

Doctor

José Luis Salazar

Jefe de División de Nuevas Creaciones (C)

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

E. _____ S. _____ D. _____

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



No. 08-095703- -00006-0000

Fecha: 2012-11-27 16:16:52 Dep. 2020 DIR.NUEVASCR
Tra. 11 PATENTEIYII Eve: 378 FASENACIONALI
Act. 523 RESPUESTA Folios: 59 60

Referencia: Expediente No. 08.095.703

Solicitud de Patente de Invención titulada: "Anticuerpos anti-5T4"

Solicitante: Wyeth

RESPUESTA AL AUTO NOTIFICADO POR FIJACIÓN EN LISTA No. 402
DEL 18 DE JULIO DE 2012, OFICIO NO. 12526 (EXAMEN DE FONDO)

ÁLVARO CORREA ORDÓÑEZ, mayor de edad y vecino de Bogotá D.C., identificado tal como aparece al pie de mi firma, en mi carácter de apoderado de la sociedad en referencia, doy respuesta al auto emanado por ese Despacho dentro del término de prórroga solicitado en mi memorial 08-095703-000050000 de fecha 8 de octubre de 2012:

1. Objeciones del Examinador

1.1 Título

Su Despacho solicita eliminar del título la referencia los usos.

1.2 Reivindicaciones

Su Despacho hace las siguientes observaciones:

"El objeto de las reivindicaciones 1, 3- 10 y 40-47 no se considera una invención, de acuerdo con el Arte citado.

No obstante, las reivindicaciones 36 - 39 se encuentran encabezadas como composición, esta se encuentra definida por su uso, por lo tanto no es patentable de acuerdo con el Art 14.

La reivindicación 11 se refiere a un anticuerpo humanizado o quimérico, sin definir sus secuencias, esta forma de definirlo no es adecuada porque no permite caracterizar al anticuerpo. Se sugiere definir al anticuerpo por su estructura completa con las secuencias que le conforman en la cadena ligera y cadena pesada, o las secuencias variables de las cadenas Ligera y pesada incluyendo las secuencias de los 6 CDR's que conforman ambas cadenas.

Las reivindicaciones 16, 17 y 18 - 25 se refieren a fragmentos de los anticuerpos nombrando diferentes secuencias para la región variable de cadena pesada y cadena ligera que no son estructuras activas, probadas, ni definidas; también se encuentra la expresión al menos 2 CDRs, la cual no es aceptable puesto que es conocido en el campo técnico que pequeñas variaciones en las secuencias del anticuerpo influyen en su unión con el antígeno, especialmente si estas se realizan en los CDRs. Adicionalmente, se da lugar a una serie de combinaciones entre las secuencias variables de cadena ligera y cadena pesada que no encuentran soporte en la descripción. No es posible definir un anticuerpo por fragmentos del mismo por lo cual se sugiere caracterizarlo a partir de su estructura completa, mencionando las secuencias que lo conforman en la cadena ligera y cadena pesada, o las secuencias variables de las cadenas ligera y pesada incluyendo las secuencias de los 6 CDR's que conforman ambas cadenas, restringiendo la solicitud a los anticuerpos que presentan alguna modificación, tales como los humanizados e quiméricos, puesto que anticuerpos murinos o fragmentos de los mismos no se consideran una invención.

No es claro a que se refiere el solicitante con un anticuerpo quimérico humanizado en las reivindicaciones 12 - 16 y 18, debido a que estas reivindicaciones son dependientes de la reivindicación 11 o de alguna reivindicación dependiente de esta, se piensa que puede ser un error de transcripción y que el solicitante se refiere a un anticuerpo quimérico o

Y en referencia a la reivindicación 26 y sus dependientes dice:

"La reivindicación es obvia, porque la persona del oficio normalmente versada en la materia enfrentada al problema de obtendría (SIC) un conjugado de un anticuerpo anti-5T4 y un fármaco como un agente citotóxico según las enseñanzas de los documentos D1 y D2, logrando de esta forma el conjugado de esta solicitud."

2. Argumentos a favor de la patentabilidad de la solicitud

2.1 Título

De acuerdo con la solicitud de su Despacho el título se ha modificada y ya no menciona el uso, quedando ahora como: " Anticuerpos anti-5T4".

2.2. Reivindicaciones

2.2.1. Su despacho considera que las reivindicaciones 1, 3-10 y 40-47 no se consideran una invención de acuerdo con el artículo 15 de la Decisión 486. En respuesta a esta observación la reivindicación 1 se ha corregido para especificar un anticuerpo humanizado o quimérico y las reivindicaciones 3 y 6-11 se han cancelado. Las reivindicaciones 40-47 también se han cancelado.

2.2.2. Su despacho considera que las reivindicaciones 36-39 están dirigidas a una composición que se define mediante su uso y como tal no son patentables de acuerdo con el artículo 14 de la decisión 486. Dichas reivindicaciones se han cancelado.

2.2.3. Su despacho considera que la reivindicación 11 se refiere a un anticuerpo humanizado o quimérico sin definición de su secuencia. Su despacho también sugiere que el anticuerpo se defina con su estructura completa que incluye las secuencias de seis CDR. En respuesta a su observación la reivindicación 1 se ha corregido para describir los anticuerpos reivindicados mediante referencia a los seis CDR. Por favor tenga en cuenta que al momento de la presentación de la presente solicitud era bien conocido que la especificidad de unión del anticuerpo era atribuible a la región de unión de antígeno formada por los seis CDR.

2.2.4. Su despacho consideró que las reivindicaciones 16, 17 y 18-25 se refieren a fragmentos de los anticuerpos que tienen diferentes secuencias que no son conocidos por tener la misma actividad de unión o que se alega que no están soportados en la descripción. En respuesta a su observación por favor note que las reivindicaciones 16-25 dependen de la reivindicación 1 la cual se corrigió para especificar los anticuerpos que tienen regiones de unión de antígeno particulares caracterizadas por los seis CDR indicados.

2.2.5. Su despacho considera que la expresión "anticuerpo quimérico humanizado" en las reivindicaciones 12-16 y 18 no es clara y que se puede deber a un error de transcripción. Dicha referencia se ha corregido en las nuevas reivindicaciones. La reivindicación 18 se ha cancelado.

2.2.6. Su despacho considera que las reivindicaciones 12-15 no son concisas por que se refieren a las regiones constantes definiéndolas por ser humanas y derivadas de una cadena constante de cadena ligera kappa humana de IgG1, IgG2, IgG3, o IgG4 humano. En respuesta a esta observación las actuales reivindicaciones 12-14 (reenumeradas como reivindicaciones 5-7) se corrigieron para suprimir el lenguaje que indicaba derivación de una secuencia de región constante proveniente de una secuencia conocida. Por favor también note que las secuencias de región constante humana eran bien conocidas en la técnica al momento de la presente invención.

2.2.7. Su despacho considera que las reivindicaciones 17, 24, 25, 26, y 47 no son aceptables por que no permiten definir una secuencia exacta ni caracterizar un anticuerpo. En respuesta a esta observación los elementos de las reivindicaciones 17, 24-25 y 46-47 (reivindicaciones reenumeradas 10, 13-14 y 26-27) que incluyen tales porcentajes se han suprimido.

2.2.8. Su despacho considera que las reivindicaciones 26-30, 34 y 36 definen componentes de un conjugado que se puede encontrar en una composición (a) como el anticuerpo de la reivindicación 1, que no está adecuadamente caracterizado, (b) un fármaco sin definición específica del fármaco o sin definición de su funcionalidad, efecto terapéutico o mecanismo de acción, y (c) un ligador que tampoco está definido. Su despacho considera que esta definición de un conjugado es insuficiente sobre la base de que el fármaco de anticuerpo y el ligador no están definidos. Se considera además que solo se encuentra

soporte en la descripción cuando el fármaco es caliqueamicina y el ligador es ácido 4-(4'acetilfenoxi)butanoico.

En respuesta a esta observación por favor note que la eficacia de los conjugados de anticuerpo/fármaco es dependiente de la selectividad de la porción de anticuerpo del conjugado. Como se describió aquí adelante, la selectividad mejorada de los conjugados de anticuerpo/caliqueamicina A1 y A3 parte del supuesto de los epitopos únicos unidos por los anticuerpos A1 y A3, que son diferentes del anticuerpo H8 de la técnica anterior. Basados en esta selectividad, una persona medianamente versada en la materia entendería fácilmente que cualquier molécula terapéutica (es decir, fármaco) se podría conjugar a los anticuerpos A1 y A3 con la expectativa razonable de que tal conjugado fuera similarmente ventajoso.

Por favor también tenga en cuenta que en la solicitud originalmente presentada existe soporte para el uso de una variedad de fármacos terapéuticos que eran conocidos en la técnica por ser eficaces. Ver, por ejemplo, descripción en la página 63, línea 9 a página 67 línea 10 (que describe, por ejemplo, que las citotoxinas representativas incluyen antibióticos, inhibidores de polimerización de tubulina, agentes alquilantes que se unen y afectan el ADN, y agentes que afectan la síntesis de proteína o la función de las proteínas celulares esenciales tales como las proteínas quinasas, fosfatasas, topoisomerasas, enzimas, y ciclinas); en las páginas 65-69 el párrafo puente (que describe citotoxinas representativas que incluyen, por ejemplo, citotoxinas que también son conocidas en la técnica como antibióticos (por ejemplo, puromicina, actinomicina, autramicina)); en las páginas 65-69, el párrafo puente (que describe radio isotopos representativos) ; en la página 70 primer párrafo completo que describe agentes inmunomoduladores representativos; en la página 36, segundo párrafo (que describe agentes antiproliferativos y proapoptóticos representativos) ; en la página 71-72 el párrafo puente (que describe agentes quimioterapéuticos representativos); y desde página 72, último párrafo (que describe agentes terapéuticos representativos adicionales).

2.2.9 Menciona su despacho que la reivindicación 32 no es clara debido a la referencia al "análogo". Por favor note que una persona medianamente versada en la materia entendería el término "análogo" en razón a que este término se utiliza para describir una molécula de caliqueamicina significando con esto versiones de una caliqueamicina específica que comparte similitudes estructurales y funcionales

2.2.10 Su despacho considera que las reivindicaciones 36-39 indican el uso que puede dársele al producto reivindicado y además hace alusión a métodos de tratamiento los cuales no son patentables. En respuesta a esta observación el solicitante ha decidido cancelar dichas reivindicaciones.

2.2.11 Su despacho ha considerado que las reivindicaciones 46 y 47 no serían patentables por que se refieren a ácidos nucleicos de murino y se sugiere limitarlo a los ácidos nucleicos humanizados solamente. En respuesta a esta observación las reivindicaciones se han corregido para establecer secuencias de anticuerpo A1 y A3 humanizadas.

2.2.12 Su despacho considera que las reivindicaciones 46 y 47 definen los ácidos nucleicos reivindicados por un resultado a alcanzar en razón a que estas reivindicaciones se refieren a un ácido nucleico que hibridiza de manera específica al complemento de cualquiera de (a)-(d) bajo condiciones de hibridización severa. En respuesta a su observación por favor note que los elementos de las reivindicaciones mencionadas que comprenden variantes hibridizantes se han cancelado.

2.2.13 Adicionalmente se han realizado otras correcciones como en la reivindicación 2 donde se ha suprimido la referencia a "un anticuerpo específico de dominio" y "anticuerpos de dominio único". La reivindicación 17 (nueva reivindicación 10) se han corregido para reemplazar las designaciones indicadas de las regiones de estructura (por ejemplo, "DP-21 (VH7)" con identificadores de secuencia específicos.

2.3. Descripción

En respuesta a las observaciones hechas por su despacho en relación con la descripción como por ejemplo los signos de interrogación, la referencia a "Kg (1)", la referencia al anticuerpo A43, se han hecho las correcciones correspondientes en la descripción.

Como complemento por favor note lo siguiente: en relación con la segunda observación a la descripción relacionada con la Tabla 1 y Tabla 2 el solicitante no ha entendido la observación, sin embargo, desea manifestar lo siguiente: para evaluar la novedad de A1, A2 y A3 las secuencias de región variable A1, A2 y A3 se hicieron búsquedas de BLASTn y

BLASTp para identificar las secuencias de nucleótido y de proteína más cercanamente relacionada, respectivamente. El algoritmo BLAST para la comparación de secuencias es bien conocido y se practica rutinariamente en la técnica. Ver también descripción en la página 62 último párrafo. Las Tablas 1 y 2 presentan los resultados de este análisis cuando se utilizan las secuencias de consulta A1, A2 o A3 indicadas (columna más a la izquierda). La secuencia públicamente conocida más cercanamente relacionada con la secuencia de consulta se lista (con una más a la derecha), y se calculó el porcentaje de identidad entre las dos secuencias (columna de la mitad). Estos porcentajes fueron la base para los porcentajes especificados en las reivindicaciones 24-25 (reivindicaciones reenumeradas 13-14), cuyos elementos se han suprimido de estas reivindicaciones.

En la Tabla 3 la referencia a "Kg (1" corresponde a "ka (1/Ms)"

La referencia al anticuerpo A43 se refiere realmente a anticuerpo "A3".

Su despacho solicita la inclusión de la definición de CMA-676 en la descripción puesto que este se define como un conjugado anti-CD3/caliqueamicina. En particular el examinador busca aclaración del anticuerpo anti-CD33. En respuesta a su observación por favor note que al momento de la presentación de esta solicitud el CMA-676 era conocido en la técnica. Ver por ejemplo, Sievers et al., *Blood*, 1999, 93: 3678-84 (Copia anexa) cuya referencia y referencias relacionadas se describen en la solicitud originalmente presentada. Ver descripción, párrafo puente entre las páginas 1 y 3.

Su despacho establece que existe una inconsistencia en la información del capítulo descriptivo por que en la página 145 (líneas 19-22) se encuentran que las secuencias SEQ ID No. 52 y 54 corresponden a regiones variables de cadena pesada A1 humanizadas y en la Tabla 10 estas aparecen como secuencias de la región variable de cadena ligera. El solicitante desea manifestar que no entiende la observación. De acuerdo con la página 145, renglones 15 y 16 se dice que "La similitud de las regiones variables de cadena pesada A1 humanizadas se muestra en la tabla 10.". Por su parte la página 146, renglones 11 y 12 dice que: "La similitud de las regiones variables de cadena ligera A1 humanizadas se muestra en la tabla 10.". La tabla 10 muestra estas similitudes porcentualmente tal y como lo menciona la descripción. De acuerdo con esto no es claro porque su Despacho considera que las secuencias 52 y 54 se consideran como de cadena pesada en un lado y de cadena ligera en

otro.

El solicitante presenta nuevamente la figura 15 en el cual se ha corregido el término "cynomolgus" que apareció mal escrito originalmente.

2.4. Nivel inventivo

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

Contrario a lo que plantea el Examinador, el documento del arte previo citado no afecta el nivel inventivo de la presente solicitud por cuanto no es posible derivar la presente invención a partir de las enseñanzas presentadas en los documentos D1 y D2.

En relación con el nivel inventivo el solicitante desea manifestar que el problema a ser resuelto por la presente invención no es simplemente el suministro de un anticuerpo anti-5T4 alternativo sino, el suministro de anticuerpos anti-5T4 que tengan selectividad y citotoxicidad mejoradas cuando se utilizan en conjugados de anticuerpo/fármaco citotóxico. Los diferentes efectos técnicos logrados son atribuibles, al menos en parte, a los diferentes epitopos 5T4 específicamente unidos por los anticuerpos de la presente invención y aquellos de los documentos citados, como se describe adelante.

Inicialmente, note que el anticuerpo anti-5T4 quimérico de D1 fue preparado utilizando regiones variables del anticuerpo H8 murino. Específicamente, D1 establece que un ScFv específico de 5T4 se amplificó mediante PCR utilizando el anticuerpo monoclonal que produce hibridoma mAb5T4, ver Shaw et al., *Biochim. Biophys. Acta*, 2000, 1524: 238-246 (copia adjunta). Ver D1, página 885, columna 2, segundo párrafo. Shaw describe la preparación de anticuerpos de cadena única y también muestra las secuencias de aminoácido amplificadas de las secuencias de región variable mAb5T4 ver Shaw figura 1B. D2 establece que el anticuerpo 5T4 o mAb5T4 como se describió en las referencias D1/Shaw es el mismo que el anticuerpo denominado como anticuerpo anti-5T4 "H8" murino. Ver D2, página 16, renglones 23-25.

Como se anotó anteriormente, los anticuerpos de la presente invención suministran selectividad mejorada cuando se utilizan en los conjugados anticuerpo/fármaco citotóxico comparado con el anticuerpo H8 humanizado. El ejemplo 6 de la presente solicitud describe los resultados de los ensayos utilizando tinte vital (MTS) para evaluar la supervivencia de las células que expresan 5T4 cuando se exponen a conjugados de anticuerpo anti-5T4/caliqueamicina. Después de 96 horas, los conjugados preparados utilizando los anticuerpos A1 y A3 reducen sustancialmente la viabilidad de las células que expresan 5T4 (MDAMB435/5T4). Para evaluar la especificidad del efecto citotóxico, se calcularon los valores de selectividad como la proporción del efecto citotóxico del conjugado contra las células que expresan 5T4 (es decir, actividad específica) sobre el efecto citotóxico del conjugado contra las células a las que le falta la expresión de 5T4 (es decir, actividad no específica). Los conjugados preparados con A1 (a1-AcBut-CalichDMH) mostraron un valor de selectividad alto de 93, mientras que los conjugados preparados con anticuerpo H8 humanizado (huH8-AcBut-CalichDMH) mostraron un valor de selectividad relativamente bajo D8. Ver descripción, Tabla 9.

Además con respecto a la selectividad de los conjugados de anticuerpo/fármaco luego de la revisión de los datos incluidos en la Tabla 9 y el texto que lo acompaña en el Ejemplo 6 hemos notado que el texto "selectividad: ...A3=1.6" incluido debajo de la Tabla 9 es una representación incorrecta y/o incompleta de los datos. Con base en los resultados de los ensayos MTS descritos en el Ejemplo 6, los inventores concluyeron que los conjugados de anticuerpo/caliqueamicina preparados utilizando los anticuerpos anti-5T4 A1 y A3 redujeron sustancialmente la viabilidad de las células que expresan 5T4. Ver descripción, página 140, renglones 1-5. El Ejemplo 6 también se refiere a la Tabla 9 que presenta resultados de los ensayos MTS y explica que los valores de selectividad se calcularon como sigue:

Los valores de selectividad se calcularon al comparar la actividad específica del conjugado con la actividad no específica. Esto es, las veces de CalichDMH para las células que expresan 5T4 se dividieron por las veces de los valores CalichDMH para las células que no expresan 5T4. Descripción página 72 renglones 5-10.

Utilizando esta ecuación, la selectividad de los conjugados preparados utilizando los varios anticuerpos se indica debajo de la Tabla 9 como sigue:

selectividad: H8=8; Hp67.6=1; A1=93; A3=1.6

Sin embargo, notamos que cada una de las entradas correspondientes en la Tabla 9 para "veces de CalichDMH" cuando se aplico a las células que expresan 5T4 (MDAMB435/5T4) es un rango de valores, y por lo tanto, los valores de selectividad calculados también deben ser un rango de valores en lugar de los valores específicos ubicados delante de la Tabla 9. Al efectuar el cálculo de la selectividad como se describió en la solicitud originalmente presentada se llega a lo siguiente:

Selektividad: H8=8-13; Hp67.6=1 (note que el valor de 1 se calcula para varios extremos de un rango); A1=87-93; A3=2-68 (note que el valor de selectividad establecido de 1.6 parece ser el resultado de dividir incorrectamente el fold CalichDMH *más bajo* para las células que expresan 5T4 por el fold CalichDMH *más alto* para las células de control).

De acuerdo con esto, un cálculo adecuado de los valores de selectividad con base en los datos presentados en la Tabla 9, indican que los conjugados preparados con el anticuerpo A3 muestran selectividad sustancial, comparados con los conjugados H8/caliqueamicina, que es la conclusión sacada de los ensayos MTS tal como se establece en el Ejemplo 6.

La citotoxicidad de los conjugados de caliqueamicina anti-5T4 también se ensayo utilizando un cultivo celular esferoide tridimensional que se aproxima más cercanamente a un ambiente celular *in vivo*. Los conjugados preparados utilizando tanto los anticuerpos A1 como A3 inhibieron significativamente el crecimiento de las células que expresan 5T4 pero no las células de control. Ver descripción, página 141, primer párrafo delante de la Tabla 9 hasta página 142, renglón 24, y Figura 8. También como se muestra en la Figura 8, los conjugados preparados con anticuerpo H8 humanizado (CME-548) fueron sustancialmente citotóxicos tanto a las poblaciones de células que expresan 5T4 como a las células de control, consistente con la baja selectividad observada en los ensayos MTC. Estos resultados también subcalifican la ventaja técnica ofrecida por los conjugados anticuerpo A1 y A3/caliqueamicina.

Los efectos técnicos mejorados exhibidos por los anticuerpos de la presente invención resultan de unir a los epitopos únicos sobre el antígeno 5T4. Shaw et al., *Biochem J.*, 2002,

363:137-145 (copia incluida) describe el mapeo del epitopo del anticuerpo mAb5T4/H8 sobre el antígeno 5T4, y que el anticuerpo mAb5T4/H8 se une a un epitopo conformacional (es decir, no lineal) que está influenciado por la glicosilación compleja del antígeno 5T4. Ver Shaw 2002, resumen. La presente solicitud caracteriza además que los residuos 213 y 214 del antígeno 5T4 humano son esenciales para la unión del mAb5T4/H8. Ver descripción, Ejemplo 4 y Tabla 6. Para identificar los epitopos a los cuales cada uno de los anticuerpos A1, A2 y A3 se une, se efectuaron ensayos ELISA utilizando (1) construcciones Fc de ectodominio 5T4 con secuencias suprimidas o mutadas y (2) construcciones quimeras 5T4 transitoriamente expresadas en células COS-1. Ver descripción, Ejemplo 4. Además, los ensayos de unión competitiva se efectuaron utilizando H8 humanizado biotinilado y cada uno de los anticuerpos A1, A2 y A3. Ver descripción, Ejemplo 4. El mapeo del epitopo se refinó adicionalmente utilizando ensayos de unión competitiva BIAcore®. Ver Ejemplo 5. Los resultados combinados dan apoyo a que cada uno de los anticuerpos A1, A2 y A3 se une a un epitopo 5T4 que es distinto de aquel unido mediante el anticuerpo H8. Ver descripción, Figura 7.

Con base en lo anterior, los anticuerpos de la reivindicación 1 tienen nivel inventivo sobre los anticuerpos citados en el estado de la técnica. Las reivindicaciones 2, 4-5, 12-17, 22-36 y 46-47 (reivindicaciones reenumeradas 2-27 y nuevas reivindicaciones 28-29) finalmente dependen de la reivindicación 1, y por lo tanto incluyen los elementos inventivos de la reivindicación 1.

3. Solicitudes concedidas en otros países para esta misma invención.

A manera informativa, pongo en conocimiento del Examinador que la patente peruana No. 6145 del 13 de septiembre de 2011 equivalentes a la presente solicitud colombiana ha sido otorgada. El nuevo capítulo reivindicatorio presentado en la presente solicitud es sustancialmente el mismo de la solicitud peruana con la excepción de que 1) las reivindicaciones que comprende anticuerpos de murino se han cancelado en las reivindicaciones de Colombia, y 2) se han agregado las nuevas reivindicaciones 26 y 27 que muestran un aspecto particular de la invención comprendida por las reivindicaciones de la patente peruana. Aún cuando entiendo que estas decisiones de ninguna manera obligan al Despacho para decidir de igual forma, considero que por tratarse de una pregunta fáctica la

determinación de la existencia de nivel inventivo, las decisiones de otras oficinas de patentes constituyen un antecedente persuasivo en la materia.

