C A

Que según Acta No. 11 del 01 de Febrero de 2001 correspondiente a la Junta Directiva en Barranquilla, de la sociedad: PROCAPS S.A. cuya parte pertinente se inscribió en esta Cámara de Comercio, el 12 de Febrero de 2001 bajo el No. 91,273 del libro respectivo, fueron hechos los siguientes nombramientos:

Cargo/Nombre	Identificación
Gerente General.	
Uribe Ochoa Guillermo Luis	CC.*****70,073,754

C E R T I F I C A

Que según Acta No. 406 del 02 de Sep/bre de 2004 correspondiente a la Junta Directiva en Barranquilla, de la sociedad: PROCAPS S.A. cuya parte pertinente se inscribió en esta Cámara de Comercio, el 09 de Sep/bre de 2004 bajo el No. 113,263 del libro respectivo, fueron hechos los siguientes nombramientos:

Cargo/Nombre	Identificación
Suplente del Gerente.	
Tanaka Tatekawa Walter	CC.*****16,251,923

C E R T I F I C A

Que según Acta No. 124 del 29 de Marzo de 1999 correspondiente a la Asamblea de Accionistas en Barranquilla, de la sociedad: PROCAPS S.A. cuya parte pertinente se inscribió en esta Cámara de Comercio,

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el 04 de Agosto de 1999 bajo el No. 82,226 del libro respectivo, fueron hechos los siguientes nombramientos:

Cargo/Nombre	Identificación
Revisor Fiscal Ppal. Vargas Pertuz Ana Isabel	CC.*****32,696,645

C E R T I F I C A

Que según Acta No. 158 del 17 de Junio de 2011 correspondiente a la Asamblea de Accionistas en Barranquilla, de la sociedad: PROCAPS S.A. cuya parte pertinente se inscribió en esta Cámara de Comercio, el 23 de Junio de 2011 bajo el No. 170,929 del libro respectivo, fueron hechos los siguientes nombramientos:

Cargo/Nombre	Identificación
Revisor Fiscal Suplente Rodriguez Rey Jose Luis	CC.*****72,001,912

C E R T I F I C A

Que por Escritura Pública No. 2,784 del 07 de Dic/bre de 2007, otorgada en la Notaria 2 a. de Barranquilla cuya parte pertinente se inscribió en esta Cámara de Comercio, el 20 de Dic/bre de 2007 bajo el No. 3,492 del libro respectivo, -----
y las inscripciones 3.492-1, 3.493, 3.494, 3.495, 3.496, 3.497, ----
3.498, 3.499, 3.500, 3.501, 3.502, 3.503, 3.504, 3.505, 3.506, ----
3.507, Consta que RUBÉN MINSKI GONTOVNIK, C.C. No. 7.463.982, ----
obrando como Presidente y Representante Legal de la sociedad C.I.
NATURMEGA S.A., que obrando en su condicion de Presidente y -----
Representante Legaql de la sociedad PROCAPS S.A.; el presidente ----
debidamente facultado por los "Estatutos" Sociales y por el Maximo
Organo Social, otorga los siguientes poderes: Otorgar poder -----
general, amplio y suficiente a los doctores JOSEFINA DEL CARMEN ----
MIELES BUELVAS CC.No.33.138.497; a MARCELA CARVAJALINO PAGANO ----
CC.No.22.579.748; CRISTINA VIOLI FRANCESCHINI CC.No.22.463.716, con
las facultades que se transcriben a continuacion: a.- Para que ----
representen a la sociedad PROCAPS S.A, ante todo tipo de -----
autoridades administrativas y jurisdiccionales, en asuntos de ----
naturaleza civil, laboral, comercial, penal, aduanera, cambiaria,
tributaria, policiva, administrativa y fiscal. b.- Contestar y ----
presentar demandas, requerimientos, recursos, pedir y aportar ----
pruebas, recibir, notificarse, ejercer la accion de tutela, -----
tramitar incidente de desacato, comparecer en los procesos que ----
cursen contra la sociedad, allanarse en nombre de la sociedad en
los asuntos que sean necesarios, transigir, desistir, sustituir,
renunciar y reasumir el poder. c.- La facultad expresa de -----
conciliar, por tanto pueden llegar a acuerdos conciliatorios, ----
transaccionales, judiciales o extrajudiciales o extrajudiciales,
cualquiera que sea su cuantia, para lo cual las apoderadas deben
aportar la autorizacion simple y escrita del Representante legal
de la sociedad. d.- Podran presentar solicitudes de devolucion y/o
compensacion de impuestos, ventas y rentas, recibir los pagos por
las devoluciones y adelantar todo tramite o proceso administrativo
ante la DIAN, de tal forma que en ningun momento quede sin -----

*****C O N T I N U A *****



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representacion legal de la sociedad. PODERES ESPECIALES: a.- Se ----
 confiere poder especial amplio y suficiente a las personas que se
 determinan seguidamente, que conforman el grupo A y B, para, para
 que conjuntamente suscriban contratos de suministros de mercancías
 y servicios con entidades oficiales, hasta la suma de OCHOCIENTOS
 MIL DOLARES DE LOS ESTADOS UNIDOS DE AMERICA (US800.000.00), -----
 liquidados a la tasa representativa del mercado vigente en el día
 que se realice la respectiva transacción. Grupo A. WALTER NANAKA
 TATEKAWA CC.No.16.251.923, CLAUDIA TANGARIFE CASTILLO -----
 CC.No.41.660.293, CARMEN ALICIA FLOREZ CC.No.22.434.044, IVAN -----
 VILLAREAL CAMACHO CC.No.91.201.749, CARMEN ALICIA HERRERA -----
 DIAZGRANADOS CC.No.22.377.540, DAVID ANTONIO CHARRIS GIRALDO -----
 CC.No.19.341.274, RICARDO DORADO HURTADO CC.No.79.715.772, JAIME
 FORERO LLINAS CC.No.19.300.593. b.- Se confiere poder especial ----
 amplio y suficiente al señor WILLIAM ALBERTO BARROS CERRA -----
 CC.No.8.715.941, para que en nombre y representación de PROCAPS ----
 S.A., suscriban declaraciones cambiarias, aduaneras y tributarias a
 que este obligada la sociedad, en el giro normal de sus negocios y
 operaciones sociales, por tanto quedan facultados para firmarlas,
 como también los demás documentos relacionados con ellas, -----
 notificarse, aportar y solicitar las pruebas que sean necesarias.
 c.- Otorgar poder especial amplio y suficiente al señor MARLON ----
 TATIS PAREDES CC.No.72.176.874 y al señor WILLIAM ALBERTO BARROS
 CERRA CC.No.8.715.941, para que en nombre y representación de ----
 PROCANPS S.A., suscriban las declaraciones, reportes y cualquier
 documento relacionado con los trámites o registros de Comercio ----
 Exterior y o cualquier entidad del Estado que haga sus veces: d)
 Otorgar poder especial amplio y suficiente a la doctora CARMEN ----
 ALICIA HERRERA DIAZGRANADOS, CC.No.22.377.540 y al doctor WALTER
 TANAKA TATEKAWA, CC.No.16.251.923, para que en nombre y ----
 representación de PROCAPS S.A.; endosen los títulos valores de la
 sociedad hasta por la suma de UN MIL MILLONES DE PESOS -----
 (1.000.000.000.00) M.L. CUARTO: El representante legal de la ----
 sociedad PROCAPS S.A.; doctor RUBEN MINSKI GONTOVNIK, manifiesta
 que la presente reforma fue aprobada por la Asamblea General ----
 Extraordinaria de Accionista de PROCAPS S.A., con observancia de
 las disposiciones legales y estatutarias.-----

C E R T I F I C A

Que por Escritura Pública No. 1,933 del 16 de Sep/bre de 2008,
 otorgada en la Notaria 2 a. de Barranquilla cuya parte pertinente
 se inscribió en esta Cámara de Comercio, el 06 de Octubre de 2008
 bajo el No. 3,748 del libro respectivo,-----
 Consta que el señor RUBEN MINSKI GONTOVNIK, C.C. No. 7.463.982, que

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comparece en su condicion de Presidente y Representante Legal de la Sociedad PROCAPS S.A., confiere por medio de este instrumento -----
publico Poder especial Amplio y Suficiente a la doctora MILENA -----
ESCAFFI PARDO, C.C. No. 32.758.420, para que en nombre y -----
representacion de PROCAPS S.A., suscriba declaraciones cambiarias,
aduaneras y tributarias a que este obligada la sociedad en el giro
normal de sus negocios y operaciones sociales, por tanto queda -----
facultada para firmar, como tambien los demas documentos -----
relacionados con ellas, notificarse, aportar y solicitar las -----
pruebas que sean necesarias.-----

C E R T I F I C A

Que por Documento Privado del 09 de Abril de 2010, otorgado en Barranquilla inscrito en esta Cámara de Comercio, el 20 de Abril de 2010 bajo el Nro 4,201 del libro respectivo,-----
Consta que el señor RUBEN MINSKI GONTOVNIK, C.C. No. 7.463.982, ----
quien obra en el caracter de Presidente y/o Representante legal de la sociedad PROCAPS S.A., que por medio de este instrumento -----
publico, otorga Poder Especial Amplio y Suficiente a la Doctora ----
MONICA DE HART DE OCHOA, identificada con C.C. No. 32.676.025, para que en nombre y representacion de la sociedad, Endose las Facturas Nacionales e Internacionales hasta por la suma de UN MIL MILLONES DE PESOS (\$1.000.000.000.00)M.L. La doctora Monica de Hart de Ochoa, queda facultada para realizar todo acto o tramite necesario para el cabal cumplimiento del presente mandato.-----

C E R T I F I C A

DE BARRANQUILLA

Que por Documento Privado del 10 de Junio de 2010, otorgado en Barranquilla inscrito en esta Cámara de Comercio, el 13 de Julio de 2010 bajo el Nro 4,228 del libro respectivo,-----
consta que el señor RUBEN MISNKI GONTOVNIK, cc. No. 7.463.982 en su condicion de Presidente y representante legal de PROCAPS S.A., ----
confiere poder especial, amplio y suficiente al señor RICARDO DORADO HURTADO cc. No. 79.715.772 para que represente a la sociedad, ante el INVIMA, Fondo Nnacional de estupefacientes e ICA, con el fin que gestione, tramite o presente las diferentes solicitudes y -----
peticiones de la empresa, segun la competencia de estas entidades.
Por lo anterior, el Señor RICARDO DORADO HURTADO, queda facultado para presentar, gestionar, tramitar y firmar las diferentes -----
solicitudes y peticiones de la empresa, segun la competencia de ----
estas entidades, como tambien presentar y firmar todo los -----
documentos relacionados con estas solicitudes, notificarse de -----
cualquier acto administrativo que expidan, aportar y solicitar las pruebas que sean necesarias, desistir, y en general a realizar todo tramite o acto necesario de tal forma que la sociedad en ningun ----
momento quede sin representacion legal ante estas entidades, en ----
cumplimiento fiel al mandato otorgado.-----

C E R T I F I C A

Que por Documento Privado del 03 de Agosto de 2010, otorgado en

***** C O N T I N U A *****



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C A M A R A D E C O M E R C I O
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CERTIFICADO DE EXISTENCIA Y REPRESENTACION LEGAL O DE INSCRIPCION DE DOCUMENTOS.

