

OLARTE RAISBECK

Industria y Comercio
SUPERINTENDENCIA

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



No. 06-127751-00004-0000

Fecha: 2009-08-12 17:39:54 Dep. 2020 NUECREACIONE
Tra. 11 PATENTE IYII Eve: 378 FASENACIONALI
Act. 329 CTOINFORMACION Folios: 12

FORMULARIO ÚNICO DE CORRECCIONES Y MODIFICACIONES

P2006/000264

SOLICITUD DE:

1

☐

Corrección de Error Material.

☒

Modificación a una Solicitud.

2

SOLICITANTE
(71)

Nombre: BAYER SCHERING PHARMA
AKTIENGESELLSCHAFT

Dirección: Müllerstrasse 178, 13353 Berlín,
Alemania

Nacionalidad o Domicilio: ALEMANIA

Lugar de Constitución: ALEMANIA

Teléfono: _____

Fax: _____

E-mail: _____

IDENTIFICACIÓN

C.C. ☐ NIT ☐

C.E. ☐ Otro ☐

Cual _____

Número _____

3

REPRESENTANTE
O APODERADO

Nombre: J. IAN RAISBECK

Dirección: World Trade Center Torre A

Calle 100 No. 8A-37 Piso 10, Bogotá

Teléfono: 601-7700 Fax: 601-7799

E-mail: _____

IDENTIFICACIÓN

C.C. ☒ NIT ☐

C.E. ☐ Otro ☐

Cual _____

Número 79.376.996 Bta
TP 135.799

4

Número de expediente:

06-127.751

5

Descripción de la corrección o modificación solicitada Me permito solicitar el
cambio de nombre del solicitante de SCHERING AKTIENGESELLSCHAFT por el de
BAYER SCHERING PHARMA AKTIENGESELLSCHAFT

6

Comprobante de pago No. _____

Fecha _____

7

Anexos

☒

Comprobante de pago de la tasa.

☒

Copia simple del Poder y copia simple del documento que soporta el cambio de nombre (original ver expediente No. 03:024.675)

☐

Documento que acredita la existencia y representación legal de las partes, cuando sean personas jurídicas.

8

NOMBRE: J. IAN RAISBECK

FIRMA: Juan Raisbeck

C.C. 79.376.996 Bta T.P. 135.799

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

NIT : 800176089-2

RECIBO OFICIAL DE CAJA : 09 - 59,462
FECHA : AGOSTO 11 DE 2009

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



No. 06-127751--00004-0000

Fecha: 2009-08-12 17:39:54 Dep. 2020 NUECREACIONE
Tra. 11 PATENTEIYII Eve: 378 FASENACIONALI
Act. 329 CTOINFORMACION Folios: 12

***** CONSIGNACION *****

DEPOSITANTE	TIPO PAGO	BANCO	CUENTA	No. PAGO	FECHA PGO	Vr. PAGO
DLARTE RAISBEK	CONSIGNACION	BANCO DE BOGOTA	062754387	105797716	11/08/2009	9,384,000.00

***** CONCEPTO *****

CANT.	RENTIS/ICO	CONCEPTO	TOTAL CONCEPTO
1	50005-01-03	CAMBIO DE MODALIDAD Y MODIFICA	
12		MARCAS Y LEMAS COMERCIALES	111,000.00
		TOTAL :	111,000.00

SON: CIENTO ONCE MIL PESOS

RESPONSABLE : _____

RECIBO DE CAJA APLICADO AL EXPEDIENTE No. _____

322

Certificado de registro según § 21 disposición notarial nacional
(ENotO)

Debido a mi verificación en el registro mercantil del juzgado Charlottenburg del número comercial HRB 283 B el día 27.04.2007 confirmo:

A través de resolución de la Asamblea General del 13.09.2006 Schering Sociedad Anónima ha cambiado la empresa (§ 1 párrafo 1 del reglamento) a Bayer Schering Pharma Sociedad Anónima.

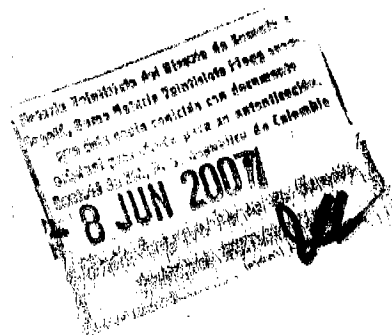
Este cambio entró en vigor el 29.12.2006. La inscripción en el registro mercantil fue efectuada el 20.12.2006.

Berlín, 27. Abril 2007

Firma

Notario

Sello – Dimitri Rempen – Notario en Berlín



Adolf Watzke

Adolf Watzke
Traductor oficial
Res. 2433 Min. Justicia

4 228

APOSTILLE
(Convención de la Haya del 5 de octubre de 1961)

1. País: República Federal de Alemania

Este documento publico

2. está firmado por Dimitri R E M P E N _____

3. en su calidad de Notario en Berlín _____

4. está proveída con el sello

del Notario _____

confirmado

5. en Berlín

6. el 02.05.2007

7. por el presidente de la audiencia provincial en Berlín

8. bajo el número. 9101 a E-F 2563/07

9. Sello

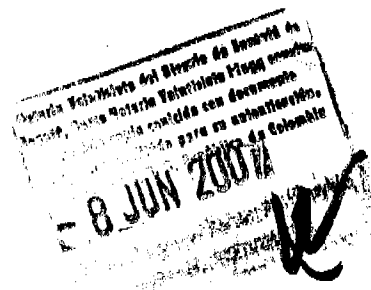
10. Firma
p.o. (por order)

Firma ilegible

Bünning

(jueza presidente de la audiencia provincial)

AVR 95a



Adolf Watzke
Traductor oficial
Res. 2433 Min. Justicia

S

Apostille
(Convention de La Haye du 5 octobre 1961)

1. Land: Bundesrepublik Deutschland

Diese öffentliche Urkunde

2. ist unterschrieben von Dimitri R e m p e n

3. in seiner Eigenschaft als Notar in Berlin

4. sie ist versehen mit dem Siegel

des Notars

Bestätigt

5. in Berlin 6. am 02.05.2007

7. durch den Präsidenten des Landgerichts in Berlin

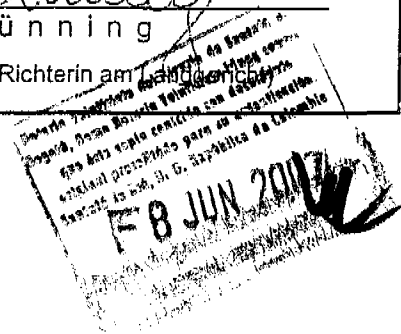
unter Nr. 9101a E-F 2563/07

9. ~~Siegel/
Stempel~~

10. Unterschrift
Im Auftrage

Bünning
B ü n n i n g

(Vorsitzende Richterin am 1. Senat)



16

Registerbescheinigung gemäß § 21 BNotO

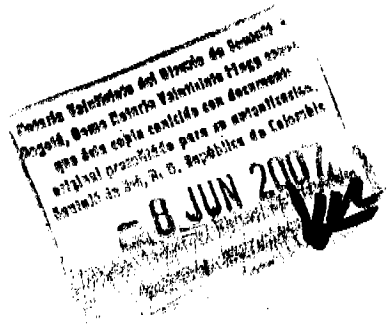
Aufgrund meiner Einsicht in das Handelsregister des Amtsgerichts Charlottenburg zur
Geschäftsnummer HRB 283 B am 27.04.2007 bescheinige ich hiermit:

Durch Beschluss der Hauptversammlung vom 13.09.2006 hat die Schering Aktiengesellschaft
die Firma (§ 1 Absatz 1 der Satzung) in Bayer Schering Pharma Aktiengesellschaft geändert.

Die Änderung wurde am 29.12.2006 wirksam. Die Eintragung im Handelsregister erfolgte am
20.12.2006.

Berlin, 27. April 2007

Notar





N 00742205

1 FOLIO 85.- PRIMERA COPIA.- ESCRITURA NÚMERO: VEINTICUATRO.
2 SUSTITUCIÓN PARCIAL DE PODER: MESSERER, Dagmar a favor de
3 OLARTE, Carlos Reinaldo y otro del Poder otorgado por BAYER SCHERING
4 PHARMA AG.- En la Ciudad Autónoma de Buenos Aires, Capital de la República
5 Argentina, el día NUEVE de MARZO del año DOS MIL SIETE, ante mí María
6 Silvina Lasarte, Escribana Autorizante, titular del Registro Notarial número 1788
7 COMPARECE: Dagmar MESSERER, argentina, nacida el 28 de mayo de 1.968
8 hija de Walter Messerer y Elsa Onelia Romanelli, soltera, titular del Documento Na-
9 cional de Identidad Número 20.383.892, Clave Única de Identificación Tributaria 27-
10 20383892-7, con domicilio especial en la Avenida Leandro N. Alem 1.134, Piso 10º,
11 de esta Ciudad de Buenos Aires.- La compareciente es mayor de edad.- INTERVIE-
12 NE: por su propio derecho.- Y la compareciente EXPRESA: PRIMERO: Que la
13 Sociedad "BAYER SCHERING PHARMA AG", con domicilio en Müllerstrasse
14 178, 13353, Berlín, Alemania, le confirió con fecha 22 de enero de 2007 en dicho pa-
15 ís, un Poder, el cual mediante escritura número 14 de fecha 16 de febrero de 2007,
16 otorgada ante la Escribana Mariela Vanina Scardaccione, al folio 44 protocolo co-
17 rriente de este Registro Notarial interinamente a su cargo, fue incorporado y transcrip-
18 to al mismo, cuya primera copia tengo para este acto a la vista hoy, fe en lo perti-
19 nente transcribiré en la presente.- SEGUNDO: Manifiesto bajo juramento que el refe-
20 rido poder se encuentra plenamente vigente en virtud de no haberle sido revocado,
21 suspendido ni limitado en ninguna de sus partes.- TERCERO: En consecuencia, DI-
22 CE: Que en uso de la facultad conferida SUSCRIBO Y AUTORIZO EN LA PRESENTE el poder
23 mencionado a favor de Carlos Reinaldo OLARTE y/o James Ian RAISEBECK, al
24 solo y único objeto de que en nombre y representación de la Sociedad "BAYER
25 SCHERING PHARMA AG", y actuando en forma individual, indistinta y/o alterna-

CECBA - LEY 404 GCBA

LEGALIZACION

070312117346



\$20.00

13:51:46

12/03/2007

CECBA - LEY 404 GCBA

LEGALIZACION

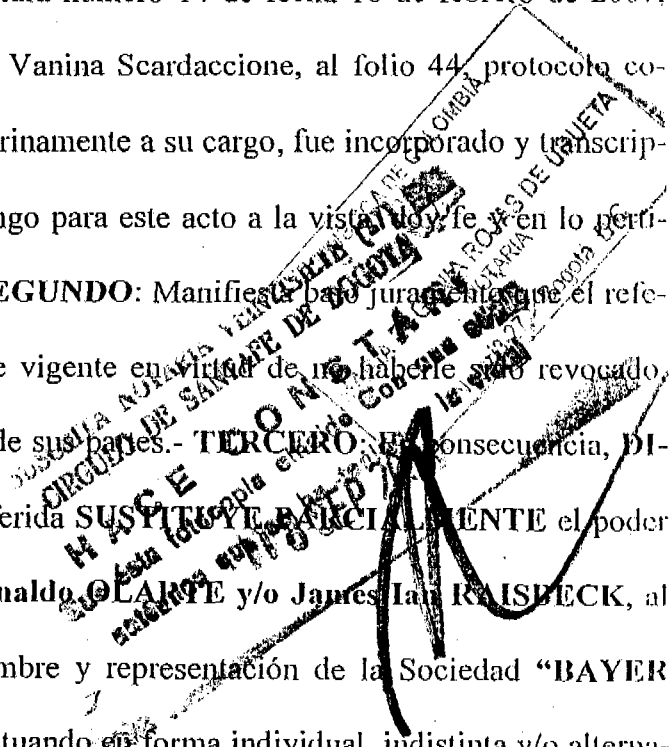
070312054713

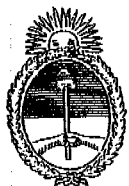


\$39.00

13:51:54

12/03/2007





N 007422051

damamente, se presente exclusivamente ante las autoridades de COLOMBIA que co-
rrespondan y realicen los actos enumerados en el citado poder y con las facultades que
surgen del mismo, el que transcripto en lo pertinente dice: "FOLIO 44.- PRIMERA
COPIA.- ESCRITURA NÚMERO: CATORCE.-- En la Ciudad Autónoma de
Buenos Aires, Capital de la República Argentina, el día DIECISEIS de FEBRERO del
año DOS MIL SIETE, ante mí ..., Escribana Autorizante, ..., COMPARECE: Dagmar
MESSERER, argentina, nacida el 28 de mayo de 1.968, hija de Walter Messerer y
Elsa Onelia Romanelli, soltera, titular del Documento Nacional de Identidad Número
20.383.892, Clave Única de Identificación Tributaria 27-20383892-7, domiciliada
legalmente en la Avenida Leandro N. Alem 1.134, Piso 10º, de esta Ciudad de Buenos
Aires.- La compareciente es mayor de edad.- INTERVIENE: por su propio derecho.-
Y la compareciente EXPRESA: Que a los efectos que hubiere lugar, solicita de mí, la
Autorizante, LA INCORPORACIÓN y TRANSCRIPCIÓN en este protocolo del
PODER que le fuera conferido en Berlín, Alemania, el 22 de enero de 2007, por la
Sociedad "BAYER SCHERING PHARMA AG", con domicilio en Müllerstrasse 178,
13353, Berlín, Alemania, sobre cuya plena vigencia responde la requirente bajo jura-
mento.- Yo, la Autorizante, hago constar: a) Que el instrumento de poder cuya incor-
poración y transcripción a este protocolo solicita la compareciente, se encuentra ex-
tendido en idioma castellano, certificadas las firmas por notario en idioma alemán
debidamente legalizado en idioma alemán (Sello de Apostilla de la Convención de La
Haya del 5 de octubre de 1961) y traducido en lo pertinente al idioma nacional por la
Traductora Pública Laura Thieberger.- b) Que la compareciente, en virtud de lo mani-
festado tiene interés legítimo en su perfeccionamiento, consecuencia de lo manifestado
en este acto del original del instrumento de poder relacionado con su respectiva tra-
ducción al idioma nacional, el que agrego a la presente y transcripto dice: "MOE-



N 007422052

a juicios verbales, declinar y prorrogar de jurisdicción, tachar, recusar, apelar, transar, 26
someter a arbitraje o mediación, hacer renunciaciones, exigir y dar cauciones, pedir embar- 27
gos preventivos, definitivos o inhibiciones y su levantamiento, pedir la venta de los 28
bienes de los deudores, solicitar y obtener el cumplimiento de sentencias, prestar ju- 29
ramentos, decir de nulidad, desistir, percibir, otorgar recibos y en general practicar 30
todos los actos y diligencias que sean precisos al desempeño de este mandato. Podrán 31
asimismo substituir el presente poder en todo o en parte.- Dado y firmado en Berlín 32
(Sigue fecha en idioma inglés). Bayer Schering Pharma AG (Sigue texto en idioma 33
inglés). ppa. Sigue una firma ilegible. (Aclarado:) Dr. Noeske-Jungblut. ppa. Sigue 34
otra firma ilegible. (Aclarado:) Dr. Klose". ES COPIA FIEL, doy fe.- En hoja adjunta 35
consta la certificación notarial de las firmas obrantes en el poder en idioma alemán, 36
efectuado el 22 de enero de 2007 por el Escribano Dimitri Rempen, de Berlín, Alema- 37
nia, y a continuación consta la respectiva legalización de la firma y sello del nombra- 38
do Notario (Sello de Apostilla de la Convención de La Haya del 5 de octubre de 39
1961), en idioma alemán, efectuada por el Presidente del Tribunal Regional de Berlín, 40
Alemania.- En hojas adjuntas sigue la referida traducción al idioma nacional la que 41
transcripta textualmente, dice: "TRADUCCIÓN PUBLICA - (Sobre papel con mem- 42
brete de "MOELLER y CO" - América Latina - Agentes de la Propiedad Intelectual" 43
figura un Poder otorgado por la Sociedad "BAYER SCHERING PHARMA AG" con 44
sede en Berlín, Alemania, redactado en idioma nacional, luego del que aparecen los 45
siguientes textos, que traducidos dicen:) "BAYER SCHERING PHARMA AG" 46
Patentes y licencias - (Firmado y Aclarado:) pp Dr. Noeske-Jungblut, pp Dr. Klose. 47
Aclarado:) Dr. Klose.- La versión en inglés es para su uso en la Unión Europea.- Re- 48
gistro de Actas N° R 17/2007.- Me he referido a la prohibición de intervención según 49
el artículo 3, párrafo 1, N° 7, de la Ley de Certificaciones, en pregunta de si ha existi- 50

REPUBLICA ARGENTINA
CIRCUITO NOTARIAL DE BUENOS AIRES
NOTARIO SAMUEL DE BOGOTA
HACE CONSTAR
esta fotocopia simple con
firmado y rubricado
19 SEP 2007



