

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO			
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DEP:	2020 DIRECCION DE NUEVAS CREACIONES	EVE:	378 FASE NACIONAL INCLUIDO CAPITULO I
TRA:	11 PCT-PATENTE DE INVENCION CAPITULOS I Y II	FOLIOS:	7
ACT:	519 PRESENTACION		

DIRECCIÓN DE NUEVAS CREACIONES

2020
Bogotá, D.C.,

Abogado(a)/Señor(a)
DILIA MARIA RODRIGUEZ D´ALEMAN
NOTIFICACIONES@CLARKEMODET.COM

Asunto: Radicación: 10-41193- -5
 Trámite: 11
 Evento: 378
 Actuación: 519
 Oficio: 21430
 Folios: 7

Patente de Invención	X			Patente de Modelo de Utilidad	
Vía Nacional					
Vía PCT (Fase Nacional)	X	Capítulo I	X	Capítulo II	
No. Solicitud Internacional	PCT/JP2008/002690				
Fecha de Presentación Internacional	26/09/2008				

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

1. Documentos estudiados

Descripción:	Folios 15 a 169	9/Abril/2010
Reivindicaciones:	Folios 170 a 189	9/Abril/2010
Listado de Secuencias:	Folios 190 a 203	9/Abril/2010
Dibujos:	Folios 204 a 212	9/Abril/2010
Prioridad:	JP 2007-256063	28/Septiembre/2007

2. Análisis de la Prioridad

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

3. Objeto de la invención

De acuerdo con lo establecido en el numeral 1.2.2.2 del capítulo primero del Título 10 de la Circular Única para efectos del examen de patentabilidad, se tendrá en cuenta la

tasa pagada por el solicitante al momento de comenzar el examen para las reivindicaciones adicionales; esto es que cuando la solicitud contenga más de diez (10) reivindicaciones y no se haya pagado la tasa complementaria, sólo se estudiarán las cubiertas por la tasa pagada.

Título de la solicitud: “Anticuerpo anti-glipican-3 que tiene cinéticas mejoradas en el plasma”.

El problema planteado en esta solicitud consiste en modular la vida media en plasma de un anticuerpo anti-glipican-3.

Para resolver este problema técnico la solicitud en estudio proporciona un método para preparar un anticuerpo anti-glipican-3 en el que el anticuerpo tiene una secuencia de aminoácido alterada para originar una modificación en la carga de al menos un residuo de aminoácido que se puede exponer sobre la superficie del anticuerpo.

El objeto de la presente Solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): A61K 39/395; A61P 35/00; C12N 1/15, 1/21, 15/09, 1/19, 5/10; C12P 21/08.

4. No es una invención (Art 15 D 486 literal (b))

El objeto de las reivindicaciones 63 y 64 no se considera una invención, de acuerdo con el Art citado, porque hace referencia a una célula de huésped que comprende un ácido nucleico de acuerdo con la reivindicación 62. Sin embargo, esta célula puede corresponder a parte de un ser vivo que comprende un ácido nucleico que codifica para un anticuerpo anti-glipican 3.

5. De la Solicitud de Patente

Reivindicaciones (Art 30 D 486, Art 22 PCT)

El capítulo reivindicatorio no es conciso porque contiene un excesivo número de reivindicaciones independientes, lo cual hace difícil definir cuál es el objeto que se busca proteger.

El capítulo reivindicatorio carece de concisión porque utiliza las expresiones “un anticuerpo anti-glipican 3”, “cultivar una célula huésped (...) bajo condiciones que permiten la expresión del ácido nucleico”, “recuperar el anticuerpo anti-glipican 3 del cultivo de la célula huésped”, “una secuencia de aminoácido alterada para originar una modificación en la carga de al menos un residuo de aminoácido que se puede exponer sobre la superficie del anticuerpo”, “sustitución de aminoácido”, “localizado en una región del anticuerpo de anti-glipican 3 diferente de la región de unión FcRn”, “donde el residuo de aminoácido cuya carga presente en la región variable de cadena pesada o en la región variable de cadena ligera”, “donde el anticuerpo comprende una región de determinación de complementariedad (CDR) derivada de un animal o humano, una región o estructura (FR) derivada del humano, y una región constante derivada del humano (...)”, “donde la modificación en la carga del residuo de aminoácido se logra mediante la sustitución de al menos un residuo de aminoácido que se puede exponer sobre la superficie (...)”, “donde la modificación en la carga del residuo de aminoácido se logra mediante al menos una sustitución en la región variable de cadena pesada (...)”, “al menos una sustitución en la región variable de cadena ligera (...)”, “donde el anticuerpo anti-glipican 3 tiene un contenido reducido de mucosa unido a la región Fc del anticuerpo”, “un anticuerpo”, “un ácido nucleico”, lo cual dificulta determinar la extensión del objeto que se busca proteger.

Para las expresiones “un anticuerpo anti-glipican 3”, “donde el anticuerpo comprende una región de determinación de complementariedad (CDR) derivada de un animal o humano, una región o estructura (FR) derivada del humano, y una región constante derivada del humano (...)”, “donde el anticuerpo anti-glipican 3 tiene un contenido reducido de mucosa unido a la región Fc del anticuerpo”, “un anticuerpo”, “un ácido nucleico”, se recuerda al solicitante que un ácido nucleico y un polipéptido se caracterizan por sus secuencias de polinucleótidos y aminoácidos, respectivamente. Así mismo, un anticuerpo se caracteriza por la secuencia de aminoácidos de las regiones variables de las cadenas pesada y ligera o la secuencia de aminoácidos de las seis regiones determinantes de complementariedad (CDRs), en su conjunto.

Para las expresiones “cultivar una célula huésped (...) bajo condiciones que permiten la expresión del ácido nucleico”, “recuperar el anticuerpo anti-glipican 3 del cultivo de la célula huésped”, se requiere definir las condiciones en las que se llevan a cabo las etapas de cultivo y recuperación del anticuerpo.

Para las expresiones “una secuencia de aminoácido alterada para originar una modificación en la carga de al menos un residuo de aminoácido que se puede exponer sobre la superficie del anticuerpo”, “sustitución de aminoácido”, “localizado en una región del anticuerpo de anti-glipican 3 diferente de la región de unión FcRn”, “donde el residuo de aminoácido cuya carga presente en la región variable de cadena pesada o en la región variable de cadena ligera”, “donde la modificación en la carga del residuo de aminoácido se logra mediante la sustitución de al menos un residuo de aminoácido que se puede exponer sobre la superficie (...)”, “donde la modificación en la carga del residuo de aminoácido se logra mediante al menos una sustitución en la región variable de cadena pesada (...)”, “al menos una sustitución en la región variable de cadena ligera (...)”, se requiere definir el tipo de sustitución y la localización en la secuencia del polipéptido.

Las reivindicaciones 2 y 16 utilizan la expresión “donde la modulación de las genéticas del plasma se incrementa o disminuye en un parámetro seleccionado desde la vida media del plasma, el tiempo de residencia medio del plasma, y la distancia del plasma”, que es un resultado a alcanzar y no define al anticuerpo puesto que la reivindicación incluiría no sólo la solución propuesta por el solicitante, sino todas las alternativas presentes o futuras que lleguen a ese resultado. Se sugiere reemplazarlo por las características esenciales o estructurales que contribuyen a la obtención de ese efecto, como la secuencia de aminoácidos de las regiones variables de las cadenas pesada y ligera o la secuencia de aminoácidos de las seis regiones determinantes de complementariedad (CDRs).

La reivindicación 45 define el anticuerpo porque comprende una región variable de cadena pesada y/o una región variable de cadena ligera. No se encuentra soporte en la descripción que un anticuerpo que comprenda solamente una región variable de cadena pesada o ligera comparta la actividad del anticuerpo reclamado. Se requiere al solicitante definir el anticuerpo por la secuencia de aminoácidos de las regiones variables de las cadenas pesada y ligera o la secuencia de aminoácidos de las seis regiones determinantes de complementariedad (CDRs).