PROCAPS S.A.-----

NIT: 890.106.527-5.

Barranquilla inscrito en esta Cámara de Comercio, el 24 de Agosto de 2010 bajo el Nro 4,258 del libro respectivo,-----
consta que RUBEN MINSKI GONTOVNIK, varón, mayor de edad, de ésta vecindad, identificado con la cédula de ciudadanía No.7.463.982, expedida en Barranquilla, en mi condición de Representante legal y/o Presidente de la sociedad PROCAPS S. A., conforme a las -----
facultades otorgadas por los Estatutos Sociales, todo lo cual -----
demuestro con el certificado de existencia y representación legal expedido por la Cámara de Comercio de Barranquilla, que adjunto a este escrito, me permito manifestarles que confiero poder especial, amplio y suficiente al señor MARIO LÓPEZ LEON, varón, mayor de -----
edad, de esta vecindad, identificado con la cédula de ciudadanía No.72.311.558, expedida en Puerto Colombia (Atlántico), para que en nombre y representación de la sociedad, realice los siguientes -----
actos: 1. Endosar las Facturas Nacionales e Internacionales de esas compañías hasta por la suma de UN MIL MILLONES DE PESOS -----
(\$1.000.000.000.00) Moneda Legal. 2. Suscribir Declaraciones -----
Cambiarias, Aduaneras y Tributarias a las que está obligada la -----
sociedad en el giro ordinario de sus negocios, quedando facultado para notificarse, aportar y solicitar pruebas relacionados con las declaraciones Cambiarias. El señor MARIO LÓPEZ LEÓN, queda -----
facultado para notificarse, aportar y solicitar pruebas, presentar escritos en caso que resulte necesario en general a realizar todo acto, diligencia o tramite que considere indispensable para el -----
cabal cumplimiento del presente mandato.-----

C E R T I F I C A

Que en esta Cámara de Comercio no aparecen inscripciones posteriores de documentos referentes a reforma, disolución, liquidación o nombramientos de representantes legales de la expresada sociedad.

C E R T I F I C A

Que su última Renovación fue el: 31 de Marzo de 2011.

La información sobre embargos de establecimiento se suministra en Certificados de Matrícula, la de contratos sujetos a registro, en Certificados Especiales.

C E R T I F I C A

Que los actos de registro aquí certificados quedan en firme cinco días hábiles después de la correspondiente inscripción, siempre que, dentro de dicho término, no sean objeto de los recursos de

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ANEXO 1



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(54) **ANTIBODY VARIANTS AND USES THEREOF**

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(57) **ABSTRACT**

The invention provides improved humanized CD20 binding antibodies for treatment of B cell malignancies and autoimmune diseases.

ANTIBODY VARIANTS AND USES THEREOF

[0001] This application claims the benefit of U.S. Provisional Application Ser. No. 60/651,111, filed Feb. 7, 2005, 60/689,404 filed Jun. 10, 2005, and 60/702,571 filed Jul. 25, 2005, which applications are incorporated herein by reference in their entirety.

FIELD OF THE INVENTION

[0002] The invention relates to anti-CD20 antibodies and their use in the treatment of B-cell related diseases.

BACKGROUND OF THE INVENTION

[0003] Lymphocytes are one of several populations of white blood cells; they specifically recognize and respond to foreign antigen. The three major classes of lymphocytes are B lymphocytes (B cells), T lymphocytes (T cells) and natural killer (NK) cells. B lymphocytes are the cells responsible for antibody production and provide humoral immunity. B cells mature within the bone marrow and leave the marrow expressing an antigen-binding antibody on their cell surface. When a naive B cell first encounters the antigen for which its membrane-bound antibody is specific, the cell begins to divide rapidly and its progeny differentiate into memory B cells and effector cells called "plasma cells". Memory B cells have a longer life span and continue to express membrane-bound antibody with the same specificity as the original parent cell. Plasma cells do not produce membrane-bound antibody but instead produce secreted form of the antibody. Secreted antibodies are the major effector molecules of humoral immunity.

[0004] The CD20 antigen (also called human B-lymphocyte-restricted differentiation antigen, Bp35) is a hydrophobic transmembrane protein with a molecular weight of approximately 35 kD located on pre-B and mature B lymphocytes (Valentine et al. *J. Biol. Chem.* 264(19):11282-11287 (1989); and Einfeld et al. *EMBO J.* 7(3):711-717 (1988)). The antigen is also expressed on greater than 90% of B cell non-Hodgkin's lymphomas (NHL) (Anderson et al. *Blood* 63(6):1424-1433 (1984)), but is not found on hematopoietic stem cells, pro-B cells, normal plasma cells or other normal tissues (Tedder et al. *J. Immunol.* 135(2):973-979 (1985)). CD20 is thought to regulate an early step(s) in the activation process for cell cycle initiation and differentiation (Tedder et al., *supra*) and possibly functions as a calcium ion channel (Tedder et al. *J. Cell. Biochem.* 14D:195 (1990)).

[0005] Given the expression of CD20 in B cell lymphomas, this antigen has been a useful therapeutic target to treat such lymphomas. There are more than 300,000 people in the United States with B-cell NHL and more than 56,000 new cases are diagnosed each year. For example, the rituximab (RITUXAN®) antibody which is a genetically engineered chimeric murine/human monoclonal antibody directed against human CD20 antigen (commercially available from Genentech, Inc., South San Francisco, Calif., U.S.) is used for the treatment of patients with relapsed or refractory low-grade or follicular, CD20 positive, B cell non-Hodgkin's lymphoma. Rituximab is the antibody referred to as "C2B8" in U.S. Pat. No. 5,736,137 issued Apr. 7, 1998 (Anderson et al.) and in U.S. Pat. No. 5,776,456. In vitro mechanism of action studies have demonstrated that RITUXAN® binds human complement and lyses lymphoid B cell lines through complement-dependent cytotoxicity

(CDC) (Reff et al. *Blood* 83(2):435-445 (1994)). Additionally, it has significant activity in assays for antibody-dependent cellular cytotoxicity (ADCC). In vivo preclinical studies have shown that RITUXAN® depletes B cells from the peripheral blood, lymph nodes, and bone marrow of cynomolgus monkeys, presumably through complement and cell-mediated processes (Reff et al. *Blood* 83(2):435-445 (1994)). Other anti-CD20 antibodies indicated for the treatment of NHL include the murine antibody Zevalin™ which is linked to the radioisotope, Yttrium-90 (IDEC Pharmaceuticals, San Diego, Calif.), Bexxar™ which is another fully murine antibody conjugated to I-131 (Corixa, Wash.).

[0006] CD20 is also a useful target antigen for treating autoimmune diseases. Rituximab has also been studied in a variety of non-malignant autoimmune disorders, in which B cells and autoantibodies appear to play a role in disease pathophysiology. Edwards et al., *Biochem. Soc. Trans.* 30:824-828 (2002). Rituximab has been reported to potentially relieve signs and symptoms of, for example, rheumatoid arthritis (RA) (Leandro et al., *Ann. Rheum. Dis.* 61:883-888 (2002); Edwards et al., *Arthritis Rheum.*, 46 (Suppl. 9): S46 (2002); Stahl et al., *Ann. Rheum. Dis.*, 62 (Suppl. 1): OP004 (2003); Emery et al., *Arthritis Rheum.* 48(9): S439 (2003)), lupus (Eisenberg, *Arthritis. Res. Ther.* 5:157-159 (2003); Leandro et al. *Arthritis Rheum.* 46: 2673-2677 (2002); Gorman et al., *Lupus*, 13: 312-316 (2004)), immune thrombocytopenic purpura (D'Arena et al., *Leuk. Lymphoma* 44:561-562 (2003); Siasi et al., *Blood*, 98: 952-957 (2001); Saleh et al., *Semin. Oncol.*, 27 (Supp 12):99-103 (2000); Zaia et al., *Haematologica*, 87: 189-195 (2002); Ratanatharathorn et al., *Ann. Int. Med.*, 133: 275-279 (2000)), pure red cell aplasia (Auner et al., *Br. J. Haematol.*, 116: 725-728 (2002)); autoimmune anemia (Zaja et al., *Haematologica* 87:189-195 (2002) (erratum appears in *Haematologica* 87:336 (2002)), cold agglutinin disease (Layios et al., *Leukemia*, 15: 187-8 (2001); Berentsen et al., *Blood*, 103: 2925-2928 (2004); Berentsen et al., *Br. J. Haematol.*, 115: 79-83 (2001); Bauduer, *Br. J. Haematol.*, 112: 1083-1090 (2001); Damiani et al., *Br. J. Haematol.*, 114: 229-234 (2001)), type B syndrome of severe insulin resistance (Coll et al., *N. Engl. J. Med.*, 350: 310-311 (2004)), mixed cryoglobulinemia (DeVita et al., *Arthritis Rheum.* 46 Suppl. 9:S206/S469 (2002)), myasthenia gravis (Zaja et al., *Neurology*, 55: 1062-63 (2000); Wylam et al., *J. Pediatr.* 143: 674-677 (2003)), Wegener's granulomatosis (Specks et al., *Arthritis & Rheumatism* 44: 2836-2840 (2001)), refractory pemphigus vulgaris (Dupuy et al., *Arch. Dermatol.*, 140:91-96 (2004)), dermatomyositis (Levine, *Arthritis Rheum.*, 46 (Suppl. 9):S1299 (2002)), Sjogren's syndrome (Somer et al., *Arthritis & Rheumatism*, 49: 394-398 (2003)), active type-II mixed cryoglobulinemia (Zaja et al., *Blood*, 101: 3827-3834 (2003)), pemphigus vulgaris (Dupay et al., *Arch. Dermatol.*, 140: 91-95 (2004)), autoimmune neuropathy (Pestronk et al., *J. Neurol. Neurosurg. Psychiatry* 74:485-489 (2003)), paraneoplastic opsoclonus-myoclonus syndrome (Pranzatelli et al. *Neurology* 60(Suppl. 1) PO5.128:A395 (2003)), and relapsing-remitting multiple sclerosis (RRMS). Cross et al. (abstract) "Preliminary results from a phase II trial of Rituximab in MS" Eighth Annual Meeting of the Americas Committees for Research and Treatment in Multiple Sclerosis, 20-21 (2003).

