UNIVERSIDAD AUTONOMA DE NUEVO LEON
FACULTAD DE CIENCIAS BIOLOGICAS
LABORATORIO DE INMUNOLOGIA Y VIROLOGIA

"EVALUACION DE LA RESPUESTA INMUNE EN UN MODELO ANIMAL DESPUES DE LA ADMINISTRACION NASAL DE CEPAS RECOMBINANTES DE *Lactococcus lactis* QUE EXPRESAN LA PROTEINA E7 DEL HPV-16 Y LA INTERLEUCINA-12 MURINA"

T E S I S

PRESENTADA COMO REQUISITO PARCIAL PARA OBTENER EL GRADO ACADEMICO DE

DOCTOR EN CIENCIAS
CON ESPECIALIDAD EN BIOTECNOLOGIA

M.C. NAIMA GISELA CORTES PEREZ

SAN NICOLAS DE LOS GARZA, NUEVO LEON
JULIO DEL 2004

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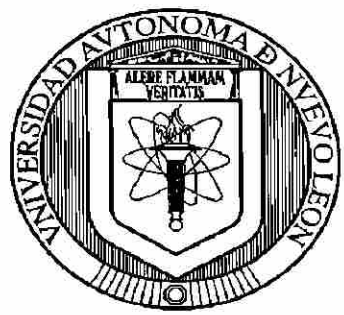

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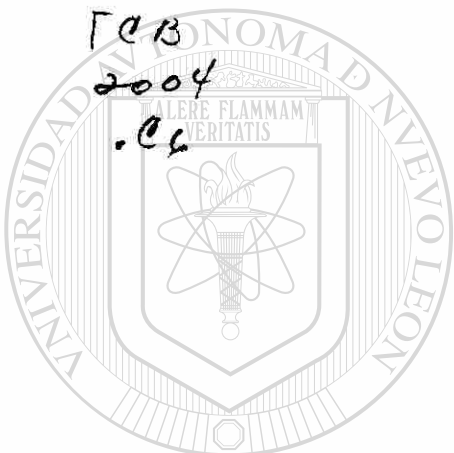

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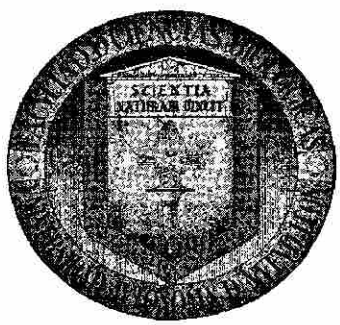

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Los miembros de esta comisión recomendamos que la presente tesis de la

M.C. Naima Gisela Cortes Perez sea aceptada como requisito

parcial para obtener el grado académico de

Doctor en Ciencias con especialidad en **Biotecnología**

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Esta tesis se realizó en el Laboratorio de Manipulación Genética, Departamento de Microbiología e Inmunología de la Facultad de Ciencias Biológicas en la Universidad Autónoma de Nuevo León, bajo la dirección del Dr. Roberto Montes de Oca Luna y la asesoría externa de los Doctores Luis Bermúdez y Philippe Langella del Instituto Nacional de Investigaciones Agronómicas (INRA, Jouy en Josas, Francia).

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RESUMEN

El virus del papiloma humano tipo 16 (HPV-16) es considerado como el principal agente etiológico del cáncer cérvico-uterino (CaCu). A la fecha, la mayoría de las estrategias para combatir la infección por este virus estan basadas en el desarrollo de vacunas dirigidas contra la oncoproteína E7. En este trabajo nosotros evaluamos la capacidad de *L. lactis* para despertar una respuesta inmune en contra de la proteína E7 del HPV-16. Primero, debido a que la localización de la proteína puede influenciar la respuesta inmune se evaluó que forma de presentación celular de la proteína E7 (citoplasma, secretada ó anclada a la superficie) por *L. lactis* es más inmunogénica. La respuesta inmune más alta fué generada por la administración intranasal de la cepa de *L. lactis* que produce en forma inducible la proteína E7 anclada a su pared celular. Por otra parte, la respuesta específica de antígeno (secreción de citocinas Th1, IL-2 e INF gama) generada por la administración de *L. lactis* recombinante que expresa la proteína E7 anclada a su superficie fue dramáticamente incrementada por la co-administración de una cepa de *L. lactis* que secreta la citocina IL-12 biológicamente activa. Los datos obtenidos sugieren que nuestra estrategia puede ser usada para aumentar una respuesta inmune específica de antígeno e estimular la inmunidad mucosal. Además nosotros analizamos la inmunogenicidad y el efecto anti-tumor de las cepas de *L. lactis* recombinantes administradas intranasalmente en un modelo de tumor TC-1. La protección contra el desarrollo del tumor y sobrevivencia se incrementan notablemente en los animales inmunizados con las cepas de *L. lactis* recombinantes. Finalmente con la idea de desarrollar una vacuna segura, con baja o nula actividad transformante, se construyó una cepa de *L. lactis* que expresa en su superficie una proteína E7 mutante. Estos prometedores resultados representan un paso al desarrollo de un nuevo y seguro vector mucosal para tratar el CaCu relacionado al HPV-16.

ABSTRACT

The human papillomavirus type 16 (HPV-16) is considered the main ethiologic agent of cervical cancer (CxCa). To date, most of the strategies to combat this infection are based on the development of vaccines directly against E7 oncoprotein. In this study we evaluated the capacity of *L. lactis* to elicit an immune response against E7 protein from HPV-16. First, as bacterial antigen location may influence the immune response, recombinant *L. lactis* strains producing E7 protein in three cellular locations, cytoplasm, secreted or cell-wall-anchored were evaluated. The highest immune response was elicited by intranasal administration of *L. lactis* producing an inducible cell-wall-anchored form of E7 protein. Moreover, an antigen-specific cellular response (i.e., secretion of Th1 cytokines, IL-2, and IFN-gamma) elicited by a recombinant *L. lactis* strain displaying E7 at cell surface was dramatically increased by the co-administration with a *L. lactis* strain secreting a biologically active IL-12 cytokine. The data obtained suggest that our strategy can be used to enhance an antigen-specific immune response and stimulate local mucosal immunity. In addition, we tested the immunogenicity and anti-tumor effect of recombinant *L. lactis* strains administered intranasally in a TC-1 tumor challenge model. Mice protection and rate survival from TC-1 tumor challenge increase significantly in animals immunized with recombinant *L. lactis* strains. Finally, to develop a safe vaccine with low or no transforming activity we produced an inducible cell-wall-anchored form of E7 protein mutant. These promising results represent a step towards the development of a new, safe mucosal vector to treat HPV-associated cervical cancer.

CAPITULO 1

INTRODUCCION

El cáncer cérvico-uterino (CaCu) permanece como la segunda neoplasia más común en la mujer alrededor del mundo. Cada año se registran globalmente 466,000 nuevos casos y 232,000 mujeres pierden la vida debido a esta enfermedad (Garland S, 2003). América latina es una de las regiones del mundo con mayor incidencia en CaCu, pero en México este padecimiento representa la primer causa de muerte por cáncer en la mujer (Mohar *et al.,* 1997). Por lo tanto, dado el gran problema de salud publica que representa esta enfermedad, el desarrollo de una vacuna profiláctica y/o terapéutica es una prioridad para prevenir o tratar, respectivamente el CaCu.

La relación causal entre las infecciones persistentes con ciertos genotipos del virus del papilloma humano (HPV) y el CaCu esta bien establecida (Furumoto & Irahara, 2002, Muñoz *et al* 2003). Recientemente Koutsky *et al.* (2002) demostraron el efecto profiláctico de una vacuna basada en partículas tipo virales (VLP's) en ensayos clínicos en un grupo de mujeres. En dicho estudio se encontró una significativa reducción en la incidencia de la infección con HPV del tipo 16 (HPV-16), así como en el desarrollo de CaCu relacionado al HPV-16. Sin embargo, el desarrollo de este tipo de vacunas no es una opción terapéutica en pacientes infectados, ya que las proteínas de la cápside del virus no son detectadas en los casos de CaCu. La proteína E7 del HPV-16 es constitutivamente producida en carcinomas cervicales y esencial para el proceso de transformación (Baker et al., 1987; Bedell et al., 1987; Dyson et al., 1989; Tanaka et al., 1989) por lo cual se considera un buen antígeno, candidato para el desarrollo de una vacuna terapéutica contra el CaCu.

En varios trabajos se ha estudiado el uso de bacterias como vectores de expresión del antígeno E7 para despertar una respuesta inmune contra el HPV-16. En estos estudios los vectores usados han sido cepas atenuadas de bacterias patógenas como *Salmonella* y *Mycobacterium* spp. (Lodoño et al,.

1996; Jabbar et al., 2000). A pesar de que estas cepas recombinantes despiertan una respuesta inmune, ciertos factores como su invasividad y el riesgo de reversión a su estado patogénico limitan su uso en grupos vulnerables, como niños y pacientes inmunosuprimidos. De ahí la necesidad de desarrollar una nueva generación de vehículos seguros. Las bacterias lácticas (BL) son prometedores vectores para la expresión de antígenos *in vivo*. Son ampliamente utilizadas en la elaboración de productos lácteos, además algunas especies exhiben efectos probióticos en humanos. Comparadas con otros vectores vivos las BL son considerados micoorganismos seguros, grado GRAS (por sus siglas en ingles, Generally Recognized As Safe). *Lactococcus lactis* es una BL trasciende, no-colonizante, del tracto digestivo, (Drouault *et al.*, 1999; Geoffroy *et al.*, 2000), lo cual disminuye el riesgo de obtener una tolerancia al antígeno, como sucede con otras bacterias.

Nuestro equipo ha logrado expresar eficientemente el antígeno E7 en *L. lactis* (Bermúdez-Humarán *et al.*, 2002, 2003a; Cortes-Perez *et al.*, 2003) lo cual ha representado un importante paso para el desarrollo de una vacuna contra CaCu. A pesar de que una alta producción de proteínas heterólogas ha sido obtenida en *L. lactis* con el uso de promotores constitutivos (de Vos, 1999), la continua expresión de altos niveles de proteína puede conducir a una acumulación intracelular o la degradación en el citoplasma, lo cual puede resultar deteriorante para la célula; En este estudio nosotros evaluaremos el uso de un sistema inducible por nisina [NICE (de Ruyter et al., 1996; Kuipers et al., 1998) en el cual la expresión de un gen puede ser regulada más de 1000 veces] para producir la proteína E7 del HPV-16 en *L. lactis*. Además, como la inmunogenicidad puede depender de la localización del antígeno, evaluamos también la respuesta inmune de tres cepas de *L. lactis* que expresan el antígeno E7 en tres localizaciones diferentes (en citoplasma, secretada en el medio de cultivo y anclada a pared celular). Una vez establecido el sistema de expresión mas eficiente, en éste trabajo se utilizará también una proteína E7 que posee mutaciones que modifican la estabilidad de la proteína y directamente afectan su capacidad transformante, esta proteína mutante es capaz de inducir una actividad citotóxica mayor y una mejor protección contra tumor que la proteína nativa, en vacunas de DNA; por lo cual se pretende

mediante ingeniería genética expresar esta mutante en *L. lactis* y proponerla como una nueva herramienta terapéutica capaz de prevenir y combatir tumores que expresan E7. Finalmente analizaremos la respuesta inmune en un modelo animal, después de la administración nasal de *L. lactis* recombinante que expresa el antígeno E7 del HPV-16, la proteína E7mutante y la Interleucina 12.