N 007422053

do una intervención previa en el sentido de la norma me fue contestaba por la negati-
va.- Las firmas trazadas ante mi por el Dr. Walter Klose, que nació el 26 de diciembre
de 1952, y de la Dra. Chistiane Noeske-Jungblut de soltera Noeske, que nació el 26 de
junio de 1957 – Ambos de mi personal conocimiento – Ambos con domicilio comer-
cial en 13353 Berlín, Müllerstrasse 178 son auténticas, de lo que doy fe.- Al mismo
tiempo certifico en base a que tuve a la vista en el día de hoy las actas del Registro de
Comercio del Tribunal de Primera Instancia de Charlottenburg, N° de Registro HR B
283, que la Sociedad “Bayer Schering Pharma Aktiengesellschaft”, con sede en Ber-
lín, está inscripta con el número mencionado arriba en el Registro de Comercio y que
la arriba mencionada Dra. Noeske-Jungblut y el arriba mencionado Dr. Walter Klose
están facultados en su carácter de mandatarios a representar en forma conjunta a la
Sociedad “Bayer Schering Pharma Aktiengesellschaft” de Berlín. Berlín, 22 de enero
de 2007.- El escribano (firma ilegible) y liquidación de derechos. (Hay un sello en
pública sobre precinto:) Dimitri Rempen, Escribano de Berlín.- 9101ª E-F.- APOSTI-
LLA – (Convención de La Haya del 5 de Octubre de 1961) - País: República Federal
de Alemania - Este instrumento público fue firmado por Dimitri Rempen, actuando en
su carácter de escribano de Berlín, estando provisto del sello del escribano.- Certifica-
do en Berlín, el 24 de enero de 2007, por el Presidente del Tribunal Regional de Ber-
lín, con el N° 9101 a E-F 536/07 - Sello (hay un sello:) el Presidente del Tribunal Re-
gional de Berlín – Firma el Funcionario autorizado (firmado y aclarado) Holthoff
Juez Principal del Tribunal Regional.- ES TRADUCCIÓN FIEB de la parte per-
tinentes en los idiomas alemán e inglés del documento que se acompaña que se vertió
al idioma nacional, en Buenos Aires, a 6 de febrero de 2007.- Hay una firma ilegible.
Hay un sello que dice: “LAURA THIEBERGER – MATRÍCULA DE TRADUCTO-
RES ALEMÁN – FRANCÉS – Tomo II Folio 254 (Cap.) Tomo III – 98 – (La Plata) –

COLEGIO DE ESCRIBANOS DE BUENOS AIRES
LAURA THIEBERGER
MATRÍCULA DE TRADUCTORES
ALEMÁN - FRANCÉS
TOMO II FOLIO 254 (CAP.) TOMO III - 98 - (LA PLATA)



N 007422053

INGLÉS – Tomo III: Folio 14 (Cap.) – Tomo III Folio 329 (La Plata) – COLEGIO 26
DE TRADUCTORES PÚBLICOS Nº 173”.- ES COPIA FIEL, doy fe.- A continua- 27
ción hay un sello que dice: “COLEGIO DE TRADUCTORES PÚBLICOS DE LA 28
CIUDAD DE BUENOS AIRES Corresponde a la Legalización Nº T4-3278/07. Sigue 29
una firma ilegible: Esteban Santiago Allegretti”.- ES COPIA FIEL, doy fe.- Al dorso 30
en el margen superior hay un sello timbrador ...”.- A continuación sigue la legaliza- 31
ción de la firma de la nombrada traductora, ...”.- ES COPIA FIEL, doy fe.- En este 32
estado la compareciente DICE: que en la forma expresada deja INCORPORADO y 33
TRANSCRIPTO en este Protocolo el Poder que agrego a la presente, solicitando de 34
mí, la Escribana Autorizante, expida primera copia de la presente para la nombrada 35
apoderada Dagmar MESSERER.- Constancias Notariales: Identidad de la compare- 36
ciente (Artículo 1.002 del Código Civil): Se justifica la misma por conocimiento de la 37
Autorizante.- ...- LEO esta escritura a la compareciente, quien así la otorga y en prue- 38
ba de conformidad, la firma ante mí, doy fe.- Dagmar MESSERER. Ante mí: MA- 39
RIELA VANINA SCARDACCIONE. Escribana. Está mi sello. CONCUERDA con 40
su escritura matriz que pasó ante mí, al folio 44 del Registro Notarial número 1788, 41
interinamente a mi cargo.- PARA LA APODERADA expido la presente PRIMERA 42
COPIA en tres (3) sellos de actuación notarial numerados correlativamente desde el 43
número N 007376153 al presente inclusive, que sello y firmo en la fecha y lugar de 44
otorgamiento, doy fe.- Sigue una firma ilegible y un sello que dice: “MARIELA V. SCARDACCIONE - Escribana - Mat. 4527.”.- ES COPIA FIEL, doy fe.- Sigue una copia cincuenta y cinco (55) folios de la 45
escritura de referencia, doy fe.- CUARTO: Finalmente la compareciente expresa: a) 46
Que el presente otorgamiento no significa renunciar al ejercicio de las facultades sus- 47
tituidas; y b) Que los nombrados apoderados sustitutos no podrán sustituir el presente 48
poder en ninguna de sus partes.- Constancias Notariales: Identidad de la Compare- 49
ciente (Artículo 1.002 del Código Civil): Se justifica la misma por conocimiento de la 50

COPIA NOTARIAL VENTIDUECO (21)
CIUDAD DE BUENOS AIRES
HACE CONSTAR:
Con una copia
vistos
14 SEP 2009
REPUBLICA DE ARGENTINA
MARIELA VANINA SCARDACCIONE
Escribana
Notaria 27 - Buenos Aires



N 007422054

1 ciente (Artículo 1.002 del Código Civil): Se justifica la misma por conocimiento de
2 la Autorizante.-Yo, la Escribana Autorizante impongo y advierto a la compareciente, a
3 tenor de lo dispuesto en el artículo 77 inciso h) de la Ley Orgánica Notarial Número
4 404, respecto de los alcances y consecuencias de las cláusulas contenidas en la presen-
5 te escritura y del derecho de leer por sí este instrumento.- LEO esta escritura a la
6 compareciente, quien así la otorga y en prueba de conformidad la firma ante mí, doy
7 fe.- Dagmar MESSERER. Ante mí: MARIA SILVINA LASARTE. Escribana. Está
8 mi sello. **CONCUERDA** con su escritura matriz que pasó ante mí, al folio 85 del Re-
9 gistro Notarial número 1788, a mi cargo. PARA LA PODERDANTE expido la pre-
10 sente **PRIMERA COPIA** en cuatro (4) sellos de actuación notarial numerados corre-
11 lativamente desde el número N 007422051 al presente inclusive, que sello y firmo en
12 la fecha y lugar de su otorgamiento, doy fe.-

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[Handwritten signature]

[Circular stamp: MARIA SILVINA LASARTE]

[Diagonal stamp: HACER CONSTAR: Esta copia fotostática coincide con la original que se ha ratado a la...]

[Date stamp: 18 SEP 2018]



N 007422054

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LEGALIZACION



L 007495419

EL COLEGIO DE ESCRIBANOS de la Ciudad de Buenos Aires, Capital Federal de la República

Argentina, en virtud de las facultades que le confiere la ley vigente, **LEGALIZA** la firma

y sello del escribano **MARIA SILVINA LASARTE**

obrantes en el documento anexo, presentado en el día de la fecha bajo

el N° **070312117346/0**

La presente legalización no juzga sobre

el contenido y forma del documento.

Buenos Aires, Lunes 12 de Marzo de 2007



[Handwritten signature]

ESC. EDDA ENRIQUETA SINELLI
COLEGIO DE ESCRIBANOS
CONSEJERA

CONSEJERO

BOGOTÁ NOVIEMBRE VEINTISIETE (27) 2007
CIRCULO DE SANTAFÉ DE BOGOTÁ
HACE CONSTAR
que esta fotocopia es una copia fiel de la original
cualitica que se le da a la vista
27 SEP 2007

APOSTILLE

REPUBLICA
ARGENTINA

(Convention de La Haye du 5 octobre 1961)

A 00115229

1. PAÍS: REPUBLICA ARGENTINA

El presente documento público

2. Ha sido firmado por EDDA ENRIQUETA SINELLI

3. Quien actúa en calidad de CONSEJERA

4. Lleva el sello / timbre de COLEGIO DE ESCRIBANOS DE LA CIUDAD DE BUENOS AIRES

5. En LA CIUDAD DE BUENOS AIRES 6. El día 12/03/2007

7. Por EL COLEGIO DE ESCRIBANOS DE LA CIUDAD DE BUENOS AIRES

MINISTERIO DE RELACIONES EXTERIORES, COMERCIO INTERNACIONAL Y CULTO

(Convenio 02/09/2003)

o el número: 070312054713

10. Firma

10. Sello / Timbre

COLEGIO DE ESCRIBANOS
LA CIUDAD DE BUENOS AIRES



[Handwritten signature]

COLEGIO DE ESCRIBANOS
LA CIUDAD DE BUENOS AIRES
CONSEJERO

COLEGIO DE ESCRIBANOS DE BOGOTÁ
BOGOTÁ - COLOMBIA
HACE CONSTAR
que esta fotocopia es una copia
fidelidad a la original
20 SEP 2007

COLEGIO DE ESCRIBANOS DE BOGOTÁ
BOGOTÁ - COLOMBIA
NOTARIA

DIRECCIÓN DE NUEVAS CREACIONES

RADICACIÓN EXPEDIENTE No. 06-127751

EL EXTRACTO DE LA PRESENTE SOLICITUD DE **PATENTE DE INVENCION**

FUE PUBLICADO EN LA GACETA DE PROPIEDAD INDUSTRIAL No **582** DE

FECHA **30 DE NOVIEMBRE DE 2007** EL TÉRMINO HÁBIL SEÑALADO POR LA

LEY EXPIRA EL **27 DE FEBRERO DE 2008**

NOTA: Dentro del plazo de los seis (6) meses siguientes a partir de la fecha de publicación en la gaceta de Propiedad Industrial, deberá solicitar y cancelar el examen de patentabilidad so pena de que la solicitud caiga en abandono, de acuerdo con lo establecido en el Artículo 44 de la Decisión 486/2000.

SE PRESENTARON OPOSICIONES POR PARTE DE TERCEROS.

SI _____

NO X

Developing Novel Oncolytic Adenoviruses through Bioselection

Wen Yan, Galila Kitzes, Farid Dormishian, Lynda Hawkins,[†] Adam Sampson-Johannes,
 Josh Watanabe, Jenny Holt, Vivian Lee, Thomas Dubensky,[‡] Ali Fattaey,
 Terry Hermiston,[§] Allan Balmain,^{||} and Yuqiao Shen*

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Mutants of human adenovirus 5 (Ad5) with enhanced oncolytic activity were isolated by using a procedure termed bioselection. Two mutants, ONYX-201 and ONYX-203, were plaque purified from a pool of randomly mutagenized Ad5 that was repeatedly passaged in the human colorectal cancer cell line HT29, and they were subsequently characterized. ONYX-201 and ONYX-203 replicated more rapidly in HT29 cells than wild-type Ad5, and they lysed HT29 cells up to 1,000-fold more efficiently. The difference was most profound when cells were infected at a relatively low multiplicity of infection, presumably due to the compounding effects of multiple rounds of infection. This enhanced cytolytic activity was observed not only in HT29 cells but also in many other human cancer cell lines tested. In contrast, the cytotoxicity of the bioselected mutants in a number of normal primary human cells was similar to that of wild-type Ad5, thus enhancing the therapeutic index (cytotoxicity in tumor cells versus that in normal cells) of these oncolytic agents. Both ONYX-201 and -203 contain seven single-base-pair mutations when compared with Ad5, four of which were common between ONYX-201 and -203. The mutation at nucleotide 8350, shared by both mutant viruses, was shown to be essential for the observed phenotypes. This mutation was mapped to the i-leader region of the major late transcription unit, resulting in the truncation of 21 amino acids from the C terminus of the i-leader protein. This work demonstrates that bioselection is a powerful tool for developing novel tumor-selective oncolytic viruses. Other potential applications of this technology are discussed.

Conditionally replicating viruses represent a promising new class of anticancer agents (2, 8, 21, 23, 24, 29). Derivatives of human adenovirus type 5 (Ad5) have been developed that selectively replicate in and kill cancer cells. The prototype of such viruses, ONYX-015, has demonstrated clinical benefit for cancer patients, particularly when combined with chemotherapy, in several phase I and phase II clinical trials to treat recurrent cancers of the head and neck or liver metastatic disease (7, 16, 20, 22, 31, 34, 35). ONYX-015 is currently being tested in placebo-controlled phase III clinical trials.

ONYX-015 replication has been demonstrated to occur selectively in tumor tissues from patient biopsy samples, and the virus has been well tolerated when directly injected into the tumor mass, administered via the hepatic artery, or injected intravenously. Based on the experience from clinical trials with ONYX-015, enhanced replication and/or spread is highly desired of adenovirus cancer therapy strategies (14, 16, 20, 22, 33–35). While no dose-limiting toxicities have been observed in clinical trials with ONYX-015, and the response rates have been encouraging, the extent of virus replication and spread was often limited in tumor tissues. A more-potent virus would presumably increase the efficacy of the oncolytic adenoviral therapy and/or the durability of the responses. One strategy to enhance the clinical efficacy is to combine ONYX-015 with

other therapies, e.g., standard chemotherapy (16, 20). An extension of such combination therapies is to arm the oncolytic adenoviruses with anticancer genes (17), such as antiangiogenesis factors, cytotoxic agents, prodrug converting enzymes, or cytokines with antitumor activity. Selective replication of the viruses in tumor cells allows targeted expression of these gene products within the cancer environment. Meanwhile, it would be highly desirable to enhance the therapeutic virus' inherent replication efficacy such that the virus would replicate faster or produce more viral progeny per unit of time. Conceivably, this would allow the therapeutic viruses to kill cancer cells more rapidly and spread more extensively within tumors, leading to efficient eradication of the cancer. Another highly desired feature of cancer therapeutic viruses is enhanced tissue and/or cell type specificity; such features would, potentially, confer systemic efficacy on viruses, allowing for specific tissue and/or cell targeting after systemic administration.

Two general approaches have been undertaken to develop novel adenoviruses with desired features. The first is targeted genetic manipulation, in which designated viral genes have been deleted or modified, therapeutic transgenes have been inserted, or strong, tissue-type selective promoters have been substituted for endogenous viral regulatory sequences. Although these approaches have been successfully utilized to construct many novel viruses (for examples, see references 13, 18, 19, 30, 42, and 51), their application has been limited by the requirement for a complete understanding of the biology of the virus life cycle. Even in the case of Ad5, one of the most extensively studied viruses, such information is not always available or complete. Thus, the consequences of targeted genetic manipulations are in many cases unpredictable. The second general approach to generate viruses with desired prop-

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erties is genetic selection under carefully controlled conditions. Viruses selected in this fashion preferentially replicate and reproduce under a predefined set of conditions (for examples, see references 4–6, 9, 38, and 46). In essence, this is an evolution process occurring under carefully controlled conditions in the laboratory.

In this study, we exploited a genetic selection approach to isolate mutants of Ad5 that replicate more efficiently in tumor cells than the parental strain. Two such viruses, ONYX-201 and -203, were derived, and their genetic makeup was analyzed in detail. This study provided a clear proof of concept for the genetic selection approach, which we have termed bioslection, for developing novel oncolytic viruses with enhanced potency and resulted in two mutant viruses that may have important clinical implications as oncolytic agents.

MATERIALS AND METHODS

Cells and viruses. All human cancer cell lines were obtained from the American Type Culture Collection and were propagated as monolayer cultures in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 μ g of nonessential amino acids (NEAA)/ml, 10 U of penicillin/ml, and 10 μ g of streptomycin/ml. Primary human normal cells were obtained from Clonetics Corp. and were propagated under conditions recommended by the manufacturer. Wild-type Ad5 was obtained from the American Type Culture Collection and propagated in 293 cells. ONYX-201 and -203 and their derivatives were propagated in HT29 cells until the last proliferation step, for which they were grown in 293 cells. All viruses were purified by the CsCl gradient banding method and titrated by plaque assay on 293 cells. In several cases plaque assays were performed on HT29 cells as well as on 293 cells, and the results from both were consistent. Infections of cancer cells were performed in DMEM supplemented with 2% FBS, 2 mM L-glutamine, 100 μ g of NEAA/ml, 10 U of penicillin/ml, and 10 μ g of streptomycin/ml. Infections of normal cells were performed in their recommended growth media.

Random mutagenesis. Random mutagenesis of Ad5 with nitrous acid was performed as described previously (12, 25, 39, 50). Briefly, wild-type Ad5 was treated with 0.7 M NaNO₂ in 1 M acetate buffer, pH 4.6. The reaction was terminated at various time points by the addition of 4 volumes of ice-cold 1 M Tris-HCl (pH 7.9). The virus was then dialyzed overnight against 20 mM phosphate-buffered saline (PBS) (pH 7.2)–10% glycerol and stored at –80°C. The infectivity of the treated virus was examined by plaque assay on 293 cells.

Bioslection. Mutagenized Ad5 was passaged repeatedly on selected human cancer cell lines representing various human cancers. In all cases, infections were carried out in T-185 tissue culture flasks containing approximately 10⁷ adherent cells in 25 ml of culture medium (2% FBS). For the first round of passaging, cells were infected at a multiplicity of infection (MOI) of 1. Tissue culture media were harvested at the very initial sign of visible cytopathic effect (CPE). In subsequent passages, 1, 0.1, or 0.01 ml of the harvested media from the previous passage was used as the inoculum. Cultures that began to reveal CPE at 3 to 5 days postinoculation were considered effective, and media were collected at the initial sign of CPE. This strategy allowed us to avoid infection with too many virus particles, which may reduce the effectiveness of bioslection, or too little virus, which would reduce the complexity of the viral population. Passaging was carried out for 6 to 20 rounds, depending on the cell lines used for selection.

Cytolytic assay. Viral cytolysis activities were examined by using the MTT assay as described previously (43). Briefly, cells were seeded into 96-well plates at a density of 3,000 cells per well in the appropriate growth media. Infections were performed at 24 h after seeding. In most cases, infections were carried out in quadruplicate with serial threefold dilutions of the viruses. A total of 10 dilutions were used for each virus, starting at an MOI of 30 and ending at an MOI of 1.5×10^{-3} . Some of the MTT assays with primary human cells (see Fig. 6) had a starting MOI of 10 and an ending point at 5×10^{-4} . Cytolytic assays described in the legend to Fig. 3 were conducted in triplicate at MOIs of 10, 1, 0.1, and 0.01. Infected cells were incubated at 37°C, and colorimetric reactions were performed at the indicated time points by using the CellTiter 96 nonradioactive cell proliferation assay (Promega) according to the manufacturer's instructions. Cells that were mock infected were used as negative controls and set as the reference (100% survival).