[0007] A Phase II clinical trial has been conducted in patients with rheumatoid arthritis (RA), providing 48-week follow-up data on safety and efficacy of Rituximab. Emery

dextrins; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (e.g. Zn-protein complexes); and/or non-ionic surfactants such as TWEEN™, PLURONICS™ or polyethylene glycol (PEG).

[0281] Exemplary hu2H7 antibody formulations are described in WO98/56418, expressly incorporated herein by reference. Another formulation is a liquid multidose formulation comprising the hu2H7 antibody at 40 mg/mL, 25 mM acetate, 150 mM trehalose, 0.9% benzyl alcohol, 0.02% polysorbate 20 at pH 5.0 that has a minimum shelf life of two years storage at 2-8° C. Another anti-CD20 antibody formulation of interest comprises 10 mg/mL antibody in 9.0 mg/mL sodium chloride, 7.35 mg/mL sodium citrate dihydrate, 0.7 mg/mL polysorbate 80, and Sterile Water for Injection, pH 6.5. Yet another aqueous pharmaceutical formulation comprises 10-30 mM sodium acetate from about pH 4.8 to about pH 5.5, preferably at pH 5.5, polysorbate as a surfactant in an amount of about 0.01-0.1% v/v, trehalose at an amount of about 2-10% w/v, and benzyl alcohol as a preservative (U.S. Pat. No. 6,171,586). Lyophilized formulations adapted for subcutaneous administration are described in WO97/04801. Such lyophilized formulations may be reconstituted with a suitable diluent to a high protein concentration and the reconstituted formulation may be administered subcutaneously to the mammal to be treated herein.

[0282] In a specific embodiment, one IV formulation of humanized 2H7 v16 is: 30 mg/ml antibody in 20 mM sodium acetate, 4% trehalose dihydrate, 0.02% polysorbate 20 (Tween 20™), pH 5.3. This IV formulation can be administered for oncology indications such as NHL, as well as for rheumatoid arthritis. One formulation for the humanized 2H7.v511 variant is 15-30 mg/ml antibody, preferably 20 mg/mL antibody, in 10 mM histidine sulfate, 60 mg/ml sucrose (6%), 0.2 mg/ml polysorbate 20 (0.02%), and Sterile Water for Injection, at pH 5.8. In yet another embodiment, the formulation for 2H7 variants and in particular 2H7.v511 is 20 mg/ml 2H7, 20 mM sodium acetate, 4% trehalose dihydrate, 0.02% polysorbate 20, pH 5.5, for intravenous administration. This formulation can be used for single i.v. infusions. One formulation for 2H7.v 114 is antibody at 15-25 mg/ml, preferably 20 mg/ml, in 20 mM Sodium Acetate, 240 mM (8%) trehalose dihydrate, 0.02% Polysorbate 20, pH 5.3.

[0283] The formulation herein may also contain more than one active compound as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect each other. For example, it may be desirable to further provide a cytotoxic agent, chemotherapeutic agent, cytokine or immunosuppressive agent (e.g. one which acts on T cells, such as cyclosporin or an antibody that binds T cells, e.g. one which binds LFA-1). The effective amount of such other agents depends on the amount of antibody present in the formulation, the type of disease or disorder or treatment, and other factors discussed above. These are generally used in the same dosages and with administration routes as described herein or about from 1 to 99% of the heretofore employed dosages.

[0284] The active ingredients may also be entrapped in microcapsules prepared, for example, by coacervation tech-

niques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methylmethacrylate) microcapsules, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in *Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. (1980).

[0285] Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semi-permeable matrices of solid hydrophobic polymers containing the antagonist, which matrices are in the form of shaped articles, e.g. films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol)), polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT™ (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid.

[0286] The formulations to be used for in vivo administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes.

Articles of Manufacture and Kits

[0287] Another embodiment of the invention is an article of manufacture containing materials useful for the treatment of autoimmune diseases and related conditions and CD20 positive cancers such as non-Hodgkin's lymphoma. The article of manufacture comprises a container and a label or package insert on or associated with the container. Suitable containers include, for example, bottles, vials, syringes, etc. The containers may be formed from a variety of materials such as glass or plastic. The container holds a composition which is effective for treating the condition and may have a sterile access port (for example the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). At least one active agent in the composition is a hu2H7 antibody, e.g., hu2H7.v511 of the invention. The label or package insert indicates that the composition is used for treating the particular condition. The label or package insert will further comprise instructions for administering the antibody composition to the patient.

[0288] Package insert refers to instructions customarily included in commercial packages of therapeutic products, that contain information about the indications, usage, dosage, administration, contraindications and/or warnings concerning the use of such therapeutic products. In one embodiment, the package insert indicates that the composition is used for treating non-Hodgkins' lymphoma.

[0289] Additionally, the article of manufacture may further comprise a second container comprising a pharmaceutically-acceptable buffer, such as bacteriostatic water for injection (BWFI), phosphate-buffered saline, Ringer's solution and dextrose solution. It may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, and syringes.

[0290] Kits are also provided that are useful for various purposes, e.g., for B-cell killing assays, as a positive control

for apoptosis assays, for purification or immunoprecipitation of CD20 from cells. For isolation and purification of CD20, the kit can contain a hu2H7.v511 antibody coupled to beads (e.g., sepharose beads). Kits can be provided which contain the antibodies for detection and quantitation of CD20 in vitro, e.g. in an ELISA or a Western blot. As with the article of manufacture, the kit comprises a container and a label or package insert on or associated with the container. The container holds a composition comprising at least one anti-CD20 antibody of the invention. Additional containers may be included that contain, e.g., diluents and buffers, control antibodies. The label or package insert may provide a description of the composition as well as instructions for the intended in vitro or diagnostic use.

EXPERIMENTAL EXAMPLES

Materials

[0291] Mice: Generation of human CD20 (hCD20)⁺ mice was accomplished through the use of bacterial artificial chromosomes (BAC) incorporating the hCD20 locus. Positive BAC clones were injected into blastocytes derived from FVB mice to generate transgenic (Tg) founder lines that expressed hCD20. See detailed description of generation and characterization of hCD20 Tg⁺ mice in Gong et al. (2005) *J. Immunol.* 174:817-826. HCD20Tg⁺ mice were subsequently bred with hCD16Tg⁺ mCD16^{-/-} to generate hCD20Tg⁺ mCD16^{-/-}hCD16Tg⁺ mice, which were used in the studies described below.

Antibodies: all antibodies used in FACS analysis were purchased from BD PharMingen.

Equipments/software: FACS analysis was performed using FACScan or FACSCalibur machines, and using CellQuest software purchased from Becton Dickinson.

General Methods

[0292] Preparation of single cell suspensions: 50 μ l of mouse blood was collected orbitally using EDTA-containing tubes, followed by a red cell lysis using ACK Lysis Buffer (purchased from Biosource). Following their removals, mouse spleens were granulized into single cell suspensions using frosted glass slides. Cell pellet was then washed, resuspended and filtered. 5 ml of cold PBS was used to wash peritoneal cavity of each mouse. Lavage fluid was recovered using 5-ml syringes followed by a centrifugation. Leukocytes from mouse blood, spleen and peritoneum were then washed, and counted before staining for FACS analysis.

[0293] FACS staining: approximately 1×10^6 were stained for the various cell surface makers (see definitions in FACS analysis) in 100 μ l of phosphate-buffered-saline plus 1% albumin (Sigma). Following a 30-minute-incubation on ice, stained cells were then washed and resuspended before being subjected for FACS analysis.

[0294] FACS analysis: FACS analysis was performed using FACScan or FACSCalibur with CellQuest software. B cells in blood were defined as CD21⁺CD23⁺; peritoneal B cells were defined as CD19⁺; splenic B cells were defined as B220⁺; MZ (marginal zone) B cells were defined as CD21⁺CD23^{low}; and FO (follicular) B cells were defined as CD21⁺CD23^{high}.

Statistics: data were expressed as the mean $\pm$ standard error (n=5). Statistical analysis was performed using two-tail unpaired T test.