1.1 Justificación y objetivos de la tesis

El CaCu es uno de los principales tumores en la mujer, y contribuye a un alto número de defunciones en todo el mundo. Sin embargo en América latina es una de las regiones del mundo que posee la mayor incidencia de CaCu. Afortunadamente el CaCu se considera una enfermedad curable gracias a que se conoce el agente etiológico, el HPV-16. En este contexto el objetivo principal de este trabajo es el de desarrollar un nuevo vector seguro y de bajo costo de producción para despertar una respuesta inmune en contra de este virus. Dicho vector es *Lactococcus lactis,* una bacteria utilizada comúnmente para la producción de productos láacteos y la cual ha sido manipulada en los últimos años para el desarrollo de nuevas vacunas. Para despertar la respuesta inmune contra el HPV-16, se eligió como antígeno la proteína E7, la cual es constitutivamente producida en casos de CaCu. Primero se analizará la respuesta inmune contra la proteína E7 expresada en varias localizaciones de *L. lactis,* una vez determinada la forma más inmunogénica, se tratará de aumentar la respuesta inmune mediante el uso de una cepa de *L. lactis* que secreta una molécula estimuladora del sistema inmune, la interleucina-12. Finalmente con la idea de usar la estrategia de este estudio en el humano se expresará una proteína E7 mutante con bajo poder transformante.

CAPITULO 2

ANTECEDENTES

2.1 Cáncer cérvico-uterino (CaCu). El cérvix o cuello uterino es la parte inferior, estrecha, del útero. El útero es un órgano hueco, en forma de pera, que está ubicado en la parte baja del abdomen de la mujer, entre la vejiga y el recto. El cérvix forma un canal que desemboca en la vagina, el cual conduce al exterior del cuerpo (Fig 1).

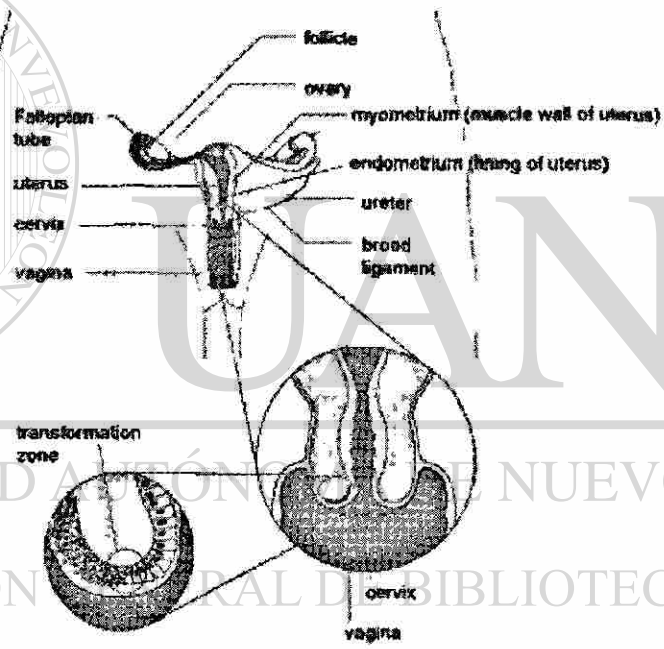

Fig. 1. Localizacion de cuello uterino. Sitio de inicio del proceso de transformación por HPV

El Cacu es el resultado de una serie de cambios progresivos que comienza con la aparición de células atípicas en las capas basales del epitelio escamoso, cambios en el núcleo, citoplasma, aumento de las imágenes mitóticas y pleomorfismo. Principalmente se reconocen dos tipos histológicos de CaCu: el epidermoide, (espinocelular o de células escamosas) y el adenocarcinoma. El primero se origina en el epitelio plano estratificado que recubre el ectocérvix y el segundo en el epitelio cilíndrico que tapiza el

canal endocervical; un tercer tipo se origina en la mucosa endometrial y constituye el adenocarcinoma del endometrio, sin embargo el 85-90% de los casos del CaCu es del tipo epidermoide y usualmente aparece en la unión entre el canal cervical y el ectocérvix. En esta área el epitelio columnar es reemplazado por el epitelio escamoso. Sin embargo no todas las células anormales que aparecen en el cérvix son cancerígenas, las lesiones intraepiteliales, las cuales son precursoras del cáncer invasor, estan formadas por tejido anormal que aparece sólo en la superficie del cérvix, suelen aparecer sobre todo en mujeres jóvenes, por abajo de los 40 años de edad y a veces desaparecen de forma espontánea. Estas lesiones precursoras son conocidas como neoplasia intraepitelial cervical (NIC I, II y III) y se caracterizan por cambios displásticos del epitelio cervical. Los casos más graves se desarrollan en mujeres de 30 a 40 años y puede tardar meses, e incluso años en provocar el cáncer. Si van más allá de la superficie y crecen hacia la profundidad del cérvix se llaman lesiones invasivas y suelen aparecer a partir de los 40. Estudios epidemiológicos han establecido la existencia de una asociación entre el cáncer del cuello uterino y múltiples factores sociales interdependientes, relaciones sexuales a temprana edad, el hábito de fumar, condición socio-económica entre otros; pero la opinión general es que la mayoría de los casos, el cáncer de cuello debe considerarse como una enfermedad de transmisión sexual, ya que la relación causal entre las infecciones persistentes con ciertos genotipos del HPV y el CaCu esta bien establecida (zur Hausen, 1996, Stern *et al.*, 2001, Steller M, 2002, Furumoto & Irahara, 2002, Muñoz *et al* 2003). DNA de HPV ha sido detectado en mas del 99% de los tumores cérvico-uterinos (Steller M 2002).

El Papanicolaou (Pap) es la principal herramienta para combatir el CaCu, consiste en la detección temprana de lesiones cervicales pre-malignas o malignas que requieren posterior evaluación. Una vez diagnosticada la enfermedad, los pacientes con NIC I generalmente no requieren tratamiento, sin embargo los pacientes con NIC II y NIC III si lo requieren para prevenir el desarrollo de lesiones invasivas. De manera general existen dos alternativas para erradicar este tipo de lesiones (NIC II y NIC III), la primera incluye el uso de crioterapia, vaporización láser y procedimiento de escisión con asa

eléctrica; la segunda, implica un procedimiento quirúrgico con resección de un área en forma cónica de tejido cervical, incluyendo parte del canal endo-cervical (conización cervical o biopsia en cono).

Cuando las condiciones precancerosas están presentes, puede que no existan síntomas aparentes o problemas (en las primeras etapas el CaCu es asintomático), pero en etapas avanzadas se puede presentar descarga vaginal, sangrado, flujo anormal, y dolor. El diagnóstico de enfermedad invasiva es normalmente realizado mediante biopsia de una lesión visible en examinación pélvica o por biopsia de una lesión observada durante el estudio colposcópico. Los estadíos del CaCu son clasificados de acuerdo a criterios puramente clínicos, el tratamiento depende de lo avanzado que esté. Si se encuentra en la primera etapa se puede extraer quirúgicamente únicamente la parte dañada. Puede que se extraiga todo el útero cuando se tiene cáncer en el cuello del útero. En una histerectomía el médico extrae todo el útero, incluyendo el cuello del útero, algunas veces inclusive los ovarios y los tubos de Falopio. El médico puede inclusive extraer los nódulos linfáticos cerca del útero para revisar si el cáncer ha sido propagado a estos órganos. Los exámenes regulares de Pap pueden detectar problemas prematuramente antes de que el cáncer progrese a una etapa avanzada. Lo cual ofrece a la mujer una mejor oportunidad de tratamiento.

2.2 Asociación entre HPV y cáncer. La infección genital por HPV esta ampliamente diseminada en el mundo, es considerada la enfermedad de transmisión sexual mas común, con una prevalencia de hasta el 50% (Holly EA. 1996, Hagensee ME. 1999). Los papilomavirus son un grupo heterogéneo de virus de DNA circular de doble cadena, el cual consiste de aproximadamente 8 Kb. (Fig 2). El genoma codifica para seis proteínas tempranas (E1, E2, E4, E5 E6 y E7) y dos tardías (L1 yL2). Los dos genes "**L**" codifican para proteínas de la cápside viral, mientras que los genes "**E**" codifican para proteínas que cumplen una variedad de funciones regulatorias (Zur Hausen H.1996, Steller M 2002,). Presentan además una región

larga de control (LCR) conteniendo aproximadamente 1000 pb en la que se han identificado secuencias estimuladoras y represoras de la transcripción viral.

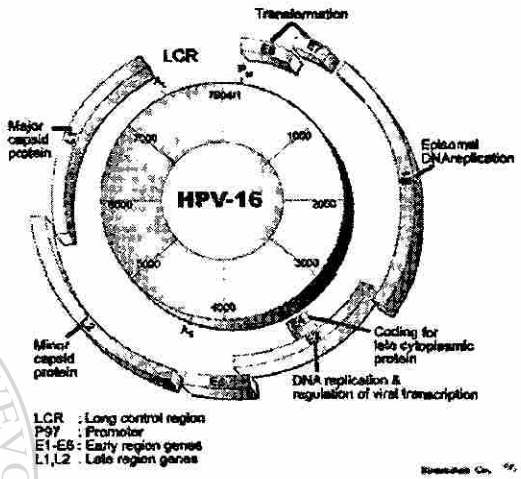

Fig. 2. Representación esquemática de la organización genómica del HPV-16.

Los HPV son epiteliotróficos, infectan varios sitios en el cuerpo, incluyendo la piel, boca, esófago y tracto anogenital. Han sido idenfiticados mas de 100 genotipos de los cuales 20 muestran tropismo por el tejido del tracto anogenital. Los HPV mucotrópicos han sido agrupados dentro de las categorías de bajo o alto riesgo, en base a la asociación de cada genotipo con el proceso de enfermedad benigna o maligna. Los tipos de bajo riesgo HPV-6 y HPV-11 han sido detectados en lesiones benignas, pero nunca en carcinoma cervical, en contraste los tipos HPV-16 y HPV-18 han sido detectados en mas del 70% de los casos de carcinomas de celulas escamosas [de ellos el HPV-16 es el más importante (Galloway, 1994)]. Otros tipos de alto riesgo incluyen HPV-31, -33, -35, -39, -45, -51, -52, -56, -58, -59, y -68. (Durst *et al.*, 1983; Boshart *et al.*, 1984; zur Hausen, 1985; McCance, 1986, Steller M 2002).

El HPV infecta las capas basales del epitelio (piel y mucosas) y tiene acceso a ellas por medio de heridas o escoriaciones. Básicamente pueden existir dos tipos de infecciones con el HPV i) Infecciones productivas, las cuales originan partículas virales, el ensamblaje de dichas partículas se lleva a cabo a medida que la célula migra hacia la superficie es por ello que las partículas virales son encontradas solo en las capas más superficiales del epitelio. (Fig. 3 y 4), y ii) Infecciones no productivas, en las cuales genoma del HPV se integra al de la célula, dando lugar a la síntesis de oncoproteínas produciendo la transformación e inmortalización de sus células blanco. (Fig. 4).