Virus production assays. HT29 cells were seeded in 24-well dishes at 4×10^4 cells per well. After attachment, cells were infected with Ad5 and ONYX-201

and -203 at the indicated MOIs. After an incubation period of 90 min, nonadsorbed viruses were removed by aspiration and cells were washed once with PBS. Infected cells were then incubated at 37°C in DMEM supplemented with 2 mM L-glutamine, 100 μ g of NEAA/ml, 10 U of penicillin/ml, 10 μ g of streptomycin/ml, and 2% FBS. At the indicated time points after infection, cells and culture media were harvested separately. Cell fractions were freeze-thawed three times to release virus particles, and lysates were cleared by centrifugation. The total virus yield (cell and medium combined) was determined by plaque assay on 293 cell monolayers.

Viral DNA replication. HT29 cells were infected with Ad5 and ONYX-201 and -203 at various MOIs. At the indicated times postinfection, cells and culture media were harvested and DNAs were extracted from combined cell and medium fractions with the Blood DNA Extraction kit (Qiagen). DNAs were digested to completion with *Hind*III, and digested DNAs were resolved on 0.8% agarose gels. After Southern transfer, DNA blots were hybridized with probes prepared with the digoxigenin High Prime DNA labeling kit (Roche Biochemicals). Purified Ad5 genomic DNA was used as a template for probe synthesis.

Western blot analysis. HT29 cells were either mock infected or infected with Ad5 and ONYX-201 and -203 at various MOIs. At indicated times postinfection, cells were harvested and lysed in sodium dodecyl sulfate (SDS) gel loading buffer (100 mM Tris-HCl [pH 6.8], 5 mM EDTA, 1% SDS, 5% β -mercaptoethanol). Proteins were fractionated by electrophoresis on 4 to 20% protein gels (Bio-Rad). After electrophoresis, the proteins were electrophoretically transferred to nylon membranes. Blots were then incubated with antibodies diluted in PBS containing 1% dry milk and 0.1% Tween 20 and visualized by enhanced chemiluminescence (Amersham). Anti-E1A antibody M73 (Calbiochem) was diluted 1:500; a polyclonal rabbit anti-Ad5 (structural protein) antibody was used at a dilution of 1:10,000. A polyclonal antibody against the i-leader protein (a generous gift from M. Green, St. Louis University) was used at a dilution of 1:1,000.

DNA sequencing. Genomic DNAs of ONYX-201 and -203 were purified from CsCl gradient-banded virus particles. Briefly, virus particles were lysed by incubation at 37°C in 10 mM Tris-HCl (pH 8.0), 5 mM EDTA, 0.6% SDS, and 1.5 mg of pronase (Sigma)/ml. Lysed particles were extracted twice with phenol-chloroform, and DNA was precipitated with ethanol. The genome of ONYX-201 was sequenced by Lark Technologies, Inc., Houston, Tex. The genomic DNA of ONYX-203 was sequenced at Onyx by using an automated sequencer (CEQ2000XL; Beckman).

Construction of recombinant viruses. Genomic DNAs of Ad5 and ONYX-201 and -203 were purified from CsCl gradient-banded virus particles. For construction of ONYX-211 and -212, genomic DNAs from Ad5 and ONYX-201 were both digested to completion with *Spe*I, which cuts only once (Ad5 nucleotide no. 27075) within the viral genome. Digested DNAs were mixed in equal amounts and ligated in the presence of T4 DNA ligase at room temperature overnight. This ligation mixture was then transfected into 293 cells with the FuGene reagent (Promega). Plaques derived from this transfection were isolated and screened by DNA sequencing. Proper clones were isolated by an additional round of plaque purification. ONYX-231 through -236 were constructed in a similar fashion, except that DNAs from Ad5 and ONYX-203 were digested with *Pme*I, *Bam*HI, or *Spe*I, respectively (see Fig. 7). All recombinant viruses were confirmed by sequencing the relevant regions surrounding the mutation sites in ONYX-201 and -203.

Dual immunofluorescent staining. HT29 cells were seeded on Lab-Tek II chamber slides the day before infection. Cells were infected at an MOI of 10 with ONYX-201 or -203 or wild-type Ad5. Twenty-four hours postinfection, cells were washed with PBS and fixed for 30 min at room temperature with 4% formaldehyde in PBS. Cells were permeabilized and blocked with PBS supplemented with 0.1% Triton X-100, 0.05% Tween 20, and 10% goat serum for 30 min at room temperature. Samples were incubated with the anti-E1A monoclonal antibody M73 (1:1,000; Calbiochem) and the anti-i-leader protein polyclonal antibody (1:1,000), followed by Alexa 594-conjugated anti-rabbit and Alexa 488-conjugated anti-mouse secondary antibodies (Molecular Probes).

RESULTS

Adenovirus mutagenesis. To generate a genetically diverse virus population for bioslection, wild-type Ad5 was mutagenized by treatment with the chemical mutagen nitrous acid (NaNO₂). Incubation with NaNO₂ for 6 min at room temperature dramatically reduced the viability of Ad5 by a factor of 2×10^3 (Fig. 1A), which was consistent with earlier observations (12, 50). To estimate the mutation frequency resulting from

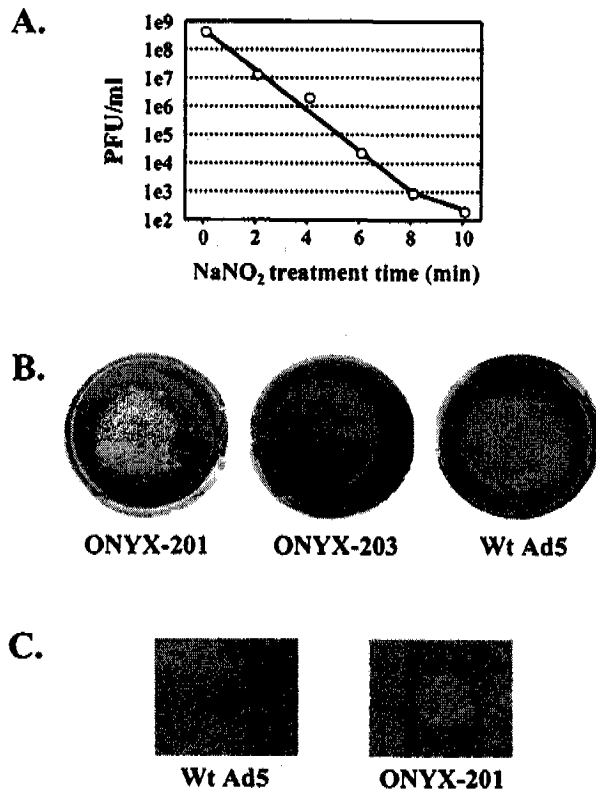


FIG. 1. (A) Wild-type Ad5 was mutagenized by treatment with NaNO₂. Infectivity of the treated virus was examined by plaque assay on 293 cells and plotted as a function of incubation time with NaNO₂. (B) Representative plaque morphology on an HT29 cell monolayer 5 days postinfection with wild-type (Wt) Ad5 or bioselection virus. (C) Microscopic view (magnification, ×40) of representative plaques formed on HT29 cells by Ad5 or ONYX-201.

this treatment, we isolated 22 individual plaques formed on A549 cell monolayers infected with this virus population, purified the viral DNAs, and amplified a 340-bp fragment within the E1A region by PCR for sequence analysis. Of the 22 individual virus isolates analyzed, two contained a single-base-pair mutation within the amplified region. We therefore estimated that mutations occurred at a frequency of 2.7×10^{-4} (2 mutations/22 × 340 nucleotides sequenced), or an average of 10 single point mutations per 36-kb viral genome. This calculation was based on the assumption that mutations occurred evenly along the virus genome. This number might have been an underestimation as the region analyzed (E1A) is essential for virus replication. Nevertheless, it appears that this estimation was consistent with the actual number of mutations identified in the two viral genomes we have fully deciphered through sequence analysis (see below).

Bioselection. To isolate viral variants with enhanced oncolytic activity, mutagenized Ad5 was serially passaged in parallel in a number of human cancer cell lines of diverse tissue origin, as described in detail in Materials and Methods. During each passage cycle, medium from infected monolayer cultures was harvested at the first sign of CPE and used to inoculate the next fresh monolayer culture of the same cell line. This strategy was adopted to enrich the selection of viruses with enhanced

replication and spread phenotypes. Depending upon the cell line used, the passing procedure was carried out between 6 and 20 cycles.

To test the effectiveness of this bioselection protocol, we performed a proof-of-concept experiment. Two viruses, wild-type Ad5 and LGM, a derivative of Ad5 that contains the green fluorescent protein gene in place of the E1B-55,000-molecular-weight protein (E1B-55K protein) gene, were mixed at a PFU ratio of 1:1 and passaged on monolayers of U2OS human osteosarcoma cells by using the protocol described above. It has been shown previously that adenovirus mutants defective in the E1B-55K gene grow poorly in U2OS cells relative to wild-type Ad5 (7, 15, 41). After each passage cycle, culture medium was harvested and the relative abundance of the two viruses was measured by Southern blot analysis (data not shown). After 3 passages on U2OS cells, Ad5 clearly became the dominant species, constituting >95% of the total viral DNA detected. By passage 5 there was no detectable LGM in the culture medium. This experiment demonstrated that the passing protocol used in this study could enrich for viruses with a selective advantage for replication and spread.

Characterization of bioselected viruses. In our studies, the human colorectal cancer cell line HT29 was relatively resistant to infection by Ad5, usually taking more than 4 days to develop significant CPE, even at an MOI of 10. In contrast, with each cycle of serial passage in HT29 cells, the harvested virus pool progressively demonstrated an improvement in its cytolitic capacity. Due to the enhanced cytolitic phenotype, this virus population, henceforth referred to as VHT29, was characterized following 19 serial passages in HT29 cells. The VHT29 pool was first analyzed by plaque assay on eight tumor cell lines: HT29 (colon), A549 and H2009 (lung), DU145 and PC-3 (prostate), MB231 (breast), Panc-1 (pancreas), and HlaC (head and neck) as well as HEK-293 cells. Wild-type Ad5 was used as a control. Approximately 50% of the total plaques formed by VHT29 on the HT29 monolayers were exceptionally large (3 to 5 mm in diameter after 7 days) compared to the plaques formed by Ad5 (<2 mm in diameter).

Twenty large plaques from the VHT29 pool were isolated for further characterization. Plaque preparations were propagated in HT29 cells and reexamined by plaque assay on HT29 cells. Two of the plaque-purified virus isolates, ONYX-201 and -203, were selected for further analysis. Each virus produced uniformly large plaques on an HT29 cell monolayer compared to Ad5 (Fig. 1B and C). To further characterize the relative replication efficacy of ONYX-201 and -203, HT29 cells were infected either with these viruses or with Ad5 at an MOI of 10. Cells infected with ONYX-201 and -203 showed CPE much more rapidly than cells infected with Ad5 (Fig. 2A), with ONYX-201 consistently demonstrating higher cytolitic activity than ONYX-203. Additionally, the morphology of HT29 cells infected with ONYX-201 and -203 was distinct from that of cells infected with Ad5. ONYX-201- and -203-infected cells were well separated from one another and cells were swollen and had a smooth surface, whereas Ad5-infected cells clumped together, displaying a classical grapevine-like shape characteristic of Ad5 infection.

The cytolitic activities of ONYX-201 and -203 and Ad5 were compared by a cell viability assay (MTT assay) (Fig. 2B). As defined by the MOI producing 50% cytolysis of the culture at

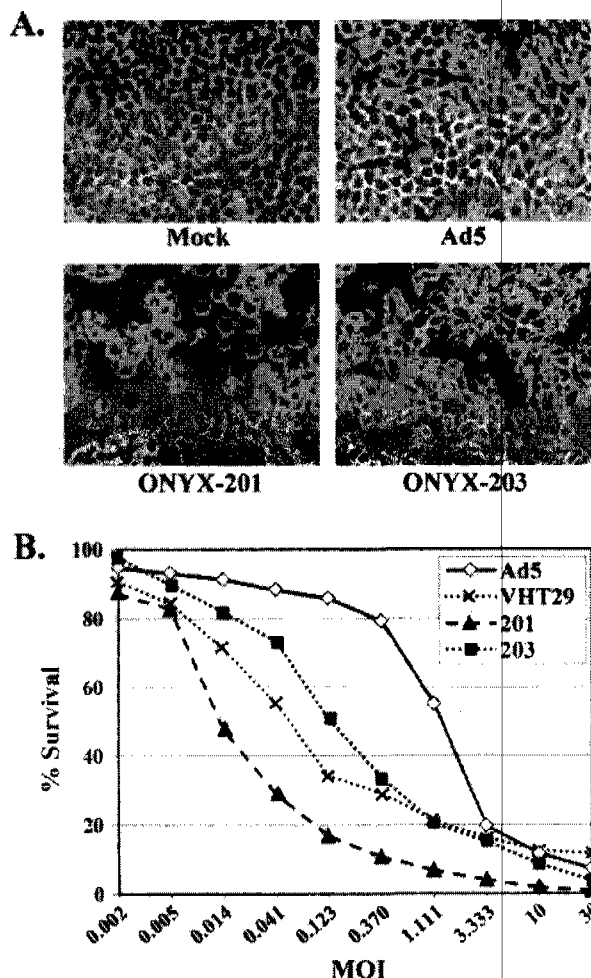


FIG. 2. (A) CPE of HT29 cells either mock infected (Mock) or infected (at an MOI of 10) with wild-type Ad5 and ONYX-201 and -203. Pictures were taken 3 days postinfection. (B) Cytolytic activity in HT29 cells was examined by using MTT assays. HT29 cells were infected with serial threefold dilutions of various viruses, with MOIs ranging from 30 to 1.5×10^{-3} . MTT assays were performed 5 days after infection as described. Results from one of the many independent experiments are shown here. Similar results were obtained in other experiments.

5 days postinfection, ONYX-201 was consistently 300- to 1,000-fold more active than Ad5, whereas ONYX-203 was approximately 30- to 50-fold more active than Ad5. The cytotoxicity of the VHT29 virus pool was between those of ONYX-201 and ONYX-203 (Fig. 2B), consistent with the fact that VHT29 was a mixture of viral mutants with various degrees of cytotoxicity.

The difference in cytolysis between bioselected viruses and the wild-type Ad5 was unlikely to be due to inaccurate assessment of virus titers. Ad5, ONYX-201 and -203 and VHT29 were titrated both in HT29 cells and in 293 cells, and the results were consistent in both cell lines with all viruses tested (data not shown). In addition, by fluorescence-activated cell sorter analyses and immunofluorescent staining studies of cells that were infected at a low MOI (0.01), the fraction of cells positive for the viral early protein E1A at early times postin-

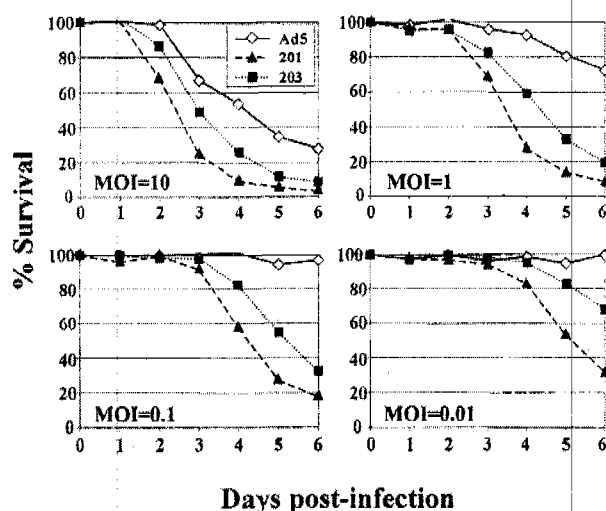


FIG. 3. Kinetics of HT29 cytotoxicity. HT29 cells were infected with Ad5, ONYX-201, and ONYX-203 at various MOIs. At different time points postinfection the percentage of viable cells was assessed by MTT assay and plotted versus time.

fection was similar for Ad5 and ONYX-201 and -203 (data not shown). The particle-to-infectious unit (particle:PFU) ratios for these viruses were similar and ranged from 10 to 50 (data not shown).

Kinetics of cell lysis and viral replication. We next examined the progression of HT29 cytolysis as a function of time and MOI. At an MOI of 10, ONYX-201 lysed cells more rapidly than ONYX-203, which killed cells more efficiently than Ad5 (Fig. 3). The cytolytic differences between the viruses were even more pronounced at lower MOIs. At MOIs of 0.1 and 0.01, cells infected with Ad5 showed no clear signs of CPE after 6 days with minimal change in cell viability. In distinct contrast, cells infected with ONYX-201 and -203 at the same MOIs displayed unambiguous CPE between days 3 and 4, and cell viability dropped dramatically (Fig. 3).