Example 1

Humanization of 2H7 Anti-CD20 Murine Monoclonal Antibody

[0295] Humanization of the murine anti-human CD20 antibody, 2H7 (also referred to herein as m2H7, m for murine), was carried out in a series of site-directed mutagenesis steps. The murine 2H7 antibody variable region sequences and the chimeric 2H7 with the mouse V and human C have been described, see, e.g., U.S. Pat. Nos. 5,846,818 and 6,204,023. The CDR residues of 2H7 were identified by comparing the amino acid sequence of the murine 2H7 variable domains (disclosed in U.S. Pat. No. 5,846,818) with the sequences of known antibodies (Kabat et al., Sequences of proteins of immunological interest, Ed. 5. Public Health Service, National Institutes of Health, Bethesda, Md. (1991)). The CDR regions for the light and heavy chains were defined based on sequence hypervariability (Kabat et al., supra) and are shown in FIG. 1A and FIG. 1B, respectively. Using synthetic oligonucleotides (Table 1), site-directed mutagenesis (Kunkel, *Proc. Natl. Acad. Sci.* 82:488-492 (1985)) was used to introduce all six of the murine 2H7CDR regions into a complete human Fab framework corresponding to a consensus sequence V_LI, V_HIII (V_L kappa subgroup I, V_H subgroup III) contained on plasmid pVX4 (see FIG. 2 in WO 04/056342 which PCT publication is incorporated herein in its entirety).

[0296] The phagemid pVX4 was used for mutagenesis as well as for expression of F(ab)s in *E. coli*. Based on the phagemid pb0720, a derivative of pB0475 (Cunningham et al., *Science* 243: 1330-1336 (1989)), pVX4 contains a DNA fragment encoding a humanized consensus κ -subgroup I light chain (V_L κ I-C_L) and a humanized consensus subgroup III heavy chain (V_HIII-C_HI) anti-IFN- α (interferon α) antibody. pVX4 also has an alkaline phosphatase promoter and Shine-Dalgarno sequence both derived from another previously described pUC119-based plasmid, pAK2 (Carter et al., *Proc. Natl. Acad. Sci. USA* 89: 4285 (1992)). A unique SpeI restriction site was introduced between the DNA encoding for the F(ab) light and heavy chains. The first 23 amino acids in both anti-IFN- α heavy and light chains are the SHI secretion signal sequence (Chang et al., *Gene* 55: 189-196 (1987)).

[0297] To construct the CDR-swap version of 2H7 (2H7.v2), site-directed mutagenesis was performed on a deoxyuridine-containing template of pVX4; all six CDRs of anti-IFN- α were changed to the murine 2H7CDRs. The resulting molecule is referred to as humanized 2H7 version 2 (2H7.v2), or the "CDR-swap version" of 2H7; it has the m2H7CDR residues with the consensus human FR residues shown in FIGS. 1A and 1B. Humanized 2H7.v2 was used for further humanization.

[0298] Table 3 shows the oligonucleotide sequence used to create each of the murine 2H7 (m2H7) CDRs in the H and L chain. For example, the CDR-H1 oligonucleotide was used to recreate the m2H7H chain CDR1. CDR-H1, CDR-H2 and CDR-H3 refers to the H chain CDR1, CDR2 and CDR3, respectively; similarly, CDR-L1, CDR-L2 and CDR-L3 refers to each of the L chain CDRs. The substitutions in CDR-H2 were done in two steps with two oligonucleotides, CDR-H2A and CDR-H2B.

TABLE 3

Oligonucleotide sequences used for construction of the CDR-swap of murine 2H7 CDRs into a human framework in pVX4. Residues changed by each oligonucleotide are underlined.	
Substitution	Oligonucleotide sequence
CDR-H1	C TAC ACC TTC ACG <u>AGC</u> TAT <u>AAC</u> <u>ATG</u> CAC TGG GTC CG (SEQ ID NO. 35)
CDR-H2A	G ATT AAT CCT GAC <u>AAC</u> <u>GSC</u> <u>GAC</u> ACG <u>AGC</u> TAT AAC CAG <u>AAG</u> TTC AAG GGC CG (SEQ ID NO. 36)
CDR-H2B	GAA TGG GTT GCA <u>GCG</u> ATC <u>TAT</u> CCT <u>GGC</u> AAC GGC GAC AC (SEQ ID NO. 37)
CDR-H3	AT TAT TGT GCT CGA GTG <u>GTC</u> <u>TAC</u> TAT <u>AGC</u> <u>AAC</u> <u>AGC</u> <u>TAC</u> <u>TGG</u> <u>TAC</u> <u>TTC</u> GAC <u>GTC</u> TGG GGT CAA GGA (SEQ ID NO. 38)
CDR-L1	C TGC ACA GCC AGC <u>TCT</u> TCT <u>GTC</u> AGC TAT ATG CAT TG (SEQ ID NO. 39)
CDR-L2	AA CTA CTG ATT TAC <u>GCT</u> <u>CCA</u> <u>TGG</u> AAC CTC <u>GCG</u> TCT GGA GTC C (SEQ ID NO. 40)
CDR-L3	TAT TAC TGT CAA CAG <u>TGG</u> <u>AGC</u> <u>TTC</u> <u>GGT</u> CCG <u>CCC</u> ACA TTT GGA CAG (SEQ ID NO. 41)

[0299] For comparison with humanized constructs, a plasmid expressing a chimeric 2H7Fab (containing murine V_L and V_H domains, and human C_L and CH₁ domains) was constructed by site-directed mutagenesis (Kunkel, supra) using synthetic oligonucleotides to introduce the murine framework residues into 2H7.v2. The resulting plasmid construct for expression of the chimeric Fab known as 2H7.v6.8, is shown in FIG. 3 in WO 04/056342. Each encoded chain of the Fab has a 23 amino acid StII secretion signal sequence as described for pVX4 above.

[0300] Based on a sequence comparison of the murine 2H7 framework residues with the human V_K1/V_HIII consensus framework (FIGS. 1A and 1B) and previously humanized antibodies (Carter et al., *Proc. Natl. Acad. Sci. USA* 89:4285-4289 (1992)), several framework mutations were introduced into the 2H7.v2 Fab construct by site-directed mutagenesis. These mutations result in a change of certain human consensus framework residues to those found in the murine 2H7 framework, at sites that might affect CDR conformations or antigen contacts. Version 3 contained V_H(R71V, N73K), version 4 contained V_H(R71V), version 5 contained V_H(R71V, N73K) and V_L(L46P), and version 6 contained V_H(R71V, N73K) and V_L(L46P, L47W).

[0301] Humanized and chimeric Fab versions of m2H7 antibody were expressed in *E. coli* and purified as follows. Plasmids were transformed into *E. coli* strain XL-1 Blue (Stratagene, San Diego, Calif.) for preparation of double- and single-stranded DNA. For each variant, both light and heavy chains were completely sequenced using the dideoxynucleotide method (Sequenase, U.S. Biochemical Corp.). Plasmids were transformed into *E. coli* strain 16C9, a

derivative of MM294, plated onto LB plates containing 5 µg/ml carbenicillin, and a single colony selected for protein expression. The single colony was grown in 5 ml LB-100 µg/ml carbenicillin for 5-8 h at 37° C. The 5 ml culture was added to 500 ml AP5-100 µg/ml carbenicillin and allowed to grow for 16 h in a 4 L baffled shake flask at 37° C. AP5 media consists of: 1.5 g glucose, 11.0 Hycase SF, 0.6 g yeast extract (certified), 0.19 g anhydrous MgSO₄, 1.07 g NH₄Cl, 3.73 g KCl, 1.2 g NaCl, 120 ml 1 M triethanolamine, pH 7.4, to 1 L water and then sterile filtered through 0.1 µm Sealkeen filter.

[0302] Cells were harvested by centrifugation in a 1 L centrifuge bottle (Nalgene) at 3000xg and the supernatant removed. After freezing for 1 h, the pellet was resuspended in 25 ml cold 10 mM MBS-10 mM EDTA, pH 5.0 (buffer A). 250 µl of 0.1M PMSF (Sigma) was added to inhibit proteolysis and 3.5 ml of stock 10 mg/ml hen egg white lysozyme (Sigma) was added to aid lysis of the bacterial cell wall. After gentle shaking on ice for 1 h, the sample was centrifuged at 40,000xg for 15 min. The supernatant was brought to 50 ml with buffer A and loaded onto a 2 ml DEAE column equilibrated with buffer A. The flow-through was then applied to a protein G-Sepharose CL-4B (Pharmacia) column (0.5 ml bed volume) equilibrated with buffer A. The column was washed with 10 ml buffer A and eluted with 3 ml 0.3 M glycine, pH 3.0, into 1.25 ml 1 M Tris, pH 8.0. The F(ab) was then buffer exchanged into PBS using a Centri-con-30 (Amicon) and concentrated to a final volume of 0.5 ml. SDS-PAGE gels of all F(ab)s were run to ascertain purity and the molecular weight of each variant was verified by electrospray mass spectrometry.

[0303] In cell-based ELISA binding assays (described below), the binding of Fabs, including chimeric 2H7Fab, to CD20 was difficult to detect. Therefore, the 2H7Fab versions were reformatted as full-length IgG1 antibodies for assays and further mutagenesis.

[0304] Plasmids for expression of full-length IgG's were constructed by subcloning the V_L and V_H domains of chimeric 2H7 (v6.8) Fab as well as humanized Fab versions 2 to 6 into previously described pRK vectors for mammalian cell expression (Gorman et al., *DNA Prot. Eng. Tech.* 2:3-10 (1990)). Briefly, each Fab construct was digested with EcoRV and BlnI to excise a V_L fragment, which was cloned into the EcoRV/BlnI sites of plasmid pDR1 (FIG. 4 of WO 04/056312) for expression of the complete light chain (V_L-C_L domains). Additionally, each Fab construct was digested with PvuII and ApaI to excise a V_H fragment, which was cloned into the PvuII/ApaI sites of plasmid pDR2 (FIG. 5 of WO 04/056312) for expression of the complete heavy chain (V_H-CH₁-hinge-CH₂-CH₃ domains). For each IgG variant, transient transfections were performed by cotransfecting a light-chain expressing plasmid and a heavy-chain expressing plasmid into an adenovirus-transformed human embryonic kidney cell line, 293 (Graham et al., *J. Gen. Virol.*, 36:59-74, (1977)). Briefly, 293 cells were split on the day prior to transfection, and plated in serum-containing medium. On the following day, double-stranded DNA prepared as a calcium phosphate precipitate was added, followed by pAdVantage™ DNA (Promega, Madison, Wis.),

and cells were incubated overnight at 37° C. Cells were cultured in serum-free medium and harvested after 4 days. Antibodies were purified from culture supernatants using protein A-Sepharose CL-4B, then buffer exchanged into 10 mM sodium succinate, 140 mM NaCl, pH 6.0, and concentrated using a Centricon-10 (Amicon). Protein concentrations were determined by quantitative amino acid analysis.