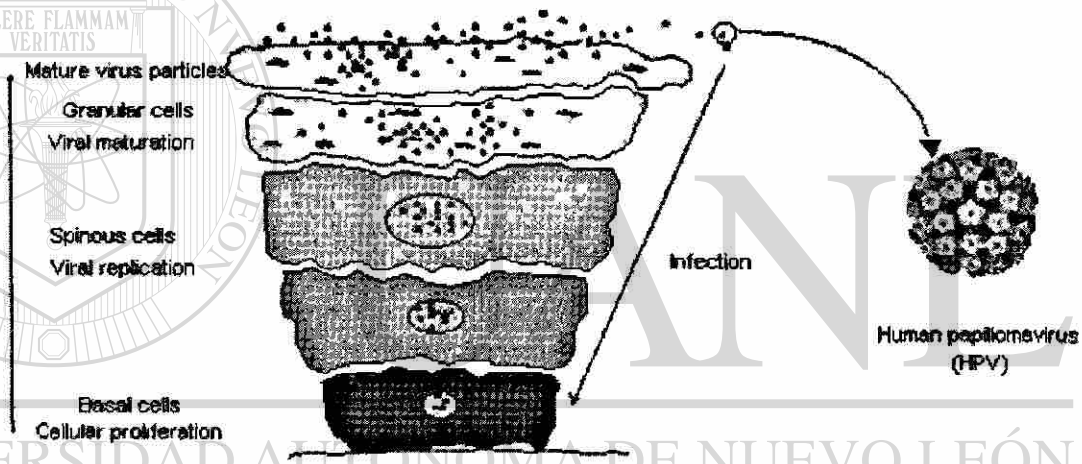

Fig 3. Infección productiva. Formación de partículas virales maduras

En general en lesiones benignas el DNA del HPV es encontrado de forma episomal pero en tumores malignos se encuentra integrado al ADN de la célula hospedera (Boshart *et al.*, 1984; McCance, 1986). En el proceso de transformación celular están involucrados algunos genes de expresión temprana del virus. Dentro de los primeros eventos en la inmortalización de las células es la desregulación del promotor P97 del HPV; el cual dirige la expresión de los oncogenes *E6* y *E7* (Jeon *et al.*, 1995). Este promotor se encuentra normalmente regulado por la proteína E2, sin embargo la expresión del gen E2 se ve interrumpida por la integración del virus al DNA del hospedero (Thierry & Yaniv, 1987). Después de

la integración las partículas virales no pueden ser producidas, pero la continua expresión de los genes E6 y E7 prolongan el ciclo de vida celular, destacando la perdida de la eficiencia de los mecanismos reparadores del DNA. Esto permite la acumulación de cambios genéticos importantes que pueden resultar en el desarrollo del cáncer (Stern *et al.,* 2001, Galloway, 2003).

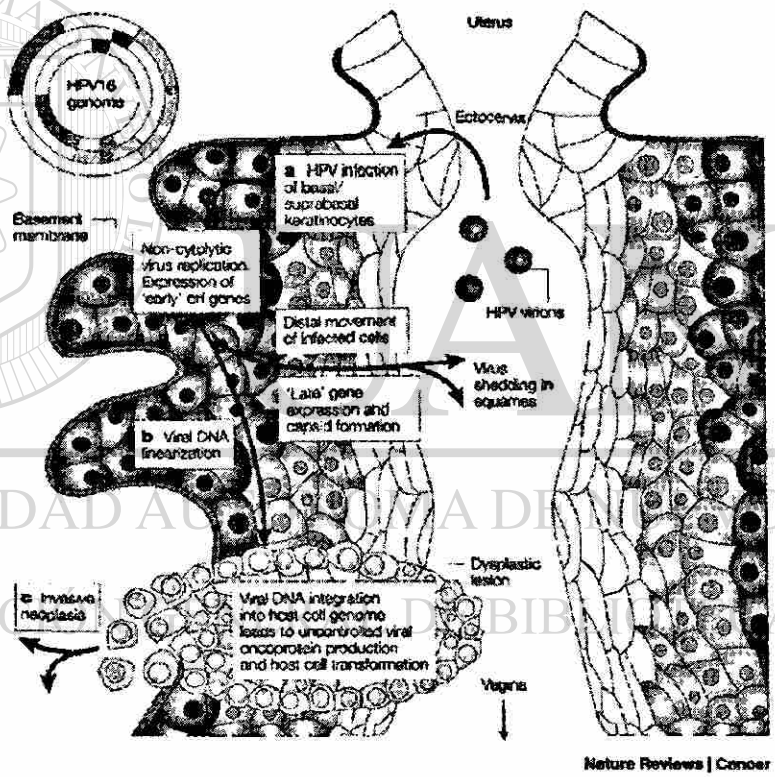

Fig 4 Infección con HPV y desarrollo del CaCu

2.3 La Proteína E7 del HPV-16. Los genes *E6* y *E7* del HPV-16 son expresados constitutivamente en carcinomas cervicales (Smotkin & Wettstein, 1986) y son requeridos para el proceso transformante. El principal mecanismo por medio del cual E6 y E7 contribuyen al desarrollo del cáncer cervical es la

interacción funcional con las proteínas celulares p53 y Retinoblastoma (Rb), estas proteínas juegan un papel pivotal en el control del crecimiento celular (Howley *et al.*, 1989; Münger *et al.*, 1989). La proteína E7 es también capaz de interaccionar con otros componentes celulares como el encogen *ras* activado, ciclinas A y E, causando la desregulación del ciclo celular y la transformación (Dyson, N *et al*, 1989, Jones, D. L., *et al.*, 1997, Kanda, T., *et al.*, 1988).

La proteína E7 del HPV-16 es una fosfoproteína nuclear de 98 aminoácidos (aa), sin actividad enzimática (Smotkin & Wettstein, 1986), En células eucariotas, tiene una vida media de 30 a 40 min. La proteína E7 es ampliamente estudiada debido a su relación con el CaCu. Su producción se ha desarrollado tanto en sistemas eucariotes como en procariotes. En células de mamíferos transformadas, solo se detecta el RNA mensajero (Bedell *et al.*, 1987, Kanda *et al.*, 1988) lo cual sugiere que la proteína se degradada después de la traducción. En levaduras, la producción de E7 nativa ha sido reportada en su forma biológicamente activa en *Schizosaccharomyces pombe* (Tommasino *et al.*, 1990) en localización nuclear, así como de forma secretada en *Saccharomyces cerevisiae* (Carter *et al.*, 1991). En los sistemas procariotes, la producción de E7 fue reportada en *Escherichia coli* como una proteína fusionada a otras (Seedorf *et al.*, 1987, Sato *et al.*, 1989, Barbosa *et al.*, 1989) y posteriormente en su forma nativa (Imai *et al.*, 1991; Pahel *et al.*, 1993). La inmunogenicidad de E7 ha sido demostrada en diferentes sistemas, (Chen *et al.*, 1991, Feltkamp et al., 1993, Lin *et al.*, 1996, Londoño *et al.*, 1996, Jabbar *et al.*, 2000). Además de que la proteína E7 es mantenida y expresada en tumores cervicales, su secuencia no muestra homología con proteínas celulares humanas, lo cual elimina el riesgo de una respuesta autoinmune (Steller, 2002). Estas observaciones soportan la idea de que la proteína E7 es un atractivo blanco para el desarrollo de vacunas e inmunoterapia contra CaCu .

Una región dentro de la proteína E7 contiene dos motivos conservados Cys-X-X-Cy en su extremo carboxilo (Barbosa *et al.*, 1989; Rawls *et al.*, 1990) que tienen la capacidad de unirse al Zn^{2+} . Las mutaciones en uno solo de los motivos Cys-X-X-Cys, los cuales son conservados entre diferentes proteínas E7, reduce la actividad transformante de la proteína. Dobles mutantes en estos motivos tienen

muy poca actividad transformante (Edmonds C *et al.*, 1989). Se cree que estos motivos forman dedos de zinc los cuales son importantes para mantener la estabilidad de la proteína (Barbosa M *et al.*, 1989, Berezutskaya, E. *et al.*, 1997) ya que estas mutaciones generan una proteína inestable, sin embargo, en un estudio realizado con vacunas de DNA, en el cual utilizaron el ORF de esta proteína E7 mutante (58 Cys→Gly, 91 Cys→Gly), observaron que esta condición favorece la inducción de una mayor respuesta inmune específica de E7 y una mejor protección contra tumor que la proteína estable E7 nativa (Shi W *et al.*, 1999)

2.4 Inmunología del CaCu. Ciertas observaciones indican que aún y cuando la detección del HPV anogenital es muy común, solamente una pequeña proporción de estas mujeres desarrolla una neoplasia intraepitelial cervical (NIC). Este fenómeno se debe a que, según datos epidemiológicos existe una respuesta inmune protectora contra el desarrollo del CaCu una vez que se ha establecido la infección con el HPV y que aquellas mujeres que llegan a desarrollar NIC carecen de tal respuesta (Sillman *et al.*, 1984; Caussy *et al.*, 1990). Además existe evidencia clínica que sugiere que los HPV establecen infecciones latentes o persistentes y que éstas están relacionadas con la progresión de la enfermedad cervical. Pacientes en estados de inmunosupresión, por transplantes o bien como resultados de la infección con el virus de la inmunodeficiencia humana (HIV), frecuentemente desarrollan lesiones asociadas a HPV, lo cual indica que el HPV establece una infección subclínica que es controlada por la inmunidad mediada por células (Galloway, 2003).

La respuesta humoral es importante sólo para infecciones productivas, en las que las partículas virales salen al espacio extracelular por exocitosis o durante la lisis celular. Estudios serológicos usando partículas tipo virales (VLP's) indican que los individuos expuestos al HPV con o sin lesiones cervicales desarrollan anticuerpos sistémicos contra la proteína L1. Además se han encontrado anticuerpos de tipo IgA en secreciones cervicales, pero estos no están relacionados con la reversión de las lesiones (Bontkes *et al* 1999). En individuos inmunocompetentes el sistema inmunológico es capaz de combatir las lesiones

previas al CaCu invasor (condilomas y NIC), y mantener al tumor localizado. Por ejemplo, durante la regresión de las verrugas genitales se presentan infiltrados locales muy importantes de células mononucleares, incluyendo linfocitos T citotóxicos (CTL), células asesinas naturales (NK) y macrófagos que invaden la epidermis y destruyen las células neoplásicas. Sin embargo no es sorprendente que en los pacientes con cáncer invasivo exista poca producción de anticuerpos anti-L1, ya que en esta etapa, no hay formación de partículas virales (Nonnenmarcher *et al* 1995)