Se sugiere al solicitante corregir errores de traducción y tipográficos como “modulación de las genéticas del plasma” y “distancia del plasma”, de la reivindicación 2, el término “numeración Rabat” de las reivindicaciones 6 y 21 y la expresión “contenido reducido de mucosa”, de las reivindicaciones 13 y 58.

Se requiere al Solicitante hacer las aclaraciones necesarias.

6. Unidad de invención (Art 25 D 486)

La solicitud no cumple con el requisito de unidad de invención porque se refiere a 8 grupos inventivos.

Los grupos inventivos están definidos por las siguientes características **esenciales**:

- 1° Método para preparar un anticuerpo anti-glipican 3 de acuerdo a la reivindicación 1. Reivindicaciones 1 – 13 y 14.
- 2° Método para preparar un anticuerpo con una cinética de plasma modulado de acuerdo a la reivindicación 15. Reivindicaciones 15 – 23.
- 3° Método para estabilizar un anticuerpo anti-glipican 3 de acuerdo a la reivindicación 24. Reivindicaciones 24 – 28.
- 4° Método para preparar un anticuerpo con citotoxicidad modulada de acuerdo a la reivindicación 29. Reivindicaciones 29 – 42, 43 y 44.
- 5° Un anticuerpo de acuerdo a la reivindicación 45. Reivindicaciones 45 – 51 y 58 – 65, parcialmente.
- 6° Un anticuerpo de acuerdo a la reivindicación 52. Reivindicaciones 52 – 55 y 58 – 65, parcialmente.
- 7° Un anticuerpo de acuerdo a la reivindicación 56. Reivindicaciones 56 y 58 – 65, parcialmente.
- 8° Un anticuerpo de acuerdo a la reivindicación 57. Reivindicaciones 58 – 65, parcialmente.

No se guarda un concepto común inventivo porque los anticuerpos de los grupos inventivos 5 – 8 y los métodos para preparar los anticuerpos de los grupos inventivos 1 – 4 no comparten una estructura esencial para la actividad común.

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

7. Determinación del Estado de la Técnica

El estado de la técnica se determinó con base en la fecha de presentación de la solicitud de prioridad: septiembre 28 de 2007, y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

Ítem	Documento	Título	Fecha de Publicación
D1	EP1816140	Anti-glypican 3 antibody having modified sugar chain	8/Agosto/2007
D2	EP0329185	Alteration of pharmacokinetics of proteins by charge modification	23/Agosto/1989

El documento D1¹ divulga un método para preparar un anticuerpo anti-glipican 3 que comprende las etapas de: a) cultivar una célula huésped que lleva un ácido nucleico que codifica al anticuerpo anti-glipican 3 bajo condiciones que permiten la expresión del ácido nucleico y b) recuperar el anticuerpo anti-glipican 3 del cultivo de la célula huésped (Pág. 2, párrafo 8 y Pág. 8, párrafo 39).

El documento D2² enseña un método para preparar un anticuerpo con cinéticas de plasma mejorada que comprende alterar la secuencia de aminoácidos del anticuerpo para originar una modificación en la carga de al menos un residuo de aminoácido (Pág. 2, líneas 3-6 y Pág. 7, líneas 1-15).

1http://worldwide.espacenet.com/publicationDetails/biblio?CC=EP&NR=1816140A1&KC=A1&FT=D&ND=4&date=20070808&DB=EPODOC&locale=en_EP
2http://worldwide.espacenet.com/publicationDetails/biblio?CC=EP&NR=0329185A2&KC=A2&FT=D&ND=4&date=19890823&DB=EPODOC&locale=en_EP

8. Examen de Patentabilidad (Art 14 D486)

Grupo Inventivo 1

Nivel Inventivo (Art18 D486)

La reivindicación 1 consiste en un método para preparar un anticuerpo anti-glipican 3 es con cinéticas de plasma modulado, dicho método comprende las etapas de:

- (a) cultivar una célula huésped que lleva un ácido nucleico que codifica al anticuerpo anti-glipican 3 bajo condiciones que permiten la expresión del ácido nucleico, en donde el anticuerpo anti-glipican 3 tiene una secuencia de aminoácido alterada para originar una modificación en la carga de al menos un residuo de aminoácido que se puede exponer sobre la superficie del anticuerpo;
- (b) recuperar el anticuerpo anti-glipican 3 del cultivo de la célula huésped.

Comparación de las características técnicas esenciales, con las del Estado de la Técnica:

Características esenciales	D1	D2
Un método para preparar un anticuerpo anti-glipican 3 es con cinéticas de plasma modulado, dicho método comprende las etapas de: (a) cultivar una célula huésped que lleva un ácido nucleico que codifica al anticuerpo anti-glipican 3 bajo condiciones que permiten la expresión del ácido nucleico, en donde el anticuerpo anti-glipican 3 tiene una secuencia de aminoácido alterada para originar una modificación en la carga de al menos un residuo de aminoácido que se puede exponer sobre la superficie del anticuerpo; (b) recuperar el anticuerpo anti-glipican 3 del cultivo de la célula huésped.	Un método para preparar un anticuerpo anti-glipican 3 que comprende las etapas de: a) cultivar una célula huésped que lleva un ácido nucleico que codifica al anticuerpo anti-glipican 3 bajo condiciones que permiten la expresión del ácido nucleico; b) recuperar el anticuerpo anti-glipican 3 del cultivo de la célula huésped. (Pág. 2, párrafo 8 y Pág. 8, párrafo 39)	Un método para preparar un anticuerpo con cinéticas de plasma mejorada que comprende alterar la secuencia de aminoácidos del anticuerpo para originar una modificación en la carga de al menos un residuo de aminoácido. (Pág. 2, líneas 3-6 y Pág. 7, líneas 1-15)

Los documentos D1 y D2 se consideran el estado de la técnica cercano a la invención definida en la reivindicación 1 porque:

El documento D1 enseña un método para preparar un anticuerpo anti-glipican 3 que comprende las etapas de: a) cultivar una célula huésped que lleva un ácido nucleico que codifica al anticuerpo anti-glipican 3 bajo condiciones que permiten la expresión del ácido nucleico y b) recuperar el anticuerpo anti-glipican 3 del cultivo de la célula huésped (Pág. 2, párrafo 8 y Pág. 8, párrafo 39).

La diferencia entre la invención, definida en la reivindicación 1 y el método del documento D1 consiste en que el anticuerpo tiene una secuencia alterada para originar una modificación en la carga.

El efecto que logra el incluir esta modificación en la secuencia de aminoácidos es que permite modular la cinética de plasma del anticuerpo.

Por lo tanto, el problema técnico objetivo que pretende resolver esta invención se puede formular así: “cómo modificar el método conocido en el documento D1 para mejorar la

cinética de plasma del anticuerpo”.

Sin embargo, la utilización de modificaciones en la secuencia de un anticuerpo para mejorar la cinética de plasma, ya está divulgada en el documento D2 que se refiere a un método para preparar un anticuerpo con cinéticas de plasma mejorada que comprende alterar la secuencia de aminoácidos del anticuerpo para originar una modificación en la carga de al menos un residuo de aminoácido (Pág. 2, líneas 3-6 y Pág. 7, líneas 1-15).

En consecuencia, la persona del oficio normalmente versada en la materia, buscaría originar una modificación de la carga de al menos un residuo de aminoácido del anticuerpo en el método del documento D1 para llegar así al objeto de la reivindicación 1 de la solicitud, por lo cual se considera obvia. La persona del oficio normalmente versada en la materia se vería motivada a inducir modificaciones en la carga del anticuerpo del documento D1 porque el documento D2 enseña que la habilidad de incrementar la vida media en suero de una proteína tiene un impacto sustancial en la eficacia terapéutica de dicha proteína (Pág. 4, líneas 38-41), lo cual se puede lograr al modificar la carga del anticuerpo hacia un punto isoeléctrico más ácido (Pág. 7, líneas 4-15) y porque es bien conocido en el estado del arte que modificaciones introducidas en las regiones variables de anticuerpos, y que disminuyen el punto isoeléctrico con respecto a la proteína nativa, permiten modular la cinética plasmática del anticuerpo³.