The enhanced cytolysis observed with ONYX-201 and -203 may result from a number of possible mechanisms, including more-efficient cell entry, faster rate of replication, larger progeny yield per cell, and more-efficient release and spread. To begin to explore the mechanism(s) for the distinct phenotypes of ONYX-201 and -203, we examined the kinetics of viral progeny production, DNA replication, and early as well as late gene expression at MOIs ranging from 0.01 to 10. Immunofluorescent staining studies indicated that Ad5 and ONYX-201 and -203 were indistinguishable with respect to infectivity, as the number of E1A-expressing cells at early times postinfection was similar after infection with equivalent MOIs of each virus (data not shown). When analyzed after infection at an MOI of 10, at which the majority of the cells were infected, the total yields of ONYX-201 and -203 and Ad5 virus progeny were similar (Fig. 4A). However, it took Ad5 considerably longer to reach the maximum virus progeny yield (5 versus 3 days for ONYX-201 and -203) (Fig. 4A). This result suggested that the total number of virus progeny produced per cell was similar but that the rates of production were significantly different. Indeed, viral DNA replication occurred more rapidly

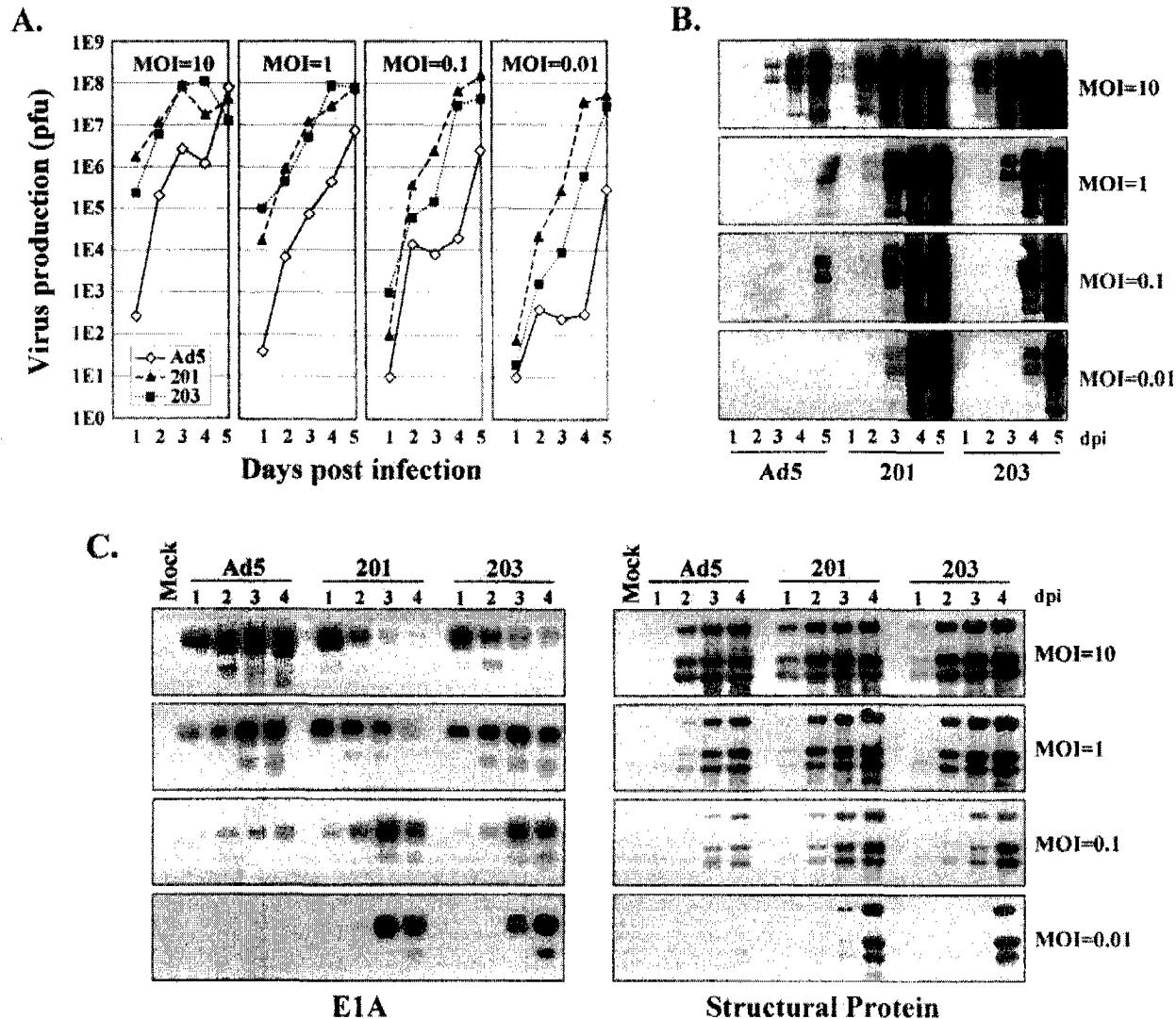


FIG. 4. HT29 cells were infected at MOIs of 10, 1, 0.1, and 0.01. At different time points after infection, cells and culture media were collected. (A) Virus yields. HT29 cells (4×10^4) were infected. Total virus yields (cells and culture media combined) were determined by plaque assay on 293 cells. (B) Viral DNA replication. DNAs were extracted by using the Blood DNA Extraction kit (Qiagen), digested with *Hind*III, and resolved on 0.8% agarose gels. After Southern transfer, the blots were hybridized with probes prepared with the digoxigenin High Prime DNA labeling kit (Roche Biochemicals). Ad5 genomic DNA served as a template for probe synthesis. (C) Viral gene expression. Cell extracts were prepared at various days postinfection (dpi) and separated by SDS-polyacrylamide gel electrophoresis. The expression of E1A and viral late proteins (structural protein) was examined by Western blot analysis. M, mock infected.

(Fig. 4B) and viral gene products accumulated faster (Fig. 4C) for ONYX-201 and -203 than for Ad5. These observations supported the hypothesis that ONYX-201 and -203 replicate more rapidly than wild-type Ad5. It is unclear why E1A disappeared in the ONYX-201- and -203-infected cells at 3 and 4 days postinfection. We speculate that, due to highly efficient virus DNA replication, these cells rapidly entered the late phase, at which time E1A ceased to be expressed. At lower MOIs, the differences between ONYX-201 and -203 and Ad5 with respect to progeny production, DNA replication, and accumulation of viral proteins was more pronounced (Fig. 4), consistent with the cytotoxicity results (Fig. 3). This can be attributed to the fact that at low MOIs (MOI < 1) multiple rounds of infection occurred, and the difference in the replication

rates between the viruses was amplified through each virus production cycle.

Cytotoxicity in diverse human cancer cells. To assess whether the enhanced cytolytic activity of ONYX-201 and -203 was restricted to HT29 cells, we examined their cell killing activity in a number of human cancer cell lines. Six tumor cell lines derived from human colorectal cancers, HT29, HCT116, CCL221, RKO, SW480, and SW620, and 6 cell lines of diverse tissue origins, A549 (lung), DU145 (prostate), MB231 (breast), Panc-1 (pancreas), U2OS (osteosarcoma), and 293 (transformed human embryonic kidney cells), were tested by MTT assay. Results from a representative experiment are shown in Fig. 5. ONYX-201 and -203 and VHT29 displayed a significantly higher cytolytic activity than Ad5 in HT29 cells, consis-

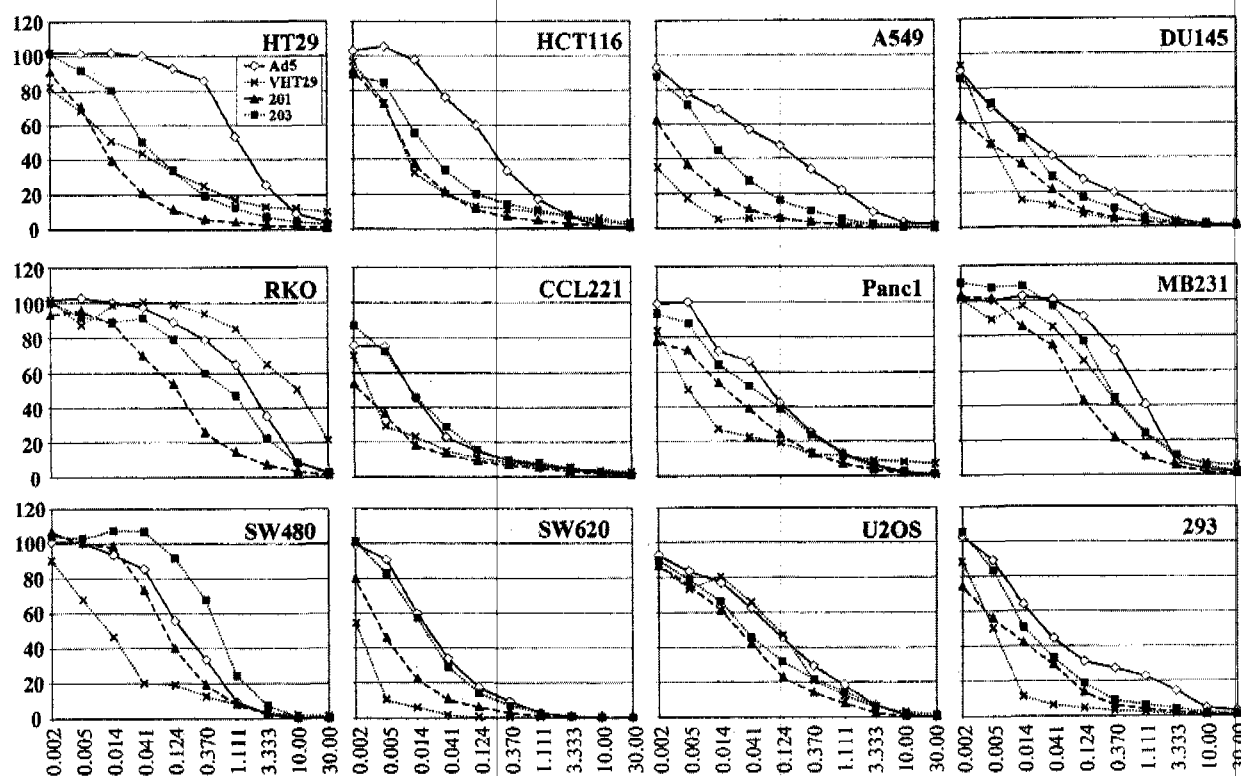


FIG. 5. Cytolytic activity in various tumor cells. Tumor cells were seeded in 96-well dishes at a density of 3,000 cells/well. Twenty-four hours after seeding, they were infected with Ad5, ONYX-201, ONYX-203, or the virus pool that was passaged in HT29 cells (VHT29). Infections were conducted by using serial threefold dilutions of each virus, starting from an MOI of 30. Results from one of the three independent experiments are shown here. Similar results were obtained in other experiments. MOIs are indicated on the x axes. Percentages of cell survival are indicated on the y axes.

tent with the results shown in Fig. 2. Additionally, the bioselected viruses showed substantially higher cytolytic activity than Ad5 in a number of, but not all, other cancer cell lines. As shown in Fig. 5, in A549 and in HCT116 cells, ONYX-201 and -203 and VHT29 were significantly more potent in cell killing than Ad5, whereas in DU145 and Panc-1 cells, their differences were marginal. It should be noted that in all cell lines tested, ONYX-201 was more active than Ad5. Interestingly, VHT29 was significantly more active in killing some cell types (for example, SW480 and SW620) than Ad5 or even ONYX-201 and -203. This observation suggests that the VHT29 pool may contain viral variants that replicate efficiently in these tumor cells. We conclude that the viruses selected on HT29 cells following chemical mutagenesis and serial passage accumulated mutations that promoted efficient replication in cancer cell lines of diverse tissue origin as well as in other cell lines derived from colon cancers.

Cytolytic activity in normal cells. Our goal is to generate and develop selectively replicating viruses as cancer therapeutic agents. One measure of the therapeutic index of oncolytic adenoviruses is the ratio of productive replication in cancer cells compared to normal cells. To determine whether the bioselection procedure affected virus replication selectivity for cancer versus normal cells, the cytotoxicity of ONYX-201 and -203 in primary human normal cell types was examined by MTT assay. In all of the primary cell types tested, which included small airway epithelial cells (SAEC), prostate epithelial cells,

mammary epithelial cells (MEC), renal epithelial cells, human umbilical vascular endothelial cells, and microvascular endothelial cells (MVEC), whether proliferating or contact inhibited, the replication properties of ONYX-201 and -203 were similar to those of Ad5. In general, ONYX-201 was only slightly more active than Ad5, and ONYX-203 was slightly attenuated compared to Ad5. Representative results from SAEC, MEC, and MVEC are shown in Fig. 6. The selectivity of each virus was assessed by comparing its cytotoxicity in normal primary MEC with that in MB468, a mammary cancer cell line. The cytolytic activity of each virus in MB468 cells and MEC was normalized to that of Ad5 and plotted as represented in Fig. 6D. ONYX-201 and -203 demonstrated a tumor-to-normal selectivity similar to that of ONYX-015, indicating that all three viruses preferentially killed tumor cells rather than normal cells. Importantly, ONYX-201 and -203 were substantially more potent in tumor cells than either ONYX-015 or Ad5. In conclusion, these bioselected viruses obtained increased tumor cell selectivity as well as enhanced cytotoxicity compared to their parent, Ad5.

Mutation mapping. To understand the underlying genetic basis for the increased cytotoxicity of ONYX-201 and -203 in tumor cells, their entire genomes were sequenced. Table 1 lists the identified mutations along with their possible consequences. ONYX-201 and -203 each contained seven single point mutations, four of which were shared by both viruses and the remaining three were unique to each virus.

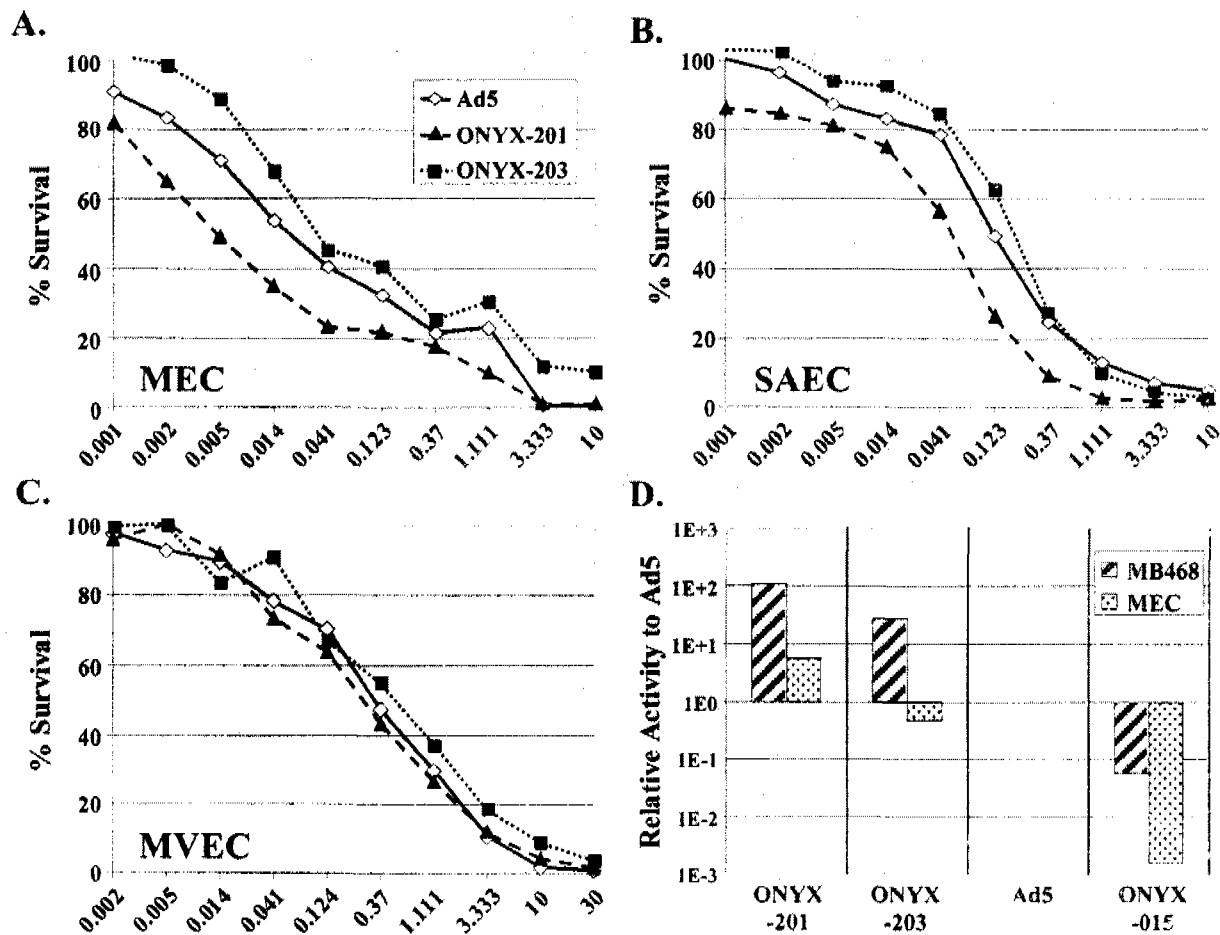


FIG. 6. Cytolytic activity in primary normal human cells. Cells were grown in 96-well dishes and were infected with Ad5 and ONYX-201 and -203 in serial threefold dilutions. (A) Quiescent MEC. (B) Quiescent SAEC. (C) Proliferating MVEC. Similar experiments were carried out multiple times in various primary normal human cells (proliferating or nonproliferating). Similar results were obtained; only three representative experiments are shown here. (D), Relative cytotoxicity in matched tumor and normal cells. MTT assays were performed on primary MEC and a mammary cancer cell line, MB468, with Ad5 and ONYX-201, -203, and -015. The 50% inhibitory concentration (IC_{50}) was defined as that MOI which resulted in 50% cell killing. These values were then plotted relative to that of Ad5 for each virus as follows: IC_{50} of Ad5/ IC_{50} of test virus. Therefore, the relative activity of Ad5 in normal and tumor cells is 1. The height of the bars represents the cytotoxicity compared to Ad5, and the difference between the striped bar and the stippled bar indicates the selectivity.