En las etapas iniciales de la enfermedad la respuesta inmune es aún efectiva, pero durante su evolución, las células adquieren nuevas modificaciones genéticas que les confieren ventajas selectivas para evadir cada vez mejor los mecanismos de control del sistema inmune hasta que éste se hace ineficiente (Stern *et al.,* 2001). Alteraciones en las células presentadoras de antígeno (CPA) a nivel cervical pueden proteger al tejido infectado del sistema inmune del hospedero. Análisis recientes muestran una reducción en el numero de células de Langerhans [células dendrítricas (CD) epidermales inmaduras] en infecciones por HPV y displasias, posiblemente influenciada por la expresión de los genes virales (Connor *et al.*, 1999); Se ha demostrado también que en lesiones cervicales existe una falta de activación de las células de Langerhans, aunado a una regulación negativa del factor de necrosis tumoral (TNFα) producido por los queratinocitos, y una sobre-expresión de la interleucina 10 (IL-10) en NIC (Mota *et al.,* 1999). Además los productos de los genes E6 y E7 de los HPV de alto riesgo modulan la ruta de señalización mediada por el interferón gamma (INF α), esto provee una manera de evadir la respuesta del sistema inmune (Barnand *et al.*, 1999, Li S *et. al*, 1999). En algunas lesiones progresivas el sistema inmune puede no ser disparado hasta que haya ocurrido la integración y otros eventos celulares que desencadenan en la transformación, comprometiendo la inmunidad en la resolución de la malignidad. Los CTL podrían dirigir la resistencia al tumor. Sin embargo es altamente frecuente que las moléculas del sistema mayor de histocompatibilidad tipo 1, (HLA-1), estén sub-reguladas en NIC lo cual esta directamente relacionado con la progresión de la enfermedad (Keating *et al.*, 1995, Bontkes *et al.,* 1998). Análisis moleculares de carcinomas cervicales muestran que estas sub-regulación de las moléculas de HLA clase I es el resultado de múltiples

mecanismos genéticos y ocurre en al 90% de los tumores, lo cual afecta fuertemente la función efectora de los CTL (Koopman *et al.*, 2000)

2.5. Estrategias de intervención inmune. El objetivo de la inmunización profiláctica y terapéutica es la estimulación de la respuesta inmune para producir moléculas y/o células efectoras para prevenir la infección o eliminar las células infectadas o transformadas. Toda estrategia de inmunización debe estar basada en identificar antígenos específicos; a pesar de que los blancos responsables de la regresión inmune de lesiones asociadas a HPV no han sido claramente identificados. Tomando en cuenta el ciclo de vida celular del HPV, los genes L1, L2 o E6 y E7 y sus productos, pueden ser excelentes candidatos para el desarrollo de vacunas. Las proteínas E6 y E7 de HPV-16 han sido reconocidas como antígenos tumorales altamente específicos dado que son capaces de inducir la generación de CTL CD8+ y estos a su vez pueden eliminar células tumorales (Chen *et al.*, 1991; Chen *et al.*, 1992; Tarpey *et al.*, 1994; Felkamp *et al.*, 1995). La capacidad de activar células CTL ha estimulado la posibilidad de desarrollar vacunas basadas en esta proteína, además, la activación de los linfocitos T ofrece la ventaja de producir memoria de largo plazo y no requiere la persistencia del antígeno (Lau *et al.*, 1994; Mullbacher, 1994). Las vacunas profilácticas y terapéuticas desempeñan un importante papel en el combate de la enfermedad asociada a HPV. Una vacuna profiláctica puede prevenir la infección al generar una respuesta inmune efectiva en el sitio y tiempo de la infección, lo cual inhibe el establecimiento de la misma y la re-infección. Por otra parte la vacunación terapéutica podría directamente eliminar la infección establecida, tanto en la enfermedad benigna como maligna.

La mucosa genital se caracteriza por ser un área de muy pobre inmunogenicidad para el huésped; principalmente por las limitaciones de los queratinocitos para funcionar como células presentadores de antígeno (Tindle, 1996). Una estrategia de vacunación profiláctica debe superar estos problemas de inmunogenicidad, por ejemplo la vacunación con subunidades simples basadas en la proteína L1 desnaturalizada no ha tenido buenos resultados en estudios animales (Pilacinski *et al.*, 1986). Dentro de

las dificultades del uso de virus completos es la falta de habilidad para propagar el virus *in vitro*, además tampoco es fácil obtener cantidades razonables de viriones de las lesiones. Afortunadamente se ha demostrado que la proteína L1 tiene la capacidad intrínseca de auto ensamblarse, para producir partículas tipo virales (VLP) libres de ADN, los cuales son morfológicamente indistinguibles de los virus infecciosos, y presentan epitopes altamente inmunogénicos (Kirnbaur *et al.*, 1992); Así también se han producido las proteínas L1 y L2 en levaduras y en otros tipos de células usando como vectores virus vaccinia, baculovirus y virus de semliki forest (Lee Cann *et al.*, 1995; Hofmann *et al.*, 1996; Heino *et al.*, 1995; Ghim *et al.*, 1996). Actualmente existen 17 vacunas virales con licencia para su uso en humanos basadas en virus muertos o atenuados (Stern *et al.*, 2001)

El desarrollo de vacunas terapéuticas se enfoca en despertar una respuesta de tipo citotóxica en contra de los oncogenes virales constitutivamente expresados en las lesiones cervicales. Como ya mencionamos la expresión de las proteínas E6 y E7 son necesarias para mantener la capacidad transformante de las células por lo cual son consideradas específicas de tumor y representan blancos potenciales para dirigir una vacuna que controle los tumores inducidos por el HPV. Una vacuna terapéutica eficaz debe de inducir la activación de CTL específicos de epitopes derivados de proteínas virales (E6 o E7) procesadas intracelularmente y presentadas en el contexto de cualquiera de las moléculas de HLA-I del huésped.

Existe una gran variedad de métodos para el desarrollo de vacunas terapéuticas, tal es el caso de la vacunación con péptidos sintéticos, las vacunas de DNA, las vacunas basadas en células dendríticas, y las mediadas por bacterias vivas. Los péptidos sintéticos no son eficientes inductores de CTL contra el E7 del HPV (Jochmus *et al.*, 1997), probablemente porque no son presentados al organismo de forma inmunogénica como lo hace la proteína endógena. La estimulación del sistema inmunológico de mamíferos mediante vacunas de ADN representa una nueva forma de vacunación, que ha mostrado en repetidas ocasiones inducir una respuesta inmune celular y humoral (Ulmer *et al.*, 1993; Robinson, 1997; Donnelly *et al.*, 1998). A pesar de ello, la falta de inducción de la respuesta inmune a nivel mucosal es una de las

principales limitaciones de este tipo de sistemas. Por este motivo las nuevas investigaciones se han enfocado en dirigir las vacunas de ADN directamente a sitios inductivos del sistema inmunológico de mucosas, tal es el caso de la inmunización vía vaginal directa, la oral y la nasal (Jones *et al.,* 1997b; Klavinskis *et al.,* 1997; Wang *et al.,* 1997).

2.6 Adyuvantes e Inmunoestimulantes. Frecuentemente los péptidos y la mayoría de los antígenos en cáncer son poco inmunogénicos; Para sobrellevar esta limitación pueden ser alterados por modificaciones en su secuencia de aminoácidos, conjugarse con moléculas inmunoestimuladoras y ser coadministradas con adyuvantes. Varios péptidos estimulantes de linfocitos T ayudadores (HTL) han sido encontrados (Alexander *et al.*, 1994), la unión covalente de estos epitopes a dos residuos de acido palmitito, y un epitope del péptido E7 del HPV-16 se encuentra en estudio de fase clínica (Steller *et al.*, 1998). El adyuvante incompleto de Freud's ha sido utilizado clínicamente por varios años, pero permanece como un adyuvante no especifico que algunas veces induce una respuesta inflamatoria local no deseada. Otros inmunoestimulantes en investigación incluyen, al complejo estimulador inmune (ISCOM) citocinas y moléculas estimulatorias.

La interleucina-12 (IL-12), previamente conocida como un factor estimulador de células NK y factor de maduración de CTL, es un heterodímero compuesto de dos subunidades (p35 y p40), producido por CPA, tales como macrófagos, células dendríticas y células de Langerhans; dentro de sus actividades biológicas se incluyen el aumento de CTL, activación y proliferación de células NK, aumento de citotoxicidad, inducción de la producción de INF-γ por células NK y linfocitos T entre otras. (Gately *et al.*, 1992; Kiniwa *et al.*, 1992; Scott, 1993). Varios estudios muestran que la IL-12 es también un adyuvante efectivo para estimular la respuesta inmune (Metzger *et al.,* 1996, 1997). El efecto de la administración nasal (i.n.) en función, tanto de la inmunidad de mucosas como sistémicas fue estudiada por Arulanandam & Metzger (1999) y concluyeron que IL-12 puede ser por lo tanto administrada *i.n.* de

una manera no invasiva para influenciar la respuesta inmune humoral de una manera similar a la administración parenteral y servir como un adyuvante efectivo y seguro en el desarrollo de vacunas.

2.7 *Lactococcus lactis* como BL modelo en el diseño de vacunas. En la mayoría de los casos los vectores bacterianos utilizados para la expresión de antígenos heterólogos son bacterias Gram negativa; probablemente debido al gran numero de herramientas disponibles para su manipulación genética y al gran conocimiento que se tiene de estos organismos. Sin embargo, dichos organismos generalmente son patógenos para el humano y aún y cuando hayan sido manipulados extensamente para reducir o eliminar su patogenicidad, mantienen ciertas características invasivas (Cadoz *et al.*, 1992; Tacket *et al.*, 1992; Tartaglia *et al.*, 1992). Por tal motivo se han desarrollado sistemas con bacterias Gram positivas no patogénicas (Iwaki *et al.*, 1990; Hansson *et al.*, 1992; Pozzi *et al.*, 1992; Wells *et al.*, 1993; Piard *et al.*, 1997; Le Loir *et al.*, 1998). En estos sistemas el antígeno ha sido expresado en citoplasma o secretado al medio de cultivo (Iwaki *et al.*, 1990; Wells *et al.*, 1993; Le Loir *et al.*, 1998;), en otros se ha optado por presentar el antígeno en la superficie celular (Hansson *et al.*, 1992; Nguyen *et al.*, 1993; Pozzi *et al.*, 1994; Medaglini *et al.*, 1995; Samuelson *et al.*, 1995; Gunneriusson *et al.*, 1996; Piard *et al.*, 1997).

Lactococcus lactis es un organismo Gram positivo perteneciente al grupo de bacterias lácticas (BL); clasificado como un microorganismo "generalmente seguro" (GRAS), no patógeno y no colonizante, Además, es un organismo ampliamente usado en la producción de productos lácteos fermentados. Son varias las propiedades de esta bacteria que la hacen un atractivo modelo para la secreción de proteínas biológicamente activas, ya que no secreta proteasas ni alguna otra proteína en cantidades significativas, característica que además puede simplificar los análisis de las proteínas secretadas. A la fecha numerosas proteínas heterólogas han sido producidas y secretadas existosamente en *L. lactis* usando péptidos señales (SP) nativos ó sintéticos (Perez-Martinez *et al.*, 1992; Simonen & Palva, 1993; van Asseldonk *et al.*, 1993; Steidler *et al.*, 1995; Norton *et al.*, 1997; Savijoki *et al.*, 1997; Le Loir *et al.*, 1998). Estudios con modelos animales y en voluntarios alimentados con lactococos vivos

han mostrado que es una bacteria transitoria del tracto digestivo, sin evidencia de colonización (Gruzza *et al.*, 1994; Klijn *et al.*, 1995) a pesar de ser no colonizante *L. lactis* es capaz de expresar antígenos y presentarlos al sistema inmune de forma inmunogénica. (Wells & Schofield, 1996; Steidler *et al.*, 1998). Además, otra ventaja que ofrece esta BL para el desarrollo de vacunas es que su pared celular es un adyuvante natural. Todas estas características podrían favorecen la aceptación de *L. lactis* como un vector de inmunización en humanos. Por lo cual ha comenzado a utilizarse como vehículo de expresión de proteínas heterólogas para inmunización vía mucosa.