De manera que la solución propuesta por esta solicitud en la reivindicación 1 y en las reivindicaciones 2 – 13 y 14 es obvia y no tiene nivel inventivo.

9. Conclusión

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

Ítem	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	EP1816140	1 – 14	Nivel Inventivo
D2	EP0329185	1 –14	Nivel Inventivo

³ G. Pavlinkova et al. Charge-modified single chain antibody constructs of monoclonal antibody CC49: generation, characterization, pharmacokinetics, and biodistribution analysis. Nuclear medicine and Biology, Vol. 26, Pág. 27-34. Publicado en 1999.

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

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

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

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



JOSÉ LUIS SALAZAR LÓPEZ
Director de Nuevas Creaciones (c)

El anterior requerimiento fue notificado por fijación en lista, de fecha 2012-11-27

Elaboró: Moncaleano Prado Juan David

Revisó: Consuelo Leguizamon Leguizamon



Charge-Modified Single Chain Antibody Constructs of Monoclonal Antibody CC49: Generation, Characterization, Pharmacokinetics, and Biodistribution Analysis

Gabriela Pavlinkova,¹ Guy Beresford,¹ Barbara J. M. Booth,¹ Surinder K. Batra²
and David Colcher¹

DEPARTMENTS OF ¹PATHOLOGY AND MICROBIOLOGY AND ²BIOCHEMISTRY AND MOLECULAR BIOLOGY, UNIVERSITY OF NEBRASKA MEDICAL CENTER, OMAHA, NEBRASKA 68198, USA

ABSTRACT. A novel strategy was developed in which an antibody scFv fragment of the monoclonal antibody (MAb) CC49 was modified by engineering DNA coding sequences to lower its isoelectric point. Negatively charged amino acids were added to the carboxy terminus of the CC49 V_H region by adding nucleotide sequences in a polymerase chain reaction (PCR) amplification of the coding sequence of CC49 scFv. Two new DNA constructs coding for CC49 scFv with lower isoelectric points of 5.8 and 5.2 were engineered. These novel strategy-generated, charge-modified antibody constructs were compared for their immunological, pharmacokinetic, and biodistribution properties in athymic mice bearing LS-174T human colon carcinoma xenografts. NUCL MED BIOL 26;1:27–34, 1999. © 1998 Elsevier Science Inc.

KEY WORDS. Single chain fragments, Isoelectric point, Antibody engineering, Radioimmunodiagnosis, Colon carcinoma xenografts

INTRODUCTION

The primary effect of antibodies is through their interaction with antigen-specific binding sites; however, antibodies also interact *in vivo* with other structures by nonspecific electrostatic interactions. The weak ionic interactions can increase the propensity for antibodies either to be phagocytosed by the reticuloendothelial cells of the liver, or to be bound to the highly charged glomerular basement membranes of the kidneys (29). It has been shown previously that IgG fragments are cleared from the blood pool primarily by glomerular filtration (30). High liver and spleen tissue levels (as compared with other organs) are observed with radiolabeled immunoglobulin molecules and high kidney levels when using antibody fragments. These interactions are potentially problematic in terms of imaging and therapy.

Several strategies are being investigated to overcome undesirable elevations in renal uptake of radiolabeled antibody fragments. Preclinical studies by some investigators showed that intravenous (IV) infusion of lysine and arginine blocked negatively charged sites on the surface of renal tubule and prevented binding and subsequent absorption of radiolabeled Fab fragments (1, 23) or a disulfide-stabilized Fv fragment (dsFv) (11). Chemically modified antibody fragment molecules, negatively charged synthetic polymer (DTPA-succinylated polylysine) coupled to antimyosin R11D10 Fab, showed similar blood clearance but reduced hepatic and renal concentrations in comparison with the parental Fab (9). Acylation of amino groups in anti-Tac dsFv antibody resulted in lowered pI and reduced renal uptake; however, the chemical reactions decreased dsFv ability to bind antigen by 25–30% (10). The standard chemical conjugation methods modify amino acid side chains in an

uncontrollable and random fashion, which can reduce the immunoreactivity of modified antibody.

The monoclonal antibody (MAb) CC49 reacts with a unique Sialyl-Tn antigen expressed on tumor-associated mucin, TAG-72 (19). CC49 exhibits high reactivity with tumor cells in lesions from colorectal, ovarian, mammary, gastric, and pancreatic cancer (17). Radiolabeled CC49 has shown excellent tumor localization in several ongoing clinical trials. Rapid clearance and efficient tumor penetration of the CC49 single chain Fv fragment (scFv) are attractive characteristics for diagnostic and possibly therapeutic applications (16, 31).

In this report, we have utilized a novel strategy to engineer the DNA coding sequences of CC49 scFv to generate two molecules with predicted isoelectric points lower than that of native CC49 scFv. Negatively charged amino acids were added to the carboxy terminus of the CC49 V_H region by adding the appropriate nucleotide sequences in a polymerase chain reaction (PCR) amplification of the CC49 V_L-linker-CC49 V_H DNA. The CC49 scFvs, with predicted pI 8.1 (native molecule: scFv[8.1]), pI 5.8 (scFv[5.8]), and pI 5.2 (scFv[5.2]), were analyzed for biochemical and immunological characteristics, as well as their pharmacokinetic and biodistribution properties.

MATERIALS AND METHODS

Purification of MAb CC49 IgG

MAb CC49 IgG was developed by the immunization of mice with purified TAG-72 as described previously (4, 19). CC49 IgG was purified from ascitic fluid using Protein G Sepharose 4 Fast Flow (Pharmacia, Piscataway, NJ). The protein concentration was determined by the method of Lowry *et al.* (13).

Construction of the Expression Vector

The CC49 scFv[8.1] was constructed through a collaborative effort of the National Cancer Institute Laboratory of the Tumor Immu-

Address correspondence to: Dr. David Colcher, Department of Pathology and Microbiology, University of Nebraska Medical Center, 600 South 42nd Street, Omaha, NE 68198-3135, USA; e-mail: dcolcher@unmc.edu.

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nology and Biology and the Dow Chemical Company (Midland, MI). The CC49 scFv cDNA (V_L -Linker- V_H) was constructed using the PCR method by combining the V_L region sequence and the V_H region sequence via a linker sequence (18). The previously published 205C linker (20) was modified (changing the terminal amino acids) resulting in the sequence: LSADDAKKDAKKDDAKKD DAKKDL and used for the scFv construction. The sequences of the variable domains of the CC49 have been reported by Rixon *et al.* (25). The fragment was cloned into the pRW83 vector that contains a chloramphenicol resistance gene for recombinant selection, *penP* gene with a *penP* promoter and terminator, and a *pelB* signal peptide (15). Two new genes for scFvs were constructed according to amino acid sequence for isoelectric points of 5.8 and 5.2 as predicted using a computer PROTEIN-TITRATE program available through DNASTAR (Madison, WI). The sequence of the constructs was confirmed by DNA sequencing.