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

Atentamente,


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

Anexos: Capítulo reivindicatorio modificado
 Recibo por reivindicaciones adicionales
 Recibo por modificación voluntaria
 Formulario de modificación
 Capítulo descriptivo modificado en versión digital
 Reemplazo figura 15
 Documento Shaw 1
 Documento Shaw 2
 Documento Sievers
 Copia de la patente Peruana

REIVINDICACIONES

1. Anticuerpo quimérico o humanizado que se une específicamente al antígeno 5T4 humano, caracterizado porque:

(a) los tres CDR de una región variable de cadena pesada que tienen una secuencia de aminoácido establecida con SEQ ID No.: 2 y tres CDR de una región variable de cadena ligera que tiene una secuencia de aminoácido como se establece en la SEQ ID No.: 4; o

(b) tres CDR de una región variable de cadena pesada que tienen una secuencia de aminoácido como se establece en la SEQ ID No.: 10 y tres CDR de una región variable de cadena ligera que tienen una secuencia de aminoácido como se establece en la SEQ ID No.: 12.

2. Anticuerpo de conformidad con la reivindicación 1, caracterizado porque el anticuerpo es un anticuerpo quimérico, un anticuerpo humanizado, un anticuerpo de cadena individual, un fragmento Fab, un fragmento F(ab)₂, un fragmento Fv, un anticuerpo tetramérico, un anticuerpo tetravalente, un anticuerpo multiespecífico, o una proteína de fusión.

3. Anticuerpo de conformidad con la reivindicación 1, caracterizado porque tiene una afinidad de unión para el antígeno 5T4 humano de al menos aproximadamente 1×10^{-7} M a aproximadamente 1×10^{-12} M.

4. Anticuerpo de conformidad con la reivindicación 1, caracterizado porque selecciona específicamente como objetivo células que expresan 5T4 *in vivo*.

5. Anticuerpo quimérico o humanizado de conformidad con la reivindicación 1, caracterizado porque comprende regiones constantes humanas.

6. Anticuerpo quimérico o humanizado de conformidad con la reivindicación 5, caracterizado porque comprende una región constante de cadena ligera kappa humana.

7. Anticuerpo quimérico o humanizado de conformidad con la reivindicación 5, caracterizado porque comprende una región constante de cadena pesada de IgG1, IgG2, IgG3 o IgG4 humana.

8. Anticuerpo quimérico o humanizado de conformidad con la reivindicación 7, caracterizado porque la región constante de cadena pesada de IgG4 humana comprende prolina en la posición 241.

9. Anticuerpo quimérico o humanizado de conformidad con la reivindicación 1, caracterizado porque comprende al menos una región variable de cadena ligera y al menos una región variable de cadena pesada que comprende:

(a) una región de estructura que comprenden residuos de una región de estructura de anticuerpo humano.

10. Anticuerpo quimérico o humanizado de conformidad con la reivindicación 9, caracterizado porque los residuos

humanos son residuos de estructuras humanas seleccionados del grupo que consiste de:

(a) una región de estructura de cadena pesada de anticuerpo humano de una cualquiera de las SEQ ID NO.: 14 - 23, 77 y 88, 93;

(b) una región de estructura de cadena ligera de anticuerpo humano de una cualquiera de las SEQ ID No.: 27 - 34, 35 - 44 y 94 - 99; y

(c) una secuencia de consenso de una región de estructura alterna de cadena pesada de (a).

11. Anticuerpo humanizado de conformidad con la reivindicación 9, caracterizado porque la región variable de la cadena pesada comprende tres CDR de la SEQ ID Nos:2.

12. Anticuerpo humanizado de conformidad con la reivindicación 16, caracterizado porque comprende las CDR de SEQ ID No:2 y 4.

13. Anticuerpo quimérico o humanizado de conformidad con la reivindicación 11, caracterizado porque la secuencia de región variable de cadena pesada comprende:

(a) una secuencia de aminoácidos de los residuos 20-138 de SEQ ID No:2;

(b) una secuencia de aminoácido de los residuos 20 - 141 de la SEQ ID No.: 10; o

(c) un secuencia de aminoácido de una cualquiera de las SEQ ID No.: 49, 51, 52, 54, 56 u 82.

19

14. Anticuerpo quimérico o humanizado de conformidad con la reivindicación 9, caracterizado porque la secuencia de región variable de cadena ligera comprende:

(a) una secuencia de aminoácidos de los residuos 21-127 de SEQ ID No:4;

(b) una secuencia de aminoácidos de los residuos 21 - 127 de la SEQ ID No.: 12;

(c) una secuencia de aminoácidos de cualquiera de las SEQ ID Nos: 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 83 u 84.

15. Conjugado de anticuerpo/fármaco para distribución de fármaco, caracterizado porque comprende:

(a) un anticuerpo de acuerdo a la reivindicación 1; y

(b) un fármaco, que está unido directa o indirectamente al anticuerpo.

16. Conjugado de anticuerpo/fármaco de conformidad con la reivindicación 15, caracterizado porque el fármaco es un agente terapéutico seleccionado del grupo que consiste de una citotoxina, un radioisótopo, un agente inmunomodulador, un agente anti-angiogénico, un agente anti-proliferativo, un agente pro-apoptótico, un agente quimioterapéutico, un ácido nucleico terapéutico.

17. Conjugado de anticuerpo/fármaco de conformidad con la reivindicación 16.27, caracterizado porque el agente terapéutico es una citotoxina.

18. Conjugado de anticuerpo/fármaco de conformidad con la reivindicación 20, caracterizado porque la citotoxina es un antibiótico, un inhibidor de polimerización de tubulina, un agente de alquilación, un inhibidor de síntesis de proteína, un inhibidor de proteína-cinasa, un inhibidor de fosfatasa o un inhibidor de topoisomerasa, o una enzima.

19. Conjugado de anticuerpo/fármaco de conformidad con la reivindicación 16, caracterizado porque la citotoxina es un antibiótico.

20. Conjugado de anticuerpo/fármaco de conformidad con la reivindicación 19, caracterizado porque el antibiótico es caliqueamicina.

21. Conjugado de anticuerpo/fármaco de conformidad con la reivindicación 20, caracterizado porque la caliqueamicina es un análogo de disulfuro o N-acetil-derivado de caliqueamicina.

22. Conjugado de anticuerpo/fármaco de conformidad con la reivindicación 21, caracterizado porque la caliqueamicina es N-acetil- γ -caliqueamicina.

23. Conjugado de anticuerpo/fármaco de conformidad con la reivindicación 15, caracterizado porque el fármaco está unido al anticuerpo mediante un ligador.

24. Conjugado de anticuerpo/fármaco de conformidad con la reivindicación 23, caracterizado porque el ligador se selecciona del grupo que consiste de ácido 4-(4'-acetilfenoxi)butanóico (AcBut), ácido 3-acetilfenil-acídico

(AcPac), ácido 4-mercapto-4-metil-pentanoico (Amida), y derivados de los mismos.

25. Una composición farmacéutica que comprende un conjugado de anticuerpo/fármaco que comprende (i) un anticuerpo anti-5T4 de la reivindicación 1, y (ii) un fármaco que está unido al anticuerpo anti-5T4 directamente o de manera indirecta.

26. Ácido nucleico aislado que codifica para una región variable de cadena pesada anti-5T4, caracterizado porque comprende una secuencia de nucleótido que codifica una cualquiera de las SEQ ID No.: 49, 51, 52, 54, 56 y 82.

27. Ácido nucleico aislado que codifica para una región variable de cadena ligera anti-5T4, caracterizado porque comprende una secuencia de nucleótido que codifica una cualquiera de las SEQ ID No.: 49, 51, 52, 54, 56 y 82.

28. El anticuerpo quimérico o humanizado de la reivindicación 9, que comprende:

(a) una región variable de cadena ligera que comprende una secuencia de aminoácido seleccionada del grupo que consiste de una secuencia de aminoácido de los residuos 21-127 de la SEQ ID No.: 4 y una cualquiera de las SEQ ID No.: 58, 60, 62, 64, 66, 68, 70, 72, 74 o 76; y

(b) una región variable de cadena pesada que comprende una secuencia de aminoácido seleccionada del grupo que consiste de los residuos 10 - 138 de la SEQ ID No.: 2 y una cualquiera de las SEQ ID No.: 49, 51, 52, 54 o 56.

29. El anticuerpo quimérico o humanizado de la

reivindicación 9, que comprende:

(a) una región variable de cadena ligera que comprende una secuencia de aminoácido de la SEQ ID No.: 70; y

(b) una región variable de cadena pesada que comprende una secuencia de aminoácido de la SEQ IN No.: 54.

RAC/BOGDMS-#1287368-V1-P2008_000392-NUEVO_CAPÍTULO_REIVINDICATORIO.DOCX



Industria y Comercio
SUPERINTENDENCIA

Espacio reservado para el adhesivo de radicación

DIRECCIÓN DE NUEVAS CREACIONES
MODIFICACIONES Y/O CORRECCIONES

1. Identificación del Trámite

☐ Error material

☒ Modificación a una Solicitud en trámite

☒ Patente de Invención

☐ Patente de Modelo de Utilidad

☐ Registro de Diseño Industrial

☐ Esquema de Trazado de Circuitos Integrados

2. Datos del solicitante

Persona Natural ☐

Persona Jurídica ☐

WYETH

Nombre o denominación / Nombre o razón social completa

Nombre representante legal

Tipo de empresa Micro ☐ Pequeña ☐ Mediana ☐ Otra ☒

Documento de identificación: ☐ C.C. ☐ C.E. ☐ NIT ☐ Otro Número _____

Nacionalidad/País de constitución	Dirección y domicilio del titular	Ciudad
Estados Unidos de América	Five Giralda Frams, Madison, NJ 07940	Madison
Dirección electrónica	No. Fax	Número telefónico
alvaro.correa@bakermckenzie.com	3762211	6341500

☐ Autorizo expresamente a la Superintendencia de Industria y Comercio para que todas las comunicaciones sean enviadas a través de la dirección de correo electrónico

3. Datos del apoderado:

Correa Ordoñez, Alvaro

79.143.366

20.281

Apellidos y Nombre

Documento de identificación

Tarjeta profesional

Dirección y domicilio		Ciudad
AV. 82 No. 10 62 Piso 6		Bogotá
Dirección electrónica	No. Fax	Número telefónico
alvaro.correa@bakermckenzie.com	3762211	6341500

En caso de haber presentado poder general para asuntos que se adelanten ante la Delegatura de Propiedad Industrial, sírvase indicar el número de su radicación o protocolo de poder general _____

4. Datos del trámite

Corrección:

Expediente No 08.095.703

Aparece _____

Debe ser _____

Modificación:

☐

Modificación al capítulo reivindicatorio.

☐

Modificación al capítulo descriptivo.

☐

Modificación al resumen.

☒

Modificación a los dibujos.

Se presenta modificación en la figura No. 15 de la solicitud con el fin de corregir error tipográfico en el término "Cynomolqus"

☐

Modificación al solicitante.

5. Anexos

☒

Comprobante de pago de la tasa para la presentación de la corrección y/o modificación

☐

Poder, si fuere el caso.

☒

Documento que contiene las modificaciones o correcciones.

☐

Copia de la solicitud y sus anexos en formato magnético.

6. Firma

Nombre y apellidos

Alvaro Correa Ordoñez

C.C. 79.143.366

Firma

Tarjeta Profesional 20.281

Dependencia:

3200

Fecha:

AAAA MM DD

Recorrido:

Tipo de Radicación:

Entrada: ☐

Salida: ☐

Traslado: ☐

Convenio: ☐

Radicator:

Planilla:

ID	No. Radicación	Consecutivo	Item
1	08-95703-002	002	
2	08-95703-	6	2
3			
4			
5			
6			
7			
8			
9			
10			
11			
12			
13			
14			
15			
16			
17			

Item	Unidad de Conservación
1	Bolsa
2	Caja
3	Libro
4	Sobre
5	Tomo
6	Otro
Item	Soporte Documental
7	CD
8	Disquete
9	DVD
10	Folleto
11	Foto
12	Periódico
13	Plano
14	Publicación Periódica
15	USB
10	Otro

Observaciones:

Folios 25 CD

FIG. 15

5T4 de mono tití de cola negra

MPGGCSRGPAAAGNGRLRLARLALVLLGWVSSSSPTSSASSSSSSAPFLASAVSAQPPLPGQCPA
LCECSEAARTVKCVNRNLTEVPTDLPPYVRNLFLTGNQLAVLPAGAFARVPPLAELAALNLSGS
RLEDVQAGAFEHLPSLRQLDL SHNPLAVLSPFAFSGSNASVSAPSPLVELILNHIVPPEDERNN
RSFEGMVVAALRAGGALHGLHRLELASNHFLYLPRDVLAQLPSLRHLDLSNNSLVSLTYVSFRN
LTHLESLHLEDNALKVLHNGTLAELQGLPHVRVFLDNNPWVCDCHMADMVAWLKETEVVQGKYQ
LTCAFPEKMRNRVLELNSADLDCDPILPPSLQTSYVFLGIVLALIG (SEQ ID NO:85)

5T4 de mono Cynomolgus

MPGGCSRGPAAAGDGRRLRLARLALVLLGWVSSSSPTSSASSFSSSAPFLASAASAQPPLPDQCPA
LCECSEAARTVKCVNRNLTEVPTDLPLYVRNLFLTGNQLAVLPAGAFARRPPLAELAALNLSGS
RLDEVVAGAFEHLPSLRQLDL SHNPLAYLSPFAFSGSNASISAPSPLVELILNHIVPPDDKRQN
RSFEGMVAAALVAGRALQGLHLELASNHFLYLPRDVLAQLPSLRYLDSNNSLVSLTYVSFRN
LTHLESLHLEDNALKVLHNGTLAELQGLPHVRVFLDNNPWVCDCHMADMVTWLKQTEVVQGKDR
LTCAFPEKMRNRVLELNSADLDCDPILPPSLQTSYVFLGIVLALIGAIFLLVLYLNRKGIKKW
MHNIRDACRDHMEGYHYRYEINADPRLTNLSSNSDV (SEQ ID NO:86)



179179



G00002

BIOCHIMICA ET BIOPHYSICA ACTA

BBA

Biochimica et Biophysica Acta 1524 (2000) 238–246

www.elsevier.com/locate/bba

Isolation of a high affinity scFv from a monoclonal antibody recognising the oncofoetal antigen 5T4

D.M. Shaw ^{a,*}, M.J. Embleton ^b, C. Westwater ^a, M.G. Ryan ^c, K.A. Myers ^c,
S.M. Kingsman ^c, M.W. Carroll ^c, P.L. Stern ^a

^a CRC Immunology Group, Paterson Institute for Cancer Research, Christie Hospital, Manchester M20 4BX, UK

^b CRC Medical Oncology Group, Paterson Institute for Cancer Research, Christie Hospital, Manchester M20 4BX, UK

^c Oxford Biomedica, Oxford Science Park, Oxford OX4 4GA, UK

Received 13 June 2000; received in revised form 19 October 2000; accepted 19 October 2000

Abstract

The oncofoetal antigen 5T4 is a 72 kDa glycoprotein expressed at the cell surface. It is defined by a monoclonal antibody, mAb5T4, that recognises a conformational extracellular epitope in the molecule. Overexpression of 5T4 antigen by tumours of several types has been linked with disease progression and poor clinical outcome. Its restricted expression in non-malignant tissue makes 5T4 antigen a suitable target for the development of antibody directed therapies. The use of murine monoclonal antibodies for targeted therapy allows the tumour specific delivery of therapeutic agents. However, their use has several drawbacks, including a strong human anti-mouse immune (HAMA) response and limited tumour penetration due to the size of the molecules. The use of antibody fragments leads to improved targeting, pharmacokinetics and a reduced HAMA. A single chain antibody (scFv) comprising the variable regions of the mAb5T4 heavy and light chains has been expressed in *Escherichia coli*. The addition of a eukaryotic leader sequence allowed production in mammalian cells. The two 5T4 single chain antibodies, scFv5T4WT19 and LscFv5T4, described the same pattern of 5T4 antigen expression as mAb5T4 in normal human placenta and by FACS. Construction of a 5T4 extracellular domain-IgGFC fusion protein and its expression in COS-7 cells allowed the relative affinities of the antibodies to be compared by ELISA and measured in real time using a biosensor based assay. MA5T4 has a high affinity, $K_D = 1.8 \times 10^{-11}$ M, as did both single chain antibodies, scFv5T4WT19 $K_D = 2.3 \times 10^{-11}$ M and LscFv5T4 $K_D = 7.9 \times 10^{-10}$ M. The small size of this 5T4 specific scFv should allow construction of fusion proteins with a range of biological response modifiers to be prepared whilst retaining the improved pharmacokinetic properties of scFvs. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Single chain antibody; scFv; Affinity; Surface plasmon resonance; 5T4 oncofoetal antigen; Tumor; Targeted therapy

1. Introduction

The use of tumour antigen specific antibodies for the delivery of therapeutic agents offers the possibility of specific therapy with reduced toxicity to normal tissues compared to conventional treatments [1]. However, the effectiveness of the use of whole monoclonal antibodies (mAbs) is restricted by several factors [2]. Repeated administration of murine antibodies generates a strong human anti-mouse immune (HAMA) response in up to 50% of patients after the first dose, and approximately 90% following a second treatment [2,3]. This causes a rapid clearance of subse-

quent doses, and immune complex formation can lead to a severe anaphylactic response [4]. The severity of the HAMA response can be lessened by the use of antibody fragments lacking the Fc domain, in particular single chain Fv antibodies (scFvs) [5,6]. ScFv antibodies may also show much greater target specificity for tumour localisation compared with whole IgG. The small size of scFv constructs allows increased diffusion into a solid tumour mass [7], and more rapid clearance from the blood [8]. Thus, although lower levels of antibody may localise at the site of the tumour, a higher labelling index (tumour: normal) can be achieved, as normal tissue labelling is minimised, in particular renal uptake [8,9]. A further advantage of scFvs is that they greatly simplify the design of genetically modified constructs for therapy, compared with whole antibody molecules, and may be expressed in vivo by recombinant viral vectors.

The oncofoetal antigen 5T4 is defined by a mouse

* Corresponding author. Fax: +44-161-446-3109;
E-mail: dshaw@picr.man.ac.uk

monoclonal antibody, mAb5T4, raised against wheat germ agglutinin purified placental glycoproteins that recognises a 72 kDa membrane molecule [10,11]. 5T4 antigen is expressed in human trophoblast and many different carcinomas [10], but shows limited expression in normal adult tissue [12]. Studies in gastric [13], ovarian [14] and colorectal carcinoma [15] have demonstrated a prognostic significance for tumour associated 5T4 expression. The shared expression of 5T4 antigen between trophoblast and tumours is thought to reflect a common function possibly including tissue invasion. The cDNA sequences encoding both human and mouse 5T4 molecules have been determined [16,17]; these predict conserved molecules comprising cytoplasmic and transmembrane domains and an extracellular domain containing two leucine rich repeat domains separated by a hydrophilic domain and seven potential *N*-linked glycosylation sites. Leucine rich repeats are involved in protein–protein interactions and are present in a wide range of proteins with diverse functions [18–24]. Overexpression of the gene in normal mouse mammary epithelial cells led to a reduction in cell–cell contacts, a rearrangement of the actin cytoskeleton, increased motility and a more dendritic morphology [25].

Here we report the isolation from the parent hybridoma of a single chain antibody with specificity for the oncofetal antigen 5T4. This was adapted for expression in eukaryotic cells via a retroviral system. Affinities of the antibodies were measured in real time by surface plasmon resonance using a fusion protein constructed from the extracellular domain of 5T4 and the Fc of human IgG. The specificity and high affinity of this scFv make it a suitable platform for the development of tumour specific targeted therapies.

2. Materials and methods

2.1. Cell culture

Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum and 2 mM L-glutamine at 37°C in a humidified atmosphere of 5% CO₂/air. Cultures were passaged as they reached visual confluence, using trypsin–EDTA. Murine L cells (A9 cells) transfected with human 5T4 (A95T4a) or control plasmid pCMV α (A9H12) [26] were maintained under antibiotic selection by the addition of 1 mg/ml G418.

2.2. Purification of monoclonal antibodies and preparation of 9E10 affinity matrix

mAb5T4 [10] was purified on protein A Sepharose in high salt loading buffer (3 M NaCl, 1.5 M glycine pH 8.9) and eluted in 100 mM pH 6 citrate buffer. 9E10 (mouse α myc tag) [27] supernatant was concentrated 40 $\times$ by ammonium sulphate precipitation and used in subsequent

assays. 9E10 affinity chromatography matrix was prepared by coupling protein G purified 9E10 to CnBr activated Sepharose (Pharmacia) at a concentration of 5 mg of antibody per ml of matrix, according to the manufacturer's instructions.

2.3. Construction and production of 5T4 extracellular domain–IgGFc fusion protein

A 974 bp fragment encoding the extracellular domain of 5T4 antigen was amplified by PCR and directionally cloned into the *Hind*III and *Bam*HI sites of the eukaryotic expression vector, sig-pIg plus (Ingenious, R&D Systems). *Escherichia coli* XL1-Blue was transformed with the plasmid, and recombinants analysed for the presence of the insert by endonuclease restriction digest. Positive clones were sequenced using a range of specific primers.

Aliquots of 5×10^6 COS-7 cells were transfected by electroporation with 10 μ g of 5T4–IgGFc plasmid DNA using the conditions 200 V, 1050 μ F. A stable bulk culture expressing the 5T4–IgGFc fusion protein was established under neomycin selection by the addition of G418 at 1 mg/ml and adapted for culture in 1% serum.

The fusion protein was precipitated from 7 day conditioned medium by ammonium sulphate at 60% saturation and dialysed against several changes of phosphate buffered saline (PBS) before loading onto a 5 ml wheat germ agglutinin (WGA) agarose column (Vector Labs.). 5T4–IgGFc fusion protein was eluted in 500 mM *N*-acetylglucosamine, the concentration determined by Fc capture ELISA and positive fractions further purified by protein G affinity chromatography using a 1 ml 'Hi-Trap' column. 5T4–IgGFc was eluted in 100 mM glycine pH 2.5 and neutralised with 1 M Tris–HCl pH 9. Positive fractions were dialysed against PBS and their protein content determined by modified Bradford assay [28], purity assessed by silver stained SDS–PAGE [29], and antigenicity by Western blot [30] and ELISA using mAb5T4.

2.4. Assembly and cloning of 5T4 scFv

Total RNA was prepared from mAb5T4 hybridoma cells using RNeasy and QIAshredder spin columns (both Qiagen), first strand cDNA synthesis was performed using MMLV reverse transcriptase (Promega) using C_{H1} and C_K specific primers.

Template cDNAs were amplified by PCR with V_H and V_K gene specific primer pairs, these were linked by PCR using primers that encoded the polylinker sequence (G₄S)₃ to form the single chain antibody cDNA. The final product was ligated into the *Sfi*I and *Not*I sites of the pHen1 vector which includes a Myc tag cassette and directs phage production in the amber suppressor strain TG1, or secretion of the scFv in the non-suppressor strain HB2151. This was electroporated into *E. coli* HB2151. Several clones were assessed for soluble production of scFv in the super-

A

[illegible]

B

EVQLQSSGPDLVKPGASVKISKASGYSTGYMHWVQSHGKSLBWIGRINPNNGV
TLYNQKFKDAILTVDKSSTTAYMELRSLTSEDSAVYYCARSTMITNYVMDVWGQVT
SVTVSSggggsgggsgggsggggSIVMQTPTFLLVSAGDRVITICKASQSVDNAWY
QQKPGQSPFTLLISYTSSRYAGVPDRFIGSGYGSDFTFTISTLQAEDLAVFYCQQDYN
SPPTFGGGTKLEIKR

Fig. 1. cDNA and amino acid sequence of scFv5T4WT19. DNA sequence of scFv5T4WT19 (a) and deduced amino acid sequence (b), scFv was constructed V_H-linker-V_L; polylinker is shown in lower case.

nant. Of those sequenced, all had the same nucleotide sequence (Fig. 1); a clone designated scFv5T4WT19 was selected.