A pesar de que una alta producción de proteínas heterólogas ha sido obtenida en *L. lactis* con el uso de promotores constitutivos (de Vos, 1999), la continua expresión de altos niveles de proteína puede conducir a una acumulación intracelular o la degradación en el citoplasma, lo cual puede resultar deteriorante para la célula, además, dado que *L. lactis* es un organismo no colonizante es recomendable el uso de un sistema de expresión *in vitro* (inducible) que garantice que la proteína de interés sea producida antes de la aplicación (inmunización, por ejemplo). En 1996 de Ruyter y colaboradores desarrollaron un sistema de expresión génica controlada (NICE); el cual es un sistema que puede regular la expresión de un gen mas de 1000 veces (de Ruyter et al., 1996). Dicho sistema esta basado en el uso de una bacteriocina (nisina) como inductor de la transcripción de genes situados bajo el control del promotor nisina.

CAPITULO 3

ESTRATEGIA EXPERIMENTAL

3.1 Bacterias y plásmidos usados en este trabajo. Las bacterias y plásmidos utilizados en este trabajo se presentan en la Tabla 1. *Lactococcus lactis* fue crecido en medio M17 (DIFCO) suplementado con 1% de glucosa (GM17) a 30°C sin agitación. *Escherichia coli* se creció en medio Luria-Bertani (LB, Sambrook *et al.*, 1989) a 37°C con agitación vigorosa. Los plásmidos fueron mantenidos por la adición de los siguentes antibióticos: para *E. coli* 100 µg/ml de ampicilina (Amp), ó 10 µg/ml de cloranfenicol (Cm) y para *L. lactis* 10 µg/ml cloranfenicol ó 5 µg/ml de eritromicina (Em).

Tabla 1: Cepas y plásmidos usados.

Cepa	Genotipo		Referencia
E. coli TG1	*supE, hsd, Δ5, thi, Δ(lac-proAB), F'(traD36 proAB-lacZΔM15)*		Gibson, 1984.
L. lactis NZ9000	MG1363 (genes *nisRK* en cromosoma). Sin plásmidos		Kuipers et al., 1998.
NZ(pCYT:E7)	Cm^r; Cepa de *L. lactis* NZ9000 que expresa la proteína E7 en su citoplasma bajo el control del promotor P_{nisA}		Bermúdez-Humarán et al., 2002
NZ(pSEC:E7)	Cm^r; Cepa de *L. lactis* NZ9000 que secreta la proteína E7 bajo el control de promotor P_{nisA}		Bermúdez-Humarán et al., 2002
NZ(E7-CWA)	Amp^r, Em^r; Cepa de *L. lactis* NZ9000 que expresa en su superficie la proteína E7 bajo el control del promotor P_{nisA}		Cortes-Perez et al., 2002
NZ(pSEC:scIL-12)	Cm^r; Cepa de *L. lactis* NZ9000 que secreta la interleucina-12 murina bajo el control del promotor P_{nisA}		Bermúdez-Humarán et al., 2002
Plásmidos	**Replicón**	**Características del plásmido y cassettes**	**Referencia**
pCYT:E7wt	pWV01	Cm^r; cassette de expresión de la proteína E7 nativa bajo el control del promotor P_{nisA}	Bermúdez-Humarán et al., 2002
pSEC:E7wt	pWV01	Cm^r; cassette de expresión del precursor SP_{Usp45}:E7 bajo el control del promotor P_{nisA}	Bermúdez-Humarán et al., 2002
pIL:E7wt-Usp-CWA	pIL252	Amp^r, Em^r; cassette de expresión del precursor SP_{Usp45}:E7-CWA bajo el control del promotor P_{nisA}	Cortes-Perez et al., 2002
pCWA:Usp-E7wt	pWV01	Cm^r; cassette de expresión del precursor SP_{Usp45}:Usp-E7-CWA bajo el control del promotor P_{nisA}	Este trabajo
pIL:E7mm-Usp-CWA	pIL252	Amp^r, Em^r; cassette de expresión del precursor SP_{Usp45}:E7mm-CWA bajo el control del promotor P_{nisA}	Este trabajo
pCWA:E7wt	pWV01	Cm^r; cassette de expresión del precursor SP_{Usp45}:E7-CWA bajo el control del promotor P_{nisA}	Este trabajo
pCWA:E7mm	pWV01	Cm^r; cassette de expresión del precursor SP_{Usp45}:E7mm-CWA bajo el control del promotor P_{nisA}	Este trabajo

3.2 Manipulaciones del DNA y enzimas usadas. Para el aislamiento del DNA plasmídico de *E. coli* se uso el método de Birnboim & Doly (1979) y el descrito por Langella *et al.* (1993) para *L. lactis*. Todas las enzimas de restricción (New England) y modificación, (Stratagene y MBI Fermentas) fueron utilizadas de acuerdo a las especificaciones del proveedor o como se ha descrito previamente (Sambrook et al., 1989). Para analizar los fragmentos de DNA, se usaron geles de agarosa 1% TBE (45 mM Tris-Borato, 1 mM EDTA) más 0.2 µg/ml de Bromuro de Etidio.

3.3 Construcción de un vector para la expresión en la superficie de *L. lactis* de una proteína E7 mutante bajo la regulación de un promotor inducible. Para construir un vector de expresión de una proteína E7 mutante (E7mm) del HPV-16, primero se amplificó por PCR un gene *E7mm* a partir del plásmido BC219-E7mm (gentilmento donado por el Dr. Lian Qiao; [Shi *et al.*, 1999]). La secuencia de los primers usados fue la sigueinte: E7/*Sal*I (5'-AGTCGACCCATTGCATGGAGATACACCTACAT TG-3') para la cadena codificante y E7/*Eco*RV (5'-CGATATCTCTGGTTTCTGAGAACAGATGGG GCA-3') para la complementaria. Posteriormente el fragmento de PCR obtenido se subclono con el kit pCR TOPO TA-Cloning (Invitrogen), para obtener TOPO:E7mm. Finalmente de este vector se purificó un fragmento de DNA con las enzimas *Sal*I y *Eco*RV y se clonó en el vector pVE5547 (Dieye et al., 2001) digerido con las mismas enzimas para obtener pIL:Usp-E7mm-CWA (Tabla 1). Esta construcción se introdujó en la cepa NZ9000 de *L. lactis* para obtener NZ(pIL:Usp-E7mm-CWA).

3.4 Construcción de un nuevo vector para la expresión en la superficie de *L. lactis* de la proteína E7 bajo la regulación de un promotor inducible. Recientemente nuestro laboratorio describió la construcción de un vector (pIL:Usp-E7wt-CWA) para la expresión en la superficie de *L. lactis* de la proteína E7 wild-type (E7wt) del HPV-16 (Cortes-Perez, *et al.*, 2003). Sin embargo, este vector es un derivado de un plásmido de alto peso molecular y de replicación de tipo theta que es muy difícil de manipular (Dieye et al., 2001), tal y como se observo en la construcción del vector pIL:Usp-E7mm-

CWA. Por lo tanto procedimos a construir un nuevo vector fácil de manipular para la expresión en superficie de *L. lactis*. Para dicho propósito se eligió un derivado del vector pGK-12, un plásmido de bajo peso molecular y de replicación shuttle, es decir que tiene la capacidad de replicar en diferentes especies de bacterias (*Escherichia coli*, *Bacillus* spp., *Lactobacillus* spp., *Enterococcus* spp., etc.) (Kok et al., 1984; Bermúdez-Humarán et al., 2002, 2003c). Primero se purificó un fragmento SPUsp-E7wt-CWAM6 del vector pIL:UspE7wt-CWA digerido con *Bgl*II/*Spe*I y se clono en el vector pSEC:E7 (derivado del pGK-12, Bermúdez-Humarán et al., 2002) digerido con las mismas enzimas. El plásmido obtenido pCWA:Usp-E7wt (Tabla 1), se introdujó por electroporación en la cepa NZ9000 de *L. lactis* resultando en NZ(pCWA:Usp-E7wt).

3.5 Construcción de vectores para la expresión en la superficie de *L. lactis* de una proteína E7 no fusionada bajo la regulación de un promotor inducible. Los vectores descritos hasta ahora para la expresión en superfice de E7 del HPV-16 (E7wt ó E7mm), están diseñados para producir una proteína E7 con 15 aminoácidos (aa) extras en su parte amino-terminal y que provenien de la proteína Usp45, de ahí su abreviación Usp-E7. En este trabajo también se construyeron dos nuevos vectores para expresar una proteína E7wt ó E7 mm no fusionada a éstos 15 aa. Para dicho propósito, se obtuvo un fragmento de DNA del vector pSEC:E7 (Tabla 1) con las enzimas *Ava*II/*Bgl*II, y se clonó en el vector pCWA:Usp-E7wt digerido con las mismas enzimas, para obtener el plásmido pCWA:E7wt. Posteriormente para construir el vector de expresión de la E7mm no fusionada a los 15 aa se procedió a amplificar un fragmento de DNA del vector pIL:Usp-E7mm-CWA con los siguientes primers: *Nsi*I/E7 (CCAGATGCATCACC ACATGGAGATACACCTACATTG) para la cadena codificante y *Eco*RV/E7 (CGATATCTCTGG TTTCTGAGAACAGATGG GGCCCAC) para la complementaria. El producto de PCR obtenido se digirió directamente con las enzimas *Nsi*I y *Eco*RV y se clono en el vector pCWA:E7wt digerido con las mismas enzimas. El plásmido obtenido, pCWA:E7mm, al igual que el

pCWA:E7wt se intodujeron en las cepa NZ9000 de *L. lactis* para obtener NZ(pCWA:E7mm) y NZ(pCWA:E7wt), respectivamente.

3.6 Caracterización de las construcciones. Todas las construcciones y fusiones de DNA, fueron caracterizadas con enzimas de restricción y posteriormente por secuenciación de ambas cadenas. La secuenciación se realizo con el kit: DYEnamic ET Terminator Cycle Sequencing Kit (Amersham, Biosciences).

3.7 Transformación de *Escherichia coli* y *Lactococcus lactis*. Para la transformación de *E. coli* se utilizó la técnica previamente descrita por Sambrook et al. (1989). Para *L. lactis* se siguió el protocolo de Langella y *et al* (1993) con las modificaciones hechas por Bermúdez-Humarán (2002). Primero se prepararon células electrocompetentes según se describe a continuación: se reactivó la cepa NZ9000 de *L. lactis* (de Ruyter *et al.*, 1996) en 5 ml de medio GM17SG (GM17, 0.5 M Sacarosa, 2% Glicina) incubando toda la noche a 30°C sin agitación, y se tomaron 4 ml de este cultivo para inocular 200 ml del mismo medio. Se incubó a 30°C sin agitación hasta que el cultivo alcanzó una densidad óptica$_{600}$ (DO)= 0.5-0.8. Se pasó inmediatamente a hielo y se incubó por 15 min. Se centrifugó el cultivo 8 min. a 7,000 r.p.m., 4°C y se lavó el botón dos veces con 100 ml de buffer de lavado (0.5M Sacarosa, 10% Glicerol). Se realizó un tercer lavado, solo que ahora con 20 ml del buffer. El botón obtenido de este último lavado se resuspendió en 1 ml de PEG 3000 (Polyethylen-Glycol 3000) y 10% Glicerol y finalmente se hicieron alicuotas de 100 µl en tubos de 1.5 ml congelando inmediatamente en nitrógeno liquido y almacenando a -80°C.