CC49 scFv Protein Purification

The expression plasmids containing CC49 scFv sequences were transformed into *Escherichia coli* strain BL21 (Stratagene, La Jolla, CA) by following the vendor's instructions. The bacteria were grown at 37°C in LB medium containing 34 µg/mL chloramphenicol. The CC49 scFv protein was purified from the periplasmic fraction of 1.0-L overnight cultures. Pelleted cells were resuspended in 100 mL of 30 mM Tris-HCl (pH 7.3), 100 mL of 30 mM Tris-HCl, 40% w/v sucrose, and 10 mM ethylene diamine tetraacetic acid (EDTA). After 10 min incubation at room temperature (RT), the cells were centrifuged at $5,000 \times g$, resuspended in ice cold 0.5 mM $MgCl_2$, incubated on ice for 10 min, and recentrifuged. The supernatant containing the *E. coli* periplasmic fraction was filtered through a 0.2-µm filter (Nalge Co., Rochester, NY). The filtrate containing scFv[8.1] was dialyzed against 20 mM bis-Tris propane (pH 9.1) and applied to a Mono-Q column (Pharmacia). The CC49 scFv was eluted with 40 mM sodium chloride in 20 mM bis-Tris propane (pH 9.1). The filtrates containing scFvs with pI 5.8 and pI 5.2 were dialyzed against 20 mM bis-Tris propane (pH 6.8) and 20 mM MES (pH 6.2), respectively, and applied to a Mono Q column. The CC49 scFv[5.8] was eluted by 75 mM sodium chloride in 20 mM bis-Tris propane (pH 6.8) and scFv[5.2] was eluted by 250 mM sodium chloride in 20 mM MES (pH 6.2). Eluted fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and by enzyme-linked immunosorbent assay (ELISA). The concentration of purified fragments was determined by the method of Lowry *et al.* (13).

SDS-PAGE

The purity and integrity of CC49 scFvs were analyzed by SDS-PAGE (15%) under reducing and nonreducing conditions (12). Proteins were visualized by staining with Coomassie blue. The molecular weights of the samples were estimated by comparison to the R_f (relative mobility) values of standards of known molecular weights.

High Performance Liquid Chromatography (HPLC) Analysis

Samples were injected onto TSK G3000SW and TSK G2000SW (Toso Haas, Japan) size exclusion columns connected in series and eluted with 0.067 M phosphate and 0.1 M KCl buffer (pH 6.8) at a flow rate of 0.5 mL/min. Elution of the proteins was monitored by

ultraviolet (UV) absorption at 280 nm or by determining the radioactivity in the eluted fractions.

pI Determination of CC49 scFvs

The pI of folded scFv proteins was determined by isoelectric focusing (IEF). Five microliters of sample (2–5 mg/mL) were applied onto IsoGel agarose IEF plates (FMC Bioproducts, Rockland, ME) of pH range 3–10 or 3–7 and IEF was performed following the manufacturer's instructions. Immediately after focusing, the protein bands were fixed, washed, and silver stained using Ag Stain™ silver stain kit (Isolab, Akron, OH). Standards with isoelectric points ranging between 9.3 and 3.6 (Sigma, St. Louis, MO) and 9.6 and 4.45 (Bio-Rad, Hercules, CA) were used to calculate the pI of the samples.

Labeling of CC49 Ig Forms and scFv

MAb CC49 IgG and the scFv forms were labeled with $Na^{125}I$ or $Na^{131}I$ using Iodo-Gen (Pierce Chemical, Rockford, IL) as described by Colcher *et al.* (5). The iodination protocol yielded specific activities of approximately 3–6 mCi/mg.

Solid Phase Competition ELISA

A competition ELISA was used to detect and quantify the specific immunoreactivity of MAb CC49 IgG and scFv forms at various steps in the purification process. The plates were coated with bovine submaxillary mucin (BSM), which contains the epitope recognized by MAb CC49, (50 ng/well) as described previously (21). Five microliters of the test samples were added to prepared plates in threefold serial dilutions and incubated with 6 ng of biotinylated CC49 IgG for 2 h at RT. After washing, the plates were incubated with alkaline phosphatase-conjugated streptavidin (Jackson ImmunoResearch, West Grove, PA) for 1.5 h and then after washing developed with *p*-nitrophenyl phosphate substrate. The optical density was measured using a Dynatech MR 5000 automatic 96-well microtiter reader. Ab levels were determined by comparison to a CC49 IgG standard.

Solid Phase Radioimmunoassay (RIA)

The immunoreactivity of radiolabeled CC49 scFv forms was assessed using an antibody capture assay with BSM as a positive antigen and bovine serum albumin (BSA) as a negative control. The proteins were attached to a solid-phase matrix (Reacti-Gel HW-65F) (Pierce, Rockford, IL). Following a 1-h incubation at RT, the unbound radiolabeled protein was removed by washing and the pellet was counted for 1 min in a gamma scintillation counter.

The plates for an antibody competitive RIA were prepared as described above using 20 ng BSM/well. The unlabeled samples were added into plates in threefold serial dilution and incubated with radiolabeled CC49 IgG (100,000 cpm in 50 µL). Following an overnight incubation at 4°C, the plates were washed with 1% BSA in phosphate-buffered saline (PBS), the individual wells were cut, and the bound radioactivity was measured in a gamma scintillation counter. Relative affinity constants were calculated by a modification of the Scatchard method (8). The final concentration for each of the dilutions of the various CC49 scFvs was calculated and the slopes were determined by linear regression of the $[Ab]_{bound}$ versus $[Ab]_{bound}/[Ab]_{free}$.

A.	5' -	GTC Val	ACC Thr	GTC Val	TCC/ Ser /	TCA Ser	TAA -3' STOP								
B.	5' -	GTC Val	ACC Thr	GTC Val	TCC/ Ser /	CCT Pro	GAG Glu	GAC Asp	TAT Tyr	GAC Asp	TCC Ser	TAA -3' STOP			
C.	5' -	GTC Val	ACC Thr	GTC Val	TCC/ Ser /	CCT Pro	GAA Glu	GAC Asp	CCT Pro	GAA Glu	GAC Asp	TAT Tyr	GAC Asp	TAA -3' STOP	

FIG. 1. The deduced carboxy terminus of CC49 V_H DNA and amino acid sequences of the CC49 species, scFv[8.1] (A), scFv[5.8] (B), and scFv[5.2] (C). Negatively charged amino acids were added to the carboxy terminus of CC49 V_H by including oligonucleotide sequences in polymerase chain reaction (PCR) amplification of the CC49 V_L-linker-CC49V_H DNA present in scFv[8.1]. The “bold” sequences indicate differences in the various constructs.

Refolding of CC49 scFvs after Denaturation with Urea

One milligram of scFv was adjusted to final concentration of 6 M urea in 500 mM NaCl and 50 mM Tris-HCl (pH 8.0). After incubation on rotary shaker overnight at 4°C, the urea was gradually dialyzed out against 50 mM Tris-HCl. The refolded scFvs were analyzed by HPLC and ELISA.

Biodistribution Studies

Female athymic mice (nu/nu) obtained from Charles River (Wilmington, MA) at 4–6 weeks of age, were injected subcutaneously (SC) on the back with 4×10^6 human colon carcinoma cells (LS-174T) (28). Dual label biodistribution studies were performed with mice injected, via the tail vein, with radiolabeled scFvs at 3–6 mCi/mg (5 μ Ci of a ^{125} I-labeled scFv and 2.5 μ Ci of a second scFv labeled with ^{131} I). At specific times, mice (groups of six) were euthanized, dissected, and the major organs were weighed and counted in a gamma scintillation counter. The pharmacokinetic studies were conducted by obtaining blood samples (5 μ L) via tail bleeds at various time points after injections of 10 μ Ci of the individual radioiodinated CC49 scFv form. For the whole body retention studies, mice bearing the LS-174T xenograft (three/group) were injected via the tail vein with 1.5 μ Ci of radiolabeled CC49 scFvs and counted using a custom built NaI crystal at various times after injection.

To compare changes in %ID/g over time among CC49 scFv constructs, statistical analysis was performed using two-way analyses of variance (ANOVA). When a significant interaction was detected, the differences between the scFv constructs at each time point were further compared using the *t* test (significance assigned at the *p* < 0.05 level). The GraphPad Prism software (GraphPad Software Inc., San Diego, CA) was used for these analyses.