To delineate which point mutation(s) was responsible for the observed phenotypes, we constructed a series of recombinant viruses harboring particular mutations identified in ONYX-201 and -203. An illustration of these recombinant viruses

and their construction strategy is shown in Fig. 7A. The cytolytic activity of these recombinant viruses was compared by MTT assay on HT29 cells. Results from the MTT assay (Fig. 7B), combined with analysis of infected HT29 cell morphology,

TABLE 1. Summary of mutations in ONYX-201 and -203

Site (nucleotide)	Mutation	ONYX virus(es) containing mutation	Gene affected (explanation) ^a
8350	C to T	201 and 203	i-leader (i-leader protein truncated, may change viral gene expression and replication)
25904	G to A	201 and 203	100K protein (silent mutation)
30191	G to A	201 and 203	E3-14.5K protein (Ser to Asn)
31405	G to A	201 and 203	Fiber (Gly to Asp)
28291	G to A	201	E5 splicing receptor (change E3 gene expression)
34756	A inserted	201	E4 ORF 2 (frameshift/truncation)
35164	A inserted	201	E4 ORF 1 (frameshift)
27406	C to T	203	pVIII (silent mutation)
33910	A to G	203	E4 ORF 6 (Leu to Ser)
35058	A inserted	203	E4 ORF 2 (frameshift/truncation)

^a ORF, open reading frame.

indicated that all recombinant viruses containing the mutation at nucleotide 8350 (C to T) displayed the enhanced-cytotoxicity phenotype. ONYX-212, -232, -234, and -236 all had activities similar to that of ONYX-203, and they produced the characteristic morphology after infection of HT29 cells. In contrast, ONYX-231, -233, and -235 were indistinguishable from wild-type Ad5. Therefore, the C-to-T transition at nucleotide 8350 appeared to be both necessary and sufficient for the increased cytolytic activity of ONYX-203. In contrast, while this mutation was necessary to account for the superior cytolytic activity of ONYX-201, it was not sufficient. An additional mutation(s) appears to be required to achieve full cytolytic activity of ONYX-201. We are currently engaged in an effort to further characterize such mutation(s).

The C-to-T mutation at nucleotide 8350 is located in the i-leader region of the major late transcription unit of Ad5 (1, 11, 26, 47, 48). The i-leader sequence is spliced to a subset of L1 mRNA, which predominantly encodes the 52/55K protein, and may modulate expression of the 52/55K protein (1, 28, 36, 45). The i-leader itself contains an open reading frame encoding the 145-amino-acid i-leader protein (1, 11, 26, 47, 48). The exact role of the i-leader protein in adenovirus replication has not been clearly defined. However, this protein may play an important role in the initiation of viral DNA replication or switch to the late phase of the viral life cycle (1, 27, 28, 47). The C-to-T mutation at nucleotide 8350 changes the Gln codon (CAG) at amino acid 125 to a stop codon UAG, thus eliminating the last 21 amino acids of the i-leader protein. The size difference between the truncated i-leader protein and the wild-type protein is clearly demonstrated in Fig. 7C. Our results indicate that the truncated protein accumulated more rapidly in the infected cells than the wild-type protein (Fig. 7D). In cells infected with ONYX-201 and -203, most of the infected cells expressed high levels of the i-leader protein at 24 h postinfection. In contrast, the full-length i-leader protein was undetectable in the vast majority of cells infected with wild-type Ad5 at 24 h postinfection. The subcellular localization of the i-leader protein did not seem to be affected by this mutation (data not shown). Given the reported function of the i-leader protein in viral DNA replication, it is possible that the rapid accumulation of the truncated i-leader protein may play a role in the early onset of viral DNA replication, accelerating the viral life cycle.

DISCUSSION

Random mutagenesis of human adenovirus followed by repeated passaging in certain cell lines or under defined culture conditions has been used to isolate temperature-sensitive (10, 12, 32, 39, 50) and host range (3, 25) mutants. Presumably, these viruses have accumulated mutations that facilitate their productive growth in particular host environments or culture conditions. We suggest that, by using the same concept, adenoviruses can also be adapted to express highly potent, tumor-selective phenotypes. The work presented here describes for the first time this biological selection approach, by which we have isolated adenoviral variants that may specifically and efficiently target certain tumor cells for infection and killing.

In this paper, we report an *in vitro* bioselection procedure to isolate two mutants of Ad5, ONYX-201 and -203, that have

significantly accelerated viral DNA replication and protein synthesis, leading to more-efficient tumor cell killing and virus spread. Both viruses were isolated by serial passage in the HT29 human colorectal cancer cell line. ONYX-201 and -203 formed larger plaques in HT29 cells (Fig. 1), replicated more rapidly, and killed HT29 cells up to 1,000-fold faster (Fig. 2 and 4) than the parental virus Ad5. The difference was more pronounced when cells were infected at lower MOIs, presumably due to the compounding effects of multiround infections (Fig. 3 and 4). More importantly, the superior cytolytic activity of ONYX-201 and -203 was not limited to HT29 cells but was observed in a number of, but not all, other human cancer cell lines of diverse tissue origin tested (Fig. 5). In contrast, in a number of primary human cells tested, their cytotoxicity was similar to that of wild-type Ad5, effectively enhancing the tumor cell selectivity of these oncolytic agents (Fig. 6) compared to wild-type virus. The genomic DNAs of ONYX-201 and -203 were sequenced, and at least one mutation was identified as responsible for the observed phenotype (Table 1 and Fig. 7).

Adenovirus mutants that induce apoptosis also display enhanced CPE and the large-plaque phenotype (37, 40, 49). However, three lines of evidence suggested that ONYX-201 and -203 do not induce apoptosis in infected cells. First, there was no evidence for DNA fragmentation (data not shown), a hallmark for apoptosis. Second, the morphology of the infected cells (Fig. 2A) suggested that they were not dying of apoptosis, as apoptosis is characterized by cell shrinking and membrane blebbing and cells infected by ONYX-201 and -203 were swollen with a smooth surface. Third, viral progeny yields often reduce dramatically when apoptosis is induced (44), and this was clearly not the case for ONYX-201 and -203 in infected tumor cells (Fig. 4A).

We suggest that one of the main reasons for the enhanced cytotoxicity by ONYX-201 and -203 is their ability to replicate more rapidly. Figures 2 and 4 clearly indicate that cell lysis, viral macromolecular synthesis, and virus production were significantly more rapid in ONYX-201 and -203 during one-step infections (i.e., MOI = 10). The vast majority of the cells were infected at this MOI; therefore, the release and spread of the virus progenies were unlikely to be important factors. Nevertheless, it is entirely possible that these viruses have also acquired the capacity to spread more efficiently in cell culture, further enhancing their cytolytic activity in the low MOI settings. It is intriguing to speculate that one of the mutations in ONYX-201 may act in this fashion, complementing the mutation in the i-leader region.

The molecular mechanism of enhanced cytotoxicity for ONYX-201 and -203 is unclear. We confirmed that all cells were infected prior to cytotoxicity (data not shown), suggesting that they were killed directly as a consequence of a full viral life cycle rather than by a diffusible factor secreted by adjacent infected cells. We identified the mutation at nucleotide 8350 as necessary and sufficient for the enhanced oncolytic activity of ONYX-203. This mutation also exists in ONYX-201 and was required, but not sufficient, for the superior activity of ONYX-201. Additional mutation(s) may be required to achieve the full activity of ONYX-201. The C-to-T mutation at nucleotide 8350 is located in the i-leader of the major late transcription unit of Ad5 (1, 11, 26, 47, 48). The i-leader sequence is spliced to a subset of L1 mRNA, which predominantly encodes the 52/55K

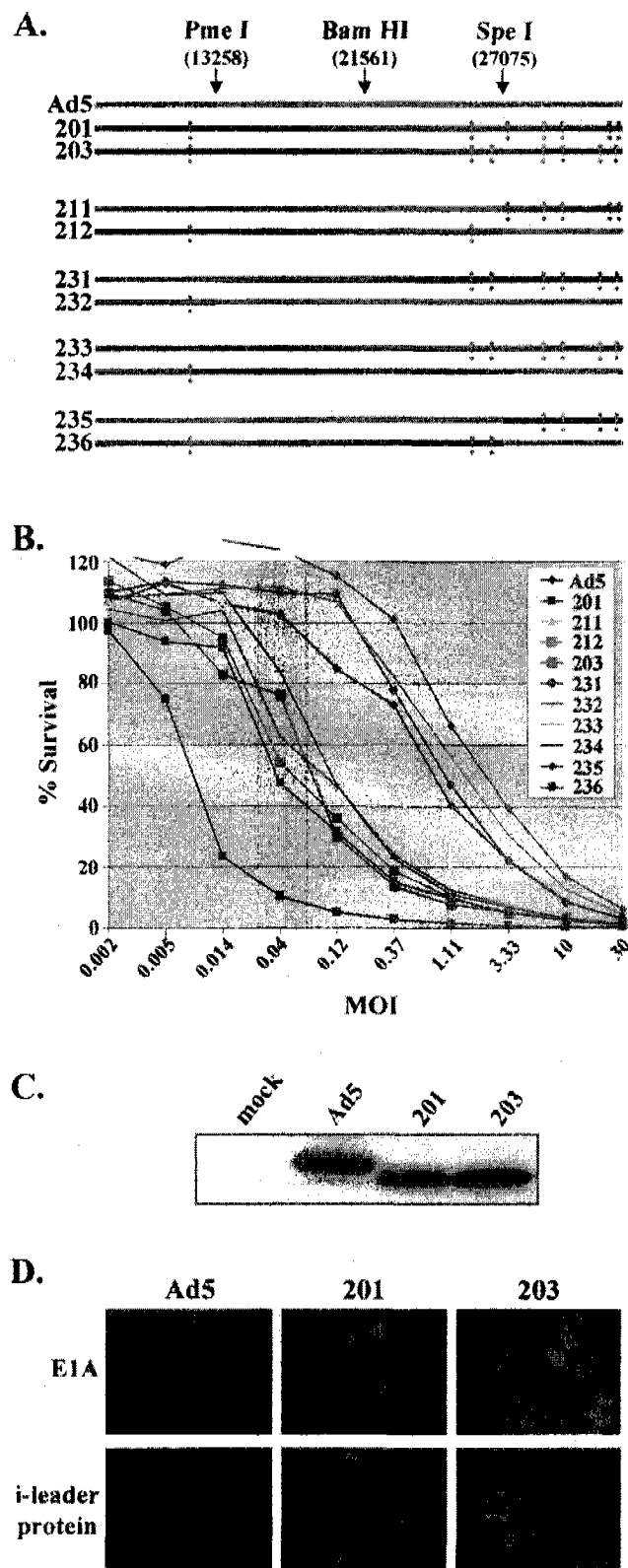


FIG. 7. (A) Recombination schemes. Various recombinant viruses were constructed as described in Materials and Methods. The exclamation marks indicate mutations present in each recombinant virus. Restriction sites for *Pme*I, *Bam*HI, and *Spe*I are indicated on the viral

protein, and may modulate expression of the 52/55K protein (1, 28, 36, 45). The i-leader itself contains an open reading frame encoding the 145-amino-acid i-leader protein (1, 11, 26, 47, 48). The exact roles of the 52/55K and the i-leader protein in adenovirus replication are not clearly defined. They are the only proteins encoded in the major late transcription unit that are expressed prior to viral DNA replication, suggesting that these two proteins may have an important role in the initiation of viral DNA replication or switch to the late phase of viral replication (1, 27, 28, 47). It is possible that early accumulation of the truncated i-leader protein can accelerate viral DNA replication through an unknown mechanism. Further analysis of the i-leader protein's function during the viral life cycle is needed in order to address this question.

ONYX-201 and -203 share 4 mutations (Table 1), suggesting that some or all 4 of the mutations are critical for the enhanced killing phenotype. Whether these mutations were independently selected, or whether the two viruses were derived from a common ancestor through differential recombination events, is not clear. The fact that ONYX-201 and -203 share more than one mutation favors the latter possibility.

ONYX-015, an adenovirus with E1B-55K deleted, efficiently replicates in and lyses tumor cells with a defective p53 pathway. In a number of human tumor cells, the loss of p53 function compensates for the loss of E1B-55K function during the viral life cycle. We may speculate that specific common genetic alterations are present in HT29 and other tumor cell lines in which ONYX-201 and -203 replicate with high efficiency and provide a cellular environment that selectively supports expression of their rapid replication phenotype. We are currently devising strategies to decipher the genetic basis for such possible mechanisms. In all of the normal human cells tested, the cytotoxicity of ONYX-201 or -203 was similar to that of wild-type Ad5. This not only lends support to the cellular genetic basis model for the ONYX-201 and -203 phenotype but also indicates that, as oncolytic agents, these viruses have an enhanced overall therapeutic index compared to wild-type virus.

Our observation that ONYX-201 and -203 replicated in, and killed, tumor cells drastically more rapidly than Ad5 at low MOIs may have profound clinical impact. Human solid tumors at clinical presentation are commonly large masses of densely populated cells. Even in the case of a direct intratumoral injection, infection by therapeutic viruses occurs at a very low multiplicity. Thus, the infection conditions in viral oncolytic therapy regimens are not optimal. Furthermore, following systemic administration, the amount of virus that reaches the tumor is estimated to be a very small fraction of the injected dose, so the multiplicity of infection would be even lower than

genomes. (B) Cytolytic activity of the recombinant viruses was examined in HT29 cells by using MTT assays. (C) ONYX-201 and -203 encode the truncated i-leader protein. HT29 cells were infected at an MOI of 10. Expression of the i-leader proteins was examined by immunoblot analysis. Mock, mock-infected cells. (D) Accumulation of the i-leader protein. HT29 cells grown on chamber slides were infected with Ad5 and ONYX-201 and -203 at an MOI of 10. At 24 h postinfection, the cells were fixed, permeabilized, and analyzed by dual immunofluorescent staining with M73 (a monoclonal antibody against E1A) (green) and a polyclonal antibody against the i-leader protein (red). Representative fields are shown.

intratumoral administration. Therefore, the rapid replication phenotype observed under low MOI described here is likely relevant to the clinical setting, for both local-regional and systemic administration. The ability to quickly replicate should give these viruses a greater chance to eliminate solid tumor masses.

The work presented here provides groundwork for using bioselection to develop highly potent, tumor-selective oncolytic viruses. To the best of our knowledge, this is the first case demonstrated for human adenovirus. ONYX-201 and -203 and their recombinant derivatives are being examined in animal models carrying human tumors. Additionally, the key mutations identified in the present study are being incorporated with other genetically engineered adenoviruses, such as ONYX-015 and -411 (19), to create new generations of cancer therapeutic adenoviruses.

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Conditionally replicative adenoviruses for cancer therapy

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Abstract

The delineation of the genetic etiology of cancer makes gene therapy a rational approach for the molecular treatment of cancer. Many gene delivery systems have been developed, with viral vectors being the most effective. Underlying cancer gene therapy protocols is the recognition that quantitative tumor transduction cannot be achieved with the vector systems available at the present time. One way to overcome this problem could be to amplify the transduction efficiency through the use of vectors capable of replicating specifically in tumor cells. We are currently developing an adenoviral vector in which viral replication will be restricted to the target tumor cells by limiting the expression of viral genes essential for the virus replication only to the tumor cells of interest. © 1997 Elsevier Science B.V.

Keywords: Gene therapy; Cancer; Adenovirus; Replication

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1. Introduction

In 1996, most malignant neoplasms remain incurable so that cancer is still one of the principal causes of death in industrialized countries [1]. Chemotherapy, radiotherapy and surgery have evolved as standard treatment modalities over the last decades; these therapeutic approaches result in reductions of

tumor burden, however in many instances the disease relapses as a result of the inability to eradicate all tumor cells and sometimes due to tumor cells becoming resistant to treatment. Based upon these considerations, there is a need for novel, alternate treatment modalities.

In this regard, gene therapy for cancer has become a rational interventional strategy [2–5]. This is based upon the discovery of a genetic etiology of many neoplastic transformation events. Further, it has been recognized that phenotypic reversion and tumor

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regression can be obtained by selective abrogation of the molecular pathways etiologic of cancer. The delineation of the genetic etiology of cancer thus makes gene therapy a rational approach for the molecular treatment of cancer. In this regard, many approaches have been utilized in experimental animal models, and promising results have already been translated into phase I human clinical gene therapy trials. To date, 86 clinical protocols employing these strategies have been proposed to the Recombinant DNA Advisory Committee (RAC) of the NIH and other agencies that control gene transfer clinical studies [6].

2. Strategies for cancer therapy

Strategies for cancer gene therapy can be divided into three groups: genetic immunopotential, mutation compensation and molecular chemotherapy [2–5]. Genetic immunopotential is defined as the genetic induction of antitumor immunity. In practice, this is accomplished by employing a vaccine consisting of tumor cells which have been genetically modified to overexpress effectors, either cytokines or immune costimulatory molecules [7–13]. This strategy has been utilized in models of metastatic disease, whereby the implantation of genetically modified tumor cells is employed to achieve systemic immune-mediated antineoplastic effects. Mutation compensation refers to the genetic modifications of malignant cells to mitigate the effects of mutations or loss of function strongly associated with neoplastic transformation. It involves replacement of wild-type tumor suppressor genes to cells bearing a deleted or mutated tumor suppressor gene or methods to diminish the production of an oncoprotein whose gene expression is aberrantly regulated. Molecular chemotherapy refers to the addition of a toxin that will lead to the death of the tumor cells. There are direct and indirect approaches for molecular chemotherapy. The direct approach utilizes the delivery of a toxic substance directly to the tumor cells. In contrast, the indirect approach relies upon the delivery of a non-toxic prodrug and a prodrug activating gene. The combination of these two elements results in the activation of the prodrug into a toxic substrate resulting in cell death. In this case, the therapeutic gene functions much like a conventional chemo-

therapy agent. However, mutation compensation and molecular chemotherapy require *in vivo* delivery of antitumor genes, and therefore they have been utilized mainly in local/regional disease models. These *in vivo* gene delivery approaches have attempted to achieve direct *in situ* tumor transduction to yield antitumor effects.