Production of scFv5T4WT19 was accomplished by growing the cells in 2×TY broth to an OD₆₀₀ of 0.9 and inducing overnight with 1 mM isopropyl-β-D-thiogalactoside at 30°C; yield was quantified by ELISA against 5T4-IgGFc. The supernatant was clarified by centrifugation, and the scFv ammonium sulphate precipitated at 65% saturation and dialysed against PBS. Dialysed material was loaded onto a 9E10 Sepharose affinity column and scFv was eluted in 100 mM glycine pH 2.5, neutralised fractions were assessed for protein content and dialysed against PBS. The purity of the preparation was assessed by silver stained SDS-PAGE and Western blot using 9E10. Reactivity toward 5T4 antigen was assessed by FACS on Calu-1 and A95T4a cells and against 5T4-IgGFc by ELISA.

2.5. Cloning and production of LscFv5T4

The sequence encoding a translation initiation sequence and the human immunoglobulin κ light chain signal peptide was cloned in frame immediately upstream of the scFv sequence in pBSH. This cassette was then cloned into

pHITLXSN, to produce a recombinant retrovirus via a three plasmid expression system [31] and used to stably transduce BHK-21 cells. The LscFv5T4 was purified from cell culture supernatant using the His tag on a 1 ml His Trap column (Pharmacia).

2.6. ELISA analysis

ELISA plates (Falcon) were coated overnight at 4°C and non-specific binding blocked using 2% casein in PBS containing 0.05% Tween 20 (PBST) at 37°C for 1 h. All subsequent steps were 1 h incubations performed at room temperature, plates were washed four times in PBST between each step. Assays were developed using tetramethyl benzidine, and the colour reaction arrested by the addition of 1 M H₂SO₄, absorbance was read at dual wavelength, 450–650 nm. For the Fc capture ELISA, plates were coated with goat α-human IgG, Fc specific, at 10 µg/ml in 100 mM sodium borate pH 8.5 containing 150 mM NaCl and developed with Fc specific goat α-human IgG horseradish peroxidase (HRP) conjugate (Sigma) at 1:15000. For the 5T4–IgGFc ELISA plates were coated with 5T4–IgGFc at 1 µg/ml in 100 mM carbonate buffer pH 9.5. MA5T4, scFv5T4WT19 and LscFv5T4 were assayed in serial dilution, for the single chain antibodies

9E10 α -myc (1:10) was used as a second layer. All antibodies were detected with rabbit α -mouse HRP (Dako, 1:1000).

2.7. FACS analysis

FACS analysis was performed on aliquots of 2×10^5 cells suspended in FACS buffer (PBS, 0.2% bovine serum albumin, 0.1% sodium azide). Antibodies were diluted in this buffer, and all steps were performed on ice. Antibodies were titrated by doubling dilution against A9 5T4a cells, or control A9H12 transfectants. Monoclonal 5T4 was used between 6 pM and 24 nM, and the single chain antibodies between 400 pM and 960 nM. For the single chain antibodies, 9E10 α -myc was used as a second layer (1:10). All antibodies were detected using rabbit α -mouse (F(ab')₂)-FITC (Dako 1:40).

2.8. Biacore kinetic analysis

The 5T4-IgG fusion protein was immobilised by amino-coupling to the surface of a carboxymethyl-dextran (CM5) biosensor chip, at a level of 500 RU. The surface was activated for 7 min with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide/*N*-hydroxysuccinimide coupling reagent at a flow rate of 5 μ l/min followed by 10 μ l of 5T4-IgGFc antigen at 50 μ g/ml in 10 mM acetate buffer pH 4.5. Surplus binding sites were blocked using 1 M ethanolamine for 7 min. Kinetic analysis was performed at a flow rate of 30 μ l/min to minimise mass transport effects. Association was measured over a 3 min injection, and dissociation for 15 min. The antigenic surface was regenerated by a 10 μ l pulse of 20 mM HCl, and a baseline re-established. MAb5T4 was assessed over the concentration range 0.25–6 nM, and scFv5T4WT19 and LscFv5T4 were assessed at 2.5–25 nM.

2.9. Immunohistochemistry

Cryostat sections (5 μ m) of normal human term placenta and gastric carcinoma were fixed in ice cold acetone for 5 min. Sections were rehydrated in PBS and blocked with normal rabbit serum (10%) for 20 min. All subsequent steps were performed at room temperature, incubations were for 1 h. Three PBS washes were performed between each antibody step. Sections were incubated with primary antibodies: mAb5T4 4 μ g/ml; scFv5T4WT19 and LscFv5T4 3 μ g/ml, mouse IgG₁ 4 μ g/ml or PBS controls. Sections stained with the scFvs or PBS control were then incubated with 9E10 α -myc (1:10). All sections were incubated with rabbit α -mouse biotin (Dako 1:400). Endogenous peroxidase was blocked by incubating for 10 min in 0.3% H₂O₂ in ethanol. Sections were then incubated with HRP conjugated avidin-biotin complex (Dako) and developed using diaminobenzidine. The sections were counterstained with Mayer's haematoxylin, dehydrated

and mounted in Pertex. Images were captured using Image Proplus software (IVC) on a Zeiss Axioskop microscope.

3. Results

3.1. Production of 5T4-IgGFc fusion protein

A fusion protein comprising the extracellular domain of human 5T4 and the Fc portion of human IgG₁ was expressed in COS-7 cells as a source of soluble 5T4 antigen. This was used to assess specificity and measure the affinities of single chain antibodies made from the mAb5T4 hybridoma. Production levels were typically 5–7 mg/l from 7 day cultures; purification on WGA-agarose and protein G Sepharose gave a yield of approximately 60%. The predicted size for the mature protein was 92 kDa. Silver stained SDS-PAGE of the purified protein showed three main bands of 95, 180 and > 220 kDa plus a diffuse band of > 70 kDa (Fig. 2). By Western blot the three higher molecular weight bands labelled with both mAb5T4 and goat α -human Fc. The lower molecular weight band labelled only with goat α -human Fc. Following reduction with β -mercaptoethanol 5T4-IgGFc was resolved as a single 95 kDa band with α -Fc and mAb5T4. This suggests that the two highest molecular weight bands represented di- and trimeric forms of 5T4-IgGFc and that the formation of aggregates was disulphide bond dependent. The immunogenicity of the 5T4-IgGFc construct was further confirmed by ELISA (Fig. 5). Plates were coated with the fusion protein, or human IgG as control (not shown), and probed with mAb5T4 at various concentrations, no binding to human IgG was observed.

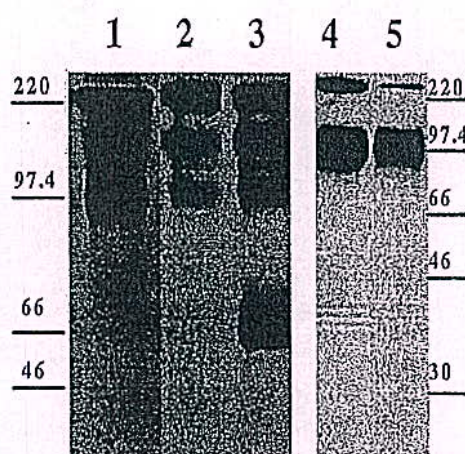


Fig. 2. SDS-PAGE and Western blot analysis of purified 5T4-IgG fusion protein. Purified 5T4-IgG was run on 8% non-reducing (lanes 1–3) and 10% reducing (lanes 4–5) acrylamide gels. Silver stain lane 1, mAb5T4 lanes 2 and 4, goat α -human IgG Fc lanes 3 and 5, positions of molecular weight markers are indicated.

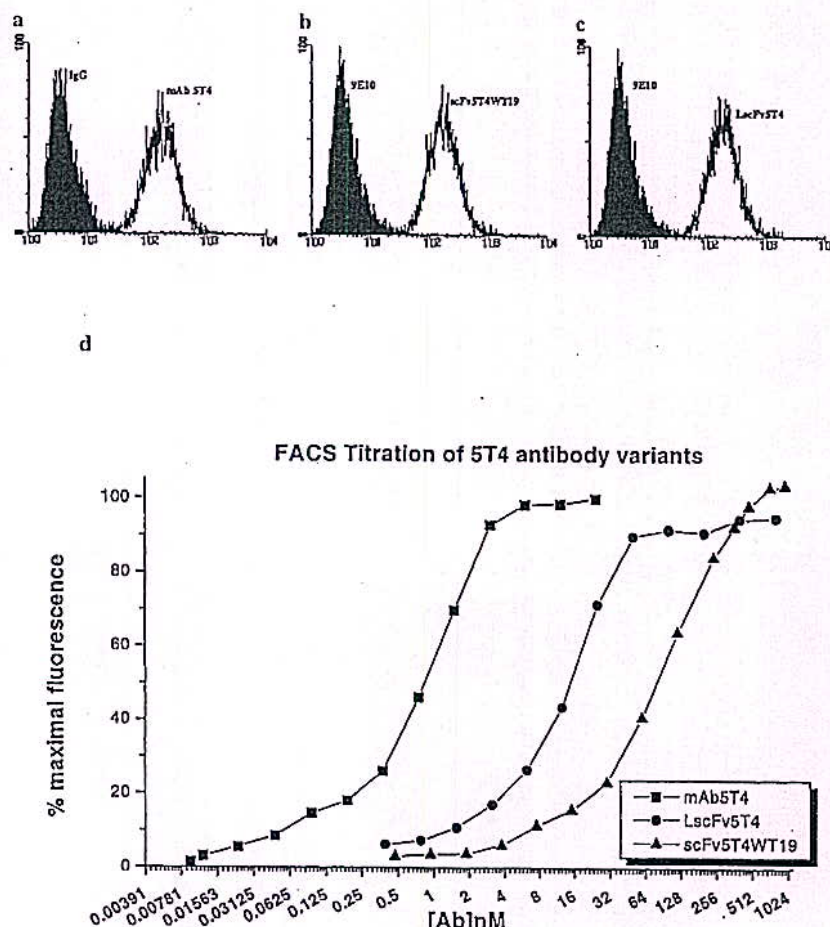


Fig. 3. FACS analysis of mAb and scFv 5T4 antibodies on A95T4a cells. 2×10^5 cells were stained with mAb5T4 or control IgG₁ at 2 μ g/ml (a), scFv5T4WT19 (b), or LscFv5T4 (c) at 16 μ g/ml, titration of 5T4 antibodies against A9 5T4a cells (d), mAb5T4 8 pM–16 nM, scFv5T4WT19 (470 pM–960 nM) and LscFv5T4 (360 pM–800 nM). 9E10 α -myc was used 1:10 as a control, rabbit α -mouse F(ab')₂-FITC was used 1:40.

3.2. Isolation and production of scFv5T4

The single chain antibody scFv5T4WT19 was constructed from the mAb5T4 hybridoma by RT-PCR, expressed as a secreted product from overnight cultures of *E. coli* HB2151, and purified from culture supernatant by 9E10 α -myc tag affinity chromatography. A retroviral expression system was used to express the construct in BHK-21 cells as LscFv5T4, and this purified by nickel affinity chromatography. Production of scFv was 858 μ g of purified protein for scFv5T4WT19, from approximately 8×10^{11} *E. coli*, and 1.25 mg of LscFv5T4 from 3 day cultures of approximately 5×10^9 BHK-21 cells. Purity of the scFvs was assessed by silver stained SDS-PAGE (data not shown). Two bands of equal intensity were detected in the scFv5T4WT19 preparation, one of the predicted size, approximately 27 kDa, and one approximately 60 kDa. Both bands labelled with 9E10 by Western blot, suggesting that the higher M_r band was due to aggregation of the scFv. In the LscFv5T4 preparation, a single band of

approximately 30 kDa was detected both in silver stain and by Western blot.

Reactivity of both versions of the single chain antibody towards the 5T4 antigen was confirmed by immunohistochemistry, ELISA and by FACS. The FACS profiles of A9 5T4a cells stained with mAb5T4, scFv5T4WT19 and LscFv5T4 were similar, having the same distribution and a mean fluorescence of approximately 200 (Fig. 3). Normal mouse IgG₁ and 9E10 α -myc controls were negative, none of the antibodies labelled A9H12 control transfectants (data not shown). Cryostat sections of normal term human placenta (Fig. 4) and gastric carcinoma (Fig. 5) were stained for 5T4 expression using mAb5T4 at 4 μ g/ml, and single chain antibodies scFv5T4WT19 and LscFv5T4 at 3 μ g/ml. In placenta, staining was localised to the syncytiotrophoblast plasma membrane. Normal mouse IgG₁ was negative, whilst 9E10 α -myc showed some background staining. 5T4 staining in the gastric carcinoma specimen showed a cytoplasmic distribution. All three 5T4 specific antibodies showed saturable binding on 5T4IgGFc in

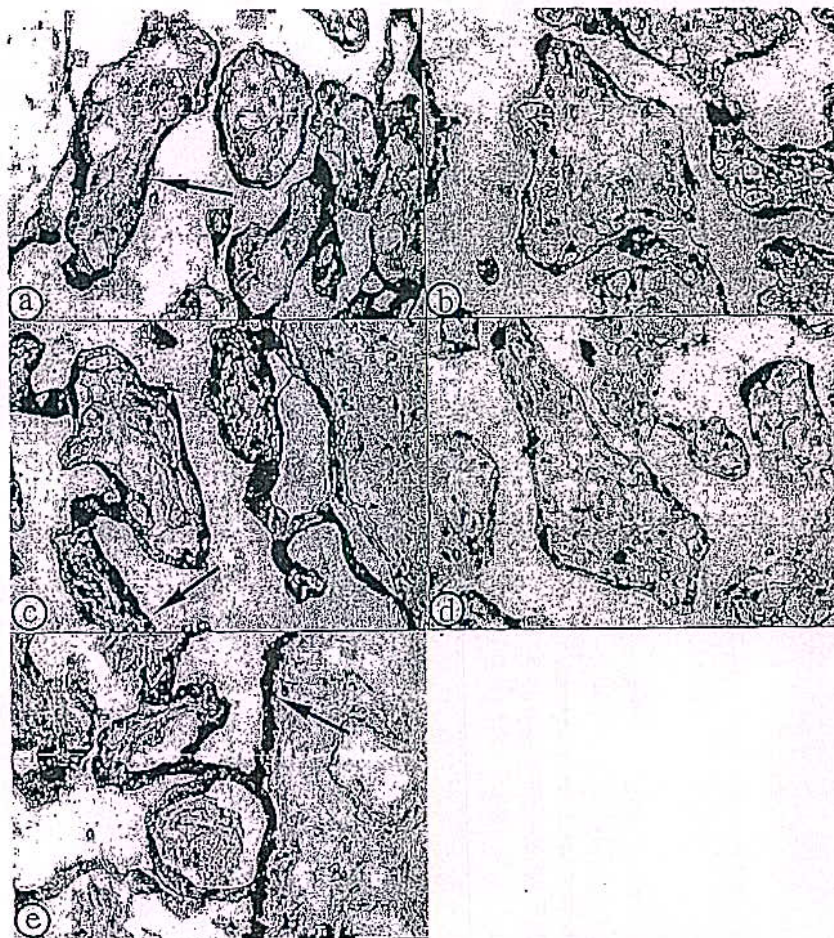


Fig. 4. Immunohistochemistry of normal term human placenta. Cryostat sections of placenta were stained for 5T4 antigen expression using mAb5T4 at 4 µg/ml (b), and scFv5T4WT19 or LscFv5T4 at 3 µg/ml (c and e). 9E10 α-myc 1:10 (d) and normal mouse IgG₁ (a) at 4 µg/ml were used as controls. Staining was localised to the syncytiotrophoblast layer (arrows). Images were captured at 40× magnification.

ELISA, at the highest concentrations used (Fig. 6). No binding was seen with human IgG control, or a mouse 5T4-IgGFc construct (data not shown).

3.3. Affinity measurements of 5T4 antibody variants

Relative affinities of the mAb5T4, scFv5T4WT19 and LscFv5T4 were compared by parallel titration in FACS, ELISA and using a Biacore based assay. MAb5T4 was titrated by doubling dilution against A9 5T4a cells, over the concentration range 6 pM to 24 nM, ScFv5T4WT19 between 470 pM and 960 nM and LscFv5T4 between 400 pM and 800 nM (Fig. 3). The geometric mean fluorescence was calculated and expressed as percentage maximal fluorescence relative to a saturating concentration of mAb5T4. All three antibodies showed saturable labelling of the cells, and titrated out to near zero fluorescence. The position of the curves indicates the relative affinities of the antibodies, decreasing from left to right. The steady state equilibrium affinity constant, K_D , can be estimated from the concen-

tration giving 50% maximal binding. In this cell based assay, scFv5T4WT19 showed an affinity of 64 nM and LscFv5T4 of 14 nM, 82× and 18× lower respectively than mAb5T4 (780 pM).

Titration of the three 5T4 specific antibodies by ELISA on the 5T4-IgGFc fusion protein (Fig. 6) showed the same overall ranking for the affinities of the antibodies. By this method, the affinities were estimated as 46 nM for scFv5T4WT19, 6 nM for LscFv5T4, and 150 pM for mAb5T4.

Affinities for the three antibodies measured in real time,

Table 1
Biacore affinity measurement of mAb5T4 and single chain 5T4 antibodies

5T4 antibody	Association rate k_a ($M^{-1} s^{-1}$)	Dissociation rate K_d (s^{-1})	Affinity K_D (M)
mAb5T4	5.6×10^5	1×10^{-5}	1.8×10^{-11}
scFv5T4WT19	6.64×10^4	1.57×10^{-4}	2.3×10^{-9}
LscFv5T4	9.88×10^4	7.81×10^{-5}	7.9×10^{-10}

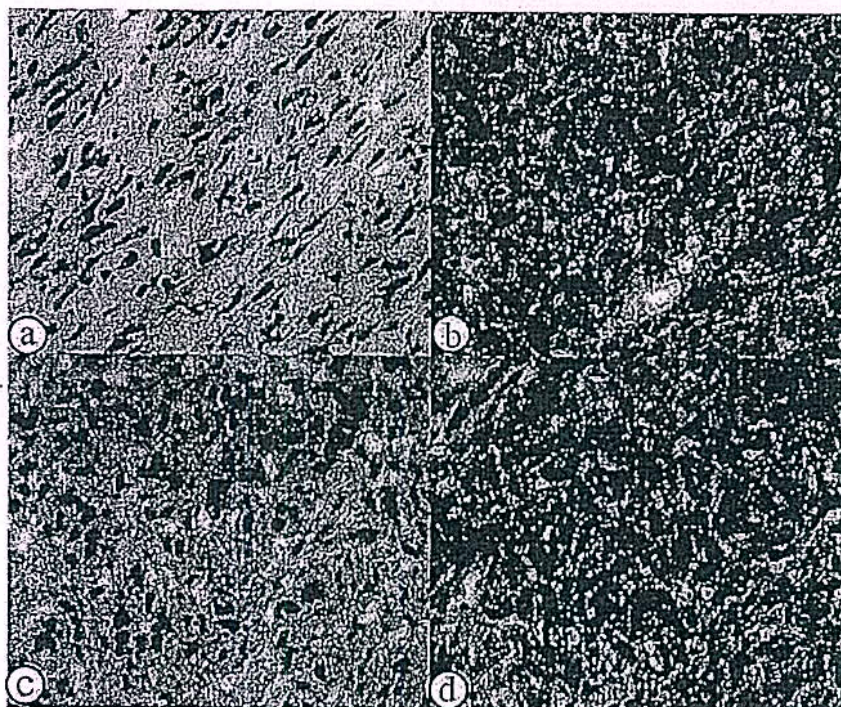


Fig. 5. Immunohistochemistry of gastric carcinoma. Cryostat sections of a gastric carcinoma were stained for ST4 antigen expression using mAb5T4 at 4 µg/ml (b), and scFv5T4WT19 at 3 µg/ml (d). 9E10 α-myc 1:10 (c) and normal mouse IgG₁ at 4 µg/ml (a) were used as controls. Images were captured at 40× magnification.

under concentration limiting conditions using a biosensor based assay with the 5T4–IgGFc fusion protein as antigen. Their affinities showed the same rank order as seen in both ELISA and FACS assays. Association rates for the two single chain antibodies were approximately 10 fold (scFv5T4WT19) and 5 fold (LscFv5T4) lower than that of mAb5T4, at 6.64×10^4 and 9.88×10^4 compared with $5.6 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ (Table 1). Their dissociation rates, however, were approximately 10 fold faster, 1.57×10^{-4} and

7.81×10^{-5} compared to $1 \times 10^{-5} \text{ M}$. Overall monoclonal 5T4 had an affinity of $1.8 \times 10^{-11} \text{ M}$ (18 pM), scFv5T4WT19 $2.3 \times 10^{-9} \text{ M}$ (2.3 nM) and LscFv5T4 $7.9 \times 10^{-10} \text{ M}$ (790 pM), approximately 130 fold and 44 fold lower than mAb5T4.

4. Discussion

The extracellular domain of human 5T4 expressed as a fusion with the Fc portion of human IgG was used to characterise single chain antibodies prepared from the mouse monoclonal antibody mAb5T4. The Fc was fused to the C-terminus of the 5T4 extracellular domain, preserving the conformation of the 5T4 molecule, and therefore its antigenicity (article in preparation).

Expressed in COS-7 cells, the fusion protein was affinity purified utilising properties of both component molecules, WGA lectin and protein G affinity chromatography. The presence of di- and trimeric forms of 5T4–IgGFc at all stages of purification was demonstrated by Western blot using antibodies against both component molecules. The presence of these multimers was disulphide bond dependent, the protein resolving as a single band of the predicted molecular weight following reduction. The formation of 5T4 aggregates has previously been noted in material isolated from placenta, this tendency being accentuated by the Fc portion of the fusion protein.

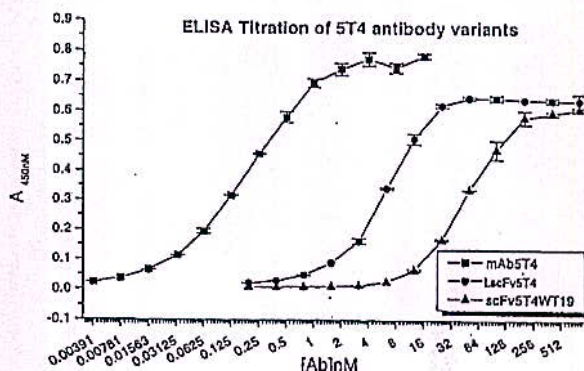


Fig. 6. ELISA titration of 5T4 antibody variants. Monoclonal 5T4, scFv5T4WT19 and LscFv5T4 were titrated by doubling dilution against 5T4–IgGFc. 9E10 (1:10) was used as a second layer for the scFvs and the plate developed with rabbit α-mouse HRP 1:1000. Colour was developed by acidified TMB.

Table 2
Relative affinity measurements of 5T4 specific antibodies by FACS, ELISA and Biacore

<i>K_D</i> (M)	FACS	ELISA	Biacore
mAb5T4	7.8×10^{-10}	1.5×10^{-10}	0.18×10^{-10}
scFv5T4WT19	640×10^{-10}	460×10^{-10}	23×10^{-10}
LscFv5T4	140×10^{-10}	60×10^{-10}	79×10^{-10}

The single chain scFv5T4WT19 was prepared by RT-PCR from the 5T4 hydridoma, and expressed in *E. coli*. It was adapted for secretion from BHK-21 cells by the addition of the human κ light chain signal peptide (LscFv5T4), stable transfectants being produced via a retroviral system.

The expression vector pHen1 directs secretion of the scFv in the *E. coli* amber non-suppressor strain, HB2151. ScFv5T4WT19 was present in the periplasm and secreted into the culture broth from where it was purified. Following affinity purification, production levels for both scFvs were found to be similar, 858 μ g/l and 830 μ g/l for scFv5T4WT19 in bacterial cultures and LscFv5T4 in BHK-21 cells respectively. However, the presence of a high molecular weight aggregate in scFv5T4WT19 suggested that not all of the scFv may be correctly folded and in an active form. Further purification or refolding would be required to recover all of the scFv or to remove the inactive fraction. For this comparison of the two scFv variants no further purification was attempted, therefore this lower effective concentration may account for the differences in the observed affinities between scFv5T4WT19 and LscFv5T4.