RESULTS

Construction and Expression of CC49 scFvs

The CC49 scFv was modified by adding amino acid sequences to generate lower isoelectric points of 5.8 and 5.2. Negatively charged amino acids were added to the carboxy terminus of the CC49 V_H by including oligonucleotide sequences in PCR amplification of the CC49 V_L-linker-CC49V_H DNA coding for the parental scFv with a pI of 8.1. Nucleotide sequence of these CC49 scFv constructs were confirmed by sequencing and the unique differences in the primary sequences are shown in Figure 1.

The CC49 fragments were cloned into the vector that contained pelB signal peptide, which directs the recombinant protein to the

periplasmic space, where refolding and renaturation of protein occur, resulting in a biologically active protein. The expression plasmids containing CC49 scFv sequences were expressed in *E. coli*. The CC49 scFv protein was purified from the periplasmic fractions using ion-exchange chromatography as described in the Materials and Methods section.

Characterization of scFvs

The purified CC49 scFvs were analyzed by SDS-PAGE. Purity of scFv[8.1], scFv[5.8], and scFv[5.2] constructs was greater than 95%, as judged by SDS-PAGE (Fig. 2). Based on amino acid composition, predicted *M_r* values were calculated. Experimentally, the *M_r* of the samples was estimated by comparison with the relative mobility values of the standards from SDS-PAGE. Modified scFv[5.8] and scFv[5.2] with estimated *M_r* of 32.7 kDa and 33.4 kDa, respectively, migrated slower than scFv[8.1] (*M_r* 30.7). IEF analysis determined the pI of expressed CC49 scFvs (Fig. 3). The observed pI of scFv[8.1] was 8.3, the pI of scFv[5.8] was predominantly 5.2 (98%), and the pI of scFv[5.2] was 4.9 with three minor bands between 4.9 and 4.8 (representing 7% of the sample). These gels demonstrated changes in pIs of the scFv constructs after modification of amino acid sequence of CC49 V_H. The experimentally measured and the computer-predicted *M_r* and pIs of each scFv construct are compared in Table 1. Each scFv construct was also assessed for capability to compete with biotinylated CC49 IgG for the binding to BSM, which contains the epitope recognized by MAb CC49 on the human tumor-associated antigen TAG-72. All three CC49 scFvs competed completely for the binding of the iodinated IgG (data not shown). Immunoreactivity and the binding properties of the CC49 scFvs were also tested in a competition RIA with the ^{125} I-labeled CC49 IgG. The CC49 scFv[8.1] and scFv[5.2] had affinity constants of 1.19×10^9 M⁻¹ and 0.97×10^9 M⁻¹, respectively, according to Scatchard analysis (see the Materials and Methods section). The affinity of CC49 scFv[5.8] for BSM was somewhat lower than the affinity of the other constructs (Table 1).

The purified scFv proteins were radiolabeled to see the effect of radiolabeling on the immunoreactivity and the stability of the CC49 scFvs. A labeling yield of nearly 60% was achieved for all three scFv molecules. Radiolabeled scFvs were analyzed by SDS-PAGE with and without exposure to β -mercaptoethanol. A single major band representing approximately 98% of incorporated radioactivity migrated with the molecular weight of the corresponding scFvs (data not shown). The labeled scFvs were also analyzed by HPLC size exclusion chromatography (Fig. 4). The scFvs were eluted as single peaks without any detectable aggregates and their

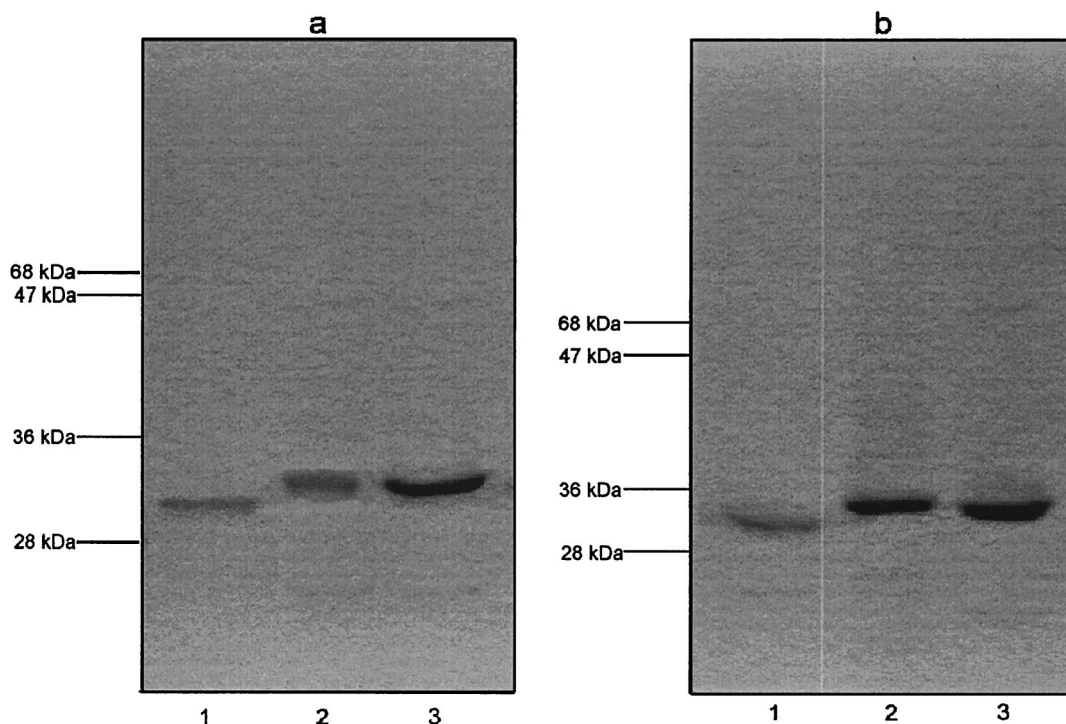


FIG. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of purified CC49 scFvs under reducing (a) and nonreducing (b) conditions. Lane 1, CC49 scFv[8.1]; Lane 2, CC49 scFv[5.2]; Lane 3, CC49 scFv[5.8]. Positions of the molecular weight marker proteins are indicated.

elution profiles corresponded to their M_r , indicating they were not altered by iodination.

The immunoreactivity of the labeled scFv proteins was determined by solid phase RIA using BSM and BSA attached to Reacti-Gel beads. The scFv[8.1] showed 69–72% binding to BSM.

Charge-modified scFv[5.2] showed similar binding (62–65%), but scFv[5.8] demonstrated lower binding (45–48%) to BSM. Furthermore, the radiolabeled scFvs were analyzed to determine the radiopurity and integrity after incubation in murine serum. The radiolabeled scFvs were mixed with murine serum and incubated at