Para la transformación se mezclaron 100 µl de células electrocompetentes con 1 µl de DNA plasmídico (10 µl en el caso de los productos de ligación), se transfirieron a cuvetas de elcctroporación (2 mm) previamente enfriadas y se expusieron a un pulso eléctrico (Gene-Pulser, BIORAD Laboratories), 25µF, 200Ω, 2.4kV. Inmediatamente después de la descarga eléctrica, se adicionaron 900

µl de medio GM17S (GM17, 0.5 M Sacarosa) y se incubó por 1 hr. para expresión. Finalmente se tomo la mezcla para sembrar en placas GM17A (GM17, 1% agar), más el antibiótico según el caso. Se seleccionaron las colonias transformantes después de 48 hrs. de incubación a 30°C.

3.8 Condiciones para la inducción a la nisina y extracción de proteínas. Para la inducción del promotor nisina, se tomo una dilución 1:250 de un cultivo de toda la noche de la cepa recombinante de *L. lactis*, se inoculo en medio nuevo y se dejo crecer hasta una DO= 0.4-0.6, una vez alcanzada esta densidad se indujo con 10 ng/ml de nisina (SIGMA).

Para preparar los extractos protéicos de *L. lactis*, se siguió el protocolo reportado por Bermúdez-Humarán *et al.* (2002), el cual incluye inhibidores de proteasas y procedimientos suaves de precipitación dada la fragilidad de E7 con algunas ligeras modificaciones. Los extractos proteicos fueron preparados a partir de 2 ml de cultivos inducidos. El botón celular y sobrenadante fueron procesados por separado. Primero se centrifugo por 5 min. 2 ml de cultivo a 14000 r.p.m. 4°C y del cual se separaron 1.4 ml en un tubo nuevo. A este sobrenadante se le adicionó 100 µL de ácido tricoloacético (TCA) 100%, 1 mM PMSF (Phenyl-Methyl-Sulfonyl-Fluoride) y 10 mM DTT (Dithiothreitol), y se incubo 10 min. en hielo. Se centrifugo 10 min. a 14000 r.p.m., 4°C, se retiro el sobrenadante y se resuspendió el botón en ½ volumen de 50 mM NaOH y ½ volumen de DTT-LB (50 mM Tris-HCl pH 6.8, 100 mM DTT, 2% SDS, 0.1% Azul de Bromofenol, 10% Glicerol) según la DO. Para la fracción celular, el pellet obtenido de la centrifugación de los 2 ml de cultivo se resuspendió en 2/6 (según DO) de buffer TES-Lys (25% Sacarosa, 1 mM EDTA, 50mM Tris-HCl pH 8.0; 10 mg/mL Lysozima), 1 mM PMSF, 10 mM DTT, se incubó por 15 min. a 37°C y después se agregó 1/6 de una solución de SDS (20%) y se dio vórtex vigorosamente. Finalmente se adicionaron 3/6 de DTT-LB. Ambas muestras (sobrenadante y fracción celular) fueron incubadas 3 min. a 98°C antes de ser analizadas en gel de poliacrilamida (SDS-Page).

3.9 Western Blot. Las proteínas fueron separadas en un gel SDS-Page 12% (Sambrook et al., 1989) y transferidas por espacio de $1^{1/2}$ hr. a una membrana de PVDF (NEN, Life Science Products). Una vez

transferidas las proteínas a la membrana, se incubó en una solución de bloqueo TBS-T (20 mM Tris pH 7.5, 5.5 mM NaCl, 5% Tween-20, 10% leche en polvo), se lavó una vez con TBS-T y se incubó 1 hr. con un anticuerpo anti-E7:HPV-16 (TVG710Y, ED17, N-21 ó C-20, Santa Cruz Biotechnology, Inc.) usando TBS-T (2% leche en polvo) para su dilución. Después de tres lavados con TBS-T, se incubó la membrana por 1 hr. con la proteína G (HRP, BIORAD). Finalmente se hicieron tres lavados con TBS-T y se reveló la membrana con el Kit ECL (Enhanced ChemiLuminescence, DuPont NEN) y exposición en un film autoradiográfico.

3.10 Ensayos de inmunofluorescencia. Para confirmación de la expresión en superficie de la proteína E7 del HPV-16 en *L. lactis* se realizaron experimentos de inmunofluorescencia por microscopia tal, como se describió anteriormente (Cortes-Perez et al., 2003) con algunas modificaciones. Una vez acabado el tiempo de inducción, se obtuvo el pellet celular de 2 ml de cultivo por centrifugación (5000 r.p.m. 2 min), y se resuspendió en 1 ml de una solución estéril de PBS-BSA 3% y el anticuerpo anti-E7 ED17 (dilución de 1:500). Se dejó en incubación toda la noche a temperatura ambiente con movimiento. Al siguiente día se centrifugó a iguales condiciones y después de tres lavados con 0.5 ml de PBS–Tween 0.05% se incubó el complejo bacteria-anticuepro, evitando la exposición a la luz y a temperatura ambiente, por 5 hr. con una dilución 1:50 del conjugado Alexa Fluor 546 dye (IgG, H+L, Molecular Probes, Europe BV). Se realizaron 3 lavados con PBS-Tween 0.05% y finalmente las células se resuspendieron en 30 µl de PBS, se realizó un frotis con 10 µl (fijando a la flama de mechero). Para observar la población completa de *L. lactis* las bacterias fueron coloreadas con 4',6-diamidino-2-phenylindole (DAPI, 2.5 µg/ml; SIGMA). Las fotografías de las bacterias se tomaron con un microscopio de inmunofluorescencia equipado con un grupo de tres filtros para caprat la emisión de luz blanca (Nikon, Tokyo, Japan) y Sensia 400 film (Fuji, Tokyo, Japan). Para la captación de la luz roja (excitación) emitida por el conjugado Alexa Fluor 546 fluorophore, se usaron filtros apropiados. Además, se tomaron imagenes de las bacterias sin excitación, esto permite que las células coloreadas

con DAPI y Alexa Fluor 546 puedan ser comparadas simultáneamente. Las imágenes se capturaron con un sistema de análisis de imágenes Visiolab 1000 (Biocom, Les Ulis, France).

3.11 Preparación de cultivos de *L. lactis* para inmunizaciones. Se cultivaron las diferentes cepas recombinantes o no de *L. lactis* en 20 ml de GM17 hasta una DO_{600}=0.5, se adicionó nisina (SIGMA) a una concentración de 10 ng/mL y se dejó incubar hasta una DO_{600}=0.8. Se recupero el pellet celular por centrifugación (5000 r.p.m. 10 min), y se hicieron 3 lavados con PBS. Finalmente las células se resuspendieron a una cincentración final de 1 X 10^9 u.f.c. (unidades formadoras de colonia). Antes de utilización de estos inoculos se realizaron conteos en placas de GM17-Agar y se corroboróla presencia de E7 por Western blot.

3.12 Protocolo de inmunización. Grupos de 3-8 ratones C57BL/6J libres de patógenos (hembras: 6-8 semanas de edad, Charles River Laboratories, France) fueron parcialmente anestesiados con una mezcla de Ketamine/Chlorobutanol (0.01 ml for 10 Kg of weight, Imalgene 1000, MERIAL, France) vía intraperitoneal (*i.p.*) e inmunizados intranasalmente (*i.n.*) con 1 X 10^9 u.f.c. de *L. lactis* (resuspendidos en 10 µl de PBS, 5 µl fueron administrados en cada nostrilo) a los días 1, 14 y 28. Como control se inmunizaron ratones con la misma cantidad de *L. lactis* wild-type (wt) o PBS solo. Todos los experimentos se realizaron de acuerdo a protocolos a probados por el comite de manejo animal de la unidad de ecología y fisiología del tracto digestivo (UEPSD, INRA Jouy en Josas, Francia).

3.13 Ensayo profiláctico y reto con la línea tumoral TC-1. La línea tumoral TC-1 es una línea epitelial del pulmón de ratón C57BL/6J transformada con los oncogenes *E6* y *E7* del HPV-16 y el oncogene *ras* humano activado (Li et al., 1996). Esta línea ha sido ampliamente utilizada para evaluar diferentes vacunas tarapéuticas y/o profilácticas dirigidas contra la proteína E7, como nuestro caso. La línea TC-1 fue crecida en medio de cultivo RPMI 1640 (GIBCO) suplementado con 10% de suero fetal

bovino (SFB), 50 unidades/ml penicilina, 50 g/ml estreptomicina y 0.4 mg/ml de G418. Grupos de 8 ratones C57BL/6J fueron inmunizados *i.n.* tal y como se describio en al parrafo anterior con $1x10^9$ u.f.c. a los días 0, 14 y 28. Siete días después los ratones fueron retados por inyección subcutánea (*s.c.*) con 5×10^4 células de la línea tumoral TC-1 en un volumen de 100 µl resuspendidos en PBS. Cada semana observaron los ratones para detectar el desarrollo de tumor. Las dimensiones del tumor se tomaron utilizando un vernier y considerando dos direcciones perpendiculares, el volumen del tumor se calculo de la siguiente forma: (largo x ancho^2)/2.

3.14 Inmunoterapia para experimentos de regresión tumoral *in vivo*. Para los experimentos terapéuticos, primero se reto a grupos de 3-8 ratones con 5×10^4 células de la línea tumoral TC-1 como se describió en el parrafo anterior, y al día 18 ó 14 se procedio a inmunizarlos con las diferentes cepas recombinantes de *L. lactis* siguiendo el mismo protocolo que para en los ensayos profilácticos (día 0, 14 y 28). Dado que a 14 días todos los ratones presentan tumores visibles en este tipo de experimento se observo la regresión tumoral.

3.15 Evaluación de la producción de citocinas de tipo Th1 (IL-2 e IFN-gamma) en células de bazo de ratones inmunizados. Siete días después de la última inmunización (día 35) los ratones fueron sacrificados por dislocación cervical. Bajo condiciones de total esterilidad se obtuvieron las células de bazo por gradiente de densidad con Ficoll-Histopaque (1083, SIGMA). Un total de 2 X 10^6 de células/ml fueron cultivadas en placas de cultivo celular de 24 pozos en medio RPMI 1640 suplementado con 10% SFB, 50 unidades/ml penicilina y 50 g/ml estreptomicina a 37°C 5% CO2. Estas células fueron estimuladas *in vitro* con 2-5 µg de un péptido sintético de la proteína E7 ($E7_{49-57}$-RAHYNIVTF) para determinar si esta re-estimulación es capaz de inducir una respuesta inmune de tipo celular específica de antígeno. Después de 24 hr. de incubación se filtro la suspensión celular y del

sobrenadante se evaluó la presencia de citocinas de tipo Th1 (IL-2 e IFN-gamma) por ELISA (R&D Systems).