The three antibodies described the same distribution of 5T4 antigen expression in placenta, localised to the trophoblast layer. Intensity of staining with LscFv5T4 was greater than that seen with scFv5T4WT19 at the same concentration (3 μ g/ml), suggesting that its effective concentration or affinity was higher. In gastric carcinoma scFv5T4WT19 showed the same distribution as mAb5T4. However, in some tumours the level of background 9E10 staining was considerably greater than that shown here and proved limiting for the use of either scFv in staining tumour specimens with higher levels of myc expression.

Three different methods, FACS, ELISA and surface plasmon resonance, were employed to measure the affinities of mAb5T4 and the two 5T4 specific scFvs (Table 2). The rank order of affinity in all three assays was mAb5T4 > LscFv5T4 > scFv5T4WT19, the extent to which these differed varied between the methods, possibly due to the nature of the antigen used in each. The oligomeric nature of 5T4IgGFc may result in a greater antigen density than is seen at the cell surface, thus offering multiple binding sites. This could stabilise the bivalent binding of mAb5T4, resulting in a higher apparent affinity due to its greater avidity [32]. In the Biacore 3000 assay, the antibodies were diluted to a range $\pm 10\times$ their estimated af-

finities as determined by FACS and ELISA. The observed affinity for mAb5T4 was near the sensitivity limit of the machine, $\sim 10^{-11}$ M. Affinities for the two single chains were lower than that of the monoclonal, as predicted by titration against cells and by ELISA, but still retained nanomolar or better affinities. Similar reductions in affinity have been reported for other single chain antibodies derived from monoclonal antibodies. Laroche et al. reported a 20 fold drop in affinity for the anti-fibrin monoclonal MA-15C5 [33] while Benedict et al. showed a similar reduction for the anti-CD34 monoclonal My10 [34]. These reductions in affinity are in part due to removal of structural regions of the heavy and light chains and the change to monovalent binding of antigen [9,32]. The affinity of scFv5T4WT19 was greatly reduced compared to that of mAb5T4 at $128\times$ lower, but can be explained by the presence of aggregates, whereas the affinity of LscFv5T4 was only reduced 44 fold.

Much tumour imaging and therapy has been performed with monoclonal antibody conjugates, their inherent specificity making them ideal targeting molecules. However, due to their size and immunogenicity whole antibodies have significant limitations [8,35]. Single chain antibodies offer better platforms for the targeted therapy of tumours, as their pharmacokinetic properties are superior to other antibody constructs. They show a faster clearance from the circulation resulting in lower normal tissue uptake, in particular the kidneys, greater tumour penetration which results in an overall greater labelling index and lower toxicity towards the patient [8,9,36,37]. In addition to radioconjugates, antibodies may be used to activate the host immune response toward the tumour. Antibodies have been used to stimulate NK cell mediated cytotoxicity and complement activation directed against tumour cells in vitro and in vivo [38]. However, the degree of host immune activation achieved by naked antibodies is limited [39]. Fusions of antibody fragments with immune effector molecules offer the potential of both reduced toxicity and higher penetration and increased immune activation at the site of the tumour. This immune activation may be antigen driven, using fusion with co-stimulatory molecules that increase the proportion of T cells activated by contact with a tumour, such as B7.2 (CD86) [40]. Antigen independent immune activation can be achieved using antibody-superantigen fusions. Superantigens non-specifically activate T cells with a very high efficiency by cross-linking MHC-class II on antigen presenting cells and a subset of T cells bearing the $v\beta 3$ TCR [41]. Such fusion constructs, including one based on a 5T4 Fab, have been used successfully to induce T cell mediated tumour therapy in experimental models [42,43] and have been taken forward to clinical trial [44,45]. Tumour necrosis factor- α , which is active against a number of tumours, and interleukin-2 have both been used successfully as antibody fusions in animal models [46,47]. In both cases, the treatment was highly selective for the tumours, and toxicity was signifi-

cantly lower than seen with systemic administration of the cytokines. However, the greatest success has been with tumours of small size, indicating that antibody therapy, including scFvs, is likely to have greatest utility in the treatment of minimal residual disease [48].

The use of recombinant vectors to deliver therapeutic genes, including scFv fusion proteins, to the site of a tumour offers an increased level of selectivity over the systemic and often toxic administration of antibodies. Additionally, placing the gene encoding the scFv fusion protein under control of a tumour specific transcriptional control element may further decrease toxicity by limiting expression to the tumour site. Furthermore, expression of the therapeutic product should be terminated when the tumour has been destroyed.

Acknowledgements

This work was funded by the Cancer Research Campaign and Oxford Biomedica.

References

- [1] G. Riethmüller, E. Scneider-Gaediche, J.P. Johnson, *Curr. Opin. Immunol.* 5 (1993) 732–739.
- [2] A.A. Epenetos, D. Snook, H. Durbin, *Cancer Res.* 46 (1986) 3183–3191.
- [3] D.L. Shawler, M.R. Bartholemew, L.S. Smith, R.O. Dilman, *J. Immunol.* 135 (1985) 1530–1535.
- [4] N.S. Courtenay-Luck, A.A. Epenetos, R. Moore, M. Larche, *Cancer Res.* 46 (1986) 6489–6493.
- [5] S.J. Kennel, R. Falcioni, J.W. Wesley, *Cancer Res.* 51 (1991) 1529–1536.
- [6] D.M. Goldenberg, H. Goldenberg, R.M. Sharkey, F.E. Higginbotham, R.E. Lee, L.C. Swayne, K.A. Burger, D. Tsai, J.A. Horowitz, T.C. Hall, C.M. Pinsky, H.J. Hansen, *Cancer Res.* 50 (1990) 909S.
- [7] T. Yokota, D.E. Milenic, M. Whitlow, J. Schlom, *Cancer Res.* 52 (1992) 3402–3408.
- [8] D. Colcher, R. Bird, M. Roselli, K.D. Hardman, S. Johnson, S. Pope, S.W. Dodd, M.W. Pantoliano, D.E. Milenic, J. Schlom, *J. Natl. Cancer Inst.* 82 (1990) 1191–1197.
- [9] D.E. Milenic, T. Yokota, D.R. Filpula, M.A. Finkelman, S.W. Dodd, J.F. Wood, M. Whitlow, P. Snoy, J. Schlom, *Cancer Res.* 51 (1991) 6363–6371.
- [10] N. Hole, P.L. Stern, *Br. J. Cancer* 57 (1988) 239–246.
- [11] N. Hole, P.L. Stern, *Int. J. Cancer* 45 (1990) 179–184.
- [12] P.J. Southall, G.M. Boxer, K.D. Bagshawe, N. Hole, M. Bromley, P.L. Stern, *Br. J. Cancer* 61 (1990) 89–95.
- [13] T. Starzynska, V. Rahi, P.L. Stern, *Br. J. Cancer* 66 (1992) 867–869.
- [14] E. Wrigley, A.T. McGowan, R. Swindell, D. Crowther, T. Starzynska, P.L. Stern, *Int. J. Gynecol. Cancer* 5 (1995) 269–274.
- [15] T. Starzynska, P.L. Marsh, P.F. Schofield, S.A. Roberts, K.A. Myers, P.L. Stern, *Br. J. Cancer* 69 (1994) 899–902.
- [16] K.A. Myers, V. Rahi-Saund, M.D. Davidson, J.A. Young, A.J. Cheater, P.L. Stern, *J. Biol. Chem.* 269 (1994) 9319–9323.
- [17] K.W. King, F.C. Sheppard, C.W. Westwater, P.L. Stern, K.A. Myers, *Biochim. Biophys. Acta* 1445 (1999) 257–270.
- [18] R. Reinke, D.E. Krantz, D. Yen, L. Zipursky, *Cell* 52 (1988) 291–301.
- [19] H. Campbell, T. Schimansky, C. Claudianos, N. Ozsarac, A. Kasprzak, J. Costell, I. Young, H.D. Coust, G. Miklos, *Proc. Natl. Acad. Sci. USA* 90 (1993) 11386–11390.
- [20] R. Medzhitov, P. Preston-Hurlburt, C.A. Janeway Jr., *Nature* 8 (1997) 394–397.
- [21] A. Gould, R. White, *Development* 116 (1992) 1163–1174.
- [22] C. Chiang, P. Beachy, *Mech. Dev.* 47 (1994) 225–239.
- [23] E. Eldon, S. Kooyer, D. D'Evelyn, M. Duncan, P. Larsvinger, J. Bostas, M. Bellen, *Development* 120 (1994) 885–899.
- [24] B. Kobe, J. Deisenhofer, *Trends Biochem. Sci.* 19 (1994) 415–421.
- [25] C.J. Carsberg, K.A. Myers, P.L. Stern, *Int. J. Cancer* 68 (1996) 84–92.
- [26] C.J. Carsberg, K.A. Myers, G.S. Evans, T.D. Allen, P.L. Stern, *J. Cell Sci.* 108 (1995) 2905–2916.
- [27] G.I. Evan, G.K. Lewis, G. Ramsey, J.M. Bishop, *Mol. Cell. Biol.* 5 (1985) 3610–3616.
- [28] J.J. Sedmah, S.E. Grossberg, *Anal. Biochem.* 79 (1979) 544–552.
- [29] U.K. Laemmli, *Nature* 227 (1970) 680–685.
- [30] H. Towbin, J. Gordon, *J. Immunol. Methods* 72 (1984) 313–340.
- [31] Y. Soncoka, P.M. Cannon, E.E. Ramsdale, J.C. Griffiths, G. Romano, S.M. Kingsman, A.J. Kingsman, *Nucleic Acids Res.* 23 (1995) 628–633.
- [32] D.M. Crothers, H. Metzger, *Immunochemistry* 9 (1972) 341–357.
- [33] Y. Laroche, M. Demayer, J.M. Strassen, Y. Gausemans, E. Demarsin, G. Matthyssens, D. Collen, P. Holvoet, *J. Biol. Chem.* 266 (1991) 16343–16349.
- [34] C.A. Benedict, A.J. Mackrell, W.F. Anderson, *J. Immunol. Methods* 201 (1997) 223–231.
- [35] J.A. Ledermann, R.H. Begent, K.D. Bagshawe, S.J. Riggs, F. Searle, M.G. Glaser, A.J. Green, R.G. Dale, *Br. J. Cancer* 58 (1988) 654–657.
- [36] D. Colcher, G. Pavlinkova, G. Beresford, B.J.M. Booth, A. Choudhury, S.K. Batra, Q. J. Nucl. Med. 42 (1998) 225–241.
- [37] A. Mayer, E. Tsiompanou, D. O'Malley, G.M. Boxer, *Clin. Cancer Res.* 6 (2000) 1711–1719.
- [38] B. Wang, Y.B. Chen, O. Ayalon, J. Bender, A. Garen, *Proc. Natl. Acad. Sci. USA* 96 (1999) 1627–1632.
- [39] G. Riethmüller, J.P. Johnson, *Curr. Opin. Immunol.* 4 (1992) 647–655.
- [40] B. Gerstmayer, M. Hoffmann, *Cancer Immunol. Immunother.* 45 (1997) 156–158.
- [41] T. Kalland, G. Hedlund, M. Dohlsten, P. Lando, *Curr. Top. Microbiol. Immunol.* 174 (1991) 81–92.
- [42] M. Dohlsten, L. Abrahmsén, P. Björk, P. Lando, G. Hedlund, G. Forsberg, T. Brodin, N. Gascoigne, C. Förberg, P. Lind, T. Kalland, *Proc. Natl. Acad. Sci. USA* 91 (1994) 8945–8949.
- [43] G. Forsberg, L. Ohlsson, T. Brodin, P. Björk, P.A. Lando, D.M. Shaw, P.L. Stern, M. Dohlsten, *Brit. J. Cancer* (2000) (in press).
- [44] B.J. Giantonio, R.K. Alpaugh, J. Schultz, C. McAleer, D.W. Newton, B. Shannon, Y. Guede, M. Kotb, L. Vitek, R. Persson, P. Gunnarsson, T. Kalland, M. Dohlsten, B. Persson, L.M. Weiner, *J. Clin. Oncol.* 15 (1997) 1994–2007.
- [45] R.K. Alpaugh, J. Schultz, C. McAleer, B.J. Giantonio, R. Persson, M. Burnite, S. Nielsen, L. Vitek, B. Persson, L.M. Weiner, *Clin. Cancer Res.* 4 (1998) 1903–1914.
- [46] C. Melani, M. Figini, *Cancer Res.* 58 (1998) 4146–4154.
- [47] U. Scherf, I. Benhar, K.O. Webber, I. Pastan, U. Brinkmann, *Clin. Cancer Res.* 2 (1996) 1523–1531.
- [48] J.Y. Douillard, J.F. Chatal, *Bull. Cancer* 85 (1998) 951–959.

Glycosylation and epitope mapping of the 5T4 glycoprotein oncofoetal antigen

David M. SHAW*, Andrew M. WOODS*, Kevin A. MYERS*¹, Caroline WESTWATER*, Veena RAHI-SAUND*, Michael J. DAVIES†², David V. RENOUEF†, Elizabeth F. HOUNSELL† and Peter L. STERN*³

*CRC Immunology Group, Paterson Institute for Cancer Research, Christie Hospital NHS Trust, Wilmslow Road, Manchester M20 4BX, U.K., and

†School of Biological and Chemical Sciences, Birkbeck College, University of London, Gordon House, 29 Gordon Street, London WC1H 0PP, U.K.

The human 5T4 oncofoetal antigen is a focus for development of several antibody-directed therapies on the basis of the murine monoclonal antibody against 5T4 (mAb5T4), which recognizes a conformational epitope. 5T4 molecules are highly N-glycosylated transmembrane glycoproteins whose extracellular domain contains two regions of leucine-rich repeats (LRRs) and associated flanking regions, separated by an intervening hydrophilic sequence. Using a series of deletion and mutated cDNA constructs as well as chimaeras with the murine homologue, we have mapped the mAb5T4 epitope to the more membrane-proximal LRR2 or its flanking region. Analysis of the glycosylation of the seven consensus Asp-Xaa-Ser/Thr sites was consistent with all of the sites being glycosylated. A combination of two high-mannose chains (predominantly octasaccharide) and five mostly sialylated bi-, tri- and tetra-antennary complex chains with minor quantities

of core fucose were detected. The two glycosylation sites, which are the most likely to have predominantly high-mannose chains, are in the only two regions that show significant differences between the human and the 81% identical mouse sequence. A site-directed mutation, which abolished glycosylation at one of these sites (position 192), did not alter antigenicity. The other, which is nearest to the N-terminus in the human, has an Asn-Leu-Thr to Asn-Leu-Leu conversion in the mouse, so cannot be glycosylated in the latter species. The large complex glycosylation at the other sites is likely to influence the antigenicity and tertiary structure generating the 5T4 epitope.

Key words: leucine-rich repeat protein, metastasis, oncotrophoblast antigen, oligosaccharide, tumour-associated antigen.

INTRODUCTION

The expression of specific trophoblast cell-surface molecules is likely to contribute to the controlled invasion of maternal tissue and/or avoid immune recognition [1]. Such functional molecules, where expressed in malignant tissues, may allow the tumours to invade surrounding normal tissue whilst avoiding host immunity. Such similarities in the properties of embryonic cells, like trophoblast and tumour cells, have stimulated the search for common functional oncofoetal antigens.

The human 5T4 oncofoetal antigen is a 72 kDa leucine-rich repeat (LRR) glycoprotein that is strongly expressed by trophoblasts throughout gestation [2–4]. This antigen is only weakly expressed in adult tissues, but strongly expressed in a number of human carcinomas [3,5–10], and its expression is prognostic in gastric, colorectal and ovarian tumours [5–7,9]. Expression of 5T4 molecules in fibroblast and epithelial cell lines localizes to microvillous projections, and overexpression of the cDNA encoding the human gene results in increased cell motility and altered morphology [11,12], consistent with a role in tumour and trophoblast invasion.

LRR proteins are a diverse family of approx. 60 members and, although no common function has been ascribed, many are involved in protein–protein interactions [13–15]. The tertiary structures of several LRR proteins have now been solved by X-ray crystallography [15–18]. Despite low levels of sequence

identity between the different proteins, a remarkable degree of structural similarity is observed [15]. The basic LRR structure is of repeating units of β -strand and anti-parallel α -helix, and this structure has been used to model other family members [16,19,20]. However, due to the presence of other functional domains and differences in the lengths of the LRRs, the precise structure varies. The short 20–22-amino-acid repeat length of the YopM protein of *Yersinia pestis* results in a conformation where most of the repeats have no α -helical component and the β -strand is less twisted than in porcine ribonuclease inhibitor, which has the longest repeat length of 29 amino acids. 5T4, having a repeat length of 24 amino acids, might be expected to show an intermediate conformation between these two extremes [4,14,17].

5T4 antigen is a highly N-glycosylated protein [21], with seven putative consensus N-linked glycosylation sites in the extracellular domain [4]. Therefore the diversity of the oligosaccharide structures may influence functional aspects both by protecting the antigen from proteolytic attack or promoting interactions with other molecules important to the invasive process.

The murine monoclonal antibody against 5T4 (mAb5T4), which defines the human protein, recognizes an epitope that is dependent upon both glycosylation of the molecule and intramolecular disulphide interactions [21], consistent with it being a conformational epitope. Comparison of the human and mouse 5T4 sequences [22] shows that the structure is highly conserved between the two species. The cytoplasmic and transmembrane

Abbreviations used: 2-AB, 2-aminobenzamide; ECL* (Amersham Biosciences), enhanced chemiluminescence; GU, glucose units; HPAEC–PAD, high-pH anion-exchange chromatography–pulsed amperometric detection; LRR, leucine-rich repeat; mAb5T4, murine monoclonal antibody against 5T4; PNGase F, peptide N-glycosidase F; RT-PCR, reverse transcriptase-PCR; SiMPM, syncytiotrophoblast microvillous membrane; SHRP, streptavidin–horseradish peroxidase; WGA, wheatgerm agglutinin.

¹ Present address: Oxford Biomedica, Medware Centre, The Oxford Science Park, Oxford OX4 4GA, U.K.

² Present address: Lonza Biologics, 228 Bath Road, Slough SL1 4DY, U.K.

³ To whom correspondence should be addressed (e-mail pstern@picr.man.ac.uk).

domains are identical and the molecules as a whole share 81% identity at the amino acid level. Six of the seven predicted N-linked glycosylation sites of the human molecule are conserved in the mouse; the most N-terminal glycosylation site is absent, but there is another in the C-terminal flanking region [22]. The murine protein contains an additional six amino acids adjacent to the glycosylation site in the hydrophilic domain, which is a direct repeat of the preceding six amino acids. Despite this high level of identity between 5T4 molecules of the two species, mAb5T4 does not recognize the murine molecule, suggesting that these small differences significantly influence the antigenicity of the molecule.

In the present study, we report the characterization of the oligosaccharide structures present on the human 5T4 antigen by a combination of chromatographic methods involving sensitive analysis of fluorescent derivatives. Furthermore, we have investigated the location of the antigenic epitope within the extracellular domain of human 5T4 by using a series of variant cDNA constructs.

MATERIALS AND METHODS

5T4 purification

Human 5T4 was purified from syncytiotrophoblast microvillous membranes (StMPM) by wheatgerm agglutinin (WGA) affinity chromatography, eluting with 0.3 M *N*-acetylglucosamine, and was purified further by mAb5T4 immunoaffinity chromatography, eluting in 8 M urea, as described by Hole and Stern [21]. Purity of the 5T4 antigen was assessed by SDS/PAGE [23]. 5T4-containing fractions were pooled and desalted using Centrifuplus 30 centrifugal concentrators (Millipore, Watford, Herts., U.K.) and proteins were quantified by a modified Bradford assay [24].

Concentrated desalted material was freeze-dried prior to further analysis.

Cloning of variant 5T4 constructs

Variant 5T4 cDNA constructs (Figure 1) were generated and expressed in various eukaryotic cell lines using the vector pCMV α neo. Truncated 5T4 variants L1, L1H and TM⁺ were prepared by PCR using sequence-specific primers encompassing the LRR1 (amino acids 1–184), LRR1 plus the hydrophilic domain (amino acids 1–200) and the entire extracellular domain (amino acids 1–335) respectively. A 5T4 variant with a truncated cytoplasmic domain CYT⁺ was made by digestion with *Nsi*I [12]. Site-directed mutations, with deletion of the consensus N-linked glycosylation site (Asn¹⁹²) and tyrosine phosphorylation site (Tyr⁴⁰¹), were made by PCR using primers introducing substitutions of phenylalanine for Asn¹⁹² and Tyr⁴⁰¹ respectively. A chimaeric 5T4/CD44 molecule was made by fusing the extracellular domain of 5T4 to the transmembrane and cytoplasmic domains of human CD44 (human CD44 cDNA provided by Professor Clare Isacke, Chester Beatty Labs, The Institute of Cancer Research, London, U.K.). The human/mouse chimaeric 5T4 molecule (H/M 5T4) was constructed by PCR, utilizing a nucleotide sequence common to both molecules encoding amino acids 166–172 (bp 499–517) as the splice site. The mouse/human chimaera (M/H 5T4) was made by digestion of both cDNAs with *Bsm*BI at nucleotide 511 (corresponding to amino acid 170) and ligating the 5' fragment of the mouse cDNA with the human 3' region.

Expression of variant 5T4 molecules

Variant 5T4 cDNA constructs were expressed variously in A9 mouse fibroblasts, CL-S1 mouse mammary epithelial cells,

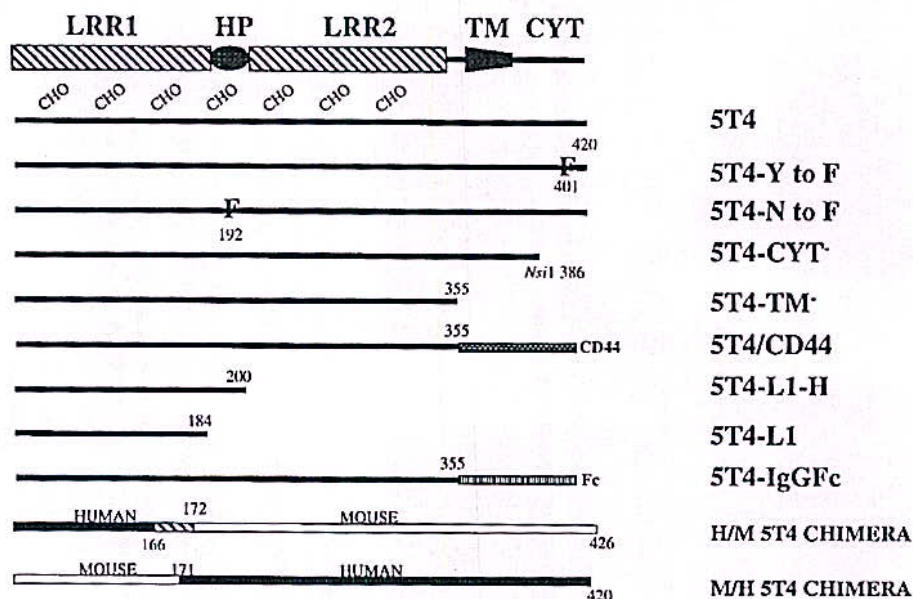


Figure 1 Construction of 5T4 variant cDNA molecules

Truncation, deletion and chimaeric 5T4 molecules were constructed by PCR using specific primers or by restriction digest. The position of carbohydrate (CHO) and hydrophilic (HP), transmembrane (TM) and cytosolic (CYT) domains are indicated. The size of each construct (in amino acids) is given. The location of the substitution of Tyr⁴⁰¹ (5T4-Y to F) and Asn¹⁹² (5T4-N to F) to phenylalanine is indicated (F).