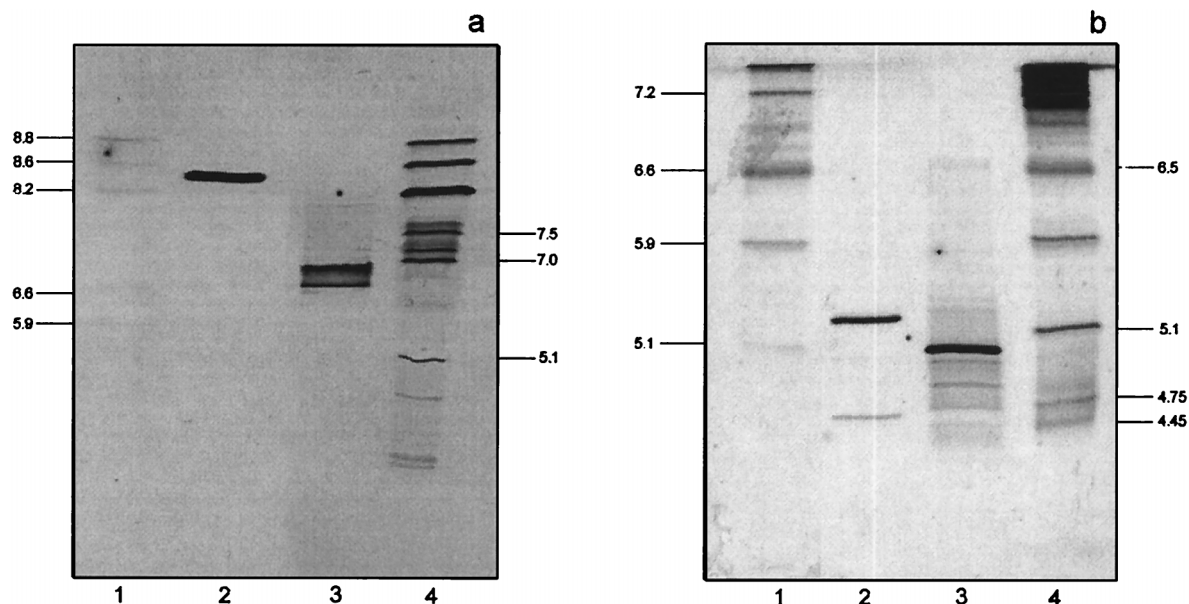


FIG. 3. Isoelectric focusing (IEF) analysis of purified CC49 scFv forms and CC49 IgG. MAbs CC49 Ig forms were analyzed using agarose IEF plates and visualized by a silver stain procedure. IEF gel with a pH range of 3–10 (a): Lane 1, Sigma's IEF standards; Lane 2, CC49 scFv[8.1]; Lane 3, CC49 IgG; Lane 4, Bio-Rad's IEF standards. IEF gel with a pH range of 3–7 (b): Lane 1, Sigma's IEF standards; Lane 2, CC49 scFv[5.8]; Lane 3, CC49 scFv[5.2]; Lane 4, Bio-Rad's IEF standards.

TABLE 1. Characterization of CC49 ScFv Species

	scFv[8.1]	scFv[5.8]	scFv[5.2]
M_r theoretical	27,749	28,369	28,623
M_r experimental	30,600	32,700	33,300
pI theoretical	8.1	5.8	5.2
pI experimental	8.3	5.2	4.9
K_A (M^{-1})	1.19×10^9	0.79×10^9	0.97×10^9

* Theoretical pIs and M_s s were determined using the computer program PROTEIN-TITRATE. Experimentally determined pI values were measured using isoelectric focusing; molecular weights were determined using sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Relative affinity constants (K_A) were calculated by a modification of the Scatchard method.

37°C. Aliquots were taken at various times and were analyzed by HPLC size exclusion chromatography. Radiolabeled charge-modified scFvs and the unmodified scFv maintained their full integrity and stability throughout the testing period (48 h) (data not shown).

Biodistribution, Pharmacology, and Tumor Targeting of the CC49 scFvs

Pharmacokinetic studies were conducted to determine the blood clearance rates of radiolabeled CC49 scFvs. As can be seen in Figure 5, the blood clearance curves of the three scFvs are quite similar. The scFv[8.1], scFv[5.8], and scFv[5.2] showed a rapid blood clearance with more than 50% cleared from the blood pool in 7, 6.7, and 10 min, respectively. Whole body clearance analysis also showed a rapid scFvs clearance, suggesting that scFvs were not being retained in the extravascular space or in any specific organ, but that

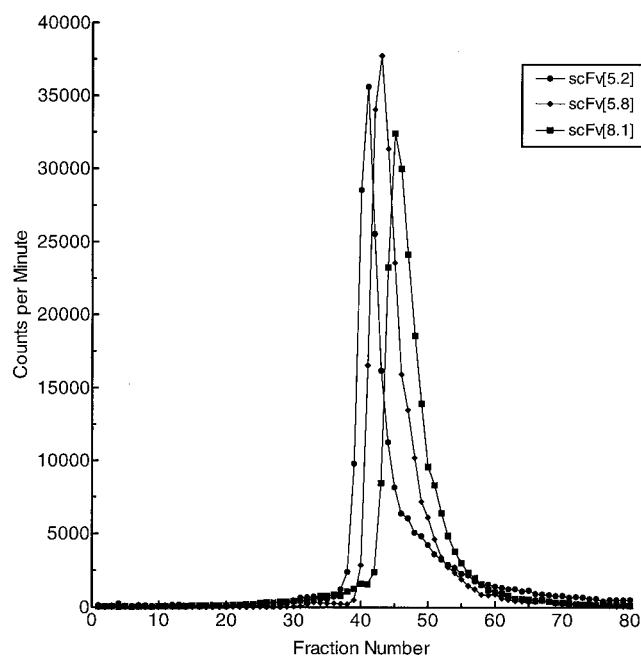


FIG. 4. High performance liquid chromatography (HPLC) size-exclusion profiles of the radiolabeled CC49 scFv[5.2], scFv[5.8], and scFv[8.1]. After radiolabeling ^{125}I -scFvs were analyzed using TSK G3000SW and TSK G2000SW size exclusion columns connected in series. The CC49 scFv[8.1] (■), scFv[5.8] (◆), and scFv[5.2] (●) were eluted as single peaks of approximately 30 kDa.

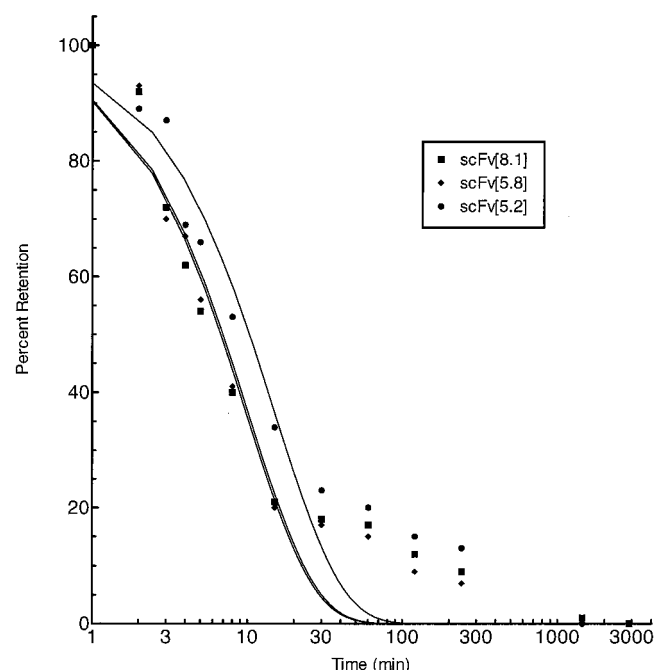


FIG. 5. Pharmacokinetics of blood clearance of CC49 scFvs. Radiolabeled scFv[8.1] (■), scFv[5.8] (◆), and scFv[5.2] (●) were injected into athymic mice (6/group) bearing LS-174T colon carcinoma xenografts. Blood samples were obtained at various times as described in the Materials and Methods section.

they were eliminated from the body apparently through the urine (Fig. 6).

Colcher *et al.* (4) showed in biodistribution competition studies that 250–500 μ g of CC49 IgG was needed to reduce the binding of the radiolabeled CC49 IgG to the antigen on LS-174T colon carcinoma xenograft. Therefore, biodistribution dual label studies can be performed to directly compare the antigen-binding ability of the scFvs *in vivo* and their efficiency to target human colon carcinoma xenograft (using less than 10 μ g of scFv). The CC49 scFvs were compared in two sets of experiments: ^{125}I -scFv[8.1] versus ^{131}I -scFv[5.8] and ^{125}I -scFv[8.1] versus ^{131}I -scFv[5.2]. At various times after injection, blood, tumor, and normal organs were analyzed to determine the amount of each radionuclide retained per gram of tissue. The biodistribution data (Table 2) showed the differential tumor binding of scFvs. The scFv[8.1] and scFv[5.2] demonstrated similar *in vivo* tumor targeting with 2.6%ID/g and 2.4%ID/g, respectively, at 6 h postadministration. Tumor uptake of scFv[5.8] was significantly lower at 24 h ($p < 0.01$) and 48 h ($p < 0.005$) postadministration than scFv[5.2] and scFv[8.1].