CAPITULO 4

RESULTADOS

4.1 Determinación de la forma más inmunogénica de la proteína E7 del HPV-16 producida por *Lactococcus lactis*. Dado que la localización de una proteína puede influenciar su inmunogenicidad (Norton et al., 1996; Reveneau et al., 2002), en este trabajo se determinó en una primera instancia la respuesta inmune en ratones generada por la administración *i.n.* de diferentes cepas recombinantes de *L. lactis* expresando el antígeno E7wt en citoplasma, secretado al medio de cultivo ó anclado a su superficie celular. Las cepas usadas fueron NZ(pCYT:E7wt), NZ(pSEC:E7wt) y NZ(pCWA:Usp-E7wt). Nuestra hipótesis es que la proteína E7 exportada en *L. lactis* (secretada al medio de cultivo o anclada a la superficie celular) debe permitir un contacto más directo con el blanco (sistema inmune, por ejemplo). La exportación de una proteína debe además ser una mejor estrategia de producción de un antígeno de interés terapeutico, comparado a una producción intracelular, la cual requiere de lisis celular para liberar la proteína. Antes de las inmunizaciones, se analizó la producción de E7 por Western blot de los diferentes cultivos inducidos de *L. lactis* recombinantes. Tal y como se reporto previamente (Bermúdez-Humarán et al., 2002), los cultivos inducidos de la cepa NZ(pCYT:E7wt) revelan una clara banda en la fracción citoplásmica (C) que corresponde a la E7wt (Fig. 1a). Ninguna banda se detecta en el sobrenadante (S, medio de cultivo). En el caso de los cultivos de la cepa NZ(pSEC:E7wt), se puede observar una banda muy tenue en la fracción C y que corresponde al precursor SP-E7 precursor (preE7), mientras que en la fracción S se observa una banda intensa y corresponde a la forma madura de E7 liberada en el medio de cultivo (Fig. 1a). En estos experimentos podemos corroborar tal y como se observo previamente (Bermúdez-Humarán et al., 2002) que la proteína E7 es eficientemente secretada, ya que aproximadamente 95% de la proteína se encuentra en la fracción S. Los resultados de los cultivos de la cepa NZ(pCWA:Usp-E7wt), revelan una banda principal en la fracción C, del tamaño esperado

para el precursor SP-Usp-E7-CWA-M6 (preCWA-E7, 38 kDa), junto con otras dos bandas de menor peso molecular y que corresponden a la forma Usp-E7-CWAM6, resultante del procesamiento del SP y la forma madura Usp-E7-CWA generada después del procesamiento de la señal de anclaje CWAM6

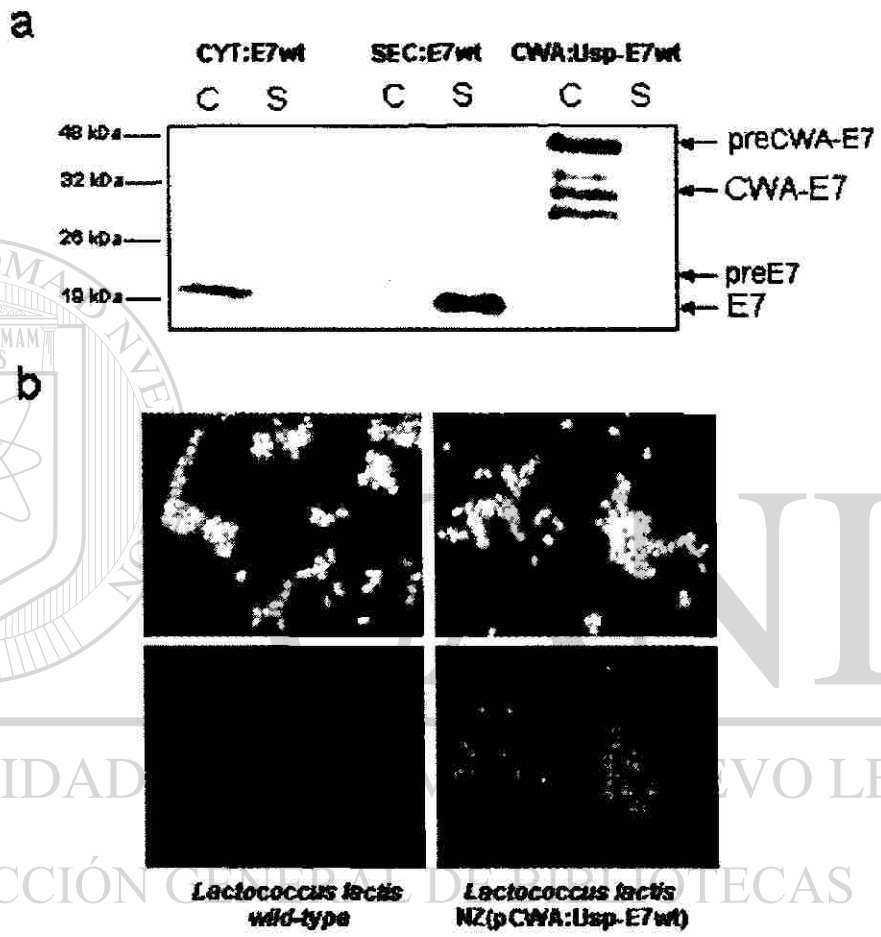

Fig. 1. Análisis de la producción de E7 en tres localizaciones celulares diferentes por *Lactococcus lactis* y detección de la proteína E7 en superficie celular por inmunofluorescencia.(a) Se analizó la producción de E7 por análisis tipo Western Blot de extractos proteicos de las cepas NZ(pCYT:E7wt) (CYT:E7wt, producción citoplásmica de E7), NZ(pSEC:E7) (SEC:E7wt, secreción de E7 al medio de cultivo) ó NZ(pCWA:Usp-E7wt) (CWA:Usp-E7, producción en superficie de E7). C, fracción celular; S, muestras de sobrenadante. A la izquierda se indican las posiciones y tallas del marcador de peso molecular. (b) Se incubaron cultivos inducidos de la cepa recombinante NZ(pCWA:Usp-E7wt) o la cepa wild-type de *L. lactis* con anticuerpos monoclonales específicos de E7 (ver estrategia experimental) y después se marcaron con un conjugado Alexa Fluor 546 dye (cuadros inferiores). En los cuadros superiores se muestran los mismos campos observados al microscopio con el conjugado pero sin teñir. Aumento, 1000X.

(enlace covalente de la proteína E7 a la pared celular de *L. lactis*) (Fig. 1a). También se puede obsrevar una tercera banda muy tenue y que puede corresponder a un producto de degradación de la proteína madura. La confirmación de la expresión de E7 en la superficie de *L. lactis* se llevo a cabo por un ensayo de inmunofluorescencia por microscopia, esencialmente como se describio previamente (Fig. 1b, Cortes-Perez et al., 2003).

Una comparación de los resultados obtenidos por Western blot de las tres formas de E7 nos indica que a pesar de que los niveles de inducción fueron similares en los tres casos, la cantidad de E7 producida en la cepa NZ(pCYT:E7wt) es significativamente menor (tres a cinco veces) que los obtenidos en la cepa NZ(pSEC:E7wt) ó NZ(pCWA:Usp-E7wt). Sin embargo en la cepa NZ(pCWA:Usp-E7wt), la mayoría de la proteína se encuentra en forma de precursor en la fracción C (Fig. 1a). En ese contexto la cantidad de proteína E7 expresado en la superficie es similar al producido por la cepa NZ(pCYT:E7wt) y que se estimó en alrededor de 2-3 µg/ml.

Una vez determinada la producción de la proteína y su localización en *L. lactis*, se procedió a deteminar la inmunogenicidad de las tres formas de E7. Grupos de cinco ratones fueron inmunizados *i.n.* con las diferentes cepas a los días 0, 14 y 28, al día 35 se sacrificaron los ratones y se midieron los niveles de citocinas Th1: IL-2 e IFN-gamma. Las células de bazo de los ratones inmunizados con la cepa NZ(pCWA:E7wt) y re-estimulados in vitro producen mayores niveles de citocinas Th1 que los obtenidos con la cepa NZ(pCYT:E7wt) (Fig. 2a y 2b). Como se acaba de discutir, en la cepa NZ(pCWA:Usp-E7wt), la cantidad de E7 anclada a la pared celular de *L. lactis* es de alrededor de 2 µg/ml, similar a la producción citoplásmica en la cepa NZ(pCYT:E7wt). Por lo tanto la alta inmunogenicidad de la proteína E7 producida por la cepa NZ(pCWA:Usp-E7wt) puede ser el resultado de la presentación en superficie de la proteína. Este tipo de resultados ha sido previamente reportado en BL (Norton et al., 1996; Reveneau et al., 2002), y se le ha atribuido a un mejor acceso del sistema inmune hacia la proteína expresada en superficie celular o a algún efecto adyuvante propio de la BL. No obstante, en estos estudios no se ha considerado la cantidad total antígeno (tomando en cuenta la

cantidad que se queda asociada a la fracción celular y el precursor) para evaluar la respuesta inmune. En este trabajo podemos concluir que aunque la cantidad de antígeno expresado en la superficie de la cepa NZ(pCWA:Usp-E7wt) es similar al producido por la cepa NZ(pCYT:E7wt) la cantidad total de E7 producido por la cepa NZ(pCWA:Usp-E7wt) es mucho mayor que el de la cepa NZ(pCYT:E7wt) (Fig. 1a). Por lo tanto la alta inmunogenicidad de E7 producido por la cepa NZ(pCWA:Usp-E7wt) puede ser debido a una combinación de presentación del antígeno en superficie celular y cantidad del antígeno expresado.

Por otro lado, la respuesta inmune de los ratones inmunizados con la cepa NZ(pSEC:E7wt) fue mucho más baja que la obtenida con los ratones inmunizados con NZ(pCYT:E7wt) y NZ(pCWA:Usp-E7wt) (Fig. 2a y b). Este resultado puede ser debido al protocolo usado para la inmunización (ver capitulo anterior). En contraste a las cepas NZ(pCYT:E7wt) y NZ(pCWA:Usp-E7wt), donde la proteína E7 se encuentra ya sea en la fracción celular o asociada a la pared celular, en la cepa NZ(pSEC:E7wt) la proteína E7 es liberada al medio de cultivo, el cual es descartado durante la preparación del inóculo (Fig. 1a). Por lo tanto, dado que en la fracción citoplásmica de la cepa NZ(pSEC:E7wt) solo encontramos una pequeña proporción del precursor de E7 (preE7 Fig. 1a), la cantidad de proteína liberada por esta cepa en los ensayos de inmunización debe ser muy baja, de ahi su falta de potecial para desperta una buena respuesta inmune.

Tomando en cuenta estos resultados concluimos que la forma más inmunogénica de E7 producida por *L. lactis*, es la de expresión en superficie celular, y por lo tanto se eligió la cepa NZ(pCWA:Usp-E7wt) para nuestro siguiente experimento.