Madin–Derby canine kidney, Chinese-hamster ovary and Cos-7 cells. Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) foetal-bovine serum and 2 mM L-glutamine in a humidified atmosphere of 5% CO₂ and 95% air at 37 °C. Transfected cells were maintained under antibiotic selection by the addition of 1 mg/ml G418. Cell lysates were prepared at 2×10^6 cells/ml in Tris-buffered saline [50 mM Tris/HCl (pH 8) and 150 mM NaCl] containing 0.5% Nonidet P40 and 0.2 μ M PMSF. Cell-culture supernatants for Western- and dot-blot analysis were collected from cultures incubated for 48 h in serum-free Dulbecco's modified Eagle's medium supplemented with L-glutamine and non-essential amino acids. The supernatant was concentrated up to 20 times using centrifugal concentrators. A secreted human 5T4–IgGFc fusion protein was expressed in Cos-7 cells and purified on WGA–agarose and Protein G (Amersham Biosciences, Little Chalfont, Bucks., U.K.) as described previously [25]. The Fc was removed by digestion with Factor Xa protease (Roche Diagnostics, Lewes, East Sussex, U.K.) and Protein G–Sepharose. Disulphide bonds in full-length 5T4, derived from transfected A9 cells, were reduced using 20 mM 2-mercaptoethanol and alkylated with 40 mM iodoacetamide.

Rat polyclonal sera

Rat anti-mouse 5T4 and -human 5T4 polyclonal sera were developed in Lou rats infected with vaccinia encoding the human or mouse 5T4 cDNA (kindly provided by Dr Miles Carroll, Oxford Biomedica, Oxford Science Park, Oxford, U.K.).

FACS analysis

FACS analysis was performed on 2×10^5 cells suspended in FACS buffer (PBS containing 0.2% BSA and 0.1% sodium azide). Antibodies were diluted in this buffer, which was used for subsequent washes; all steps were performed on ice. Cells were post-fixed in 1% paraformaldehyde before analysis. MAb5T4 was used at 1 μ g/ml and detected using FITC-conjugated rabbit F(ab')₂ anti-mouse immunoglobulins (1:40; Dako, Denmark House, Ely, Cambs., U.K.). Rat polyclonal anti-mouse 5T4 or -human 5T4 were used at 1:300 dilutions and detected with FITC-conjugated rabbit F(ab')₂ anti-rat immunoglobulins (1:40 dilution; Dako).

Reverse transcriptase (RT)-PCR

Total RNA was extracted from cells using RNazol B according to the manufacturer's instructions (Biogenesis, Poole, Dorset, U.K.). Removal of genomic DNA was ensured by re-extraction of the RNA with RNazol B. cDNA was prepared from 5 μ g of the RNA using AMV RT (Promega, Chilworth, Southampton, U.K.) in a total reaction volume of 100 μ l. For constructs, the presence of 5T4 mRNA in the cells was detected by PCR on 5 μ l of the cDNA using primers in the 5T4 LRR1 region, yielding a 338 bp 5T4-specific band.

SDS/PAGE, Western and dot blotting

SDS/PAGE was performed by the method of Laemmli [23] using a mini-gel system. Separated proteins were transferred on to nitrocellulose as described by Towbin and Gordon [26] using a semi-dry transfer system. Membranes were blocked using PBS containing 2% (w/v) non-fat dried milk for at least 1 h before developing, and probed for 1 h with each antibody. MAb5T4 (1 μ g/ml) was detected using biotinylated rabbit anti-mouse

immunoglobulins (1:3000 dilution; Dako) followed by strept-avidin–horseradish peroxidase (SHRP; 1:6000 dilution; Dako). Membranes were developed by using enhanced chemiluminescence (ECL[®]; Amersham Biosciences) according to the manufacturer's instructions.

Samples were applied to nitrocellulose by vacuum dot blotting and developed as for Western blots.

Monosaccharide analysis

Neutral and amino monosaccharides were released by hydrolysis with 100 μ l of 2 M HCl for 2 h at 100 °C, and sialic acids were released from the protein with 100 μ l of 0.1 M HCl for 1 h at 70 °C. The samples were dried and washed three times with 100 μ l of water prior to analysis by high-pH anion-exchange chromatography–pulsed amperometric detection (HPAEC–PAD) [27]. HPAEC–PAD of neutral and amino monosaccharides was carried out using a CarboPac PA1 column (4 mm $\times$ 250 mm; Dionex, Camberley, Surrey, U.K.) fitted with amino trap [28] and borate trap guard columns [29]. Sialic acids were analysed with a CarboPac PA1 guard column and a borate trap guard column. Neutral and amino monosaccharides were eluted in 2% 50 mM NaOH/1.5 mM sodium acetate and 98% H₂O for 30 min, followed by regeneration in 100 mM NaOH for 10 min; 300 mM NaOH was added as a post-column reagent to increase sensitivity and baseline stability. Detection was with a PED-2 detector (Dionex). Potentials for the gold electrode were as follows: $E_1 = 0.1$ V; $E_2 = 0.7$ V; and $E_3 = -0.3$ V. Sialic acids were eluted in a gradient from 100 mM NaOH to 300 mM sodium acetate/100 mM NaOH in 30 min, followed by regeneration in 100 mM NaOH for 10 min. All neutral and amino monosaccharides samples were analysed with 2-deoxyglucose added as a reference peak after hydrolysis and quantified against hydrolysed monosaccharide mixtures.

Oligosaccharide release

Portions of affinity-purified 5T4 were desalted further and concentrated on Microcon 10 centrifugal concentrators (Millipore). 5T4 glycoprotein was denatured by boiling for 2 min in 200 μ l of a solution containing 1% (w/v) SDS, 0.5% 2-mercaptoethanol, 20 mM Na₂HPO₄, 50 mM EDTA and 0.02% sodium azide (pH 7.5). To this, 2 units of peptide N-glycosidase F (PNGase F; Oxford GlycoSciences, Abingdon, Oxon., U.K.) and 10 μ l of 10% (v/v) Nonidet P40 were added prior to incubation for 72 h at 37 °C. The digested glycoproteins were centrifuged briefly at 13000 g and transferred to Microcon 10 centrifuge concentrators, pre-spun with 500 μ l of water to remove glycerol. The samples were centrifuged at 13000 g for 20 min and the retentate was washed with a further three times with 100 μ l of water. The retained material was freeze-dried and assayed for monosaccharides by HPAEC–PAD as described above. The filtrate and washes were pooled, concentrated and purified on a Biogel P2 column (2 ml bed volume; Bio-Rad, Hemel Hempstead, Herts., U.K.). Glycans were eluted in the void volume.

Fluorescence labelling of oligosaccharides

Oligosaccharides released from 500 μ g–2 mg of 5T4 were labelled with 2-aminobenzamide (2-AB) (Oxford GlycoSciences) as described by Bigge et al. [30]. Briefly, the oligosaccharides were suspended in 5 μ l of a solution containing 0.35 M 2-AB, 0.1 M sodium cyanoborohydride and 30% (v/v) acetic acid in DMSO, and incubated for 2 h at 60 °C. Labelled oligosaccharides were purified on a cellulose membrane (supplied with labelling kit) by eluting with decreasing proportions of acetonitrile, and the

samples were freeze-dried. Samples for GlycoSep C HPLC were then analysed directly. Following labelling and Biogel P4 (Bio-Rad) chromatography, neutral oligosaccharides, either naturally occurring or produced by mild acid hydrolysis to remove sialic acid, were then desalted further on a 1 ml mixed bed anion-exchange column of AG1 (triethylamine form) and AG50 in water.

GlycoSep C HPLC

Fluorescently labelled oligosaccharides were fractionated on the basis of charge by chromatography on a GlycoSep C HPLC column (4.6 mm × 100 mm; Oxford GlycoSciences) [31]. Oligosaccharides were eluted with a gradient of 20% acetonitrile/80% water to 20% acetonitrile/30% water/50% 0.3 M ammonium acetate (pH 4.5) over 40 min at a flow rate of 0.4 ml/min, and detected by fluorescence at 330 nm and 420 nm for excitation and emission respectively.

Biogel P4 chromatography

Analytical Biogel P4 chromatography was performed using a RAAM 2000 GlycoSequencer (Oxford GlycoSciences) at a temperature of 55 °C in water at a flow rate of 30 µl/min for 11 ml, ramped to 160 µl/min over 27 ml and held for 7 ml. Detection was by fluorescence at 330 nm and 420 nm for excitation and emission respectively. Data were analysed by the Glyco-link software supplied. Preparative Biogel P4 chromatography was carried out on the same instrument using a constant flow program (45 µl/min). Assignment of glucose units (GU) was made by comparison with a dextran hydrolysate run either concurrently (analytical runs) or separately (preparative runs) and detected by refractive-index detection. Peak assignments were achieved using comparison with the New-Bio2 database of the Eye software supplied with the RAAM 2000 instrument and relating these to the results of the monosaccharide analysis and GlycoSep C.

Glycopeptide mapping

5T4 was desalted as described above and then reduced and carboxymethylated. Trypsin digestion was performed for 5 h at 37 °C in 50 mM ammonium bicarbonate buffer (pH 8) using a 1:100 (w/w) 5T4/enzyme ratio. After freeze-drying, the digest was dissolved in 20 µl of buffer A [0.1% aq. trifluoroacetic acid]. Peptides were separated by HPLC on a graphitized carbon column (HyperCarb, 4.6 mm × 100 mm; Thermo Hypersil, Runcorn, Cheshire, U.K.) using a gradient of 2% buffer B (0.1% trifluoroacetic acid in acetonitrile) in buffer A for 2 min, followed by a linear gradient to 82% buffer B over 80 min at 1 ml/min. The order of elution of the peptides was predicted from their hydrophobicity index [32]. Fractions (1 ml) were collected over 80 min and a sample was added to the wells of a microtitre plate. This was followed by 25 µl of 4% aq. phenol and, after 5 min, 200 µl of concentrated sulphuric acid was added. The absorbance was read at 490 nm and compared with a hexose standard curve. Oligosaccharides from selected fractions were released with 0.01 unit of PNGase F in 40 mM potassium phosphate buffer (pH 7.4) containing 10 mM EDTA for 48 h at 37 °C. The oligosaccharides were desalted, desialylated and fluorescently labelled as described above, prior to analysis by Biogel P4 chromatography.

RESULTS

Epitope mapping

To investigate the contribution of different regions of the 5T4 molecule in the presentation of the epitope for mAb5T4, a number of deletion and mutation variant molecules were constructed (see Figure 1). These were cloned into the vector pCMV α neo and expression was assessed in various stable and transiently transfected mammalian cell lines by RT-PCR, SDS/PAGE and Western blotting, dot blotting and FACS analysis, as summarized in Table 1. All constructs were tested for expression of the mAb5T4 epitope in at least two cell lines.

Deglycosylation of 5T4 leaves a 42 kDa core protein [21], with removal of the carbohydrate by either chemical or enzymic means leading to a loss of mAb5T4 reactivity, but the core protein is still recognized by polyclonal sera. Additionally, disruption of the intramolecular disulphide bonds leads to a loss of mAb5T4 reactivity (Figure 2); these data are consistent with the 5T4 epitope being conformational in nature.

Table 1 Expression of the monoclonal 5T4 epitope in variant 5T4 molecules

The presence (+) of the mAb5T4 epitope was determined at the cell surface by FACS, and in cell lysates or culture supernatants by Western and dot blotting. The presence (+) of mRNA was confirmed by RT-PCR. nt, not tested; —, absence.

5T4 construct	RT-PCR	FACS (cell surface)	Western blot (kDa)		
			Cell lysates	Supernatant	Dot blot
Full-length	+	+	72	72	+
5T4-Cyt ⁺	+	+	67	67	+
5T4-Y to F	+	+	72	nt	+
5T4-N to F	+	+	69	69	+
5T4/CD44	+	+	80	nt	nt
5T4-TM ⁺	+	—	nt	64	—
5T4-IgGFc	+	—	nt	92	+
5T4-L1	+	—	—	—	—
5T4-L1-H	+	—	—	—	—
H/M 5T4 chimaera	+	—*	—*	nt	—
M/H 5T4 chimaera	+	+	75	nt	nt

* Human/mouse chimaera positive with polyclonal sera against both species.

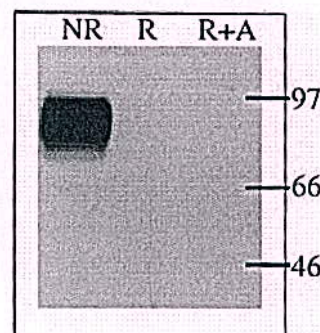


Figure 2 Reduction and alkylation of full-length 5T4

Lysates from A9 cells expressing 5T4 were separated by SDS/PAGE [10% (w/v) gel] under non-reducing (NR), reducing (R) or reducing and alkylating conditions (R + A). Western blots were probed with mAb5T4 (1 µg/ml), biotinylated rabbit anti-mouse immunoglobulins (1:3000 dilution) and SHRP (1:6000 dilution). Blots were developed using ECL[®]. The positions of molecular-mass markers (in kDa) are indicated on the right.

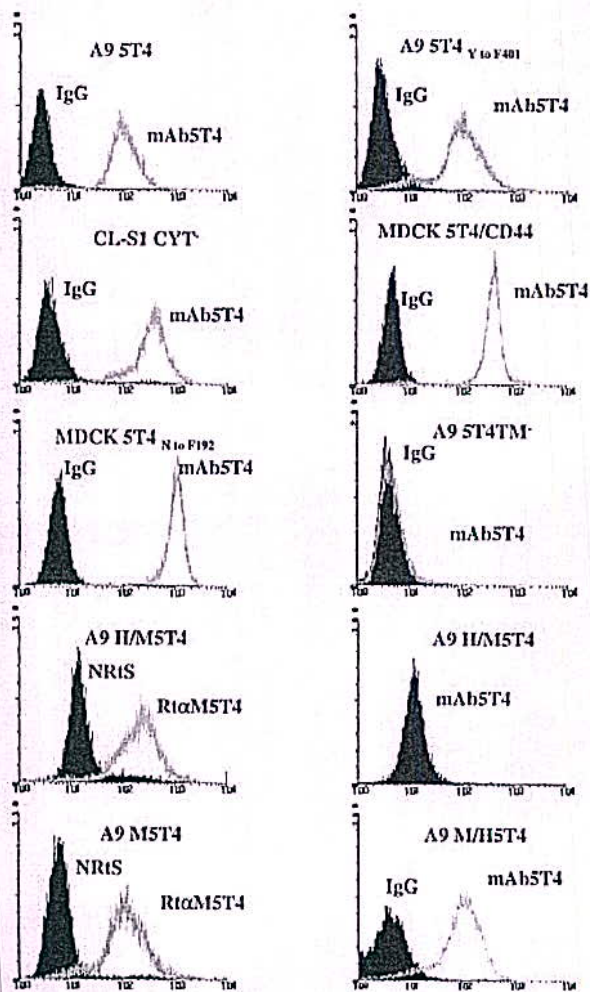


Figure 3 FACS profiles of variant human 5T4 transfectant cell lines

Cells (A9, CL-S1 and Madin–Darby canine kidney (MDCK); 2×10^5) were labelled using mAb5T4 or mouse IgG, (IgG) at 1 μ g/ml, followed by FITC-conjugated rabbit F(ab')₂ anti-mouse immunoglobulins (1:40 dilution). Wild-type 5T4, 5T4-N to F (5T4_{NtoF192}) and 5T4-Y to F (5T4_{YtoF401}) site-directed mutants, 5T4-CYT⁻, 5T4/CD44 and human/mouse chimaeric 5T4s (H/M5T4 and M/H5T4) were labelled with mAb5T4. Mouse 5T4 (M5T4) and human/mouse chimaeric 5T4 (H/M5T4) were labelled using rat anti-(mouse 5T4) (RαM5T4) or normal rat serum (NRIS) at a 1:300 dilution, followed by FITC-conjugated rabbit F(ab')₂ anti-rat immunoglobulins (1:40 dilution).

Human 5T4 variants predicted to be expressed at the cell surface were assessed by FACS using mAb5T4 or an isotype-matched control. The full-length wild-type 5T4 stained with mAb5T4, but not the control antibody (Figure 3); a neo control transfectant was negative with both antibodies (results not shown). Mutation of the putative tyrosine phosphorylation site in the cytoplasmic tail of 5T4 to phenylalanine (5T4-Y to F) did not alter the expression of the epitope at the cell surface, nor did removal of the entire cytoplasmic domain (5T4-CYT⁻). A 5T4/CD44 chimaera, comprising the extracellular domain of 5T4 and transmembrane and cytoplasmic domains of CD44, also expressed the mAb5T4 epitope. Together, these data show that there is no 'inside-out' influence on 5T4 conformation mediated specifically by the 5T4 transmembrane or cytoplasmic domains.

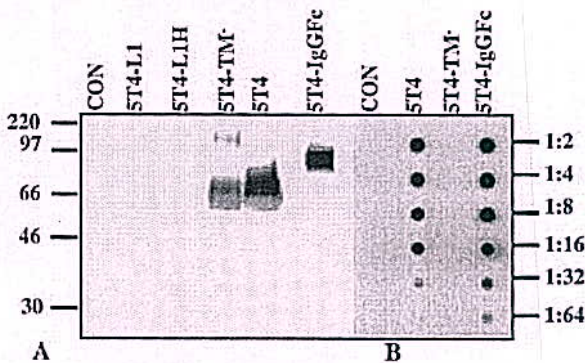


Figure 4 Western and dot blots of key 5T4 variant constructs

(A) Full-length 5T4 and H12 (CON), 5T4-TM⁻, 5T4-IgGfc, 5T4-L1 and 5T4-L1H, expressed in A9 cells, were analysed by Western blotting. The position of molecular-mass markers (in kDa) is indicated on the left. (B) Full-length 5T4 and H12 (CON), 5T4-TM⁻ and 5T4-IgGfc were serially doubly diluted (1:2–1:64) and analysed by dot blotting. Both Western and dot blots were probed with mAb5T4 (1 μ g/ml), biotinylated rabbit anti-mouse immunoglobulins (1:3000 dilution) and SHRP (1:6000 dilution). Blots were developed using ECL[®].

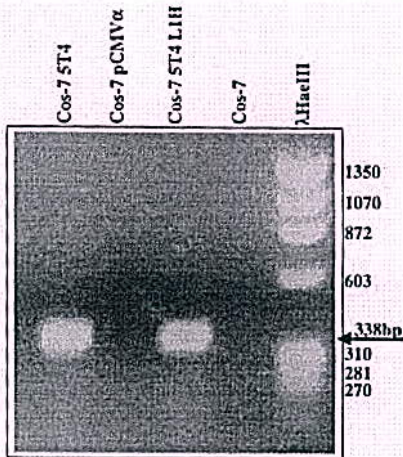


Figure 5 RT-PCR of variant 5T4 transient expression in Cos-7 cells

RT-PCR products were run on a 1% (w/v) agarose gel. Full-length 5T4 and 5T4-L1H contained the 5T4-specific band of 338 bp, whereas Cos-7 and a neo transfectant control (Cos-7 pCMVα) did not. The position of the markers (λHaeIII) is indicated on the right.

Expression of the monoclonal 5T4 epitope was not detected in the supernatant or lysate from stable transfectants of the two extracellular deletion constructs 5T4-L1 and 5T4-L1H by either Western (Figure 4A) or dot blot (results not shown). Transient expression, over 48 h, of full-length 5T4 and 5T4-L1 and 5T4-L1H cDNAs in Cos-7 cells confirmed expression of the full-length molecule at the cell surface (results not shown), but neither deletion construct could be detected using mAb5T4. The presence of 5T4 mRNA in all of the 5T4 transfectants was shown by RT-PCR using primers specific for the L1 region (LRR1) of the gene, yielding a 338 bp 5T4 band (Figure 5).

The transmembrane deletion construct (5T4 TM⁻) was not detected at the cell surface, but was detected using mAb5T4 in

the supernatants of transfected cells as a 64–67 kDa band by Western blot (Figure 4A). However, it could not be detected in the same samples, or 20 times concentrates thereof, by dot blot (Figure 4B). A fusion protein comprising the extracellular domain of human 5T4 and human IgGfC (5T4-IgGfC) was detected by both Western and dot blotting techniques. Removal of the Fc domain by protease Xa cleavage and purification on Protein G resulted in a product essentially identical with the 5T4-TM-construct, which retained no human Fc immunoreactivity. However, unlike 5T4-TM⁺, this molecule was recognized by mAb5T4 by dot-blot analysis (Table 1).

Murine 5T4 is not recognized by the monoclonal antibody to the human molecule, but can be labelled at the cell surface by polyclonal anti-mouse 5T4 sera in transfected (Figure 3) and naturally expressing cells. A difference in sequence within the hydrophilic domain, where an additional six amino acids are inserted adjacent to the N-glycosylation site in mouse 5T4, suggested that this site might be important in the recognition of human 5T4 by mAb5T4. However, mutation of Asn¹⁹² within this region to phenylalanine (5T4-N to F), deleting the potential glycosylation site, did not affect mAb5T4 binding by FACS analysis, Western or dot blotting. The size of product recognized by mAb5T4 in Western blot was approx. 3–5 kDa smaller than full-length 5T4, confirming that this site is normally glycosylated (Table 1).

A human/mouse 5T4 chimaera (H/M 5T4) was made by replacing the first LRR, LRR1, of mouse 5T4 with that of the human molecule. This construct was not recognized by mAb5T4 by either FACS or Western blotting, but the molecule was detected at the cell surface with polyclonal sera specific for both human and mouse 5T4. The reciprocal mouse/human 5T4 chimaera (M/H 5T4) was shown to express the mAb5T4 epitope, as it was detected at the cell surface of transfected cells by FACS (Figure 3) and in lysates by Western blot.

Thus these data show that the mAb5T4-recognized epitope is dependent solely on the extracellular domain of the molecule, and it is possible to positively assign the epitope to the membrane-proximal LRR2. The 5T4 molecule has been shown to be highly glycosylated, probably exclusively through N-linked structures. The extent and complexity of the glycosylation of the seven potential sites in the extracellular domain was investigated in trophoblast-derived 5T4 molecules.

Carbohydrate studies

The initial extraction of nine full-term placentae yielded 12.95 g of StMPM. Purification of 5T4 from StMPM by lectin affinity chromatography on a WGA column and mAb5T4 column yielded pure 5T4 in 81.1 ml fractions. Two representative fractions were assayed by SDS/PAGE under non-reducing conditions to confirm the isolation of 5T4. The major band eluted at 72 kDa for both fractions, confirming the isolation of the 5T4 antigen. Higher-molecular-mass bands seen in the non-reducing gels include multimeric forms of 5T4, as they were not observed in reducing gels and drop out at 72 kDa in non-reduced/reduced two-dimensional gels (results not shown). Bradford assay revealed that 400 µg of 5T4 antigen had been purified, although this value must be regarded as approximate, due to the lack of quantified 5T4 standards with which to build a calibration curve.

Monosaccharide analysis by HPAEC-PAD revealed the presence of fucose, galactose, glucosamine, mannose and *N*-acetylneuraminic acid (Table 2). The absence of galactosamine in 5T4 confirms the absence of oligosaccharides linked via GalNAc 1-O-Ser/Thr [21]. Oligosaccharides were therefore released from 5T4

Table 2 Monosaccharide compositional analysis of 5T4 before and after PNGase F treatment

Quantification was performed by comparison with a mixture of hydrolysed monosaccharides prepared simultaneously with the glycoprotein hydrolysates. 2-Deoxyglucose was added to each sample prior to injection as a reference peak. nt, not tested.

Sample	Monosaccharide	[Monosaccharide] (nmol)	Monosaccharide/5T4 ratio
5T4 before PNGase F	Fucose	0.04	negligible
	Galactose	16.69	4.62
	Glucosamine	25.67	7.11
	Mannose	10.83	3.00
	<i>N</i> -Acetylneuraminic acid	5.64	1.56
5T4 after PNGase F	Fucose	0	0
	Galactose	2.25	4.00
	Glucosamine	2.98	5.40
	Mannose	1.67	3.0
	<i>N</i> -Acetylneuraminic acid	nt	—

by PNGase F treatment under reducing conditions and purified by centrifugation through 10 kDa molecular-mass-cut-off filters and Biogel P2 chromatography. In addition to releasing the oligosaccharides from untreated 5T4, the oligosaccharides were released from a sample of 5T4 that had been mildly acid hydrolysed to generate a library of neutral oligosaccharides. The efficacy of enzymic release was shown to be 87% by performing HPAEC-PAD monosaccharide compositional analyses on the PNGase F-treated 5T4 protein (Table 2).