Another important evaluation of scFv species was their retention by the kidneys. Unmodified scFv[8.1] showed 43%ID/g at 0.5 h postadministration. The scFv[5.8] uptake by kidneys was significantly lower (33%ID/g at 0.5 h) than that observed for scFv[8.1] ($p < 0.03$). The negatively charged CC49 scFv[5.2] exhibited highest uptake (53%ID/g at 0.5 h) ($p < 0.04$) as compared with scFv[5.8] and scFv[8.1]. The level of renal uptake of CC49 scFvs was elevated only at the earliest time points (0.5 h and 1 h). The %ID/g in the kidneys observed with the scFv[8.1], scFv[5.8], and scFv[5.2] was only approximately twice the levels found in the other major organs by 4 h postadministration.

The charge-modified scFvs did not show atypical levels in the liver or spleen when compared with scFv[8.1]. The small differences

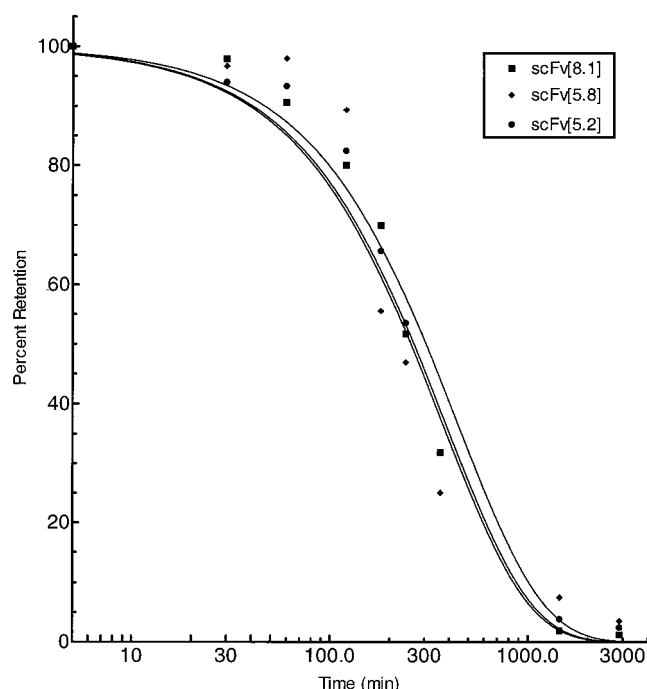


FIG. 6. Whole body clearance of CC49 scFvs. The whole body clearance of ^{125}I -scFv[8.1] (■) was compared with that of ^{131}I -scFv[5.8] (◇) or ^{131}I -scFv[5.2] (●) in athymic mice bearing LS-174T tumors. The mice (3/group) received intravenous injections, and the radioactivity was measured using a custom-built NaI gamma scintillation counter at the indicated times.

observed in the relative rates of blood clearance in the biodistribution studies were not statistically significant ($p > 0.05$).

An equally important parameter is the radiolocalization index (RI: ratio of the %ID/g in tumor to the %ID/g in normal tissue). The scFv[5.8] exhibited generally lower RI values than scFv[5.2] and scFv[8.1] due to the lower tumor uptake of scFv[5.8] (Table 3). The scFv[5.8] showed a tumor:blood ratio of 9:1 at 24 h post administration as compared with scFv[8.1] (27:1) and scFv[5.2] (25:1) at the same time point. RIs for well-vascularized organs were also lower for scFv[5.8] with a tumor:liver and tumor:spleen ratios of 7:1 and 9:1, respectively, compared with 16:1 and 22:1 for scFv[8.1] at 24 h. RIs of scFv[5.2] were similar to that seen with the scFv[8.1] with a tumor:spleen ratio of 18:1. The RI of kidney and liver were lower for scFv[5.2] with 7:1 and 14:1 respectively, as compared with 9:1 and 22:1 for scFv[8.1] at 24 h.

DISCUSSION

The major limitations of intact immunoglobulins for targeting tumor antigens are their relatively poor penetration into tissues (3, 16, 31) and the development of human antimouse immunoglobulin immune response (24). Antibody fragments such as F(ab')_2 , Fab' or scFvs have the advantages of more rapid biodistribution and improved tumor:normal organ uptake as compared with intact IgG. Furthermore, fragments are expected to be of lower immunogenicity due to their short persistence in the circulation and the lack of the Fc portion of the antibody molecule. However, high renal uptake is a common problem when dealing with low molecular weight proteins, including enzymes, tissue proteins, and antibody

TABLE 2. Comparative Biodistribution of CC49 ScFvs with pl 8.1, pl 5.8, and pl 5.2 (Percent Injected Dose per Gram)

Tissue	Time (hour)					
	0.5	1	4	6	24	48
scFv[8.1]						
Tumor	5.29	4.92	3.45	2.55	1.09	0.89
Blood	4.84	3.59	1.62	1.05	0.04	0.04
Liver	1.83	1.49	0.68	0.48	0.07	0.04
Spleen	2.31	2.00	0.99	0.65	0.05	0.03
Kidneys	43.07	14.10	2.09	1.18	0.12	0.06
scFv[5.8]						
Tumor	4.87	3.82	2.05	1.63	0.35	0.30
Blood	5.27	3.10	2.00	1.34	0.04	0.04
Liver	1.60	1.11	0.77	0.55	0.05	0.04
Spleen	2.22	1.70	1.19	0.84	0.04	0.03
Kidneys	32.83	10.65	2.10	1.22	0.11	0.08
scFv[5.2]						
Tumor	5.59	5.56	3.85	2.42	1.27	0.98
Blood	5.59	4.43	1.25	0.75	0.05	0.05
Liver	2.96	1.98	0.61	0.42	0.09	0.05
Spleen	3.00	2.36	0.74	0.40	0.07	0.03
Kidneys	52.94	15.19	1.81	1.09	0.19	0.13

^{125}I -CC49 scFv[8.1] and ^{131}I -CC49 scFv[5.2] or ^{125}I -CC49 scFv[8.1] and ^{131}I -CC49 scFv[5.8] were coinjected into athymic mice (6/group) bearing LS-174T tumors. The mice were sacrificed at the indicated times and the %ID/g of the tumor and selected normal tissues were determined. The standard error of the mean (SEM) for the samples averaged approximately 10% of the %ID/g of the corresponding tissues. No significant interaction between scFv[8.1] constructs was detected and the differences in the curves were not statistically significant, therefore the scFv[8.1] values from both experiments were averaged. Statistically significant differences in the scFv[5.2] and scFv[5.8] biodistribution curves were found for tumor at 24 h and 48 h postadministration and for kidneys at 0.5 h postadministration ($p < 0.05$).

fragments, where 30–60% or more of the administered dose can be found in the kidneys (14). High renal uptake of antibody fragments can reduce their bioavailability, with a possible reduction in tumor uptake, and may result in renal radiotoxicity (7). This disadvantage may limit the usefulness of antibody fragments for radioimmunodetection and radioimmunotherapy.