[0305] To measure relative binding affinities to the CD20 antigen, a cell-based ELISA assay was developed. Human B-lymphoblastoid WIL2-S cells (ATCC CRL 8885, American Type Culture Collection, Rockville, Md.) were grown in RPMI 1640 supplemented with 2 mM L-glutamine, 20 mM HEPES, pH 7.2 and 10% heat-inactivated fetal bovine serum in a humidified 5% CO₂ incubator. The cells were washed with PBS containing 1% FBS (assay buffer) and seeded at 250-300,000 cell/well in 96-well round bottom plates (Nunc, Roskilde, Denmark). Two-fold serially diluted standard (15.6-1000 ng/ml of 2H7 v6.8 chimeric IgG) and threefold serially diluted samples (2.7-2000 ng/ml) in assay buffer were added to the plates. The plates were buried in ice and incubated for 45 min. To remove the unbound antibody, 0.1 mL assay buffer were added to the wells. Plates were centrifuged and supernatants were removed. Cells were washed two more times with 0.2 mL assay buffer. Antibody bound to the plates was detected by adding peroxidase conjugated goat anti-human Fc antibody (Jackson ImmunoResearch, West Grove, Pa.) to the plates. After a 45 min incubation, cells were washed as described before. TMB substrate (3,3',5,5'-tetramethyl benzidine; Kirkegaard & Perry Laboratories, Gaithersburg, Md.) was added to the plates. The reaction was stopped by adding 1 M phosphoric acid. Titration curves were fit with a four-parameter nonlinear regression curve-fitting program (KaleidaGraph, Synergy software, Reading, Pa.). The absorbance at the mid-point of the titration curve (mid-OD) and its corresponding concentration of the standard were determined. Then the concentration of each variant at this mid-OD was determined, and the concentration of the standard was divided by that of each variant. Hence the values are a ratio of the binding of each variant relative to the standard. Standard deviations in relative affinity (equivalent concentration) were generally +/-10% between experiments.

[0306] As shown in Table 4, CD20 binding of the CDR-swap variant (v.2) was extremely reduced compared to chimeric 2H7 (v.6.8). However, versions 3 to 5 showed improved binding. To determine the minimum number of mutations that might be required to restore binding affinity to that of chimeric 2H7, additional mutations and combinations of mutations were constructed by site-direct mutagenesis to produce variants 7 to 17 as indicated in Table 5. In particular, these included V_H mutations A49G, F67A, I69L, N73K, and L78A; and V_L mutations M4L, M33I, and F71Y. Versions 16 and 17 showed the best relative binding affinities, within 2-fold of that of the chimeric version, with no significant difference (s.d. +/-10%) between the two. To minimize the number of mutations, version 16, having only 4 mutations of human framework residues to murine framework residues (Table 5), was therefore chosen as the humanized form for additional characterization.

TABLE 4

Relative binding affinity of humanized 2H7 IgG variants to CD20 compared to chimeric 2H7 using cell-based ELISA. The relative binding is expressed as the concentration of the chimeric 2H7 over the concentration of the variant required for equivalent binding; hence a ratio < 1 indicates weaker affinity for the variant. Standard deviation in relative affinity determination averaged +/-10%. Framework substitutions in the variable domains are relative to the CDR-swap version according to the numbering system of Kabat (Kabat et al., supra).			
2H7 version	Heavy chain (V _H) substitutions	Light Chain (V _L) substitutions	Relative binding
6.8	(Chimera)	(Chimera)	-1-
2	(CDR swap)	(CDR swap)	0.01
3	R71V, N73K	(CDR swap)	0.21
4	R71V	(CDR swap)	0.21
5	R71V, N73K	L46P	0.50
6	R71V, N73K	L46P, L47W	0.58
7	R71V	L46P	0.33
8	R71V, L78A	L46P	0.19
9	R71V, F67A	L46P	0.07
10	R71V, F67A, I69L	L46P	0.12
11	R71V, F67A, L78A	L46P	0.19
12	R71V	L46P, M4L	0.32
13	R71V	L46P, M33I	0.31
14	R71V	L46P, F71Y	0.25
15	R71V	L46P, M4L, M33I	0.26
16	R71V, N73K, A49G	L46P	0.65
17	R71V, N73K, A49G	L46P, L47W	0.67

[0307]

TABLE 5

Oligonucleotide sequences used for construction of mutations V _H (A49G, R71V, N73K) and V _L (L46P) in humanized 2H7 version 16 (2H7.v16). Underlined codons encode the indicated amino acid substitutions. For V _H (R71V, N73K) and V _L (L46P), the oligos are shown as the sense strand since these were used for mutagenesis on the Fab template, while for V _H (A49G), the oligo is shown as the anti-sense strand, since this was used with the pRX (IgG heavy chain) template. The protein sequence of version 16 is provided above under Compositions.	
Substitution	Oligonucleotide sequence
V _H (R71V, N73K)	GT TTC ACT ATA AGT <u>GTC</u> GAC <u>AAG</u> TCC AAA AAC ACA TT (SEQ ID NO. 42)
V _H (A49G)	GCCAGGATAGATGGCGCCAACCCATTCAGGGC (SEQ ID NO. 43)
V _L (L46P)	AAGCTCCGAACCACTGATTACGCT (SEQ ID NO. 44)

Example 2

Additional Mutations within 2H7CDR Regions

[0308] Substitutions of additional residues and combinations of substitutions at CDR positions that were identified as important by Ala-scanning were also tested. Several combination variants, particularly v.96 appeared to bind more tightly than v.16.

1. A humanized 2H7 antibody that binds human CD20, or an antigen-binding fragment thereof, the antibody comprising the L chain Variable region (V_L) sequence of SEQ ID NO. 25 and the H chain Variable region (V_H) sequence of SEQ ID NO. 8 but with amino acid substitution of D56A in VH-CDR2, and N100 in VH-CDR3 is substituted with Y or W.
2. The antibody of claim 1, wherein N100 is substituted with Y.
3. The antibody of claim 1, wherein the N100 is substituted with W.
4. The antibody of claim 1, further comprising the substitution S100aR in VH-CDR3.
5. The antibody of claim 4, further comprising at least one amino acid substitution in the IgG Fc region that improves ADCC and/or CDC activity.
6. The antibody of claim 5, comprising an IgG1 Fc comprising the amino acid substitutions S298A, E333A, K334A, K326A.
7. The antibody of claim 4, further comprising at least one amino acid substitution in the Fc region that improves ADCC but decreases CDC activity.
8. The antibody of claim 7, comprising at least the amino acid substitution K322A.
9. The antibody of claim 8, further comprising the amino acid substitutions S298A, E333A, K334A.
10. The antibody of claim 6, comprising the light chain sequence of SEQ ID NO.26 and heavy chain sequence of SEQ ID NO. 34.
11. The antibody of claim 4, which binds human CD20 with at least 3 fold increased affinity relative to antibody 2H7.v16.
12. The antibody of claim 4, which binds human CD20 with at least 6 fold increased affinity relative to antibody 2H7.v16.
13. The antibody of claim 6, which exhibits at least 20 fold greater antibody dependent cellular cytotoxicity (ADCC) than 2H7.v16.
14. The antibody of claim 6, which exhibits at least 25 fold greater complement cytotoxicity than 2H7.v16.
15. The antibody of claim 1, conjugated to a cytotoxic agent.
16. The antibody of claim 15, wherein the cytotoxic agent is a radioactive isotope or a toxin.
17. The antibody of any of claim 1, which antibody is produced in CHO cells.
18. An isolated nucleic acid that encodes the antibody of claim 1.
19. The nucleic acid of claim 18 which is an expression vector
20. A host cell comprising the nucleic acid of claim 18.
21. (canceled)
22. The host cell of claim 20 that produces the antibody comprising the light chain sequence of SEQ ID NO.26 and heavy chain sequence of SEQ ID NO. 34.
23. The host cell of claim 22 which is a CHO cell.
24. A method of producing the antibody of claim 10, comprising culturing the host cell of claim 22 and recovering the antibody from the cell culture.
25. A composition comprising the antibody of claim 1 and a carrier.
26. The composition of claim 25 comprising the antibody of claim 10 and a pharmaceutically acceptable carrier.
27. An article of manufacture comprising a container and a composition contained therein, wherein the composition comprises an antibody of claim 10.
28. The article of manufacture of claim 27, further comprising a package insert indicating that the composition is used to treat non-Hodgkin's lymphoma.
29. The article of manufacture of claim 27, further comprising a package insert indicating that the composition is used to treat rheumatoid arthritis.
30. A method of treating a CD20 positive cancer, comprising administering to a patient having the cancer, a therapeutically effective amount of the humanized 2H7 antibody of claim 10.
31. The method of claim 30 wherein the CD20 positive cancer is a B cell lymphoma or leukemia.
32. The method of claim 31 wherein CD20 positive cancer is non-Hodgkin's lymphoma (NHL).
33. The method of claim 32 wherein the cancer is chronic lymphocytic leukemia (CLL) or SLL.
34. (canceled)
35. The method of claim 31, wherein the antibody is administered via intravenous infusion.
36. The method of claim 35, wherein the antibody is administered at a dosage in the range of about 100 mg/m² to 375 mg/m² per dose.
37. The method of claim 36, wherein the antibody is administered at a dosage of 375 mg/m² per dose weekly for at least 4 doses in the treatment of non-Hodgkin's lymphoma.
38. The method of claim 30, further comprising administering to the patient at least one chemotherapeutic agent.
39. The method of claim 38, wherein the cancer is non-Hodgkin's lymphoma (NHL) and the chemotherapeutic agent is selected from the group consisting of doxorubicin, cyclophosphamide, vincristine and prednisolone.
40. A method of alleviating an autoimmune disease, comprising administering to a patient suffering from the autoimmune disease, a therapeutically effective amount of the humanized 2H7 antibody of any one of claims 6-10.
41. The method of claim 40, wherein the antibody comprises the light and heavy chain amino acid sequence of SEQ ID NO. 26 and 34, respectively.
42. The method of claim 41, wherein the autoimmune disease is selected from the group consisting of rheumatoid arthritis and juvenile rheumatoid arthritis, systemic lupus erythematosus (SLE) including lupus nephritis, Wegener's disease, inflammatory bowel disease, ulcerative colitis, idiopathic thrombocytopenic purpura (ITP), thrombotic thrombocytopenic purpura (TTP), autoimmune thrombocytopenia, multiple sclerosis, psoriasis, IgA nephropathy, IgM polyneuropathies, myasthenia gravis, ANCA associated vasculitis, diabetes mellitus, Reynaud's syndrome, Sjogren's syndrome, Neuromyelitis Optica (NMO) and glomerulonephritis.
43. The method of claim 42, wherein the autoimmune disease is rheumatoid arthritis.
44. The method of claim 43, further comprising administering to the patient a second therapeutic agent.
45. The method of claim 44, wherein the second therapeutic agent is an immunosuppressive agent.
46. The method of claim 45, wherein the immunosuppressive agent is methotrexate.
47. The method of claim 43, wherein the antibody is administered intravenously or subcutaneously.