Fluorescently labelled oligosaccharides released from 5T4 and purified into charged groups by GlycoSep C HPLC were shown to have one to four (a1–a4) sialic acid residues. Neutral oligosaccharides were collected in the void volume (Figure 6A). The latter were purified further by preparative Biogel P4 chromatography (Figure 6B). Calculation of GU by comparison with an external dextran standard showed an oligosaccharide of approximately a dodecasaccharide, probably a triantennary complex-type chain with core fucosylation (peak 4) and Man₆-type high-mannose chains (peak 2). In addition, the presence of small amounts of Man₅ and Man₇ high-mannose chains was shown (peaks 1 and 3 respectively).

The neutral glycan library obtained by PNGase F digestion of 5T4 after mild acid hydrolysis was analysed by Biogel P4 chromatography (Figure 6C) after fluorescent labelling. Calibration of the resulting peaks with respect to a dextran hydrolysate, detected by refractive index run simultaneously, allows the description of the N-glycans in terms of GU. Comparison of these values with a database of N-linked structures and their GU, coupled with the monosaccharide compositional data (Table 2) and peak area ratios, allowed assignment of the glycan structures as approximately the follows: two high-mannose oligosaccharides of the Man₆-type (with minor amounts of other high-mannose varieties); one each of triantennary oligosaccharides with (compared with Figure 6B) and without core fucose; one each of tetra-antennary oligosaccharides with and without core fucose; and an additional biantennary chain with 50% core fucosylation. The monosaccharide ratios for mannose:galactose were consistent with this ratio of chains.

Other interpretations of the GU data are possible due to the great potential in variation of different fucosylation, branching patterns, bisecting *N*-acetylglucosamine and hybrid chains etc. [33]. The amount of sialylation found (Table 2 and Figure 6) was

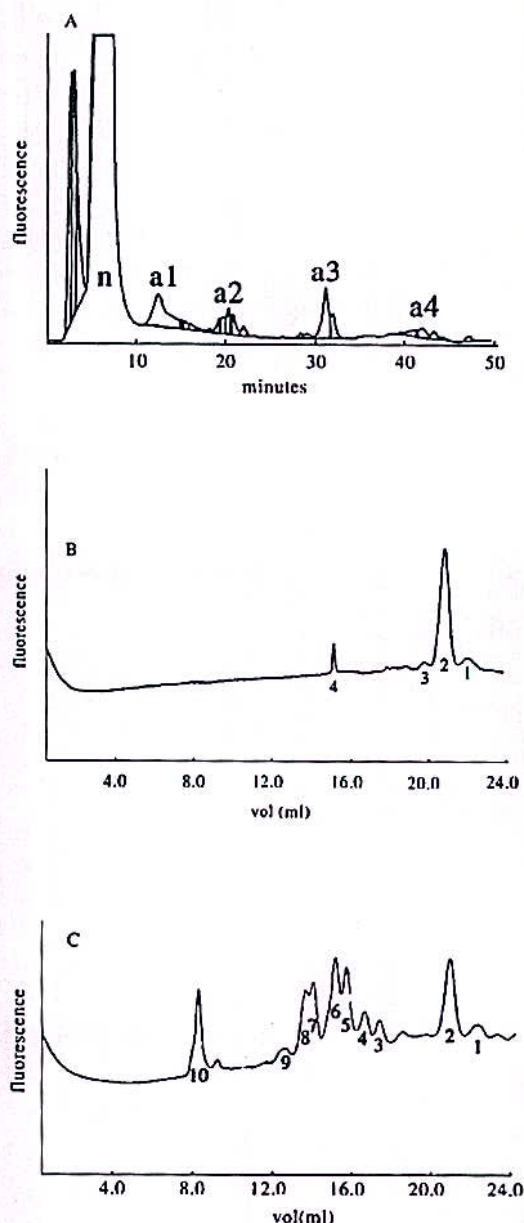


Figure 6 Chromatographic analysis of 5T4 carbohydrate structures

(A) GlycoSep C HPLC of neutral 2-AB-labelled oligosaccharides released from 5T4 by PNGase F calibrated against a dextran hydrolysate. Fractions n and a1–a4 represent neutral and mono- to tetra-sialylated structures. (B) Biogel P4 chromatography of the neutral fraction (n) from (A). Peaks were as follows: 1, Man₅ (6.6 GU); 2, Man₆ (7.6 GU); 3, Man₇ (8.6 GU); and 4, triantennary complex chain (14.4 GU), where GU was calibrated against a dextran hydrolysate. (C) Biogel P4 chromatography of the oligosaccharide pool represented in (A) after the release of sialic acid. Peak assignments were made by comparison with a structural database (Eve New-Bio2; Oxford GlycoSciences), with calibration against a dextran hydrolysate, and from the monosaccharide compositional data as follows: 1, Man₅ (6.4 GU); 2, Man₆ (7.5 GU); 3, biantennary (11.2 GU); 4, biantennary + core fucose (12.2 GU); 5, triantennary (13.5 GU); 6, triantennary + core fucose (14.5 GU); 7, tetra-antennary (16.5 GU); 8, tetra-antennary + core fucose (17.3 GU); 9, penta-antennary + core fucose (19.7 GU); and 10, unidentified.

consistent with mainly complex rather than hybrid chains. As Biogel P4 is primarily a sizing column, we can be sure of the extent of the hydrodynamic mass of the oligosaccharides if

Table 3 Position of carbohydrate chains within the 5T4 sequence

Compositions of the carbohydrate structures at each of the seven N-linked glycosylation sites (N) in the extracellular domain of 5T4 were determined.

HPLC fraction	Peptide	Carbohydrate structure
11–12	81-MLTEVPTDL	Penta-antennary + N-acetylglucosamine
14–16	256-NLTHLESLHLED	Penta-antennary + fucose
25–31	116-PLAELAAALMLSGSRL	High-mannose or penta-antennary ± fucose
25–31	238-HLDLSNNSLVSL	
25–31	267-HLEDNALKVLHMG	
67–70	166-WASVSAPS ...	Bi-, tri- or tetra-antennary + fucose
67–70	183-IVPPEDERQWRS	

information is also known about the occupancy of the N-glycosylation sites. Further information on the latter can be gained by analysis of glycopeptides and peptides from the tryptic digests fractionated by relative hydrophobicity on reverse-phase HPLC. Their predicted cleavage sites and hydrophobicity [32] can be used to assign them to the carbohydrate-containing fractions, as determined by hexose content (Table 3). The results for 5T4 showed that the most hydrophobic peptides having N-glycosylation sites were QNR(S), followed by NLTEVPTDL-PAYVR (Asn¹⁰² and Asn⁸¹ respectively; where single-letter amino-acid notation has been used). These were assigned to the early eluting hexose-positive peaks, which were shown after PNGase F treatment, mild acid hydrolysis, fluorescence labelling and Biogel P4 analysis to have only high-mannose chains.

The largest hexose-containing peak eluted next on the reverse-phase HPLC and was shown to contain predominantly biantennary chains with and without fucose, together with high-mannose chains. From the amount of hexose and the hydrophobicity read-out, it was likely that this would contain the glycopeptides NLTHLESLHLEDNALK, HLDLSNNSLVSL-TYVSFR, VLHNGTLAELQGLPHIR, RPPLAELAAALNLS-GSR (where single-letter amino-acid notation has been used), corresponding to amino acid residues 256–271, 238–255, 272–288 and 114–129, all of which are very close in hydrophobicity. This identified complex N-glycosylation at all, or one or more, of the Asn¹²⁴, Asn²⁴³, Asn²⁵⁶ and Asn²⁷⁵ sites.

Hexose was spread thinly over the later fractions of the reverse-phase HPLC and was available in two small amounts for adequate analysis by Biogel P4, but from the hydrophobicity read-out and the above results on mono/oligosaccharide composition, we would predict that they will contain other glycopeptides containing Asn¹²⁴, Asn²⁴³, Asn²⁵⁶ and Asn²⁷⁵ with different peptide cleavage sites [34,35] and the tri-, tetra- and penta-antennary or equivalent size chains discussed above.

There were few trypsin cleavage sites around the seventh N-glycosylation site at Asn¹¹⁶, and therefore it was predicted that glycopeptides containing this site were very large and with much greater hydrophobicity. A late-running cluster of hexose-positive peaks in the reverse-phase HPLC was consistent with these large glycopeptides, and Biogel P4 analysis gave some indication of oligosaccharides of tri- and tetra-antennary size.

DISCUSSION

MAb5T4 is a high-affinity monoclonal antibody that recognizes the oncofoetal antigen 5T4 [2,25]. The restricted expression of this molecule in normal adult tissue and its more widespread expression in malignancy means that it is a suitable vector for the development of antibody-targeted tumour therapy. Strategies using 5T4 specificity on the basis of Fab fragments and single-

chain antibodies to deliver *Staphylococcus aureus* superantigen ('SEA') and other immunological response modifiers to the site of a tumour are under development [25,36]. A better understanding of the nature of the 5T4 antigen, which is dependent on the appropriate folding and its carbohydrate structure, is important in optimizing these approaches.

The epitope for mAb5T4 is conformational in nature, being dependent on both glycosylation and the presence of intact intramolecular disulphide bonds. Despite human and mouse 5T4 sharing 81% identity and a conserved domain structure [22], the monoclonal against the human molecule does not cross-react with the mouse. Comparison of the sequences highlights only two significant differences between the two species, as discussed further below.

We have shown that the 5T4 epitope lies entirely within the extracellular domain, as both the 5T4-TM⁻ construct and 5T4-IgGFc fusion protein, which include only the extracellular domain of 5T4, were recognized by mAb5T4 by Western blot. Further analysis of the soluble molecule, 5T4-TM⁻, has given some insight into the conformational nature of the 5T4 epitope. Although recognized by Western blot, the construct was not detectable by dot blot. In contrast, the fusion protein, 5T4-IgGFc, was recognized in both Western and dot blots. Moreover, proteolytic removal of the Fc yields a protein essentially identical with the 5T4-TM⁻ construct, but that retains the 5T4 epitope by dot blot. Taken together, these results suggest that the most thermodynamically stable conformation of the truncated molecule, when newly synthesized, is one in which the epitope is obscured. Furthermore, the denaturation and subsequent refolding of the mature fully glycosylated molecule during SDS/PAGE and Western blotting leads to the molecule assuming its correct conformation, thus exposing the epitope. In addition, the influence of the transmembrane domain on the correct folding of the protein can be substituted by other sequences, here immunoglobulin Fc, and once this folding has been imprinted on the molecule during translation, it remains stable when the additional sequence is removed.

The extracellular domain deletion constructs, 5T4-L1 and 5T4-L1H, were not expressed at the cell surface and could not be detected in culture supernatants by either Western or dot blotting. Although these constructs were transcribed, the proteins were either not translated, unstable, or did not present the 5T4 epitope through either misfolding or its being located within another region of the extracellular domain.

Analysis of the two human/mouse chimaeric 5T4 constructs indicates that the epitope lies within the more membrane-proximal region of human 5T4. The lack of effect upon expression of the epitope by the removal of the glycosylation site within the hydrophilic domain indicates that the epitope must reside in either LRR2 or its C-terminal flanking domain.

The remaining challenge is to determine which of the carbohydrates are likely to contribute to the final structure and what role, if any, they play in the function of 5T4. Co-translational addition *en bloc* of the core glycan to nascent polypeptides confers stability and regulates folding both directly and through interaction with lectin chaperones [37]. These in turn are involved in the sorting through the Golgi apparatus of the correctly processed mature protein. Incorrect glycan processing may in turn lead to an inability to interact correctly with protein disulphide-isomerase and a failure of disulphide-bond formation [38].

From the studies presented in this report, high-mannose chains predicted at Asn¹⁹² in the hydrophilic domain do not appear to be necessary for presentation of the epitope. This is one of the two sites which differ significantly in mouse and human; the

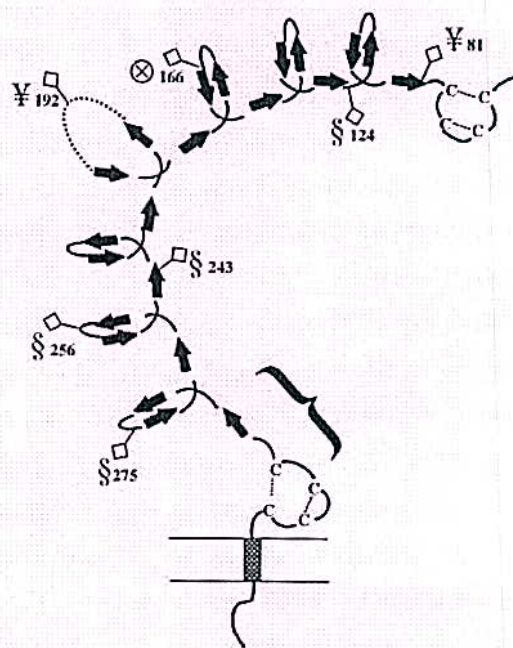


Figure 7 Cartoon diagram of 5T4 structure

Deduced position of the carbohydrate chains and proposed location of the mAb5T4 epitope are indicated. □, asparagine residues; Y, high-mannose; S, biantennary (with and without fucose) and larger complex oligosaccharides; }, mAb5T4 epitope; hatched box, transmembrane domain; dotted line, hydrophilic sequence; ⊗, large sialated complex oligosaccharides. Disulphide bonds (C·····C) are also indicated.

other is the putative high-mannose occupying site at Asn⁸¹, which is absent in the mouse. The mouse has an extra N-glycosylation consensus sequence in the C-terminal membrane-proximal region. Other differences in protein sequence are slight, the only other non-conservative amino acid differences being an increase in acidity in the mouse, with aspartic acid replacing human Phe¹⁷ and human Gly³¹⁷ and glutamic acid replacing human Val¹⁷⁶. There are some changes in the position of the basic amino acid arginine in residues 50–250 (human numbering), but no differences in number. Thus the major differences between the mouse and the human are likely to be found in the structure of the oligosaccharides. No O-glycosylation was found in the present study in placental 5T4, but two regions of the amino acid sequence in both mouse and human show several close serine and proline residues, which are indicators of potential O-glycosylation sites. One of these is near the N-terminus of the human before the cysteine-rich region and the second leads up to the hydrophilic loop. The complex oligosaccharides discussed in the present study (Figure 7) are candidates for carrying oligosaccharide-recognition motifs, which may vary with different sources of 5T4. Oligosaccharide sequences are well known as tumour-associated antigens and with functions in cell-cell interactions [39]. 5T4 appears to influence cellular motility and organization of the cytoskeleton. This function is dependent on the cytoplasmic domain and may be mediated through a recently identified cytoplasmic-interacting protein [40]. The conformation of the extracellular domain may influence its interaction with other extracellular molecules, either at the plasma membrane or in the extracellular matrix, which may in turn modulate the effects of 5T4 on the cells. From our present results, the site at Asn¹⁶⁶ is

likely to be glycosylated with relatively large sialylated oligosaccharides. This and glycosylation at Asn²⁵⁶ and Asn²⁷⁵, the sites of which are within the LRRs (Figure 7), are most likely to be important in overall structure and antigenicity

We thank the Medical Research Council (U.K.) (M.J.D., D.V.R. and E.F.H.) and the Cancer Research Campaign for financial support.

REFERENCES

- 1 Stern, P. L., Beresford, N., Thompson, S., Johnson, P. M., Webb, P. D. and Hole, N. (1986) Characterization of the human trophoblast-leukocyte antigenic molecules defined by a monoclonal antibody. *J. Immunol.* **137**, 1604–1609
- 2 Hole, N. and Stern, P. L. (1988) A 72 kD trophoblast glycoprotein defined by a monoclonal antibody. *Br. J. Cancer* **57**, 239–246
- 3 Southall, P. J., Boxer, G. M., Bagshawe, K. D., Hole, N., Bromley, M. and Stern, P. L. (1990) Immunohistological distribution of 5T4 antigen in normal and malignant tissues. *Br. J. Cancer* **61**, 89–95
- 4 Myers, K. A., Rahi-Saund, V., Davison, M. D., Young, J. A., Cheater, A. J. and Stern, P. L. (1994) Isolation of a cDNA encoding 5T4 oncofetal trophoblast glycoprotein. An antigen associated with metastasis contains leucine-rich repeats. *J. Biol. Chem.* **269**, 9319–9324
- 5 Starzynska, T., Rahi, V. and Stern, P. L. (1992) The expression of 5T4 antigen in colorectal and gastric carcinoma. *Br. J. Cancer* **66**, 867–869
- 6 Starzynska, T., Marsh, P. J., Scholfield, P. F., Roberts, S. A., Myers, K. A. and Stern, P. L. (1994) Prognostic significance of 5T4 oncofetal antigen expression in colorectal carcinoma. *Br. J. Cancer* **69**, 899–902
- 7 Starzynska, T., Wiechowska-Kozłowska, A., Marlicz, K., Bromley, M., Roberts, S. A., Lawniczak, M., Kolodziej, B., Zyluk, A. and Stern, P. L. (1998) 5T4 oncofetal antigen in gastric carcinoma and its clinical significance. *Eur. J. Gastroenterol. Hepatol.* **10**, 479–484
- 8 Mulder, W. M., Stern, P. L., Stukart, M. J., de Windt, E., Butzelaar, R. M., Meijer, S., Ader, H. J., Claessen, A. M., Vermorken, J. B., Meijer, C. J. et al. (1997) Low intercellular adhesion molecule 1 and high 5T4 expression on tumor cells correlate with reduced disease-free survival in colorectal carcinoma patients. *Clin. Cancer Res.* **3**, 1923–1930
- 9 Wrigley, E., McGowan, A. T., Swindell, R., Crowther, D., Starzynska, T. and Stern, P. L. (1995) 5T4 oncofetal antigen expression in ovarian carcinoma. *Int. J. Gynecol. Cancer* **5**, 269–274
- 10 Ali, A., Langdon, J., Stern, P. and Partridge, M. (2001) The pattern of expression of the 5T4 oncofetal antigen on normal, dysplastic and malignant oral mucosa. *Oral Oncol.* **37**, 57–64
- 11 Carsberg, C. J., Myers, K. A., Evans, G. S., Allen, T. D. and Stern, P. L. (1995) Metastasis-associated 5T4 oncofetal antigen is concentrated at microvillus projections of the plasma membrane. *J. Cell Sci.* **108**, 2905–2916
- 12 Carsberg, C. J., Myers, K. A. and Stern, P. L. (1996) Metastasis-associated 5T4 antigen disrupts cell–cell contacts and induces cellular motility in epithelial cells. *Int. J. Cancer* **68**, 84–92
- 13 Kobe, B. and Deisenhofer, J. (1994) The leucine-rich repeat: a versatile binding motif. *Trends Biochem. Sci.* **19**, 415–421
- 14 Kobe, B. and Deisenhofer, J. (1995) Proteins with leucine-rich repeats. *Curr. Opin. Struct. Biol.* **5**, 409–416
- 15 Hillig, R. C., Renault, L., Vetter, I. R., Drell, IV, T., Wittinghofer, A. and Becker, J. (1999) The crystal structure of rna1p: a new fold for a GTPase-activating protein. *Mol. Cell* **3**, 781–791
- 16 Kobe, B. and Deisenhofer, J. (1993) Crystal structure of porcine ribonuclease inhibitor, a protein with leucine-rich repeats. *Nature (London)* **366**, 751–756
- 17 Endokimov, A. G., Anderson, D. E., Routzahn, K. M. and Waugh, D. S. (2001) Unusual molecular architecture of the *Yersinia pestis* cytotoxin YopM: a leucine-rich repeat protein with the shortest repeating unit. *J. Mol. Biol.* **312**, 807–821
- 18 Liker, E., Fernandez, E., Izaurralde, E. and Conli, E. (2000) The structure of the mRNA export factor TAP reveals a *cis* arrangement of a non-canonical RNP domain and an LRR domain. *EMBO J.* **19**, 5587–5598
- 19 Janosi, J. B., Ramsland, P. A., Mott, M. R., Firth, S. M., Baxter, R. C. and Delhanty, P. J. (1999) The acid-labile subunit of the serum insulin-like growth factor-binding protein complexes. Structural determination by molecular modeling and electron microscopy. *J. Biol. Chem.* **274**, 23328–23332
- 20 Kajava, A. V., Vassart, G. and Wodak, S. J. (1995) Modeling of the three-dimensional structure of proteins with the typical leucine-rich repeats. *Structure (London)* **3**, 867–877
- 21 Hole, N. and Stern, P. L. (1990) Isolation and characterization of 5T4, a tumour-associated antigen. *Int. J. Cancer* **45**, 179–184
- 22 King, K. W., Sheppard, F. C., Westwater, C., Stern, P. L. and Myers, K. A. (1999) Organisation of the mouse and human 5T4 oncofetal leucine-rich glycoprotein genes and expression in foetal and adult murine tissues. *Biochim. Biophys. Acta* **1445**, 257–270
- 23 Laemmli, U. K. (1970) Cleavage of structural protein during the assembly of bacteriophage T4. *Nature (London)* **227**, 680–685
- 24 Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254
- 25 Shaw, D. M., Embleton, M. J., Westwater, C., Ryan, M. G., Myers, K. A., Kingsman, S. M., Carroll, M. W. and Stern, P. L. Isolation of a high affinity scFv from a monoclonal antibody recognising the oncofetal antigen 5T4. *Biochim. Biophys. Acta* **1524**, 238–246
- 26 Towbin, H. and Gordon, J. (1984) Immunoblotting and dot immunobinding – current status and outlook. *J. Immunol. Methods* **72**, 313–340
- 27 Townsend, R. R. (1995) Analysis of glycoconjugates using high pH anion-exchange chromatography. *J. Chromatogr. Libr.* **58**, 181–210
- 28 Weitzhandler, M., Pohl, C., Rohrer, J., Narayanan, L., Slingsby, R. and Avdalovic, N. (1996) Eliminating amino acid and peptide interference in high-performance anion-exchange pulsed amperometric detection glycoprotein monosaccharide analysis. *Anal. Biochem.* **241**, 128–134
- 29 Weitzhandler, M., Slingsby, R., Jagodzinski, J., Pohl, C., Narayanan, L. and Avdalovic, N. (1996) Eliminating monosaccharide peak tailing in high pH anion-exchange chromatography with pulsed amperometric detection. *Anal. Biochem.* **241**, 135–136
- 30 Bigge, J. C., Patel, T. P., Bruce, J. A., Goulding, P. N., Charles, S. M. and Parekh, R. B. (1995) Nonselective and efficient fluorescent labeling of glycans using 2-amino benzamide and anthranilic acid. *Anal. Biochem.* **230**, 229–238
- 31 Davies, M. J. and Hounsell, E. F. (1998) HPLC and HPAEC of oligosaccharides and glycopeptides. *Methods Mol. Biol. (Totowa, N.J.)* **76**, 79–100
- 32 Browne, C. A., Bennett, H. P. I. and Solomon, S. (1982) The isolation of peptides by high performance liquid chromatography using predicted elution positions. *Anal. Chem.* **124**, 201–208
- 33 Prime, S. and Merry, T. (1998) Exoglycosidase sequencing of N-linked glycans by the reagent array analysis method (RAAM). In *Methods in Molecular Biology*, vol. 96, Glycoanalysis Protocols (Hounsell, E. F., ed.), pp. 53–69, Humana Press, Totowa, NJ
- 34 Smith, K. D., Davies, M. J., Bailey, D., Renouf, D. V. and Hounsell, E. F. (1996) Analysis of the glycosylation patterns of the extracellular domain of the epidermal growth factor receptor expressed in Chinese hamster ovary fibroblasts. *Growth Factors* **13**, 121–132
- 35 Wong, N. K. C., Kanu, N., Thandrayan, N., Rademaker, G. J., Baldwin, C. I., Renouf, D. V. and Hounsell, E. F. (2000) Microassay analysis of protein glycosylation. *Mol. Biotechnol.* **14**, 147–155
- 36 Forsberg, G., Ohlsson, L., Brodin, T., Bjork, P., Lando, P. A., Shaw, D., Stern, P. L. and Dohlsten, M. (2001) Therapy of human non-small-cell lung carcinoma using antibody targeting of a modified superantigen. *Br. J. Cancer* **85**, 129–136
- 37 Drickamer, K. and Taylor, M. E. (1998) Evolving views of protein glycosylation. *Trends Biochem. Sci.* **23**, 321–324
- 38 Mirazimi, A. and Svensson, L. (1998) Carbohydrates facilitate correct disulfide bond formation and folding of rotavirus VP7. *J. Virol.* **72**, 3887–3892
- 39 Hounsell, E. F., Young, M. and Davies, M. J. (1997) Glycoprotein changes in tumours: a renaissance in clinical applications. *Clin. Sci.* **93**, 287–293
- 40 Awan, A., Lucic, M. R., Shaw, D. M., Sheppard, F., Westwater, C., Lyons, S. A. and Stern, P. L. (2002) 5T4 interacts with TIP-2/GIPC, a PDZ protein, with implications for metastasis. *Biochem. Biophys. Res. Commun.* **290**, 1030–1036

Received 14 September 2001/19 December 2001; accepted 24 January 2002

CLINICAL OBSERVATIONS, INTERVENTIONS, AND THERAPEUTIC TRIALS

Selective Ablation of Acute Myeloid Leukemia Using Antibody-Targeted Chemotherapy: A Phase I Study of an Anti-CD33 Calicheamicin Immunoconjugate

By E.L. Sievers, F.R. Appelbaum, R.T. Spielberger, S.J. Forman, D. Flowers, F.O. Smith, K. Shannon-Dorcy, M.S. Berger, and I.D. Bernstein

Leukemic blast cells express the CD33 antigen in most patients with acute myeloid leukemia (AML), but this antigen is not expressed by hematopoietic stem cells. We conducted a study to determine whether normal hematopoiesis could be restored in patients with AML by selective ablation of cells expressing the CD33 antigen. In a dose escalation study, 40 patients with relapsed or refractory CD33⁺ AML were treated with an immunoconjugate (CMA-676) consisting of humanized anti-CD33 antibody linked to the potent antitumor antibiotic calicheamicin. The capacity of leukemic cells to efflux 3,3'-diethylloxycarbocyanine iodide (DIOC₂) was used to estimate pretreatment functional drug

resistance. Leukemia was eliminated from the blood and marrow of 8 (20%) of the 40 patients; blood counts returned to normal in three (8%) patients. A high rate of clinical response was observed in leukemias characterized by low dye efflux *in vitro*. Infusions of CMA-676 were generally well tolerated, and a postinfusion syndrome of fever and chills was the most common toxic effect. Two patients who were treated at the highest dose level (9 mg/m²) were neutropenic >5 weeks after the last dose of CMA-676. These results show that an immunoconjugate targeted to CD33 can selectively ablate malignant hematopoiesis in some patients with AML. 1999 by The American Society of Hematology.