Various strategies are being examined to overcome undesirable elevations in renal uptake of antibody fragments. Preclinical studies have shown that a variety of basic amino acids, amino sugars, and cationic peptides were capable of inhibiting the tubular reabsorption

TABLE 3. Comparative Biodistribution Studies of CC49 ScFv[8.1], ScFv[5.2] and ScFv[5.8] (Radiolocalization Index) at 24 h after Administration

Tissue	scFv[8.1]	scFv[5.8]	scFv[5.2]
Blood	27.3	8.8	25.4
Liver	15.6	7.0	14.1
Spleen	21.8	8.8	18.1
Kidneys	9.1	3.2	6.7
Lungs	18.2	8.8	25.4

^{125}I -CC49 scFv[8.1] and ^{131}I -CC49 scFv[5.2] or ^{125}I -CC49 scFv[8.1] and ^{131}I -CC49 scFv[5.8] were coinjected into athymic mice (6/group) bearing LS-174T tumors. The mice were sacrificed at 24 h postadministration and Radiolocalization Index (%ID/g of the tumor divided by %ID/g of normal tissues) for each organ was determined. The SEM for the samples averaged <10% of the %ID/g of the corresponding tissues.

tion and degradation of antibody fragments of various MABs (1, 6, 11, 22). Other investigators have chemically modified antibody molecules, using negatively charged chelating agents (9), acetylation (27), low molecular weight dextran (7), and acylation (10) to reduce the MAB fragment's renal uptake. Unfortunately, depending on the number and location of the reactive groups on antibodies, chemically modified immunoglobulin molecules represent heterogeneous molecular species. Furthermore, the non-site-restricted nature of the conjugation can, with some antibodies, lead to modification of residues in the antigen-binding site or residues in the close proximity to the binding site, which can reduce or abolish antigen binding. For example, chemical modifications have resulted in a 50% drop in immunoreactivity of an anti-CEA Fab' fragment (27) and a 35% decrease in the immunoreactivity of anti-Tac dsFv (10).

Antibody engineering has renewed interest in the field of radioimmunotargeting by helping investigators manipulate the molecular structure of the single-chain antibodies to study the effect of affinity, avidity, and size on specific tumor targeting, kinetics, and penetration into tissues. In this study, we investigated whether the lowering of the isoelectric point of CC49 scFv would change its pharmacokinetic properties and result in reduced kidney uptake. The lowering of the isoelectric point by 1.7–4 pI units was one of the most important characteristics of chemically modified antibody molecules that showed reduced renal retention *in vivo* (7, 9, 10, 27). We modified the DNA sequence of CC49 scFv according to a computer-predicted amino acid sequence to result in CC49 scFvs with reduced isoelectric points of theoretically 5.8 and 5.2. The pIs of folded scFv proteins were determined by IEF, indicating that the pIs of modified CC49 scFvs were decreased by 3.4 pI units for scFv[5.2] and 3.1 pI units for scFv[5.8], as compared with parental CC49 scFv with an experimentally determined pI of 8.3.

The immunoreactivity is another important characteristic in the evaluation of modified CC49 scFv. All three CC49 scFvs could completely compete for the binding of the iodinated IgG. Nevertheless, according to Scatchard analysis of the results from a competition RIA, the scFv[5.8] demonstrated 20–30% lower binding affinity than the affinity of scFv[8.1]. The immunoreactivity of charge-modified scFv[5.2] was similar to that of unmodified scFv[8.1]. The reduced immunoreactivity of the scFv[5.8] may have been due to alteration of the structure. However, this complication is difficult to fully explain, especially when the immunoreactivity of other charge-modified scFv (scFv[5.2]) was unchanged in comparison with unmodified scFv. The differences in amino acid sequences between scFv[5.8] and scFv[5.2] were minimal with a change of three amino acids at the CC49 V_H carboxy terminus (Fig. 1). Additional experiments showed no change in the immunoreactivity of scFv[5.8] after denaturation (with urea) and refolding of the construct. This finding may indicate that structural complexities, and not simply problems in the folding of scFv molecule, are responsible for the reduced immunoreactivity.

The biodistribution and tumor targeting characteristics of CC49 scFvs were compared in the athymic mouse/LS-174T xenograft system. The blood clearance rates of radiolabeled fragments were similar, with more than 50% cleared from the blood pool in less than 10 min. However, scFv[5.2] with the lowest pI was cleared from blood stream at the slowest rate. This slow clearance could indicate lower uptake by kidneys and better bioavailability of this modified scFv. The biodistribution studies showed, however, that the renal retention levels at 0.5 h were significantly highest with scFv[5.2] (53%ID/g) and the lowest with scFv[5.8] (33%ID/g). The unmodified scFv[8.1] reached 43%ID/g at 0.5 h. Nevertheless, the

levels of renal uptake of CC49 scFvs were elevated only at the earliest time points (0.5 h and 1 h). Furthermore, the differences in renal uptake of all three CC49 scFvs were relatively low when compared with previously published studies (2, 10, 11), which evaluated anti-Tac disulfide-stabilized Fv (dsFv). The renal uptake of unmodified dsFv was 203–220%ID/g at 15 min and 150%ID/g at 45 min. Lowering the pI (by 1.3–4.4 pI units) of dsFv through the chemical modification reduced the renal uptake to 88.4%ID/g at 15 min (10). The renal uptake of unmodified CC49 scFv was low when compared with unmodified anti-Tac dsFv, which could make any significant improvement with modified CC49 scFvs difficult to detect.

One possible explanation for the contrasting renal uptake could be the structural differences between dsFv and scFv. The Fv chains of scFvs are linked by a covalent peptide bond; whereas the Fv chains of dsFv are linked by an interchain disulfide bond. Charge-modification of the CC49 scFv fragments did not decrease their renal retention, as predicted from published reports. This finding was surprising given that the negative charge should have reduced the electrostatic interactions with the negatively charged glomerular cells of the kidney. It is possible that the sequence modification of the carboxy terminus of the V_H region of CC49 scFv could not prevent the electrostatic binding of positively charged basic amino acid side chains with the negatively charged glomerular cells of the kidney. One could also argue that our estimation of pI value is based on silver-stained protein bands that might not be identical to the pI value of radiolabeled proteins. However, IEF of the radiolabeled scFv[8.1] did not show any major changes in the pI of this protein. The radiolabeled scFv was represented by three bands with pIs in the range of 8.6–8.1.

Another important parameter in the evaluation of modified antibody molecules is antigen-binding ability and the efficiency of tumor targeting *in vivo*. The biodistribution dual label studies allowed direct comparison of unmodified and genetically charge-modified antibody fragments. The ability to target antigen *in vivo* of the scFv[5.8] was significantly lower than that observed for modified scFv[5.2] or unmodified scFv[8.1]. Tumor uptake of unmodified scFv[8.1] and the modified scFv[5.2] were similar.

RI is an important parameter for diagnostic applications of the radiolabeled scFv, in which signal:noise or tumor:normal tissue ratios are paramount (3, 16, 31). Due to lower tumor uptake, the RI values obtained with modified scFv[5.8] were generally lower than those obtained with unmodified scFv[8.1] or modified scFv[5.2] (Table 3). Charge-modified scFv[5.2] showed an excellent tumor:blood ratio (25:1) and good RIs for well-vascularized organs with tumor:liver and tumor:spleen ratios of 14:1 and 18:1 at 24 h postadministration. The type of radionuclide, which can influence the biodistribution of radiolabel, is another potentially important factor. It is possible that the effect of modifications to the scFv molecule would be more evident with intracellularly retained radionuclides (e.g., radiometals) such as ¹¹¹In, ⁶⁷Cu, ¹⁷⁷Lu, or ⁹⁰Y than with iodine-labeled scFvs. The liver and kidney activities of radioiodinated antibodies are considerably lower than that seen with chelated antibodies labeled with radiometals. The radiometals are retained by the tissues metabolizing the radiolabeled antibodies, whereas iodinated antibodies are rapidly dehalogenated and the iodine excreted (1, 22, 23, 26, 31).

CONCLUSION

We showed that it was possible to generate CC49 scFvs with desired isoelectric points reproducibly as compared with other chemical conjugation methods. These charge-modified CC49 scFv constructs

were comparable to unmodified CC49 scFv construct in pharmacokinetic and biodistribution analyses in the athymic mice; however, the decrease in pI did not lower the renal uptake of these modified fragments. Unmodified parental CC49 scFv showed relatively low renal retention when compared with the other published studies using MAb fragments. It is possible that either decreases in the pI of the CC49 scFv may not significantly affect the renal retention of modified fragments or an additional decline in pI is required for modification in the renal uptake of this antibody fragment. Nevertheless, our novel strategy can be applied to other MAbs, which could benefit from this type of charge-modification, particularly those under clinical investigation, in which reproducibility plays an important role.

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