3. Gene delivery systems for cancer

A common set of gene delivery systems have been employed for the different cancer gene therapy strategies: viral (retrovirus, adenovirus), non-viral (liposomes, naked DNA injection) and physical methods (electroporation). The choice of gene delivery system depends on the nature of the intervention strategy and the context of the target disease, i.e., local/regional versus metastatic tumors. In this regard, genetic immunopotential approaches, which have been employed in cases of metastatic diseases, utilize viral vectors for *in vitro* transduction and selection of stably transduced cells. Although retroviral vectors have been used to achieve *ex vivo* transduction, constraints relevant to *in vivo* stability have not permitted its wide spread employment for other cancer gene therapy approaches. In contrast, mutation compensation and molecular chemotherapy strategies require direct *in vivo* delivery of antitumor genes and therefore have been utilized mainly in local/regional disease models. These *in vivo* gene delivery approaches thus attempt to achieve direct *in situ* tumor transduction to yield antitumor effects.

For these gene therapy approaches, vector strategies must therefore accomplish relatively efficient *in vivo* gene delivery. In this regard, the adenoviral vector has been employed for a variety of these cancer gene therapy approaches due to its ability to accomplish high efficiency *in vivo* gene delivery. Hence, it has a broad host range, it can transfer genes to non-dividing cells, it is resistant to host complement factors, and it can be generated to very high titers. For example, in the molecular chemotherapy strategy, the herpes simplex virus thymidine kinase (HSV-tk) gene has been successfully delivered with an adenovirus vector to several types of cancer cells *in vitro* and *in vivo* [14–21]. In a study by Ross et al. [15], a primary glioma in rat was shown to be sensitive to Ad/HSV-tk killing *in vivo*. Tumor

volumes decreased following administration of ganciclovir (GCV) as measured by MRI, resulting in prolonged survival. In similar experiments by Çolak et al., a metastatic breast cancer model to brain was generated by injecting MAT-B cells into the caudate nucleus of Fisher rats [16]. They demonstrated that this MAT-B cell line could be efficiently transduced *in vitro* by a replication-defective adenoviral vector bearing the HSV-tk gene and that the Ad/HSV-tk infection could render the cells sensitive to GCV killing in a dose-dependent manner. *In vivo*, when the Ad/HSV-tk was injected into tumors and the animal treated with GCV for 6 days, no visible tumors could be detected at the site of tumor injection 16 days after the GCV treatment, whereas control animals exhibited large tumors. In survival studies, animals treated with Ad/HSV-tk and GCV survived a significantly longer time than control animals. In a breast cancer model, Chen et al. employed a tumor-specific promoter, DF3/MUC1, to express the HSV-tk gene in an adenoviral vector [21]. They first demonstrated efficient *in vitro* and *in vivo* transduction of several DF3-positive breast carcinoma cell lines in a tumor-specific manner. Using an intraperitoneal breast cancer metastases model, they demonstrated that intraperitoneal injection of Ad/DF3-HSV-tk followed by GCV resulted in inhibition of tumor growth. Additional work by Eastham et al. was accomplished in the context of prostate cancer, whereby subcutaneous tumor nodules were injected with Ad/HSV-tk followed by GCV treatment for 6 days [17]. The mean tumor volume in treated animals was 18% of that in control animals, and histologically treated animals demonstrated cell death which was not seen in control animals. Using the mouse prostate reconstitution model, similar results were obtained when primary site lesions were injected with Ad/HSV-tk and treated with GCV. These studies demonstrate that, in the context of breast cancer, metastatic breast cancer to the brain and prostate cancer, adenovirus-mediated transfer of the HSV-tk gene in conjunction with a GCV regimen can efficiently inhibit tumor cell growth *in vitro* and *in vivo*.

The studies of Hwang et al. established the effectiveness of the Ad/HSV-tk/GCV system in causing tumor regression in animals inoculated intraperitoneally with a mesothelioma or a lung adenocarcinoma cell line, and showed that animals with

bulky disease could also be successfully treated [18]. Effective killing could be achieved using low titer Ad/HSV-tk and clinically safe GCV doses, and HSV-tk gene expression remained localized following infection with Ad/HSV-tk. Çolak et al. also reported successful killing of tumors in a spinal cord tumor animal model [19]. They generated spinal cord tumors by stereotaxic intramedullary injection of 9L gliosarcoma cells into Fisher 344 rats. Seven days later, intratumoral injection of Ad/HSV-tk was performed followed by GCV treatment for 6 days. Eighteen days after injection of tumor cells, all control animals had paraplegia and large tumors. In contrast, no tumors were detected in Ad/HSV-tk- and GCV-treated animals: two out of five of these animals remained tumor-free and healthy at 6 months, whereas control animals became paraplegic within 18 days. Again these studies demonstrated the efficacy of the adenovirus to mediate HSV-tk gene transfer *in vivo* to different neoplasias for inhibition of tumor growth following GCV treatment.

Rosenfeld et al. utilized Ad/HSV-tk and demonstrated efficient *in vitro* transduction of a panel of different human ovarian cancer cell lines and primary cultures of ovarian cancer cells obtained from patients with ovarian carcinoma [20]. In a SCID mouse model, SKOV3.ip1 ovarian cancer cells were injected intraperitoneally and allowed to establish tumors; significant tumor reduction and diminished overall tumor burden was achieved. These studies provided the basis for a human clinical protocol that was submitted to the RAC and accepted for a phase I human clinical trial. Thus, in the context of molecular chemotherapy, the recombinant adenovirus can be utilized successfully to deliver genes encoding pro-drug activating enzyme both *in vitro* and *in vivo*.

Mutation compensation strategies involving dominant oncogene knock-out and tumor suppressor gene replacement have also employed the adenovirus as a vector. For example, Liu et al. showed growth suppression of human head and neck cancer cells by the introduction of a wild-type p53 gene via a recombinant adenovirus [22]. *In vitro* growth assays revealed growth arrest following adenovirus carrying p53 gene (Ad/p53) infection as well as cell morphological changes consistent with apoptosis. *In vivo* studies in nude mice with established subcutaneous squamous carcinoma nodules showed significantly reduced tumor volumes in mice that received

peritumoral injection of Ad/p53. Furthermore, they demonstrated that inhibition of tumor growth occurred through induction of apoptosis [23]. Replacement of p53 in melanoma cells has been investigated by Cirielli et al. [24]. In vitro growth inhibition was observed with human B16.G3.26 and murine SK-MEL-24 melanoma cells when transduced with Ad/p53 and both underwent apoptosis. Nude mice injected subcutaneously with these cell lines developed localized tumors, however, when tumors were injected with Ad/p53 1 week later, tumors were 2.5-fold smaller than in control animals and they exhibited DNA fragmentation consistent with apoptosis. Another interesting study by Toshiyoshi et al. demonstrated induced chemosensitivity of human lung cancer cells in vivo by adenovirus-mediated p53 replacement [25]. In this study, they employed the non-small cell lung cancer cell line H358 that has both p53 alleles deleted. Their hypothesis was that the absence of p53 protein in the cancer cells caused resistance to chemotherapeutic drugs. The Ad/p53-transduced H358 cells treated with cisplatin underwent apoptosis with specific DNA fragmentation in vitro. In the in vivo context, direct injection of Ad/p53 into H358 tumors subcutaneously implanted into *nu/nu* mice, followed by i.p. administration of cisplatin, induced massive apoptotic destruction of the tumors. These results support the clinical application of a regimen combining gene replacement using replication-defective wild-type p53 encoding adenovirus and DNA-damaging drugs for the treatment of human cancer. Other studies have shown in vitro growth inhibition of tumor cells by replacement of p53 gene, notably for glioma [26], osteosarcoma [27], cervical carcinoma [28], ovarian carcinoma [29] and prostate cancer cells [30]. These studies have established the efficacy of the adenovirus to successfully mediate in vivo p53 gene transfer to a variety of disease models in order to achieve p53 gene replacement.

In a similar approach, Jin et al. employed gene replacement of p16^{INK4} into lung cancer cells carrying a homozygous deletion of this tumor suppressor gene. Their results demonstrated that expression of the adenovirally introduced p16^{INK4} blocked tumor cell entry into the S phase of the cell cycle and inhibited tumor proliferation both in vitro and in vivo [31]. Tumor suppressor genes such as p53 like Bcl-Xs and C-CAM-1 have also been targeted as thera-

peutic gene replacement strategies for prostate cancer [32] and different disseminated cancers using bone marrow purging [33], and have been shown to inhibit cell growth in vitro. Thus, the adenovirus vector can be successfully employed to accomplish tumor suppressor gene replacement.

In an alternate mutation compensation approach, Deshane et al. proposed a targeted anti-cancer strategy to selectively eradicate erbB2 tyrosine kinase receptor overexpressing tumor cells by knocking-out the erbB2 protein with an anti-erbB2 intracellular single-chain antibody (anti-erbB2 sFv) [34]. They showed that expression of an endoplasmic reticulum (ER) form of the anti-erbB2 sFv resulted in a profound down-regulation of cell surface erbB2 receptors in erbB2 overexpressing ovarian cancer cell lines and this led to a marked inhibition of tumor cell proliferation in vitro. Moreover, whereas stable transfectants expressing the anti-erbB2 sFv could be derived from non-erbB2 overexpressing cancer cell lines, expression of the intracellular antibody was incompatible with long-term survival of the erbB2 overexpressing tumor cells in vitro. Using an adenoviral vector encoding the ER form of the anti-erbB2 sFv, targeted tumor cell killing could be achieved in vivo and significant prolongation of survival of animals carrying a human ovarian carcinoma tumor burden within their intraperitoneal cavities could be obtained [35,36]. It was also demonstrated that the highly specific targeted eradication of the erbB2 overexpressing tumor cells by the intracellular antibody occurred through the induction of programmed cell death [37]. Thus an adenovirus vector can efficiently deliver genes in vitro and in vivo genes to mediate dominant oncogene knock-out.

In these in situ schemas, the vector was administered intratumorally, peritumorally, or alternatively, into an anatomic compartment containing the tumor mass. The logic of these delivery modes is based upon the achievement of a high local vector concentration which favors tumor cell transduction and simultaneously limits vector dissemination. Nevertheless underlying all of these approaches is the recognition that quantitative tumor cell transduction cannot be achieved with the vector systems available at the present time. Thus, for the gene therapy strategies without significant amplification effects, suboptimal tumor correction or killing is achieved. A

good example is presented in the studies of Hwang et al. where a molecular chemotherapy treatment could accomplish a dramatic effect in a xenogenic heterotopic mouse model of human mesothelioma [18]. In contrast, when the same strategy was undertaken in a syngenic system using orthotopic disease localization, response rates were significantly lower [38]. These studies demonstrate the fact that, in spite of effective results in a model system, optimal vector delivery could not be reached in order to translate these findings to an orthotopic model more closely mimicking human disease state. Therefore, although several viral vectors have shown their ability to accomplish direct *in vivo* gene delivery to tumors, their transduction efficiencies are presently inadequate to modify a sufficient number of tumor cells to achieve an efficacious treatment.

4. Vectors for *in situ* amplification for increased efficacy

A means to circumvent this issue would be to design a vector that would amplify tumor transduction, in spite of suboptimal anatomical or delivery-related conditions in the *in vivo* disease model. One way to achieve this amplification effect would be via replication of the delivered viral vector. In this approach, a replication-competent virus would be used to selectively replicate within the transduced tumor cells and not in normal tissues. Production of virus progeny from transduced tumor cells would then allow infection of the neighboring tumor cells, resulting in an increased viral transduction that could be of therapeutic utility in two ways. First, tumor-specific replication of a recombinant viral vector encoding a therapeutic gene would augment the intratumoral virus inoculum, thus increasing the viral concentration at the tumor interface and spreading the virions into the tumor mass. This would increase tumor transduction efficiency and thus augment therapeutic efficacy of the delivered antitumor gene. Second, and most importantly, the utilization of viruses which replicate through a lytic cycle would result in viral-mediated oncolysis. This effect would be mediated regardless of the delivered transgene and so the benefit would be additive. Through the use of viruses which possess the ability to replicate specifically in tumor cells, direct tumor cell lysis

would occur while leaving normal cells unaffected. In both cases, amplification of the antitumor effect would be achieved.

This viral vector system could be derived from two types of viruses; wild-type, attenuated or tissue culture-adapted viruses that possess the ability to replicate in specific tissues, and recombinant viruses genetically engineered to replicate only within the target tissue. In the former instance, tissue culture-adapted viruses have been extensively used in the past as oncolytic agents for experimental cancer therapy. These studies conducted in the 1950s through the 1970s involved utilization of wild-type viruses possessing the capacity to selectively replicate within tumor cells. Such studies included adenovirus [39], mumps virus [40,41], vaccinia virus [42], myxovirus [43–46], West Nile virus [47,48], Newcastle disease virus [49] and others that were administered by several routes. The three most promising and informative studies were those of Southam and Moore [47], Smith et al. [39] and Asada [40]. Southam and Moore studied the utilization of the Egypt 101 strain of West Nile virus to treat 34 patients suffering from advanced neoplasms not responsive to any established method of treatment such as surgery, roentgen-ray irradiation or chemotherapy [47]. Inocula consisting of bacteriologically sterile tissues (mouse brain, chick embryo or human tumor tissue) were injected intramuscularly or intravenously. Viral infection was definitely established in 27 of the patients. This viral infection did not exert a curative effect but in at least four, and possibly nine, of these patients a transient inhibitory effect of tumor growth was observed. In 14 of these patients, virus was localized in tumor tissue and, in five patients, it was preferentially concentrated in tumor tissues compared to normal tissues.

4.1. Replication-competent virus as a therapeutic tool for cancer therapy

Smith et al. used adenovirus as an experimental therapy in patients with advanced cervical carcinomas [39]. Their study was based on the luxuriant growth of adenoviruses on HeLa cells. Patients were *a priori* screened for the presence of neutralizing antibodies and then treated with an Ad serotype to which they were not immune by intratumoral or

intraarterial injection of virus-containing culture supernatant. Sixty-five percent of the patients showed necrosis and cavity formation in the pelvic tumors but total tumor regression never occurred.

Asada selected the mumps virus for its capacity to thrive in actively multiplying germ cells [41]. Tissue culture suspensions were administered to patients by local application to the tumor surface, by intratumoral, oral, rectal or intravenous inoculation or by inhalation. Tumors disappeared or decreased to less than half their initial size in 37 of 90 patients with only minimal toxicity. The titer of anti-mumps antibodies increased and long-term tumor regression was attributed to stimulation of antitumor immunity. Thus, these studies established the potential utilization of oncolytic viruses to treat cancer.

More recently, in 1992, Reichard et al. undertook a study to evaluate the direct effects of Newcastle disease virus (NDV) strain 73-T on a variety of human tumors *in vitro* and *in vivo* and to establish the NDV's specificity for tumor cells versus normal cells [50]. They were inspired by the work of Cassel and Garrett who suggested that a mouse tumor-adapted strain of the NDV, 73-T, was directly oncolytic to murine tumor cells both *in vitro* and *in vivo* [49]. Plaque assays determined the cytolytic activity of NDV 73-T on six human tumor cell lines (HT1080 fibrosarcoma, KHOS osteosarcoma, HCV29T bladder carcinoma, KB8-5-11 cervical carcinoma, IMR32 neuroblastoma and G104 Wilm's tumor cells) and on nine normal human fibroblast cell lines. Plaques formed on all the tumor cell lines tested, whereas no plaques could be detected on any of the normal fibroblast lines. Virus yield increased 10 000-fold within 24 h in tumor cells but remained near zero in normal fibroblast supernatants. Anti-tumor effect was evaluated *in vivo* in athymic nude Balb-c mice by subcutaneous injection of 9×10^6 tumor cells followed by intratumoral injection of 1×10^6 plaque-forming units (PFU) of live or heat-inactivated NDV or medium. After live NDV treatment, tumor regression occurred in 10 of 11 mice bearing the cervical carcinoma cells, eight of eight with fibrosarcoma cells and six of seven with neuroblastoma cells. In contrast, no tumor regression occurred after treatment with heat-inactivated NDV or medium. No toxicity was observed in healthy mice injected with 1×10^8 PFU of NDV. This study demonstrated the efficiency of NDV to selectively

replicate in tumor cells without affecting normal cells and its potential to cause tumor regression *in vivo*. The parvovirus H-1 has also been employed in several tumor models based upon its ability to replicate selectively in cultured human tumor cell lines of various tissue types. In these models, viral replication has resulted in suppression of oncogenicity and tumor killing [51,52]. An important feature of the NDV and parvovirus H-1 is that they are not pathogenic to humans; therefore viral toxicity would be expected to be minimal in clinical contexts. Thus, the studies on the utilization of the Newcastle virus and H-1 parvovirus have demonstrated that killing of human tumor cells and tumor regression can be achieved by viral oncolysis.

4.2. HSV-1 mutants

Even though development of herpes vectors for tumor therapy is at an early stage, another attractive virus for experimental tumor therapy is the herpes simplex virus type 1 (HSV-1). This concept is based on the ability of certain HSV-1 mutants to selectively replicate within tumor cells. A limited number of studies in rodents have explored the utilization of herpes vectors in experimental tumor therapies, and these works have mostly employed HSV-1 mutants which have reduced neurovirulence and replicate efficiently in dividing cells but poorly or not at all in non-dividing cells. HSV-1 vectors have been shown to infect a broad variety of tumor cell types, including most rodent tumor lines and all lines from human brain tumors tested to date, gliomas [53–57], medulloblastomas [57], meningiomas [55,57] and neurofibrosarcomas [57] and most rodent tumor lines. The first generation of HSV-1 vectors used for tumor therapy contain a single gene mutation or deletion that permits them to replicate more efficiently in some cells compared to others. HSV-1 mutants in genes for $\gamma 34.5$ [58], ribonucleotide reductase [59] and thymidine kinase [60] have been evaluated for brain cancer gene therapy. The first studies using an HSV mutant were conducted by Martuza et al. [53]. They employed a thymidine-kinase gene deleted mutant, *dlsp_{tk}*, and have shown infectivity and toxicity for two glioma cell lines *in vitro*. Intratumoral injection of mutant virus into subcutaneous tumors implanted in the subrenal capsule or intracranial tumor in nude mice produced significant growth

inhibition of the tumors. Some evidence of encephalitis was observed in these animals without being lethal. Chambers et al. compared two HSV mutants, one carrying a deletion of the $\gamma 34.5$ gene and the other producing a truncated form of that gene product, for their ability to replicate and cause cytolysis in a SCID mouse glioma model [56]. Their results showed that both mutants could replicate and cause oncolysis of glioma cells of murine and human origin and could significantly prolong survival in a dose-dependent manner. Martuza et al. employed the hrR3 ribonucleotide reductase-deficient (RR-) HSV mutant in which the *E. coli lacZ* gene was inserted into the ICP6 gene that encodes the large subunit of the RR [53]. The hrR3 mutant caused a cytopathic effect on the U-87MG human glioblastoma cell line in vitro, and drug sensitivity assays demonstrated that hrR3 was hypersensitive to ganciclovir. In vivo studies with animals harboring U-87MG tumors subcutaneously, demonstrated significant inhibition of tumor growth in animals treated intratumorally with 5×10^6 PFU and *lacZ* expression could be detected in treated tumors. Thus these studies demonstrated the concept that tumor cell killing and tumor regression can be achieved by the oncolytic property of the first generation of HSV-1 mutants.