PATIENTS WITH refractory or recurrent acute myeloid leukemia (AML) have a dismal prognosis. Toxic effects associated with additional conventional chemotherapy are often life-threatening, and few patients achieve a complete remission (CR). A therapy that more specifically targets leukemia is likely to be safer, and possibly more effective than current nonspecific chemotherapeutic agents.

During hematopoietic development, stem cells capable of establishing long-term multilineage hematopoiesis give rise to progenitors with diminished self-renewal capacity and a greater degree of differentiation. During this process, hematopoietic cells express distinct cell surface antigens that are also expressed by their malignant counterparts. Some of these antigens (eg, CD33) are present on maturing normal hematopoietic cells and on AML cells, but not on normal hematopoietic stem cells.¹⁻⁴ These findings raise the possibility that an antibody can be used to deliver cytotoxic agents to selectively ablate

malignant myeloid and immature normal cells while sparing normal stem cells.

The myeloid cell surface antigen CD33 is an attractive target for this approach, as it is expressed on AML blast cells from about 90% of patients.^{2,3} CD33 also may be expressed by most, if not all, of the malignant precursors in at least some cases of AML, as precursor cells that lack CD33 from some patients give rise to normal granulocyte/monocyte precursors in a marrow long-term culture system.^{5,6} The possibility that anti-CD33 antibody could be used to deliver a cytotoxic agent to malignant cells was suggested by studies showing rapid saturation of leukemic blast cells in peripheral blood and marrow after intravenous administration of approximately 5 mg/m² radioiodinated anti-CD33 antibodies.^{7,8} Further, *in vitro* studies showed rapid internalization of the antibody by the target cell.⁹ To test the concept of selective ablation of malignant hematopoiesis and to evaluate the safety of this approach, we treated patients with relapsed or refractory AML with escalating doses of CMA-676, antibody-targeted chemotherapy consisting of an engineered human anti-CD33 antibody linked with the potent antitumor antibiotic calicheamicin.¹⁰

MATERIALS AND METHODS

Patients. Patients were required to have AML that was either refractory to standard therapy or that had recurred after remission. Patients whose leukemia relapsed after a marrow or peripheral blood stem cell transplant were required to have had successful engraftment (platelets $\geq 20 \times 10^3/\mu\text{L}$ without transfusion and an absolute neutrophil count [ANC] $\geq 500/\mu\text{L}$) before relapse. Each prospective patient underwent a bone marrow aspirate to confirm that morphologic evidence of leukemia was present and to document leukemic blast cell surface expression of the CD33 antigen. Patients were required to have a Karnofsky performance status $\geq 60\%$, a white blood cell count $\leq 30 \times 10^3/\mu\text{L}$, serum creatinine ≤ 2.0 mg/dL, and serum bilirubin ≤ 1.5 mg/dL, to have recovered from toxic effects of previous antineoplastic therapy, and to be able to give informed consent. Patients were ineligible if they had previously received anti-CD33 antibody treatment, were pregnant or nursing, had a prior malignancy, or had active symptomatic central nervous system leukemia or an uncontrolled life-threatening infection. Recombinant humanized anti-CD33-calicheamicin drug conjugate, CMA-676 was produced and provided for clinical study by Wyeth-

From the Clinical Research Division, Fred Hutchinson Cancer Research Center, Seattle; the Departments of Pediatrics and Medicine, University of Washington, Seattle, WA; the Department of Hematology and Bone Marrow Transplantation, City of Hope National Medical Center, Duarte, CA; and Wyeth Ayerst Research, Radnor, PA.

Submitted August 19, 1998; accepted January 29, 1999.

Supported by Wyeth-Ayerst Research, E.L.S. is supported by an American Cancer Society Clinical Oncology Career Development Award. I.D.B. is an American Cancer Society Clinical Research Professor.

Presented in part at the 1997 Annual Meeting of the American Society of Clinical Oncology, Denver, CO; the 1997 European Cancer Conference, Hamburg, Germany; and the 1997 Annual Meeting of the American Society of Hematology, San Diego, CA.

Address reprint requests to E.L. Sievers, MD, Fred Hutchinson Cancer Research Center, 1100 Fairview Ave N, D5-280, PO Box 19024, Seattle, WA 98109-1024; e-mail: esievers@fhcrc.org.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1999 by The American Society of Hematology.

0006-4971/99/9311-0018\$3.00/0

Ayerst Research (Radnor, PA) under an Investigational New Drug Application. Celtech Therapeutics, Ltd (Slough, UK) transformed the murine-derived IgG monoclonal antibody (p67.6)⁸ into an engineered human IgG4 antibody (hP67.6), which was then conjugated to the enediyne calicheamicin¹⁰ using a Nac-gamma linker molecule (Hamann et al, manuscript in preparation). Calicheamicin binds in a sequence-specific manner to the minor groove of DNA and induces double-strand DNA breaks that ultimately induce cell death.¹⁰ Specific cytotoxicity of CMA-676 against CD33⁺ leukemia cells was documented in colony-forming assays in vitro, against leukemic cell lines in vitro, and in vivo in nude mice (data not shown).

Protocol design. In this open, single-arm, phase I dose escalation study, CMA-676 was administered to patients with relapsed or refractory CD33⁺ AML at two study sites. Informed consent was obtained from all patients in accordance with the institutional review boards of the participating institutions. In a single-dose tolerance study with CMA-676 in chimpanzees, an intravenous dose of 0.5 mg/m² (twice the initial clinical dose of 0.25 mg/m²) was well tolerated, with no compound-related clinical signs of clinical pathology changes evident throughout a 15-day postinfusion period (data not shown). In previous clinical studies in patients using radiolabeled mP67.6 antibody,⁸ dose-limiting toxicity was not observed at doses up to and including 17 mg/m² antibody trace-labeled with ¹²⁵I. However, saturation of CD33 binding sites was achieved at a dose level of approximately 5 mg/m². Hence, at least three patients were treated at each dose level of CMA-676 with up to three infusions of 0.25, 0.5, 1, 2, 4, 5, 6, and 9 mg protein per square meter of body surface area. If one or two of the first three of these patients experienced a grade III toxic effect (see below), three additional patients were to be treated at the same dose. The maximum tolerated dose was defined as one dose level below the dose that was associated with unacceptable CMA-676-related toxicity. Dose-limiting toxicity was defined as severe marrow hypoplasia of more than 6 weeks duration after a single dose of CMA-676, one grade IV study drug-related toxic effect or two grade IV toxic conditions of ambiguous relationship to CMA-676.

CMA-676 was administered as a single 2-hour intravenous infusion. All cytokines or chemotherapeutic agents were withdrawn before treatment. Patients without leukemic progression and drug-related, nonhematologic toxic effects judged to be grade III or less could receive one or two subsequent cycles of CMA-676 with at least 14 days between cycles. Patients were treated with acetaminophen 650 mg orally and diphenhydramine 25 to 50 mg intravenously 15 to 30 minutes before infusion of CMA-676. Patients who achieved a CR and subsequently had a relapse of their leukemia could receive retreatment at the dosage level being evaluated at the time of retreatment.

Patient evaluation. Patients were examined for acute toxic effects in accordance with the World Health Organization toxic effect grades. Peripheral blood specimens were obtained to study the pharmacokinetics of CMA-676, to evaluate for evidence of CMA-676 binding to CD33-positive cells, and to measure the effects of CMA-676 on hematologic variables and serum chemistry parameters. A complete blood count was obtained before the initial dose and each day for 2 weeks thereafter, or until recovery of granulocyte and platelet levels to the prestudy levels. Hepatic and renal function were measured before initial dose administration, three times per week thereafter, and on day 28 after the last dose of CMA-676.

Bone marrow aspirates were obtained before initial dose administration with CMA-676 on days 1, 7, and 14 of each treatment cycle and on days 1, 7, 14, and 28 of the last treatment cycle. All marrow specimens were examined by light microscopy to estimate cellularity and to detect any residual leukemic blast cells. Patients with progressive disease were removed from the study. Disappearance of leukemia was defined as the absence of peripheral blasts and the presence of $\leq 5\%$ blast cells in the bone marrow by light microscopic evaluation. CR was defined as

disappearance of leukemia in addition to an ANC $> 1,500/\mu\text{L}$ and a platelet count $> 100 \times 10^3/\mu\text{L}$, without transfusions.

Laboratory investigations. Total hP67.6 antibody concentrations in plasma samples were determined using an enzyme-linked immunosorbent assay (ELISA). Formation of antigen-antibody CMA-676 bound to peripheral blood mononuclear cells was detected by flow microfluorimetry. Cells were incubated with biotinylated goat monoclonal anti-human IgG₄, followed by avidin-fluorescein isothiocyanate. Cells incubated with avidin-fluorescein isothiocyanate alone comprised the negative control. The saturation percentage was defined as 100 times the ratio of the fluorescence intensity of patient mononuclear cells (minus the negative control) over the maximum fluorescence intensity (minus the negative control). Maximum achievable saturation was determined by incubating patient mononuclear cells from the same time point with saturating amounts of CMA-676 in vitro before the addition of the anti-human IgG₄ antibody. The efflux of 3,3'-diethyloxycarbocyanine iodide (DiOC₂) from CD33-positive blast cells was measured as an indication of functional drug efflux.¹¹ Serum samples obtained from each patient before CMA-676 administration, on day 7 after initial doses, and on days 7, 14, 21, and 28 after administration of the final dose of CMA-676 were analyzed for anti-hP67.6 (humanized mouse antibody) or anti-calicheamicin/linker immune response by ELISA.

RESULTS

Patient characteristics. A total of 40 patients with relapsed or refractory AML were enrolled. Patient characteristics are shown in Table 1. Three to eight patients were treated at each of eight dose levels of CMA-676: 0.25, 0.5, 1, 2, 4, 5, 6, and 9 mg protein per square meter of body surface area.

Nonhematologic toxicity. The most frequently reported drug-related adverse events are summarized in Table 2. The most commonly observed nonhematologic side effect, a syndrome of fever and chills, occurred in 32 of 40 (80%) patients beginning 2 to 4 hours after the start of the 2-hour intravenous infusion. The syndrome was limited to grade I to II except in three instances. Grade III fever and chills occurred in two patients after receiving CMA-676 at 6 and 9 mg/m², respectively. The third patient (COH-008) who had asymptomatic

Table 1. Patient Characteristics (N = 40)

Age: median (range)	54 (24-73)
Sex	21M:19F
Stage of disease	
First relapse	20
$\geq$ Second relapse	20
Prior stem cell transplant	
Allogeneic	14
Autologous	4
None	22
Cytogenetic abnormality	
t(8:21)	1
Inv(16)	2
t(4:11)	1
t(6:11)	1
t(9:11)	2
Complex abnormality	22
None detected	10
Unknown	1
Prior myelodysplastic phase	
Present	9
Absent	31

Table 2. Most Frequently Reported Drug-Related Adverse Events (No. of Patients)

Event*	CMA-676 Dose (mg/m ²)							
	0.25 (n = 4)	0.5 (n = 3)	1 (n = 4)	2 (n = 3)	4 (n = 6)	5 (n = 6)	6 (n = 8)	9 (n = 7)
Grade 0-I fever/chills	4	2	1	1	4	1	3	3
Grade II fever/chills	—	1	3	2	2	5	3	3
Grade III† fever/chills	—	—	—	—	—	—	1†	1
Grade IV fever/chills	—	—	—	—	—	—	1§	—
Grade III hepatic enzyme elevation (AST or ALT)	—	1	1	—	—	4	1	1
Grade IV hepatic enzyme elevation (AST or ALT)	—	—	—	—	—	—	1	—

Abbreviations: AST, alanine serum transaminase; ALT, alanine aminotransferase.

*Grades are defined according to modified World Health Organization toxicity grading scale.

†Modified to include fever <40°C with asymptomatic hypotension.

‡This patient (FH-007) is represented in the table twice. He experienced grade II fever/chills after his initial treatment at 1 mg/m². He developed shortness of breath in association with retreatment with CMA-676 after his leukemia relapsed, presumably caused by an immune reaction to CMA-676.

§This patient (COH-008) who had asymptomatic hypotension and was receiving low-dose dopamine before CMA-676 administration, had a temperature >40°C and reversible symptomatic hypotension 5 hours after the initiation of the CMA-676 infusion at 6 mg/m².

This patient (FH-020) with documented concurrent cholelithiasis had a pretreatment AST of 13 U/L that increased to 304 U/L 4 days after receiving the first dose of CMA-676, and rapidly declined to the normal range within a week. Immediately before receipt of the second dose of CMA-676, her AST increased to 1,804 U/L and again rapidly returned to the normal range. Elevated enzymes were thought to be primarily due to the patient's cholelithiasis.

hypotension and was receiving low-dose dopamine before CMA-676 administration, had a temperature >40°C and reversible symptomatic hypotension 5 hours after the initiation of the CMA-676 infusion at 6 mg/m². Another patient (FH-007) developed transient shortness of breath in association with retreatment with CMA-676 after his leukemia relapsed, presumably caused by an immune reaction to CMA-676 (see below). Nausea and fatigue were among other less frequent toxic effects thought possibly related to CMA-676.

Reversible, possibly drug-related hepatic transaminase elevations from 5 to 10 times the normal range were observed in eight patients (Table 2). One additional patient with documented concurrent cholelithiasis had a pretreatment alanine serum transaminase (AST) of 13 U/L that increased to 304 U/L 4 days after receiving the first dose of CMA-676, and rapidly declined to the normal range within a week. Immediately before receipt of the second dose of CMA-676, her AST increased to 1,804 U/L and again rapidly returned to the normal range. In this patient, elevated enzymes were thought to be primarily due to the patient's cholelithiasis. No patient had a drug-related bilirubin elevation of greater than five times the normal range. No significant study drug-related central nervous system, cardiac, or renal toxic effects were observed. With regard to nonhematologic toxic effects, a maximum tolerated dose was not reached, and dose-limiting toxicity was not observed.

Hematologic toxicity. Because most patients had concurrent neutropenia and thrombocytopenia caused by active AML at the time of enrollment, whether hematologic toxic effects were directly attributable to CMA-676 was often impossible to ascertain. However, among nine patients with neutrophil counts >1,500 cells/μL before treatment, eight had severe neutropenia (<200 cells/μL) within 14 days of CMA-676 infusion regardless of dose level. Figure 1 shows the decline and recovery of the neutrophil count from a representative patient during the 2 weeks after each infusion. Although dose-limiting toxicity was

not formally observed, two of seven evaluable patients had prolonged drug-related neutropenia after treatment at a dose level of 9 mg/m². The first patient (FH-023) required 38 days to recover to an ANC >500 cells/μL after the third dose of CMA-676, and the second patient (COH-015) did not achieve neutrophil recovery and died of sepsis 50 days after receiving the second dose (Table 3).

Elimination of blast cells from the peripheral blood and bone marrow. Blast cells were identified on a peripheral blood smear in 31 (78%) patients before infusion of CMA-676. One week after treatment, 22 (71%) of these patients had fewer blast cells and in six (19%) of these patients, blast cells had completely disappeared from the peripheral blood smears. The proportion of patients who experienced reductions in peripheral blast cell counts was highest among those who received higher

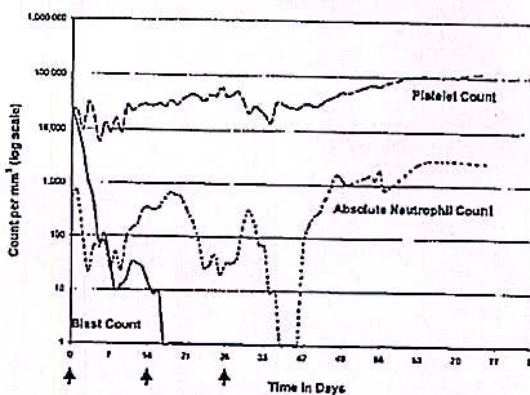


Fig 1. Relationship between hematologic parameters and time for a representative patient (FH-012) who received CMA-676 at 4 mg/m² per dose. Arrows denote infusions of CMA-676. All counts refer to peripheral blood counts.

Table 3. Clinical Responses and Recovery of Hematologic Function in Patients Who Received Treatment With CMA-676

Clinical Response	Patient No.	Age (yr)	Cytogenetic Abnormality	Dose Level (mg/m ²)	Peripheral Blasts (per μ L)	Percentage of Marrow Blasts	Time in Days From Last Dose to:			Site of Recurrence
							ANC >1,500/ μ L	Platelet Count >100 $\times$ 10 ³ / μ L	Relapse	
CR*	FH-007	43	t(6;11)	1	12	50	4	7	132	Bone marrow
	FH-012†	29	t(6;9)	4	22,377	83	20	35	188	Testes; central nervous system
	FH-023‡	34	None	9	748	60	42	160	>623	None
Elimination of blast cells from peripheral blood and marrow‡	FH-013	74	None	5	1,912	88	Not attained	Not attained	70	Marrow
	FH-019§	53	t(8;21)	6	3,560	67	Not attained	Not attained	Not applicable	Not applicable
	FH-022	65	None	9	0	8	18	Not attained	40	Marrow; central nervous system
	FH-024	52	t(1;7); t(12;17)	9	2,445	40	20	Not evaluable¶	14	Marrow
	COH-015§	34	Trisomy 11; del(15)	9	0	23	Not attained	Not attained	Not applicable	Not applicable
	FH-007	43	t(6;11)	8	300	95	8	Not attained	56	Marrow

*Patient achieved transfusion independence with ANC >1,500/ μ L, platelet count >100 $\times$ 10³/ μ L, and <5% blasts in the bone marrow aspirate.

†Patient received donor lymphocyte infusion after attaining CR (FH-012) or before achieving platelet count >100 $\times$ 10³/ μ L (FH-023).

‡Patient had <5% blasts in the bone marrow aspirate with various degrees of hematopoietic recovery.

§Patients died of infection 12 (FH-019) and 50 (COH-015) days after receiving a third dose of CMA-676.

¶Patient attained CR after receiving CMA-676 at 1 mg/m² and then was retreated with CMA-676 at a dose of 6 mg/m².

‡Patient received a bone marrow transplant shortly after leukemia disappeared from the bone marrow and eventually recovered a normal platelet count.

doses of CMA-676. Reduced numbers of peripheral blast cells were observed in 10 of 11 patients treated with either 6 or 9 mg/m² of CMA-676 compared with 12 of 20 patients treated at dose levels from 0.25 to 5 mg/m².

Morphologic evidence of leukemia within the bone marrow (>5% blast cells) was seen in all patients before treatment. After treatment with one to three doses of the drug conjugate, 8 (20%) of 40 patients had <5% leukemic blast cells on morphologic examination of bone marrow aspirate and biopsy specimens (Table 3). In one of these patients (FH-007), blast cells were also eliminated from the bone marrow on a second occasion after retreatment with CMA-676 (see below).

Recovery of normal blood counts. Recovery of blood neutrophil counts to greater than 1,500 cells/ μ L was observed in five of eight patients who had <5% leukemic blasts by morphologic examination of bone marrow aspirate and biopsy specimens (Table 3). In addition, three of these five patients achieved normal platelet counts. The first patient, FH-007, achieved a complete hematologic and cytogenetic remission 7 days after receiving the third dose of CMA-676 at 1 mg/m². After 140 days, local leukemia recurrences in his femurs and iliac wing were identified by magnetic resonance imaging and confirmed by surgical biopsy. He was treated with conventional chemotherapy and local radiation and achieved another CR. He experienced relapse again 293 days after completing the first course of CMA-676, was treated with two additional doses of CMA-676 at 6 mg/m², and his leukemia disappeared again for 56 days. His leukemic cells expressed CD33 at the time of each of his relapses.

The second patient, FH-012, achieved a complete hematologic and cytogenetic remission 35 days after the third dose of 4 mg/m². In an attempt to consolidate his remission, he was given an infusion of lymphocytes from his bone marrow donor 40 days after infusion of the third dose of CMA-676. He remained

in CR for 214 days after treatment until testicular and central nervous system relapses occurred.

The third patient, FH-023, received three doses of CMA-676 at 9 mg/m², and his ANC recovered to >1,500/ μ L 42 days after he received the third dose of CMA-676. His platelet count reached a peak of 36 $\times$ 10³/ μ L 23 days after he received the third dose of CMA-676, but the patient subsequently became platelet transfusion-dependent for approximately 3 weeks. Evidence of antibody on the patient's platelet surfaces was documented. Megakaryocytes were present and platelet maturation appeared normal on examination of the marrow aspirate. In an attempt to consolidate his remission, he was given an infusion of lymphocytes from his bone marrow donor 54 days after receiving the third dose, and his platelet count eventually rose to >100 $\times$ 10³/ μ L 106 days later. At the time of this report, he remained in continuous cytogenetic and hematologic remission >623 days after receiving the third dose of CMA-676.