48. The method of claim 43, wherein the antibody is administered intravenously at a dosage in the range of 10 mg to 500 mg per dose.

49. The method of claim 48, wherein the antibody is administered at a dosage of 200 mg/dose for at least two doses.

50. The method of claim 40, further comprising administering to the patient a second therapeutic agent.

51. The method of claim 50, wherein the second therapeutic agent is a BAFF antagonist.

52. The method of claim 51, wherein the BAFF antagonist is an anti-BR3 antibody or a BR3-Fc fusion protein.

53. The method of claim 30, further comprising administering to the patient, a VEGF antagonist.

54. A liquid formulation comprising humanized 2H7.v511 antibody at about 20 mg/ml, in 20 mM sodium acetate, pH 5.5, 4% trehalose dihydrate, 0.02% polysorbate 20, for intravenous administration.

55. A liquid formulation comprising humanized 2H7.v114 antibody at about 20 mg/ml, in 20 mM sodium acetate, pH 5.3, 240 mM (8%) trehalose dihydrate, 0.02% Polysorbate 20.

56. A liquid formulation comprising humanized 2H7.v16 antibody at about 30 mg/ml in 20 mM sodium acetate, pH 5.3, 4% trehalose dehydrate, 0.02% polysorbate 20, for intravenous administration.

57. The method of claim 36, wherein the antibody is administered in weekly doses of 200 mg/m² per dose, for at least 4 weeks in the treatment of non-Hodgkin's lymphoma.

58. The method of claim 48, wherein the antibody is administered at a dosage of 500 mg/dose for at least two doses.

* * * * *

Anexo 2

United States Patent [19]

Taupier et al.

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[45] Date of Patent: Mar. 28, 1989

[54] SERUM FREE CELL CULTURE MEDIUM

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[52] U.S. Cl. 435/240.31; 435/240.3;

435/240.2; 435/240.1

[58] Field of Search 435/240, 241, 948, 68,
435/240.2, 948, 68, 240.3, 240.31

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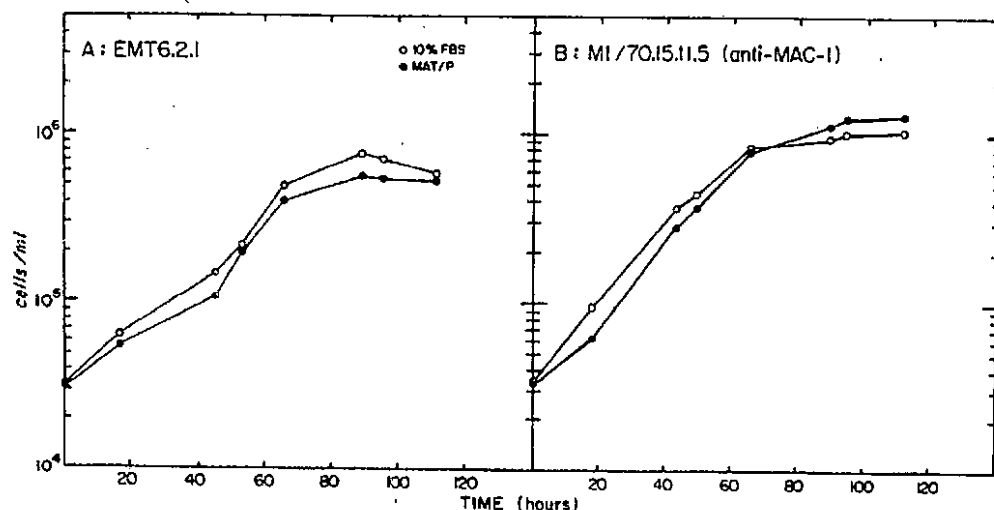
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[57]

ABSTRACT

A serum free cell culture medium containing less than 10 ug/ml protein is disclosed. The medium comprises a basal media supplemented with levels of 2-aminoethanol substantially higher than previously disclosed and supplemented with 2-mercaptoethanol, transferrin, insulin and free essential amino acids. Unlike other serum free media, the media disclosed herein will support the growth and long term culture of a large variety of cell types including hybridomas and tumor cell lines while maintaining both growth potential and the differentiated characteristics of the particular cell line.

12 Claims, 6 Drawing Sheets



SERUM FREE CELL CULTURE MEDIUM

This invention relates to the field of long term cell culture in serum free very low protein media. It provides for a composition comprising a basal medium supplemented with levels of 2-aminoethanol substantially higher than previously recognized and supplemented with 2-mercaptoethanol, transferrin, insulin, and free essential amino acids. Where the medium is exposed to wavelengths of light of less than 500 nm, a mixture of trace elements or selenium alone is added to the composition.

Since the advent of growth of mammalian cells in culture, efforts have been made to better define the requirements necessary for optimal cell growth. However, traditional supplementation of these media with serum of equine or bovine origin, which contain a myriad of undefined components, has presented complications in many areas of study. For a review of the use of serum free media, see Barnes and Sato.

Recently, however, much effort has been made to overcome these complications by replacing the serum in cell culture media with more defined protein and hormonal mixtures. In hormonal and nutritional studies, it is essential to eliminate undefined or unnecessary components to be able to define the hormonal and nutritional requirements necessary for the growth and activation of cells. In addition, the nutrients required for the growth of neural cells in the absence of serum have been analyzed primarily out of necessity, since traditional serum containing media selectively allow the growth of fibroblastoid cells to the detriment of neural cells.

In order to facilitate monoclonal antibody purification, much work also has been done to develop a serum free medium that will grow hybridoma cells. Unfortunately, most of these media contain quite high levels of exogenous protein necessitating purification steps such as ammonium sulfate precipitation or acid elution from an affinity column, either of which may inactivate some of the antibody desired. In addition, since these media were developed specifically for the culture of hybridoma cells, these formulations often will not grow other types of tumor cells. Those serum free media which have been developed to grow a particular tumor cell will only grow a limited number of other tumor cell types optimally, possibly because they were specifically tailored to the needs of a single cell line.

Recently, both Ziegler and Milstein (1979) and van Agthoven and Terhorst (1982) have reported that a new type of β -2 microglobulin molecule was associated with murine T cells. Further study however, revealed that this new molecule was bovine β -2 microglobulin derived from the fetal bovine serum (FBS) that the cells were cultured in. This illustrates the problems in data interpretation that can arise when protein components from the media bind to the membrane or membrane proteins of a cell.

In a similar manner, studies on the immune reactions to activated and tumor cells grown in culture can be complicated by components of FBS adsorbed onto the cells. Many reports have detailed a response to these FBS components rather than to tumor or activation antigens when these cultured cells are subsequently injected into animals. This has resulted in anti-FBS antibodies or polyclonal non-specific cytolytic responses.

To avoid the problems associated with high levels of protein and lipids and those associated with the use of a defined medium incapable of sustaining long-term growth or growth only in a single or limited number of cell lines, a serum free very low protein medium for long term cell culture (MAT/P) is disclosed containing less than 10 ug/ml total protein which will support the growth of a wide variety of cell cultures.

An object of this invention is to allow a wide variety of cell lines to be cultured.

A further object of this invention is to provide a medium which will sustain cell cultures in a manner as near to cell culture in serum as possible.

An advantage of this invention is that the cells can be cultured over a long term.

Another advantage is that the low protein concentration will not mask antigens.

Another advantage is that the low protein concentration will not generate spurious immune responses upon injection into animals.

A further advantage of this invention is that the media is relatively inexpensive compared to media containing serum.

A final advantage is that purification of monoclonal antibodies and biological response modifiers is simplified.