Further improvement in vector design has resulted in the so-called second generation of HSV-1 vectors. These vectors contain multiple mutations. Mineta et al. have created a multigene mutant of HVS-1, G207, with deletions at both $\gamma 34.5$ loci and a *lacZ* gene insertion in the ICP6 gene [61]. This double mutation makes reversion to wild-type unlikely and confers important advantages that need to be considered for an eventual translation into clinical trials: ganciclovir hypersensitivity, temperature sensitivity and attenuated neurovirulence. G207 killed human glioma cells in vitro and in nude mice harboring subcutaneous or intracerebral U87-MG glioma cells; intratumoral inoculation with G207 caused decreased tumor growth and/or prolonged survival. Injection of G207 was not toxic to mice and HSV-sensitive non-human primates. The study of Yazaki et al. demonstrated that the G207 HSV mutant could replicate within and kill three human malignant meningioma cell lines in vitro [62]. In a nude mouse model, where human malignant meningioma F5 cells were implanted subcutaneously, G207 could inhibit growth of tumor in a dose-dependent manner. In

nude mice harboring intracranial subdural F5 cells, one injection of G207 caused significant decrease in tumor growth and one apparent cure without neurological dysfunction or pathological changes in the surrounding brain.

Although these studies utilizing HSV-1 vectors for tumor therapy have shown very encouraging results, some issues still need to be addressed and these may prove limiting in the translation to human clinical trials. First, the use of HSV-1 mutants has been studied for the treatment of brain tumors and based upon their specific ability to replicate in brain tumor cells. But some brain tumor cells may be resistant or poorly infectable as seen in the rat C6 glioma cell line [63]. Second, in these therapeutic models, the virus infected all cells at the site of injection, including tumor cells and normal cells. One must consider that there are many dividing cells in the body that could serve as host cells for the virus to replicate. Even though most individuals can have an active HSV infection without serious consequences, the exceptions to this are very severe and should be considered even more in the case of cancer patients who are frequently at least partially immunocompromised. Another concern is the possible reactivation of a latent wild-type virus by the HSV-1 vector or mutant. The full spectrum of cell types that can harbor the virus in latency is not known. Fourth, it is not known whether infection with a HSV vector or mutant can reactivate other members of the herpes family like Epstein-Barr virus (EBV), cytomegalovirus (CMV), herpes simplex virus type 2 (HSV-2) and varicella zoster, even though infection with HSV-1 has not been associated with reactivation of other herpes viruses, so far. Thus, because the biology of replication versus latency, reactivation, and tropism are not fully understood, HSV-1 vectors may not be the best viruses to be utilized for tumor therapy at the present juncture.

4.3. Replication-competent restricted adenovirus

Adenoviruses may be suitable candidate vectors for cancer therapy because their biology is well characterized and understood and they possess in vivo stability. Recombinant adenoviruses have shown great promise for the in vivo transduction of tumors after in situ or vascular delivery [64-66]. Thus the use of these viral vectors in the context of

conditional replication would be logical. They are already prevalent in the human population and are generally of low pathogenic potential, that is infections are mild and self-limiting illnesses in most individuals [67]. Adenovirus stocks can be prepared at much higher titer than other viruses and between 10 000 and 100 000 virions can be produced per infected cell. Adenoviruses possess a lytic life cycle and this oncolytic property could be exploited by engineering the virus to replicate specifically within tumor cells (Fig. 1). Adenoviruses have a broad host range and are not cell cycle-dependent since they can infect dividing, quiescent and terminally-differentiated cells such as neurons and hepatocytes [65–68]. Use of conditionally replicative adenoviral systems would permit considerable flexibility in the treatment of tumors from a variety of histological types. Moreover, their tropism can be altered by genetic modification of the fiber gene and the penton base gene in order to redirect their binding and internalization to a specific subset of target cells [69–71]. Because they are non-enveloped viruses, they are less sensitive to complement-mediated inactivation,

and this is of importance since virus spread is desired. Unlike the situation with the retroviruses, the adenovirus genome rarely integrates into the host cell chromosomes, thus its genome does not persist for a long time in the infected cells. Moreover, there is no latency phase in contrast to herpes viruses. Most of the adenoviral vectors are derived from the non-oncogenic subgroup C and more particularly from the serotype 5. Therefore adenoviruses represent a much safer viral vector system to be utilized for cancer therapy.

Development of an adenoviral vector system in which viral replication is restricted to the targeted tumor cells could be achieved by limiting the expression of genes essential for adenovirus replication only to the tumor cells of interest. The proof of concept could be demonstrated by transcomplementation (Fig. 2). The concept of transcomplementation has been demonstrated with cell lines that stably express an adenoviral gene of interest. Examples of this are the 293 cell line that expresses the E1A protein [72,73], the gmDBP cell line expressing the AdPol [74,75], the 293-tTA-pTP

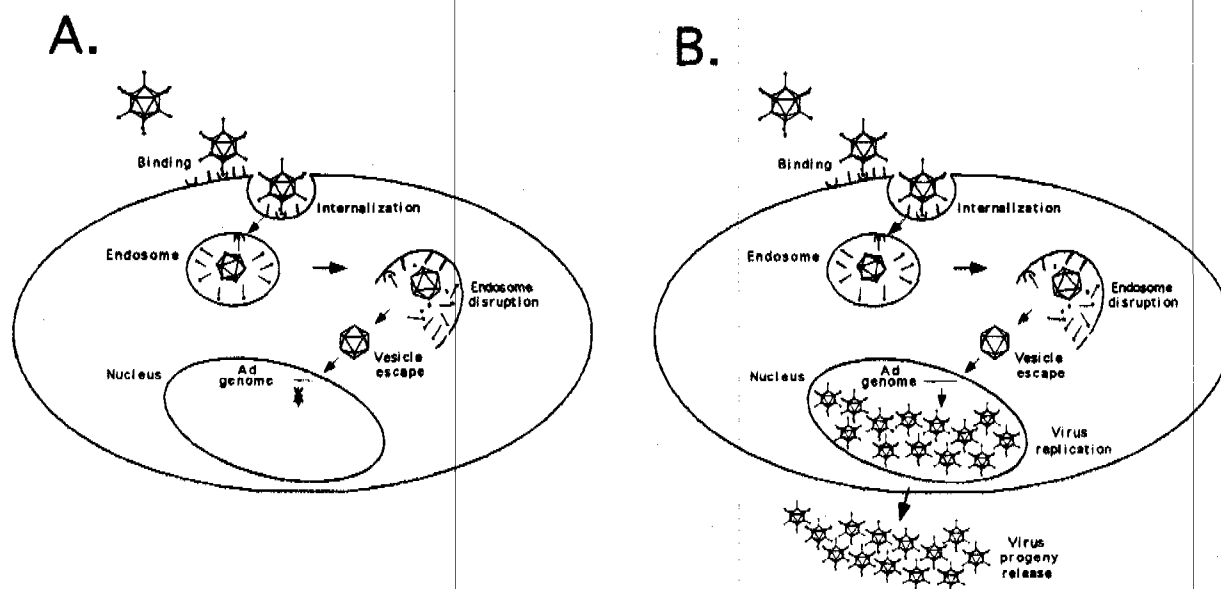


Fig. 1. Conditionally replicative adenovirus. The adenovirus first binds to an unknown cellular receptor by virtue of the fiber protein. The adenovirus is then internalized by receptor-mediated endocytosis through an interaction of the penton base Arg-Gly-Asp (RGD) motif with the $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin receptors. Disruption of the endosome occurs by pH acidification, the nucleocapsid is transported to the nucleus and the adenoviral genome enters the nucleus. (A) In an infected normal cell, the genetically modified adenovirus genome is not transcribed or replicated, therefore preventing virus production. (B) In an infected tumor cell, the genetically modified adenovirus genome is transcribed and replicated, resulting in production of virus progeny. Cell lysis occurs to release the newly made virions which in turn will be able to infect neighboring tumor cells.

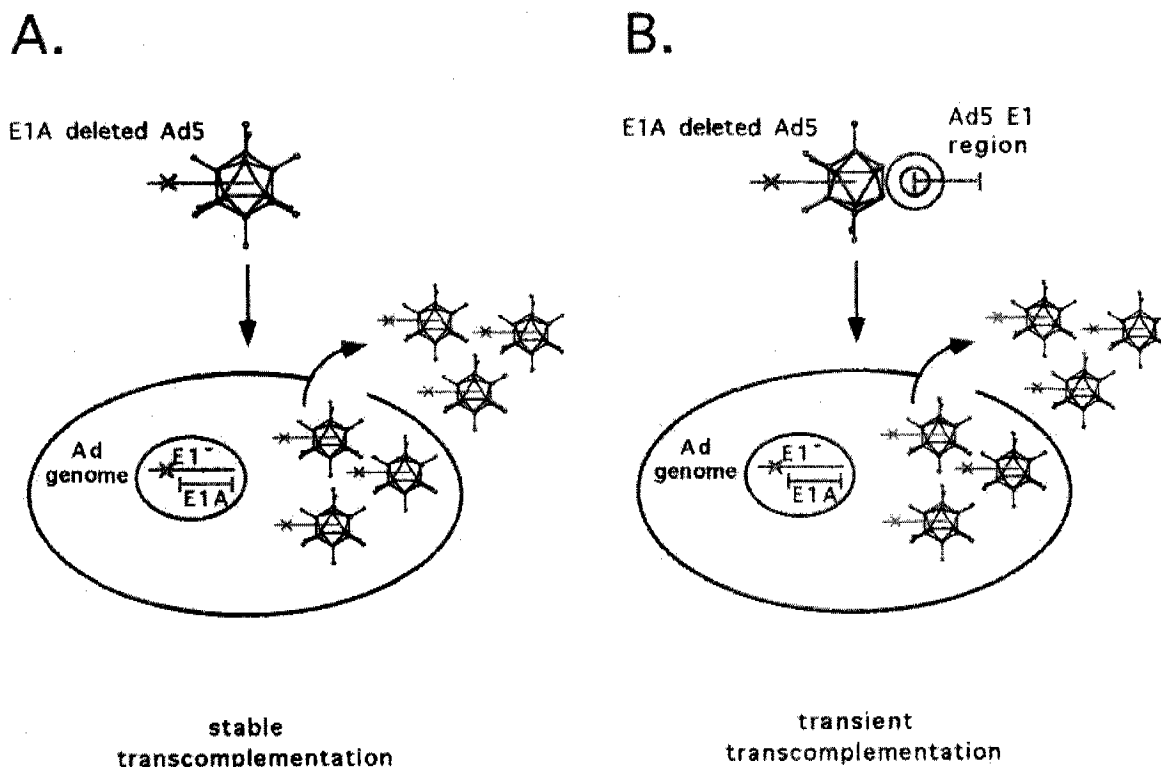


Fig. 2. Transcomplementation of adenoviruses. Schematic representation of the E1A-deleted adenovirus type 5. (A) In the stable transcomplementation, the E1A sequences provided in trans are integrated in the host chromosomes as in the 293 cell line [72]. The E1A proteins expressed from the integrated sequences in the host chromosomes allow transcription and replication of E1A-deleted adenoviruses, thus leading to production of new virions. (B) In the transient transcomplementation, the E1A sequences are provided in trans by a plasmid which is codelivered with the virus. In contrast to (A), the E1A proteins are expressed from the codelivered E1A-encoding plasmid. This approach also permits transcription and replication of E1A-deleted adenoviruses leading to production of virus progeny as in stable transcomplementation.

encoding the precursor to the terminal protein [76]. All of these cell lines have supported growth of appropriate defective adenoviruses [73,75,76]. Graham et al. were the first to construct an adenovirus complementing cell line. They transformed human embryonic kidney cells by exposing them to sheared fragments of adenovirus type 5 DNA [72]. The resultant transformed cells, designated 293, expressed a portion from the conventional left end of the Ad genome. Spector et al. demonstrated that the adenoviral integrated sequences in this cell line could complement the E1 deletions of mutant viruses and permit replication of these E1-deleted adenoviruses [73]. Klessig et al. constructed cell lines, gmDBP, which inducibly express the adenovirus DNA-binding protein [74]. They used a retroviral construct in

which the MMTV promoter was driving the expression of the DBP coding sequences to stably transfect HeLa cells. The resultant cell lines supported viral growth at a non-permissive temperature of severely restricted thermosensitive mutants like Ad2ts400 and Ad5ts125 even though the complementation was not complete. Expression of late proteins from the viruses was not as efficient at non-permissive temperature as that at permissive temperature or compared to wild-type virus infection. Moreover, these cell lines did not support plaque formation at non-permissive temperature, and this was attributed to an insufficient level of DBP expression. Following this work, Brough et al. constructed cell lines that inducibly express the DBP by using the same retroviral vector in which they inserted the SV40

enhancer to augment transcription of the DBP coding sequences from the dexamethasone-inducible MMTV promoter [75]. These cell lines permitted transcomplementation of the mutant DBP gene and allowed plaque formation of the severely defective DBP mutants. Schaack and co-workers have made HeLa and 293 cell lines that express biologically active adenovirus type 5 precursor terminal protein (pTP) [76]. HeLa and 293 pTP-expressing cell lines permitted growth of a temperature-sensitive terminal protein mutant *sub* 100r at restrictive temperature and the 293-pTP allowed plaque formation of *sub* 100r. Other adenovirus complementing cell lines such as W162 and 293-E4 have been constructed to permit growth of deletion mutants in the E4 or both E1 and E4 regions, respectively [77-79]. These works demonstrated that transcomplementation can be accomplished using stable clones expressing adenoviral proteins.

Transcomplementation can also be achieved in a transient fashion. Goldsmith et al. have demonstrated that E1A-deleted adenovirus can be transcomplemented with codelivery of E1A sequences to make adenoviral producer cells [80]. In their work, replication-enabling E1A sequences were cotransduced into human prostate carcinoma cells infected with an E1A-deleted adenovirus encoding a luciferase reporter gene. The replication-enabling plasmid was cotransduced by ionic linkage to the adenovirus capsid. Cells cotransduced with this replication-enabling plasmid produced new virions that had the ability to transfer luciferase activity to new cells. This work established that simultaneous addition of a replication-incompetent adenovirus and a replicative-enabling plasmid in a *trans* configuration could convert some of the cotransduced cells into recombinant adenovirus-producing cells. A similar experiment was undertaken by Scaria and co-workers in which an expression plasmid containing the E4-open reading frame was conjugated by ionic linkage to an E4-deleted replication-incompetent adenovirus (dl1014) and codelivered with the virus into non-permissive 293 cells [81]. New viral progeny was made but did not replicate on 293 cells as expected, however, they grew on W162 cells since they express E4 proteins. Thus these studies demonstrated that transient transcomplementation can be achieved.

The specific genetic elements necessary for adenovirus replication are well defined and characterized.

In this regard, the adenovirus early region genes would be the best candidate genes to be exploited for the derivation of a conditionally replicative adenovirus because they are expressed in the early phase of the lytic cycle and they encode proteins involved in transactivation of viral promoters (E1A), DNA replication (E2), host immune surveillance escape (E3) and post-transcriptional control of adenoviral gene expression (E1B and E4). Our laboratory proposes to modify the expression of the E2 and E4 regions. We believe these are the ideal candidate genes for this purpose because they are absolutely essential for viral replication *in vitro* and *in vivo* and, so far, no cellular protein has been reported to complement these gene products. The E2 region encodes proteins involved in DNA replication, such as the DNA-binding protein (DBP), the DNA polymerase and the precursor to the terminal protein [82]. The E2 region is separated as two transcription units, E2A and E2B. The DBP is abundantly expressed from the E2A region and binds to single-stranded DNA and to the extremity of double-stranded DNA [83,84]. The DBP is needed for DNA replication both *in vivo* and *in vitro* [85-87]. The E2B region encodes the adenoviral DNA polymerase and the precursor to the terminal protein [88], the latter serving as a primer for initiation of the viral DNA replication [89,90]. The E4-encoded proteins function in the shut-down of the host gene expression to favor that of the virus and also play a role in the up-regulation of transcription from the E2 region [91]. Therefore, transcomplementation of these regions could be accomplished transiently or in a complementing packaging cell line.