Pharmacokinetics and saturation of peripheral CD33 sites. Detectable plasma levels of CMA-676 were identified in all patients immediately after intravenous administration and the half-life of CMA-676 was estimated to be 38 $\pm$ 21 hours for patients receiving the 9 mg/m² dose. Figure 2 shows the relationship between the total number of cell surface CD33 sites and CD33 sites bound with CMA-676 in peripheral blood cells for a characteristic patient over time. Thirty minutes after infusion, CD33 sites were almost completely saturated with CMA-676. Twenty-four hours after administration, fewer CD33 sites and bound drug conjugate were evident, suggesting internalization of the CD33-immunoconjugate complex. In the patients who received the maximum dose (9 mg/m²), a median of 92.2% (range, 79.2% to 100%) of CD33 binding sites on peripheral blood blast-sized cells were bound by the drug conjugate 30 minutes after infusion of a dose. Peak CD33 saturation levels in relation to treatment response are shown in

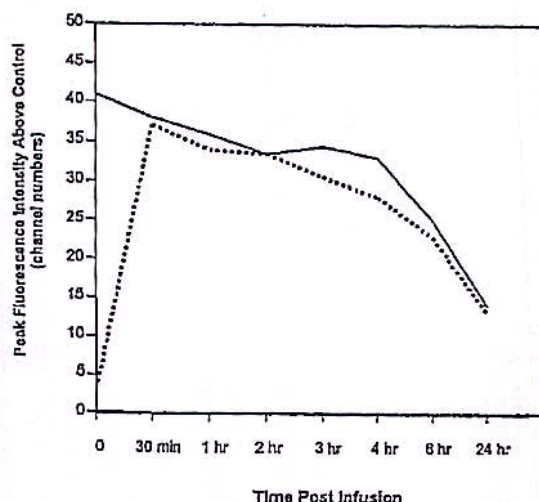


Fig 2. Relationship between total number and CMA-676-bound CD33 sites on peripheral blood cells in a characteristic patient over time. The solid line represents the number of CD33 sites available for binding to CMA-676 as estimated by the maximal fluorescence intensity obtained by incubating an aliquot of cells in vitro with excess CMA-676. The dashed line represents the fluorescence intensity of bound CMA-676 to cell surfaces. Near-complete saturation is seen 30 minutes after the start of the infusion.

Fig 3. These results show that saturation of sites is not, by itself, sufficient to insure treatment response.

Response correlation with drug efflux. We evaluated the efflux of DiOC₂ from pretreatment leukemic blast cells as a measure of functional drug resistance. Figure 3 shows the

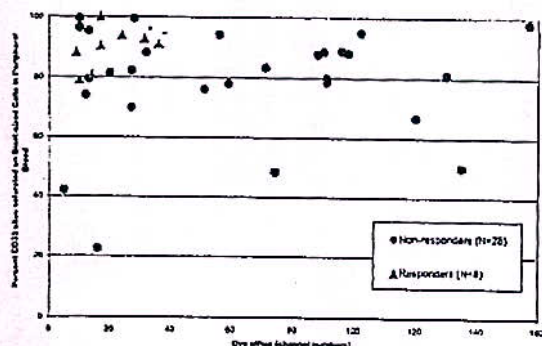


Fig 3. Relation of leukemic blast cell dye efflux and maximum CMA-676 saturation of CD33 sites on peripheral blood blast-sized cells with treatment response (N = 36). (▲) Denotes patients who had <5% leukemic blasts by morphologic examination of bone marrow aspirate and biopsy specimens after treatment. (●) Denotes patients whose leukemia did not disappear. Peripheral blood samples from four patients were unavailable for analysis. The efflux from the dominant population is represented in the five instances in which efflux profiles were bimodal. *Leukemic cell specimens from patients FH-023 and FH-024 were 2 and 3 days old, respectively, at the time of efflux measurement. Because blast-sized cells from each showed uncharacteristically low DiOC₂ loading, obtained efflux values may underestimate true efflux.

relationship between measured leukemic blast cell drug efflux levels and saturation of CD33 sites with response. Elimination of leukemia appeared to be correlated with a low capacity by leukemic blast cells to extrude DiOC₂. For example, of the 30 patients evaluated with the assay in whom doses of CMA-676 saturated >75% of available CD33 sites on peripheral blood blast cells, 8 of 17 patients with leukemic blast cells that showed ≤40 channel numbers of DiOC₂ efflux had <5% blasts in the bone marrow after treatment. In contrast, none of the 13 patients with leukemic blast cells expressing >40 channel numbers of DiOC₂ efflux entered remission.

Immune responses. No humoral responses to anti-hp67.6 antibody were detected. A humoral response to the calicheamicin-linker complex was documented in one patient after receiving a third dose of CMA-676 and in a second patient during retreatment with CMA-676.

DISCUSSION

This study describes the use of an antibody-drug conjugate capable of targeting and safely eliminating target leukemic cells in vivo. Disappearance of AML cells from the bone marrow and peripheral blood of eight patients with complete restoration of normal hematopoiesis in three patients was observed after treatment with antibody-targeted chemotherapy. These results show that eradication of CD33⁺ AML cells can allow restoration of normal hematopoiesis by remaining CD33⁻ precursors. For some patients, we hypothesize that the CD33⁻ precursors are predominantly or completely nonmalignant. This is based on findings that, in some cases of AML, the clonal abnormality originated in either a committed progenitor or an early multipotent cell whose proliferative expression is mainly restricted to the granulocyte/monocyte lineage.¹² Because selection of CD33⁻ precursors from some of these leukemias allowed normal hematopoietic growth in culture, the malignant clone may involve few, if any, CD33⁻ precursors.^{5,6}

In other cases of AML, particularly in older patients, the clonal abnormality has been found in both the erythroid and myeloid lineages, showing malignant involvement of multipotent precursors.¹² In addition, clonal karyotypic abnormalities have been found in primitive precursor cells from some AML patients.^{13,14} It is conceivable that normal hematopoiesis could also be restored in these patients even if a substantial portion of CD33⁻ precursors were malignant because normal precursors express at least a short-term proliferative advantage. While this explanation appears inconsistent with the observed leukemic growth in a immunodeficient mouse model after infusion of isolated primitive (CD34⁺ CD38⁻) precursors from human leukemia specimens,¹⁵ it is consistent with the report that a patient undergoing allogeneic transplantation who was inadvertently given an infusion of donor AML cells initially recovered with normal donor hematopoiesis.¹⁶

Each patient whose blood counts returned completely to normal had experienced relapse after an allogeneic bone marrow transplant (data not shown). Therefore, it is possible that a graft-versus-leukemia effect eliminated the remaining CD33⁻ leukemic cells after elimination of the bulk of AML cells. However, in a recently initiated phase II study of CMA-676, normal hematopoiesis was restored in some patients

who had not received a transplant.¹⁷ Molecular remissions have been observed in a portion of patients with acute promyelocytic leukemia who received unconjugated anti-CD33 antibody (HuM195).¹⁸ Although an AML patient with 8% blasts in his bone marrow achieved a CR with HuM195 given at a supersaturating dose, complete clinical responses have not been reported in patients with large tumor burdens.¹⁹ Hence, the observed elimination of leukemia after treatment with CMA-676 was not likely due to antibody-mediated effects alone.

Myelosuppression was the most clinically significant adverse event. The severe marrow hypoplasia observed in two patients who received 9 mg/m² is consistent with depletion of CD33⁺ hematopoietic progenitor cells and with the time required for CD33⁺ cells to restore hematopoietic function.²⁰ To prevent prolonged neutropenia, two (instead of three) scheduled doses of CMA-676 at 9 mg/m² are generally administered in the phase II study. In that study, a third dose can only be administered if bone marrow cellularity is >15% after two doses of CMA-676. The 9-mg/m² dose level was selected for the phase II study because consistently >75% of CD33 sites were saturated at this dose and nonhematologic toxicity was not considered to be dose-limiting in the phase I study.

Modest and reversible hepatic transaminase elevations and hyperbilirubinemia were observed in some patients who received CMA-676 at high dose levels. Only two patients developed a positive immune response to the calicheamicin-linker complex after receipt of several doses of CMA-676, in contrast with findings in other studies in which frequent immune responses occurred after administration of immunconjugates containing either murine-derived monoclonal antibodies or naturally occurring toxins.⁷

Only patients with relapsed or refractory disease were eligible for this clinical trial. In this setting, the leukemia cells in many patients expressed functional drug resistance that may have prevented killing by the drug conjugate (a known substrate of p-glycoprotein, data not shown). This was suggested by the correlation between clinical response and low levels of dye efflux by leukemic blast cells. This observation raises the possibility that the complete response rate might be improved by using agents that specifically block p-glycoprotein activity (eg, cyclosporine) in combination with CMA-676.

In summary, we found that CMA-676 selectively ablated malignant hematopoiesis in some patients with refractory or relapsed AML and that therapy was associated with few toxic effects. Studies are currently underway to evaluate this agent in pediatric and elderly populations. It is anticipated that CMA-676 will be evaluated in the setting of newly diagnosed and minimal residual disease. Because CMA-676 was associated with few nonhematologic toxic effects, it potentially could be studied as a replacement for anthracycline in combination chemotherapy regimens and combined with existing conditioning regimens for hematopoietic stem cell transplant.

ACKNOWLEDGMENT

We are indebted to Drs Dan Schochat, Dennis Parenti, Geoff Yarrington, Phillip Hamann, and Lois Hinman for efforts in initiating and supporting this work; Dr Irene Georgieff, Melissa Auen, and

Rosalane Dacanay for excellent technical assistance; and Penny Hoeltzel for providing superb editorial comments.

REFERENCES

1. Andrews RG, Torok-Storb B, Bernstein ID: Myeloid-associated differentiation antigens on stem cells and their progeny identified by monoclonal antibodies. *Blood* 62:124, 1983
2. Griffin JD, Linch D, Sabbath K, Larcom P, Schlossman SF: A monoclonal antibody reactive with normal and leukemic human myeloid progenitor cells. *Leuk Res* 8:521, 1984
3. Dinndorf PA, Andrews RG, Benjamin D, Ridgway D, Wolff L, Bernstein ID: Expression of normal myeloid-associated antigens by acute leukemia cells. *Blood* 67:1048, 1986
4. Andrews R, Singer J, Bernstein I: Precursors of colony-forming cells in humans can be distinguished from colony-forming cells by expression of the CD33 and CD34 antigens and light scatter properties. *J Exp Med* 169:1721, 1989
5. Bernstein ID, Singer JW, Andrews RG, Keating A, Powell JS, Bjornson BH, Cunner J, Najfeld V, Reaman G, Raskind W, Sutton DMC, Fialkow PJ: Treatment of acute myeloid leukemia cells in vitro with a monoclonal antibody recognizing a myeloid differentiation antigen allows normal progenitor cells to be expressed. *J Clin Invest* 79:1153, 1987
6. Bernstein ID, Singer JW, Smith FO, Andrews RG, Flowers DA, Petersens J, Steinmann L, Najfeld V, Savage D, Fruchtman S, Arlin Z, Fialkow PJ: Differences in the frequency of normal and clonal precursors of colony-forming cells in chronic myelogenous leukemia and acute myelogenous leukemia. *Blood* 79:1811, 1992
7. Scheinberg DA, Lovett D, Divgi CR, Graham MC, Bernan E, Pentlow K, Feirt N, Finn RD, Clarkson BD, Gee TS, Larson SM, Oettinger HF, Old LJ: A phase I trial of monoclonal antibody M195 in acute myelogenous leukemia: Specific bone marrow targeting and internalization of radiolabeled. *J Clin Oncol* 9:478, 1991
8. Appelbaum FR, Matthews DC, Eary JF, Badger CC, Kellogg M, Press OW, Martin PJ, Fisher DR, Nelp WB, Thomas ED, Bernstein ID: The use of radiolabeled anti-CD33 antibody to augment marrow irradiation prior to marrow transplantation for acute myelogenous leukemia. *Transplantation* 54:829, 1992
9. van der Jagt RH, Badger CC, Appelbaum FR, Press OW, Matthews DC, Eary JF, Krohn KA, Bernstein ID: Localization of radiolabeled antimyeloid antibodies in a human acute leukemia xenograft tumor model. *Cancer Res* 52:89, 1992
10. Zein N, Sinha AM, McGahren WJ, Ellestad GA: Calicheamicin gamma II: an antitumor antibiotic that cleaves double-stranded DNA site specifically. *Science* 240:1198, 1988
11. Leith CP, Chen IM, Kopecky KJ, Appelbaum FR, Head DR, Godwin JE, Weick JK, Willman CL: Correlation of multidrug resistance (MDR1) protein expression with functional dye/drug efflux in acute myeloid leukemia by multiparameter flow cytometry: Identification of discordant MDR-efflux+ and MDR1+efflux-cases. *Blood* 86:2329, 1995
12. Fialkow PJ, Singer JW, Raskind WH, Adamson JW, Jacobson RJ, Bernstein ID, Dow LW, Najfeld V, Veith R: Clonal development, stem-cell differentiation, and clinical remissions in acute nonlymphocytic leukemia. *N Engl J Med* 317:468, 1987
13. Mehrotra B, George TI, Kavanau K, Avet-Loiseau H, Moore DN, Willman CL, Slovak ML, Atwater S, Head DR, Pallavicini MG: Cytogenetically aberrant cells in the stem cell compartment (CD34+lin-) in acute myeloid leukemia. *Blood* 86:1139, 1995
14. Haase D, Feuring-Buske M, Konemann S, Fonatsch C, Troff C, Verbeek W, Pekrun A, Hiddemann W, Wormann B: Evidence for malignant transformation in acute myeloid leukemia at the level of early

hematopoietic stem cells by cytogenetic analysis of CD34+ subpopulations. *Blood* 86:2906, 1995

15. Bonnet D, Dick JE: Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nature Med* 3:730, 1997

16. Niederwieser DW, Appelbaum FR, Gastl G, Gersdorf E, Meister B, Geissler D, Tratkiewicz JA, Thaler J, Huber C: Inadvertent transmission of a donor's acute myeloid leukemia in bone marrow transplantation for chronic myelocytic leukemia. *N Engl J Med* 322:1794, 1990

17. Sievers EL, Larson RA, Estey E, Stadmauer E, Berger M, Eten C, Bernstein I, Appelbaum F: Interim analysis of the efficacy and safety of CMA-676 in patients with AML in first relapse. *Blood* 92:613a, 1998 (suppl)

18. Jurcic JG, DeBlasio A, Dumont L, Warrell RP Jr, Scheinberg DA: Molecular remission induction without relapse after anti-CD33 monoclonal antibody HuM195 in acute promyelocytic leukemia (APL). *Blood* 90:416a, 1997 (suppl)

19. Caron PC, Dumont L, Scheinberg DA: Supersaturating infusion of humanized anti-CD33 monoclonal antibody HuM195 in myelogenous leukemia. *Clin Cancer Res* 4:1421, 1998

20. Robertson MJ, Solifer RJ, Freedman AS, Rabinowe SL, Anderson KC, Ervin TJ, Murray C, Dear K, Griffin JD, Nadler LM, Ritz J: Human bone marrow depleted of CD33-positive cells mediates delayed but durable reconstitution of hematopoiesis: Clinical trial of MY9 monoclonal antibody-purged autografts for the treatment of acute myeloid leukemia. *Blood* 79:2229, 1992

Registro de la Propiedad Industrial

Dirección de Invenciones y Nuevas Tecnologías

TITULO N° 6145

La Dirección de Invenciones y Nuevas Tecnologías del Indecopi certifica que por mandato de la Resolución N° 001597-2011/DIN-INDECOPI de fecha 13 de septiembre de 2011, ha quedado inscrita en el Registro de Patentes de Invención, la siguiente invención:

Denominación : ANTICUERPOS ANTI-5T4

Clasificación : C07K 16/44; A61K 39/395; C07K 16/30

Solicitud : 000262-2007

Fecha de Presentación : 09 de marzo de 2007

Fechas de Prioridades : 10 de marzo de 2006; 23 de febrero de 2007

Titular : WYETH

País : Estados Unidos de América

Inventores : ERWIN R. BOGHAERT; NITIN K. DAMLE; PHILIP
ROSS HAMANN; KIRAN KHANDKE; ARTHUR KUNZ;
KIMBERLY A. MARQUETTE; LIUDMILA
TCHISTIAKOVA; DAVINDER GILL; KODANGATTIL R.
SREEKUMAR

Vigencia : 09 de marzo de 2027



BRUNO MÉRCHOR VALDERRAMA
Director de Invenciones y
Nuevas Tecnologías
INDECOPI

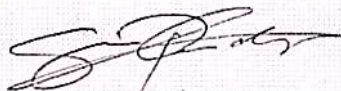


INDECOPI
DIRECCION DE INVENCIONES Y NUEVAS TECNOLOGIAS
CERTIFICA:

Que la presente copia es exactamente
igual al original que he tenido a la vista y
confrontado.

Lima,

03 ABR. 2012



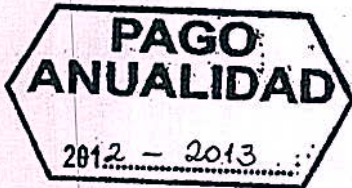
SERGIO RODRIGUEZ SORIA
Sub Director de Invenciones
y Nuevas Tecnologías
INDECOPI



PERÚ

Presidencia
del Consejo de Ministros

INDECOPI



TITULO N° 6145

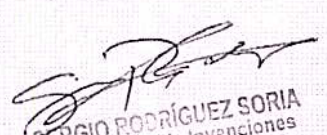


INDECOPI
DIRECCION DE INVERSIONES Y NUEVAS TECNOLOGIAS
CERTIFICA:

Que la presente copia es exactamente
igual al original que he tenido a la vista y
confrontado.

Lima,

03 ABR:2012..


SERGIO RODRÍGUEZ SORIA
Sub Director de Inversiones
y Nuevas Tecnologías
INDECOPI



PERÚ

Presidencia
del Consejo de Ministros

INDECOPI

000825

DIRECCIÓN DE INVENCIÓNES Y NUEVAS TECNOLOGÍAS

EXPEDIENTE N° 000262-2007/OIN

RESOLUCION N° 001597-2011/DIN-INDECOPI

Lima, 13 de septiembre de 2011

Patente de invención: Concedida

Mediante expediente N° 000262-2007/OIN, iniciado el 09 de marzo de 2007, con prioridades de fechas 10 de marzo de 2006 y 23 de febrero de 2007, WYETH de Estados Unidos de América, solicita patente de invención para "ANTICUERPOS ANTI-5TA Y SUS USOS", C.I.P.7 C07K 16/44; A61K 39/395; C07K 16/30, cuyos inventores son ERWIN R. BOGHAERT; NITIN K. DAMLE; PHILIP ROSS HAMANN; KIRAN KHANDKE; ARTHUR KUNZ; KIMBERLY A. MARQUETTE; LIOUDMILA TCHISTIAKOVA; DAVINDER GILL y KODANGATTIL R. SREEKUMAR.

1. CUESTION PREVIA

Mediante Informe Técnico CJE N° 27-2010 que corre de fojas 715 a 750 del expediente, se considera que el título de lo solicitado debe ser "ANTICUERPOS ANTI-5T4", por definir mejor el objeto a proteger mediante sus reivindicaciones

2. EXAMEN DE PATENTABILIDAD

La invención solicitada reúne los requisitos establecidos en la Decisión 486 de la Comisión de la Comunidad Andina que aprueba el Régimen Común sobre Propiedad Industrial, conforme aparece del informe que corre de fojas 816 a 822 del expediente.

La presente resolución se emite en aplicación de la norma legal antes mencionada y en uso de las facultades conferidas por los artículos 37 y 40 de la Ley de Organización y Funciones del Instituto Nacional de Defensa de la Competencia y de la Protección de la Propiedad Intelectual (INDECOPI) sancionada por Decreto Legislativo N° 1033, concordado con el artículo 4 del Decreto Legislativo 1075 que aprueba las disposiciones complementarias a la Decisión 486 de la Comisión de la Comunidad Andina.

INSTITUTO NACIONAL DE DEFENSA DE LA COMPETENCIA Y DE LA PROTECCIÓN DE LA PROPIEDAD INTELECTUAL
Calle De la Prosa 104, San Borja, Lima 41 - Perú Telf: 224 7800 / Fax: 224 0348
E-mail: postmaster@indecopi.gob.pe / Web: www.indecopi.gob.pe

M-DIN-14/1C



INDECOP
DIRECCIÓN DE INNOVACIONES Y NUEVAS TECNOLOGÍAS
CERTIFICA:

Que la presente copia es exactamente
igual al original que he tenido a la vista y
confrontado.

Lima,

03 ABR. 2012


SERGIO RODRÍGUEZ SOTUA
Sub Director de Innovaciones
y Nuevas Tecnologías
INDECOP

Y NUEVAS TECNOLOGÍAS
:OPI



PERÚ

Presidencia
del Consejo de Ministros

INDECOPI

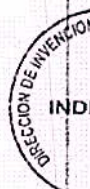
000826

3. RESOLUCION DE LA DIRECCION DE INVENCIONES Y NUEVAS TECNOLOGIAS

OTORGAR, patente de invención para "ANTICUERPOS ANTI-5T4", C.I.P.7 C07K 16/44; A61K 39/395; C07K 16/30, a favor de WYETH de Estados Unidos de América, por un plazo de veinte (20) años, contados desde el 09 de marzo de 2007, fecha de presentación de la solicitud, aprobándose las 28 reivindicaciones que corren de fojas 811 a 813 del expediente.

Regístrese y Comuníquese

BRUNO MÉRCHOR VALDERRAMA
Director de Invenciones y
Nuevas Tecnologías
INDECOPI



INSTITUTO NACIONAL DE DEFENSA DE LA COMPETENCIA Y DE LA PROTECCIÓN DE LA PROPIEDAD INTELECTUAL
Calle De la Prosa 104, San Borja, Lima 41 - Perú Telf: 224 7800 / Fax: 224 0348
E-mail: postmaster@indecopi.gob.pe / Web: www.indecopi.gob.pe

M-DIN-14/1C

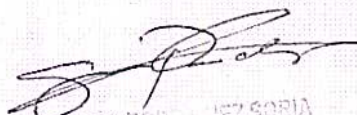


INDECOPI
DIRECCION DE INVENCIONES Y NUEVAS TECNOLOGIAS
CERTIFICA:

Que la presente copia es exactamente igual al original que he tenido a la vista y confrontado.

Lima,

03 ABR. 2012


SERGIO RODRÍGUEZ SORIA
Sub Director de Invencciones y Nuevas Tecnologías
INDECOPI

INVENIONES Y NUEVAS TECNOLOGIAS
COPI

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



RECIBO DE CAJA

No. 12 - 122628

Bogotá D.C., Noviembre 27 de 2012 - 12:10:02

RECIBIDO DE : BAKER & MCKENZIE

NI 900.034.906

RE

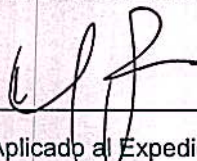
*** Soporte del Pago ***

TIPO PAGO	BANCO	CUENTA	No. PAGO	FECHA PAGO	VR PAGO
RECIBO DE CA	999999999	118537	15/11/2012		590.000.00
RECIBO DE CA	999999999	118651	15/11/2012		100.000.00

*** Conceptos Pagados ***

CANT. RENTISTICO	CONCEPTO	Vr.UNDITARIO	Vr.CONCEPTO
23 50005-01-01 SOLICITUDES	1607 REIVINDICACION UNITARIA ADICIONAL A LAS 10 INICIALES	30.000.00	690.000.00
			\$690.000.00

SON: **SEISCIENTOS NOVENTA MIL PESOS MONEDA CORRIENTE**

Responsable: 

Recibo de Caja Aplicado al Expediente No. _____

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



No. 08-095703- -00006-0000

Fecha: 2012-11-27 16:16:52
Tra. 11 PATENTEIYII
Act. 523 RESPUESTA

Dep. 2020 DIR.NUEVASCR
Eve: 378 FASENACIONALI
Folios: 59 60

Sede Centro: Carrera 13 No. 27 - 00 Pisos 3,4,5 y 10 Bogotá, D.C.- Colombia

Web: www.sic.gov.co e-mail: info@sic.gov.co Conmutador: (571) 5870000 Fax: (571) 5870284 Línea: 018000-910165 Call Center: (571) 6513240

Noviembre 27 de 2012 - 12:10:06