These and other objects and advantages will become apparent from a reading of the following detailed description of the invention and associated Figures in which:

FIG. 1 is a plot of cell growth, as measured by tritiated thymidine, uptake against time for a variety of cell types in several different media wherein media used were: ● RPMI 1640 with $5 \times 10^{-5}M$ 2-mercaptoethanol and 10% FBS; ◇ HB101 @ supplemented with sodium pyruvate and glutamine (as recommended by the manufacturer); ○ Iscove's modified Dulbecco's media:Ham's F12 (IMDM/F12) at a 1:1 ratio; ◆ RPMI 1640:Dulbecco's modified Eagle's media:Ham's F12 (RPMI 1640/DMEM/F12) at a 2:1:1 ratio; ▲ RPMI 1640; ■ DMEM/F12 at a 1:1 ratio; □ RPMI 1640/IMDM/F12 at a 2:1:1 ratio; ○, ◆, ▲, ■, □ were all supplemented with the trace element mix, and the concentrations of progesterone, insulin, and transferrin described by Mosier (1981), as well as 20 uM 2-aminoethanol;

FIG. 2 is a plot similar to FIG. 1 wherein various substances were tested for their ability to enhance the growth of cells above that found in RPMI/DMEM/F12 supplemented as described in FIG. 1 with vitamins and minimal essential medium amino acids (EAA) used at the concentration recommended by the manufacturer, bovine serum albumin (BSA) used at 500 ug/ml and putrescine used at $5 \times 10^{-5}M$;

FIG. 3 is a plot similar to FIG. 1 wherein transferrin, 2-aminoethanol, and essential amino acids were tested to determine optimal concentrations: A and D) transferrin ● 50 ug/ml, ▲ 10 ug/ml, □ 2 ug/ml, ◆ none; B and E) 2-aminoethanol ● 900 uM, ▲ 300 uM, □ 100 uM, ◆ 20 uM; C and F) minimal essential medium amino acids ● 40 ml/L, ▲ 20 ml/L, □ 10 ml/L, ◆ 5 ml/L;

FIG. 4 is a plot similar to FIG. 3 wherein various trace element mixtures were tested on cell lines EMT6.S and YAC-1:○ trace element mix described by Mosier (1981), □ trace element mix described by Cleveland (1983), ▲ $2 \times 10^{-8}M$ Na_2SeO_3 only; or ◇ no trace elements;

FIG. 5 is a plot of cell concentration against time wherein EMT6.S and M1/70 were tested for growth in MAT/P vs. RPMI 1640 with 10% FBS and 2-mercaptoethanol; and

FIG. 6 is a 10% SDS-PAGE analysis of the results of concentrating hybridoma supernatants grown in MAT/P, over a 100,000 MW cut-off filter in a Millipore Minitan®.

In order to deplete FBS components, cell lines EMT6.S, EMT6.R.1, YAC-1, Line 1, RIF-1, and the hybridoma M1/70.15.11.5 were grown for at least four passages in Mosier's modification of Iscove's medium further supplemented with 20 uM 2-aminoethanol. The growth of some of these cell lines, especially YAC-1, M1/70, and RIF-1 was clearly sub-optimal in that the doubling time was much longer, and the cells had to be passed at a much higher density than was usual in FBS containing media. In addition, about 2-3 days after the initial passage into serum free media a greater lag was often noticed in cell doubling time by microscopic examination, possibly due to depletion of FBS components carried over on the surface of the cells. Therefore, a better basal media to grow this variety of tumor cells was sought. A variety of basal media were tested with the results presented in FIG. 1.

In some cases several of the serum free formulations were quite similar in their ability to support the growth of one cell line and yet distinct differences were seen with the same formulations on other cell lines (e.g., RPMI/IMDM/F12 and RPMI both support the growth of EMT6.S.R.1 to a similar extent, however, only RPMI/IMDM/F12 and not RPMI will support the growth of YAC-1). Since the requirement is for a media that would grow all of these tumor cells, RPMI/DMEM/F12 was chosen as the basal media.

Others have reported that supplementation of serum free media with BSA, lipid-protein extracts, other proteins, nutrients, or hormones were necessary to grow various types of cells in serum free media. The next step was to determine what the effect of these additives would be on the cell lines under study. EMT6.S, YAC-1 and M1/70 were chosen as representative cell lines.

Cells that were in log phase, and growing in RPMI/DMEM/F12 plus the additives described in FIG. 1 for at least 3 passages, were placed in media containing these other additives. See FIG. 2. The control cells, assayed in RPMI with 10% FBS were continually passaged in 10% FBS, since it became apparent that as the cells became fully adapted to serum free media they required a re-adaptation to serum containing media before optimal growth was achieved. To minimize the possibility of divergence in growth characteristics between the cultures being maintained in medium containing serum versus serum-free media, the serum free cultures were only assayed between four and ten passages out of serum.

The effects of these additives fall into several categories. See FIG. 2. First, additives that have little or no effect on one cell line but are somewhat helpful to another (i.e., essential amino acids). Second, additives which appear to be detrimental to several cell lines. These are BSA especially in the case of EMT6.S and RIF-1. While putrescine had little effect on EMT6.S or YAC-1, it was somewhat toxic to M1/70, and therefore, no additional putrescine was added the formulation. Other compounds tested and found to have no effect or to be toxic were catalase, alpha cyclodextrin, dexamethasone, sodium pyruvate, non-essential amino acids and

progesterone. No additives were tested that were beneficial to one cell line and detrimental to another.

Once the necessary additives were established, 2-aminoethanol, transferrin, minimal essential medium amino acids, and insulin were then titrated on EMT6.S and YAC-1 to determine the optimal concentrations for cell growth. FIG. 3 shows the results of these experiments.

Both cell lines show an absolute requirement for iron saturated transferrin. However, both cell lines did well whether the concentration was 2 ug/ml or 50 ug/ml. 2 ug/ml has been chosen as the preferred concentration in order to minimize protein content in the medium.

While EMT6.S shows no significant difference in its requirements for 2-aminoethanol, YAC-1 grew poorly when the concentration dropped below 100 uM. The optimal range is from 400-800 uM with a preferred concentration of 600 uM. The toxicity of 2-aminoethanol reported by Murakami, et al. (1982) at levels of greater than 20 uM was not seen in concentrations as high as 1200 uM.

Insulin worked well in both cell lines regardless of whether the concentration was 2 ug/ml or 20 ug/ml.

The highest level of essential amino acids (i.e., 40 ml/L) consistently, although not significantly, augmented the growth of EMT6.S and had a greater effect on YAC-1. Two trace element mixes, selenium alone or no trace elements also were tested. As can be seen in FIG. 4, all three selenium containing mixes worked approximately the same for both EMT6.S and YAC-1. What was surprising, however, was that the absolute requirement for selenium to grow cells in serum free media reported by others, and seen in preliminary experiments done with EMT6.S, was no longer present for either EMT6.S or YAC-1. Since selenium is a co-factor for peroxidase, it may have been due to elimination of phototoxic products in the media by eliminating wavelengths of less than 500 nm. Using the media described above, selenium and other trace elements are not necessary to grow the cell lines described for short periods of time but Mosier's trace element mixture has been retained in the formulation since it does appear to be helpful in long term culture.

Given these results, the following preferred embodiment was derived. Glutamine free RPMI 1640 is combined 2:1:1 with glutamine free Dulbecco's modified Eagle's medium and Ham's F12. To this is added 40 ml/L of minimal essential media amino acids and either Mosier's trace element mixture or 2×10^{-8} M Na_2SeO_3 . 1.6 gm/L of NaHCO_3 is added and the pH is adjusted to 7.2. At this point the medium is sterilized by filtration and may be stored in the dark at 4° C. for up to 2 months.

At the time of use, 600 uM 2-aminoethanol, 50 uM 2-mercaptoethanol, 332.5 mg/L L-glutamine, 5 ug/ml insulin and 2-10 ug/ml one third iron saturated transferrin are added. The medium is buffered to be used in a 5% CO_2 culture conditions. Table I provides a complete breakdown of all the components of MAT/P.

TABLE I

MAT/P:Formulation	
	mg/liter
Salts	
$\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$	50.
CaCl_2	58.3
$\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$	0.25

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TABLE I-continued

MAT/P:Formulation	mg/liter
KCl	355.9
MgSO ₄ (Anhydrous)	48.84
NaCl	6497.975
NaHCO ₃	1600.
NaH ₂ PO ₄ (Anhydrous)	31.25
CuSO ₄ ·5H ₂ O	0.00065
FeSO ₄ ·7H ₂ O	0.2085
MgCl ₂	14.32
Na ₂ HPO ₄	435.51
ZnSO ₄ ·7H ₂ O	0.216
<u>Other Components</u>	
Sodium Hypoxanthine	1.195
Linoleic Acid	0.021
D-glucose	2575.5
Lipoic Acid	0.0525
Putrescine·2HCl	0.0405
Thymidine	0.1825
Phenol red	6.55
DL-Thioctic acid	0.525
Glutathione (reduced)	0.5
HEPES	1787.25
Sodium Pyruvate	27.5
<u>Amino Acids</u>	
L-Alanine	2.225
L-Asparagine	25.
L-Asparagine·H ₂ O	3.75
L-Arginine free base	100.
L-Arginine·HCl	326.55
L-Aspartic Acid	13.325
L-Cysteine·HCl·H ₂ O	8.78
L-Cystine	48.
L-Cystine·2HCl	48.22
L-Glutamic Acid	13.675
Glycine	14.375
L-Histidine (free base)	7.5
L-Histidine·HCl·H ₂ O	99.74
L-Hydroxyproline	10.
L-Isoleucine	157.235
L-Leucine	159.325
L-Lysine·HCl	210.625
L-Methionine	46.325
L-Phenylalanine	91.24
L-Proline	18.625
L-Serine	28.125
L-Threonine	131.925
L-Tryptophan	27.41
L-Tyrosine	72.
L-Tyrosine·2Na	14.415
L-Tyrosine·2Na·2H ₂ O	27.875
L-Valine	130.025
<u>Vitamins</u>	
Biotin	0.10175
D-calcium pantothenate	1.245
Choline Chloride	5.99
Folic Acid	1.825
i-Inositol	23.8
Nicotinamide	0.5
Para-aminobenzoic acid	0.5

6

TABLE I-continued

MAT/P:Formulation	mg/liter
Pyridoxine·HCl	0.5155
Riboflavin	0.2095
Thiamine·HCl	1.585
Vitamin B12	0.3425
Niacinamide	1.01
Pyridoxal·HCl	1.0
<u>Trace Elements</u>	
MnCl ₂	0.5 nM
(NH ₄) ₆ Mo ₇ O ₂₄	0.5 nM
NiSO ₄	0.25 nM
H ₂ SeO ₃	15. nM
Na ₂ SeO ₃	50. nM
Na ₂ SiO ₃	250. nM
SnCl ₂	0.25 nM
NaVO ₃	2.5 nM
CdSO ₄	50. nM
<u>Added at Time of Use - Unstable Components</u>	
L-Glutamine	332.5 mg/L
2-mercaptoethanol	5 × 10 ⁻⁵ μM
2-aminoethanol (ethanolamine)	600 uM
Transferrin	1 to 5 mg/L
Insulin	5 mg/L

25 In this state, MAT/P has a shelf life of 3 weeks when stored in the dark at 4° C. Without the materials added at the time of use, shelf life is 2 months when stored in the dark at 4° C.