We have decided to utilize transient transcomplementation of the defective viruses as a proof of concept. The chosen defective viruses are the thermosensitive mutants for pTP, Pol and DBP, such as Ad5sub100r, H5ts149, and H5ts107, respectively [76,92,93], or deletion mutants, like H5dl1014, lacking the E4 ORFs 3, 4 and partially 6 [94]. Transcomplementation will be achieved by means of a plasmid encoding the appropriate wild-type correcting gene codelivered with the virus during infection. These mutant adenoviruses do not replicate or form plaques under restrictive conditions except for the Ad5sub100r, which replicates some and gives a titer of less than 10^5 PFU/ml [76]. Transcomplementation will be demonstrated by titrating the cell lysates

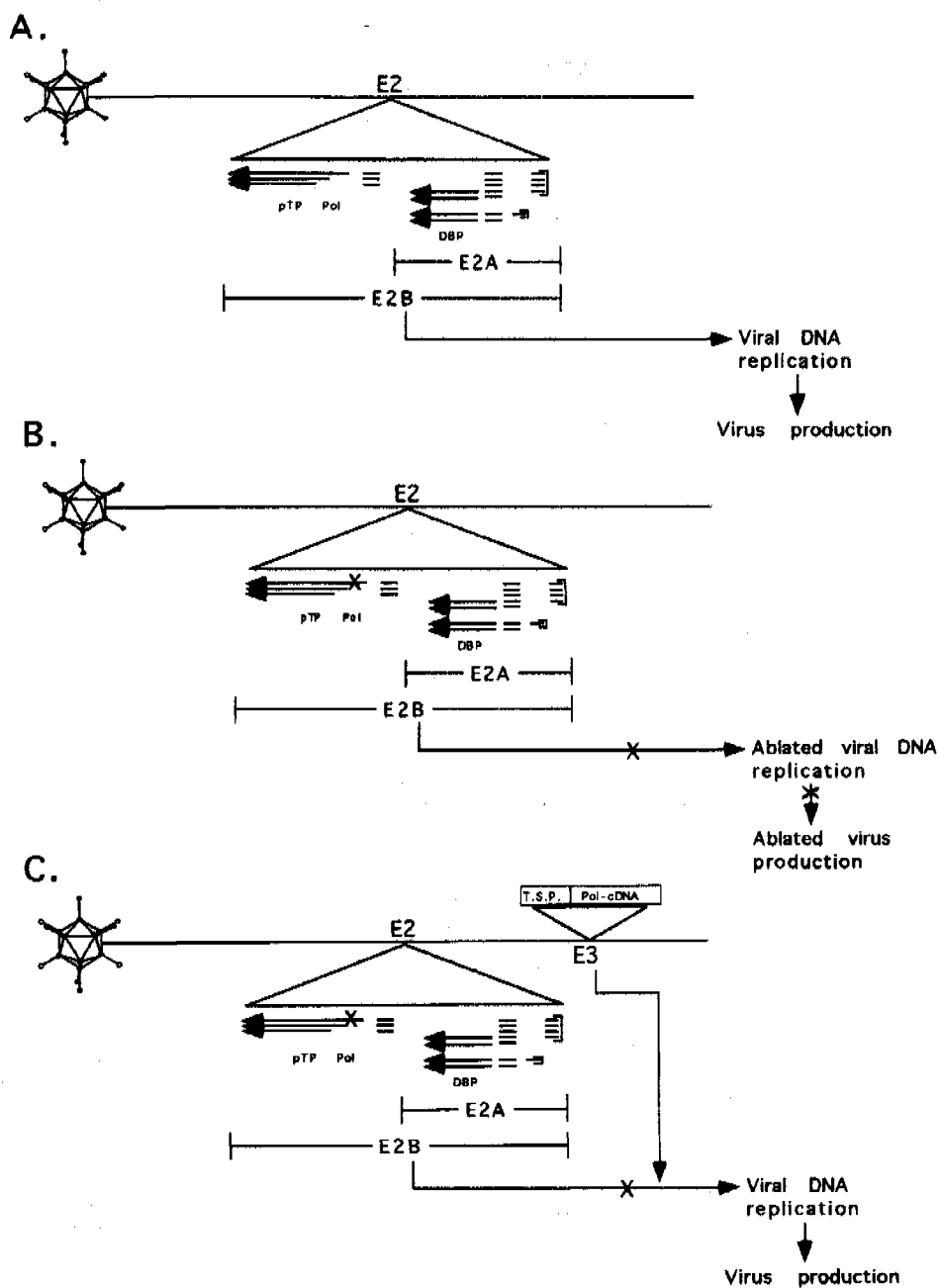


Fig. 3. Idealized conditionally replicative adenovirus. Schematic representation of the ideal conditionally replicative adenovirus, genetically modified in the E2 region in order to restrict its DNA replication to a tumor-specific context. (A) In the context of an infection with a wild-type adenovirus, the E2 region is transcribed and the three essential viral proteins for DNA replication, preterminal protein, polymerase and DNA binding protein (pTP, Pol and DBP) are synthesized from their native promoter. Viral DNA replication occurs and new virions are made. (B) A polymerase-defective adenovirus (Pol-defective adenovirus) could be engineered by site-directed mutagenesis to create a stop codon in a region of the Pol coding sequence that is not shared by Pol, pTP and DBP. The result of this would be a truncated Pol protein that would not be functional. During an infection with this Pol-defective adenovirus, DNA replication would be ablated due to the defective Pol protein and no virions would be made. (C) The same Pol-defective adenovirus as in (B) would be genetically modified by inserting in the non-essential E3 region a cassette expressing the Pol-cDNA under the control of a tumor-specific promoter. During an infection of an inductive tumor cell with this adenovirus, the inactivity of the mutated Pol protein would be transcomplemented by the active Pol protein expressed from the tumor-specific promoter, thus resulting in viral DNA replication and virus production.

of the cotransduced cells at permissive (33°C) and restrictive temperatures (39°C) for the thermosensitive mutants or on permissive (W162) and non-permissive (293) cell lines. If transcomplementation occurs, plaques should develop in permissive conditions and not in the restrictive context, since the newly made virions would still be mutant viruses.

Once transient transcomplementation is demonstrated, the expression of the E2 and E4 genes will be driven by a tissue-specific promoter in a transient transcomplementation on permissive and non-permissive cell lines. In this regard, many tissue or tumor-specific promoters have been defined and employed to target a variety of genes' expression in a specific tissue, such as the promoters for the human phenylalanine hydroxylase [95], the prostate-specific antigen [96], the carcinoembryonic antigen [97], the α -fetoprotein [98], the polymorphic epithelial mucin (MUC-1) or episialin or DF3 antigen [99], the erbB2 cellular receptor [100], the surfactant protein B [101] and the CD11b [102]. Some of them have even been used in the context of cancer gene therapy [98,100], moreover with the adenovirus-mediated gene delivery approach [21,103,104]. Then recombinant adenoviruses will be engineered to contain a tissue-specific promoter to substitute for the E2A, E2B (Fig. 3) and E4, respectively, and tested in vivo in animal models.

Therefore we propose to derive a conditionally replicative adenoviral vector designed to replicate specifically in tumor cells. The basis of this selectivity will not rest on uncharacterized biology factors as for the herpes vectors. In our system, designed elements which limit expression of essential adenoviral genes will be the basis of tumor-specific replication. The preliminary studies we have presented, as well as the work of others, have suggested the feasibility of this approach. Such a system, possessing the capacity for multiple rounds of tumor cell killing post-transduction would thus address the requirement for an amplification effect within the context of cancer gene therapy strategies.

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**SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
DIRECCIÓN DE NUEVAS CREACIONES**

Abogado
J. Ian Raisbeck

ASUNTO	Radicación	06-127751
	Trámite	011
	Evento	001
	Actuación	430
	Oficio	32
	Folios	014

Patente de Invención ☒ Patente de Modelo de Utilidad ☐

Vía Nacional ☐

Vía PCT (fase nacional) ☒ Cap. I ☒ Cap. II ☐

No. Sol. Internacional PCT/US2005/018301
Pub. Internacional WO2005/118825

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 le comunica:

1. Documentos estudiados

<u>Descripción:</u>	Folios: 58 a 116	20/12/2006
<u>Reivindicaciones:</u>	Folios: 117 a 123	20/12/2006
<u>Figuras:</u>	Folios: 124 a 132	20/12/2006
<u>Secuencias:</u>	Folios: 133 a 176	20/12/2006
<u>Prioridad:</u>	US 60/574,851	26/05/2004

2. Objeto de la invención

Título de la solicitud: "Adenovirus quiméricos para uso en el tratamiento del cáncer".

El objeto de la presente solicitud de patente de invención consiste en suministrar adenovirus quiméricos, o variantes o derivados de los mismos, de utilidad para la terapia contra el cáncer. También se divulga método para obtener estos adenovirus.

El problema planteado en esta solicitud es la necesidad de adenovirus con mayor poder oncolítico que los adenovirus Ad5 empleados usualmente en terapia viral.

Para solucionar este problema técnico la solicitud en estudio proporciona un adenovirus quimérico que posee un genoma con una región E2B derivada de dos

serotipos adenovirales diferentes. También se proporciona el método de purificación de estos adenovirus quiméricos.

El objeto de la solicitud definida anteriormente se clasificó en la IPC: C12N 15/861, 7/01, 7/02; A61K 48/00.

3. De la solicitud de Patente

3.1. Reivindicaciones (artículo 30 D 486)

El capítulo reivindicatorio presentado carece de claridad y concisión por cuánto:

- 1) Hay tres reivindicaciones independientes que se refieren a un adenovirus quimérico con modificaciones similares. Se recomienda dejar una única reivindicación independiente de esta clase, que contenga todas las características técnicas fundamentales del adenovirus y colocar en reivindicaciones dependientes las características opcionales del mismo (reivindicaciones 1, 9 y 24);
- 2) Para complementar la descripción de los adenovirus quiméricos desarrollados debe aportarse el número de depósito de las diferentes poblaciones virales creadas;
- 3) La reivindicación 1 y/o similares deben definir la estructura del adenovirus modificado, puesto que tal como es reclamado puede dar lugar a múltiples opciones de estructura de la región E2b, donde no todas ellas tiene sustento en la descripción. Se debe indicar específicamente los serotipos que comprenden la región e indicar para cada uno de ellos la secuencia que lo conforma y que aporta a la región recombinante;
- 4) La reivindicación 1 contiene términos ambiguos que no permiten definir las propiedades del adenovirus desarrollado sino que corresponden a resultados a alcanzar con el mismo, este es el caso de la expresión "presenta un índice terapéutico mejorado para la célula tumoral"
- 5) La reivindicación 2 que se refiere a regiones que codifican proteínas de fibras, exones y pentones, debe ser caracterizada indicando la(s) secuencia(s) nucleótida que la(s) conforma(n). Adicionalmente, es necesario aclarar si la indicación de que "proviene del mismo serotipo adenoviral", se refiere al serotipo adenoviral progenitor o al serotipo introducido;
- 6) Las reivindicaciones 12 a 14 que se refieren a la introducción de un gen heterólogo en el adenovirus quimérico carecen de sustento en la descripción;
- 7) En general, se recomienda caracterizar cada modificación realizada en el genoma del adenovirus indicando el origen (serotipo empleado) y secuencia de las regiones introducidas (reivindicaciones 3, 4, 12-14);
- 8) La reivindicación 24 repite las características aportadas por las reivindicaciones 1 y 19, resultando en una falta de concisión del capítulo reivindicatorio;

De esta manera, el capítulo reivindicatorio presentado no cumple con los requisitos establecidos en el art. 30 de la Decisión 486.

4. Excepción a la patentabilidad (artículo 20 D486 literal d))

Las reivindicaciones 15 a 18 que se refieren a un método para inhibir el crecimiento celular (reivindicación 15) y a un método para administrar una proteína terapéutica (reivindicación 18), corresponden a formas de usar el adenovirus reclamado en tratamientos terapéuticos para humanos o animales.

De acuerdo con el artículo 20, literal d) de la D486 los métodos terapéuticos o quirúrgicos para el tratamiento humano o animal, así como los métodos de diagnóstico aplicados a los seres humanos o a animales no se consideran patentables. De esta manera, el contenido de las citadas reivindicaciones deberá excluirse.

5. Determinación del Estado de la Técnica

Para determinar el estado de la técnica se ha tenido en cuenta la fecha de presentación de la solicitud prioritaria reivindicada con la solicitud: 26/05/2004.

Teniendo en cuenta la fecha indicada se realizó la búsqueda en el estado de la técnica de documentos relacionados con la solicitud, encontrándose los siguientes documentos relevantes a la misma.

Nº	Documento	Título	Fecha de publicación
D1	Yan W. et.al. Journal of Virology, vol 77 (4):2640-2650	"Developing Novel Oncolytic Adenoviruses through Bioselection"	Feb 2003
D2	Rancourt C, Curiel D. Advanced Drug Delivery Reviews 27 (1997): 67-81	"Conditionally replicative adenoviruses for cancer therapy"	1997

Resumen de los documentos citados:

D1: Mutantes de adenovirus humano 5 (Ad5) con actividad oncolítica incrementada fueron aislados usando un procedimiento denominado bioselección (resumen, folio 238 D1).

D2: Se utilizan vectores adenovirales en los cuales la replicación viral puede ser restringida a células tumorales blanco por limitar la expresión de genes virales esenciales para la replicación del virus solo en las células tumorales de interés (resumen, folio 243 D2).

6. Análisis de patentabilidad (artículo 14 D486)

6.1. Nivel inventivo (artículo 18 D486)

La solicitud reclama un adenovirus quimérico recombinante, o una variante o derivado del mismo que tiene un genoma que comprende una región E2B

- en el que dicha región E2B comprende una secuencia de ácido nucleico derivada de un primer serotipo adenoviral y un secuencia de ácido nucleico derivada de un segundo serotipo adenoviral;
- en el que dicho primer y segundo serotipos se seleccionan cada uno entre los subgrupos adenovirales B,C,D,E, o F y son distintos entre sí; y
- en el que dicho adenovirus quimérico es oncolítico y presenta un índice terapéutico mejorado para la célula tumoral

En el estado del arte se encuentra el documento de Yan, *et.al* (D1) el cual divulga mutantes de adenovirus humanos 5 (Ad5) con actividad oncolítica incrementada. Dos mutantes, ONYX-201 y ONIX-203, fueron purificados en placa de una mezcla de Ad5 mutagenizados aleatoriamente con pasajes repetidos en líneas de cáncer colorrectal HT29 (pág 2641 columna izq), de forma equivalente a como se hace en la solicitud en estudio (folio 109).

ONYX-201 y -203 contienen siete mutaciones puntuales cuando se compara con Ad5 silvestre, cuatro de las cuales eran comunes entre los dos mutantes. Una mutación fundamental era en el nucleótido 8350, que resultaba en una truncación de 21 aminoácidos del C-terminal de la proteína líder-i (resumen, pág 2640).

Para diversas líneas celulares el mutante ONIX-203 mostró una mayor actividad citolítica (fig 6), evidenciando el efecto sobre esta actividad que tenía principalmente la mutación 8350 localizada en la proteína i-líder y en menor proporción las mutaciones 30191 en el transcripto E3 y la mutación 31405 en la fibra (tabla 1, pág 2646).

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

Características esenciales	D1	D2
adenovirus quimérico recombinante, que comprende una región E2B	Adenovirus mutantes en regiones i-líder, E3 y E4 (tabla 1)	Adenovirus mutante con replicación condicional (resumen, pág 76)
E2B comprende una secuencia de ácido nucleico derivada de un 1er serotipo adenoviral y un 2do serotipo adenoviral		modificación del transcripto E2B por mutación de parada (pág 76, fig 3)
serotipos se seleccionan entre los subgrupos B,C,D,E, o F y son distintos entre sí	Adenovirus del serotipo 5 – grupo C (resumen)	Serotipo 5 (grupo C) (pág 76 columna der último párrafo)
adenovirus quimérico es oncolítico	Adenovirus oncológico con actividad citolítica incrementada (resumen, fig 6)	Adenovirus oncolítico, tumor-específico (resumen, pág 76-78)

La diferencia entre la materia reclamada en la solicitud y D1 es que esta última no modifica el transcripto E2B, y los adenovirus modificados son mutantes ensayados por mutaciones puntuales pero no por segmentos con regiones provenientes de diferentes serotipos.

El efecto que logra esta construcción quimérica es que incrementa la capacidad citolítica del vector adenoviral.

El problema resuelto por la solicitud a partir de la información divulgada por D1 es incrementa la capacidad citolítica del vector adenoviral, modificando otra región de transcripto.

La modificación en el transcripto E2B había sido sugerida y ensayada en el documento de Rancourt (D2) el cual divulga el efecto sobre la capacidad de replicación del adenovector al modificar por medio de mutaciones la región E2B produciendo además que éste tenga una infección tumor-específica (pág 76-78, fig 3).

La modificación de esta región logra que la replicación del adenovector este totalmente condicionada a la célula blanco a infectar. Logrando de esta manera que los efectos nocivos del virus sean mínimos y que el tratamiento sea dirigido.

De esta manera, el método de bioselección de virus recombinantes, con el cual se obtiene la población de virus de la solicitud había sido divulgado por D1 y aunque la construcción de un adenovirus quimérico conformado por dos serotipos de adenovirus para la región E2B, no había sido realizada ni sugerida en el estado del arte, sin embargo la modificación particular de esta región había sido divulgada por D2.

De esta manera, a partir de la combinación de D1 y D2, la invención habría podido ser realizada por la persona del oficio normalmente versada en la materia, carece de nivel inventivo e incumple el artículo 18 de la D486.

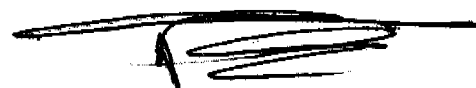
7. Conclusión

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

Item	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	Yan W. <i>et.al.</i> Journal of Virology, vol 77 (4):2640-2650	1-27	Nivel inventivo
D2	Rancourt C, Curiel D. Advanced Drug Delivery Reviews 27 (1997): 67-81	1-27	Nivel Inventivo

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de 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.

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 N° 10 de 2001.



JOSE LUIS SALAZAR LÓPEZ
Director de Nuevas Creaciones (C)

El anterior requerimiento fue notificado por fijación en lista, de fecha

05 ENE. 2012

Elaboró: N.Lamprea *NLB.*

Revisó: Consuelo Leguizamón L. *ly*