30 Growth then was tested for in MAT/P. FIG. 5 shows that the growth kinetics of EMT6.S and M1/70 are the same in RPMI containing 10% FBS as in MAT/P. EMT6.S, M1/70, YAC-1, RIF-1 and Line 1 have since been continuously cultured for up to five months in this formulation, with no changes in growth or other characteristics.

35 To determine the breadth of use of MAT/P, cell lines were tested for long term growth. Table II illustrates the variety of cells isolated from mouse, rat and human that have been tested and will grow in MAT/P. Most importantly, as well as supporting the growth of these cells, MAT/P also supports differentiated functions (e.g., secretion of monoclonal antibody by hybridoma cells). In addition, the immunosuppressive factor produced by the parental EMT6.S line and some of the subclones of EMT6.S is still produced at similar levels in MAT/P as in serum containing media. Other cellular characteristics such as response to stimuli are maintained (e.g., Line 1 expresses similar levels of H-2 antigen when cultured in the presence of DMSO in serum containing medium or in MAT/P; PC 12 responds to neural growth factor in serum containing media or MAT/P).

TABLE II

CELL LINES THAT HAVE THE SAME GROWTH CHARACTERISTICS IN MAT/P AS IN FBS CONTAINING MEDIA:

Cell Line	Origin	Length of Time Cultured
<u>Sarcomas (adherent)</u>		
EMT6.S/Ro & subclones .2.1 .8 .R.1 .P	BALB/c spontaneous mammary sarcoma(g) (secretes immunosuppressive factor in MAT/P as in FBS) each clone has differing growth characteristics which are maintained in MAT/P	6 months
Line 1	BALB/c spontaneous lung tumor(h) (Expresses H ₂ in response to DMSO in MAT/P as in FBS).	6 months
Meth A.4	Methyl-cholanthrene induced sarcoma(i) (non-adherent variant) of BALB/c mice	2 months
RIF-1	C3H radiation induced fibrosarcoma(j)	6 months
WEHI 164	NC susceptible cell line of BALB/c mice(k)	4 months
<u>Hybridoma Lines</u>		

TABLE II-continued
CELL LINES THAT HAVE THE SAME GROWTH CHARACTERISTICS IN
MAT/P AS IN FBS CONTAINING MEDIA:

Cell Line	Origin	Length of Time Cultured
M1/70.15.11.5 (anti-MAC-1)	NS-1/rat spleen(a)	4 months
GK1.5 (anti-L3/T4)	SP2-0/rat spleen(b)	3 months
AF3-12.1 (anti-H-2K ^k)	SP2-0/BALB/c spleen(c)	3 months
53-6 (anti-Lyt 2)	NS-1/rat spleen(d)	2 months
T24/4017 (anti-Thy1)	S194/5/Lewis rat spleen(e)	3 months
MK-D6 (anti Ia ^k)	P3X63/(C57BL/6xA/J)F ₁ (f)	3 months
AF4-62.4 (anti-H-2D ^d)	SP2-0/C3H(e)	4 months
OR-689.2.4 (anti-opiate receptor)	P3/BALB/c spleen(r)	9 months
Lymphomas		
YAC-1	A/Sn T-cell lymphoma(l)	6 months
EL4	C57BL/6 T-cell lymphoma(m)	4 months
S49.1	T cell lymphoma(n)	1 month
F815	DBA/2 Mastocytoma(o)	6 months
Other Tumor Cell Lines		
NG108-15	Rat Neuroblastoma/glioma fusion(p)	1 month
PC12	Rat pheochromocytoma(q) (chromaffin tumor) (differentiates in presence of NGF in MAT/P as in FBS containing media)	1 month
Human Cell Lines		
HT 1080	Human Colon cancer	2 weeks

Note:
Cultures were terminated at the times specified due to experimental design rather than loss of growth potential.

In addition, primary cultures were tested for growth in MAT/P. Five-day cultures of mice spleen cells were found to proliferate in response to concanavalin A or tumor antigens. Culture in this medium of spleen cells from mice immunized to tumor antigens also allowed the generation of specific cytolytic T cells.

Finally, an advantage of having very low amounts of exogenous protein in the media is the ease of purification of secreted products. This is illustrated by FIG. 6. Hybridoma supernatant was concentrated forty fold over a 100,000 m.w. cut-off filter on a Millipore Minitan. The concentrate was assayed for purity by SDS poly-acrylamide gel electrophoresis. Lane 4 contains the transferrin control, lane 5 the insulin control and lanes 1 and 6 are molecular weight standards. The hybridoma supernatants in lanes 2 and 3 yielded two major peaks representing heavy and light chains. No detectable insulin was found retained in either preparation. Although a small amount of transferrin was detected in the GK1.5 hybridoma supernatant, the preparation was still 80 to 90% immunoglobulin as judged by molecular weight. This low protein content has also been useful in not only purifying but also characterizing poorly defined biological response modifiers such as the immunosuppressive factor secreted by EMT6.S.

The examples provided herein are for purposes of explanation only and should not be taken in any limiting sense. Variations and modifications, therefore, of the above-described invention may suggest themselves to those skilled in the art within the scope of this invention.

- We claim:
1. A serum-free culture composition containing less than 10 µg/ml of protein comprising a basal medium supplemented with a solution of 2-aminoethanol to a concentration of at least 100 µM, a solution of 2-mercaptoethanol, a solution of transferrin, a solution of insulin and an added solution of essential amino acids.
 2. The composition as set forth in claim 1 wherein the basal media comprises a 2:1:1 ratio mixture of RPMI 1640:Dulbecco's modified Eagle's Medium:Ham's F12.
 3. The composition as set forth in claim 1 wherein the 2-aminoethanol concentration is 600 µM.

4. The composition as set forth in claim 1 wherein the 2-mercaptoethanol concentration is 50 µM.
5. The composition as set forth in claim 1 wherein the transferrin concentration is 1 to 5 µg/ml of one-third iron saturated transferrin.
6. The composition as set forth in claim 1 wherein the insulin concentration is 5 µg/ml.
7. The composition as set forth in claim 6 wherein the trace element solution comprises a trace element mixture of compounds of Mn, Mo, Ni, Se, Si, Sn, V and Cd.
8. The composition as set forth in claim 7 wherein the selenium comprises 2×10⁻⁸M Na₂SeO₃.
9. The composition as set forth in claim 1 wherein 40 ml/L of minimum essential medium essential amino acids is added.
10. A serum free cell culture composition containing less than 10 ug/ml protein comprising the following compounds in the following proportions:

	mg/liter
Salts	
Ca(NO ₃) ₂ ·4H ₂ O	50.
CaCl ₂	58.3
Fe(NO ₃) ₃ ·9H ₂ O	0.25
KCl	355.9
MgSO ₄ (Anhydrous)	48.84
NaCl	6497.975
NaHCO ₃	1600.
NaH ₂ PO ₄ (Anhydrous)	31.25
CuSO ₄ ·5H ₂ O	0.00065
FeSO ₄ ·7H ₂ O	0.2085
MgCl ₂	14.32
Na ₂ HPO ₄	435.51
ZnSO ₄ ·7H ₂ O	0.216
Other Components	
Sodium Hypoxanthine	1.195
Linoleic Acid	0.021
D-glucose	2575.5
Lipoic Acid	0.0525
Putrescine·2HCl	0.0405
Thymidine	0.1825
Phenol red	6.55
DL-Thioctic acid	0.525
Glutathione (reduced)	0.5
HEPES	1787.25
Sodium Pyruvate	27.5

9		10
-continued		-continued
	mg/liter	mg/liter
<u>Amino Acids</u>		
L-Alanine	2.225	5
L-Asparagine	25.	
L-Asparagine.H ₂ O	3.75	
L-Arginine free base	100.	
L-Arginine.HCl	326.55	
L-Aspartic Acid	13.325	10
L-Cysteine.HCl.H ₂ O	8.78	
L-Cystine	48.	
L-Cystine.2HCl	48.22	
L-Glutamic Acid	13.675	
Glycine	14.375	
L-Histidine (free base)	7.5	15
L-Histidine.HCl.H ₂ O	99.74	
L-Hydroxyproline	10.	
L-Isoleucine	157.235	
L-Leucine	159.325	
L-Lysine.HCl	210.625	20
L-Methionine	46.325	
L-Phenylalanine	91.24	
L-Proline	18.625	
L-Serine	28.125	
L-Threonine	131.925	
L-Tryptophan	27.41	25
L-Tyrosine	72.	
L-Tyrosine.2Na	14.415	
L-Tyrosine.2Na.2H ₂ O	27.895	
L-Valine	130.025	
<u>Vitamins</u>		
Biotin	0.10175	
D-calcium pantothenate	1.245	
		Choline Chloride 5.99
		Folic Acid 1.825
		i-Inositol 23.8
		Nicotinamide 0.5
		Para-aminobenzoic acid 0.5
		Pyridoxine.HCl 0.5155
		Riboflavin 0.2095
		Thiamine.HCl 1.585
		Vitamin B12 0.3425
		Niacinamide 1.01
		Pyridoxal.HCl 1.0
		<u>Trace Elements</u>
		MnCl ₂ 0.5 nM
		(NH ₄) ₆ Mo ₇ O ₂₄ 0.5 nM
		NiSO ₄ 0.25 nM
		H ₂ SeO ₃ 15. nM
		Na ₂ SeO ₃ 50. nM
		Na ₂ SiO ₃ 250. nM
		SnCl ₂ 0.25 nM
		NaVO ₃ 2.5 nM
		CdSO ₄ 50. nM
		<u>Added at Time of Use - Unstable Components</u>
		L-Glutamine 332.5 mg/L
		2-mercaptoethanol 5×10^{-3} μ M
		2-aminoethanol (ethanolamine) 600 μ M
		Transferrin 1 to 5 mg/L
		Insulin 5 mg/L
		11. A composition as set forth in claim 1 wherein the concentration of 2-aminoethanol is 400 to 800 μ m.
		12. A composition as set forth in claim 1 which is also supplemented with a selenium-containing solution of trace elements or with a solution of selenium.
		* * * * *