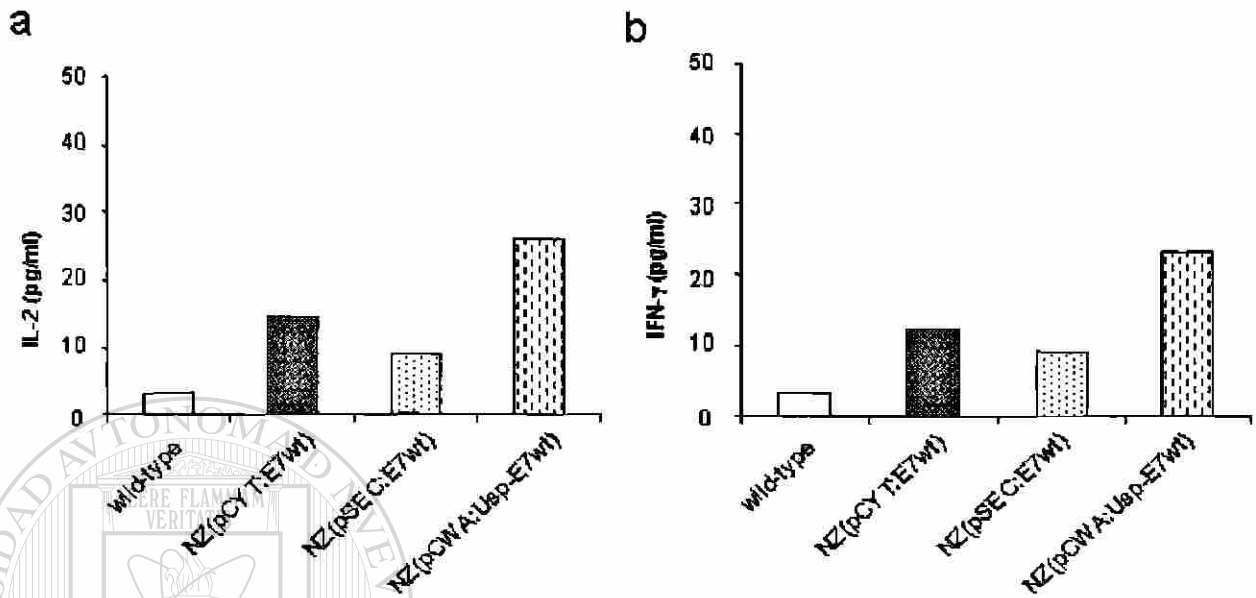

Fig. 2. Evaluación de la respuesta inmune en ratones inmunizados con las cepas recombinantes de *L. lacis* produciendo la proteína E7 en diferentes localizaciones celulares. Después de la inmunización intranasal (*i.n.*) con las diferentes cepas de *L. lactis*: *wild-type*, NZ(pCYT:E7wt), NZ(pSEC:E7wt) o NZ(pCWA:Usp-E7wt) se obtuvieron las células de bazo de cada ratón y se midio *in vitro* la producción de las citocinas IL-2 (a) e IFN-gamma (b). Los valores fueron registrados como la media y desviación estándar de *n*= cinco ratones por grupo.

4.2 Evaluación del potencial adyuvante de una cepa secretando una interleucina-12 activa cuando es co-administrada con la cepa de *L. lactis* expresando en su superficie la proteína E7. Previos estudios han desmotrado que cuando se co-expresan antígenos en *L. lactis* con citocinas, el nivel de la respuesta inmune es considerablemente aumentado (Steidler et al., 1998; Bermúdez-Humarán et al., 2003). La interleucina-12 (IL-12) es una potente molécula estimuladora del sistema inmune que juega un papel importante en la inmunidad celular contra muchos agentes microbianos y que ha sido usada exitosamente en inmunoterapia contra el cáncer. Además la IL-12 ha sido también utilisada como adyuvante cuando es co-expresada con vacunas de DNA. En un intento por mejorar la respuesta inmune en contra de la proteína E7 del HPV-16 en este trabajo se analizó el efecto de la co-administración de

una cepa de *L. lactis* que secreta una interleucina-12 de fusión activa, NZ(pSEC:scIL-12) (Bermúdez-Humarán et al., 2003), con la cepa NZ(pCWA:Usp-E7). Grupos de 3 ratones C57BL/6J fueron inmunizados *i.n.* con tres dósis (día 0, 14 y 28) de las cepa NZ(pCWA:Usp-E7) sola o en combinación con la NZ(pSEC:scIL-12) y siete días después de la última inmunización se analizó la producción de IL-2 e IFN-gamma. Los ensayos de inmunización y análisis de citocinas se hicieron tal y como se describió en la estrategia experimental (capítulo 3). Como se puede observar en la Fig. 3 los animales co-inmunizados con la cepa NZ(pCWA:Usp-E7) y NZ(pSEC:scIL-12) producen significativamente más altos niveles de IL-1 (Fig. 3a) e IFN-gamma (Fig. 3b) que los inmunizados con la cepa NZ(pCWA:Usp-E7) sola. Estos resultados confirman el efecto adyuvante de la IL-12 secretada por *L. lactis* cuando es co-administrada con el antígeno E7 anclado a la superficie de *L. lactis*.

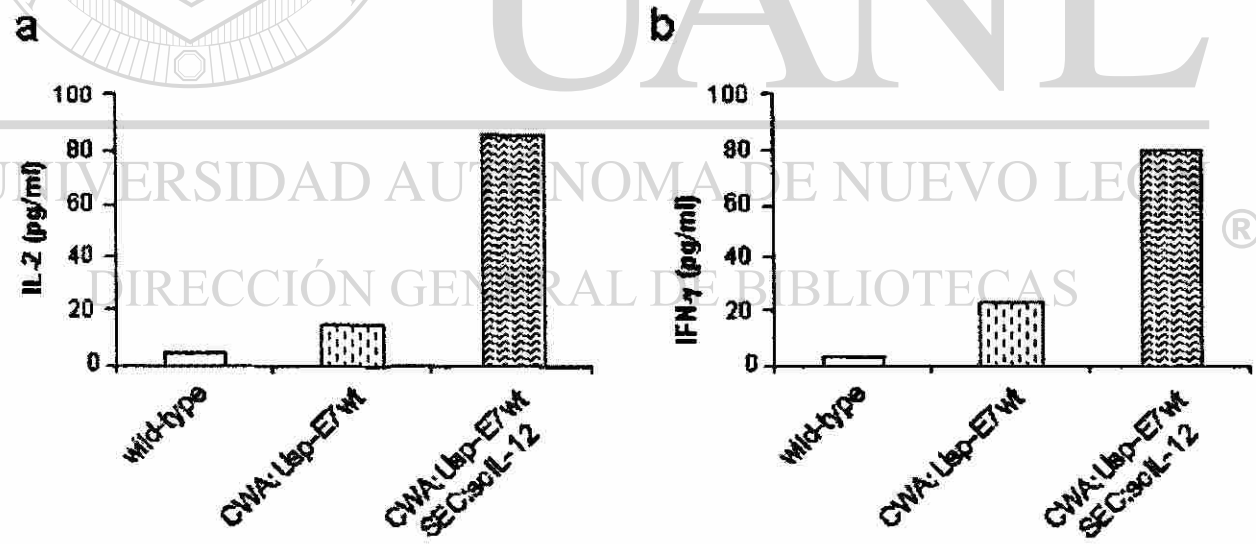

Fig. 3. Producción de citocinas Th1 en ratones co-inmunizados con una cepa de *L. lactis* que expresa el antígeno E7 en su superficie y una cepa de L. lactis que secreta una interleucina-12 activa. Después de inmunizar a grupos de cinco ratones con la cepa NZ(pCWA:Usp-E7) sola o en combinación con la cepa NZ(pSEC:scIL-12) se midieron los niveles de citocinas Th1: IL-2 (a) e IFN-gamma (b). Esta figura es es representativa de tres diferentes experimentos que se realizaron con resultados similares. Los valores fueron registrados como la media y desviación estándar de *n*= tres ratones por grupo.

4.3 Expresión de una proteína E7 mutante del HPV-16 en *Lactococcus lactis*. Dado los resultados positivos que hemos obtenido con la expresión de proteína E7 anclada a superficie de *L. lacis* y en vista de una posible utilización para inmunizar humanos se decidió construir un vector de expresión en superficie de *L. lactis* de una proteína E7 mutante (pIL:Usp-E7mm-CWA). E7 es una oncoproteína, la cual interactua con varios componentes celulares, tales como el oncogen *ras* y *pRB* (Kanda *et al.*, 1988; Vousden *et al.*, 1988), por lo cual puede tener efectos co-laterales en aplicaciones en humanos. E7 posee una región que codifica para dos motivos Cys-X-X-Cys; alteraciones en dichos motivos reducen considerablemente la actividad transformante de E7 (Edmonds & Vousden 1989) y la vuelven una proteína inestable (E7mm) la cual tiene la capacidad de generar una mayor respuesta inmune que la obtenida con la E7wt (Shi *et al.*, 1999). En este contexto nuestro siguiente objetivo fue el de expresar esta proteína E7mm anclada a la superficie de *L. lactis*. Análisis por Western blot de la cepa NZ(pIL:Usp-E7mm-CWA) nos muestran que *L. lactis* es capaz de expresar la proteína E7mm del HPV-16; sin embargo, comparación con extractos proteicos de la cepa de *L. lactis* que expresa la proteína

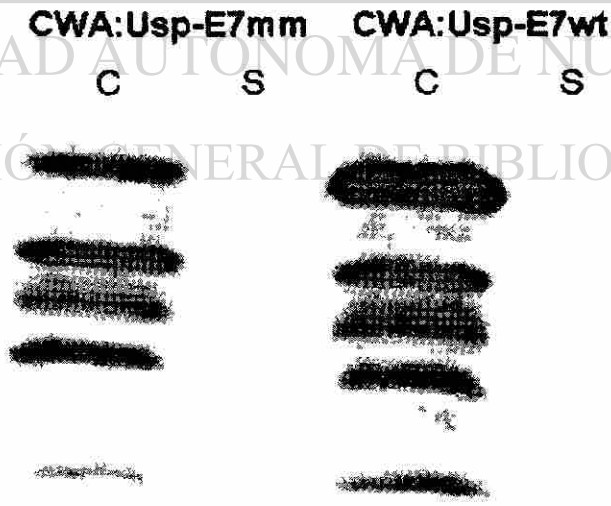

Fig. 4. Análisis de la producción de E7wt y E7mm en *Lactococcus lactis*. Se analizó la producción de E7wt y E7mm por análisis tipo Western Blot de extractos proteicos de las cepas NZ(pCWA:Usp-E7wt) y NZ(pIL:Usp-E7mm-CWA). C, fracción celular; S, muestras de sobrenadante.

E7wt nos muestran que la producción total de la E7mm (precursor y forma madura) es ligeramente menor (aproximadamente dos veces) que su contraparte wt. No obstante, la cantidad de proteína que corresponde a la forma madura (anclada a superficie) es similar, así pues se prosiguió a comparar la respuesta inmune en ratones inmunizados con las dos diferentes cepas de *L. lactis* expresando la E7wt o la E7mm.

4.4 Ensayos profilácticos y terapéuticos contra una línea tumoral que expresa el antígeno E7 del HPV-16. Para evaluar la capacidad de *L. lactis* expresando la proteína E7 en despertar una respuesta inmune en contra del HPV-16 se realizaron inmunizaciones *i.n* a los días 0, 14 y 28 en grupos de 3 ratones con los siguientes tratamientos: *L. lactis* wild-type: wild-type; NZ(pCWA:Usp-E7wt): E7wt, sola o en combinación con NZ(pSEC:scIL-12): E7wt/IL-12; NZ(pSEC:scIL-12): IL-12 o NZ(pIL:Usp-E7mm-CWA): E7mm sola o en combinación con NZ(pSEC:scIL-12) (E7mm/IL-12). Al día 35 se hizo el reto con la línea tumoral TC-1 que expresa el antígeno E7 y se monitoreo la presencia o ausencia de tumor. Los resultados nos muestran un 100% de protección contra el desarrollo del tumor en los ratones inmunizados con la cepa de *L. lactis* que expresa la E7wt (Fig. 5a) mientras que con la cepa que expresa la E7mm no se observo alguna protección (Fig. 5b). De manera interesante los ensayos de co-inmunización con la cepa de *L. lactis* que secreta la IL-12 nos muestran un 50% de protección cuando esta es administrada con la E7mm pero baja a un 25% la protección de la E7wt, mismo porcentaje observado cuando es administrada sola la IL-12 (Fig. 5a y 5b).

En otro tipo de ensayos analizamos el impacto terapéutico de la administración *i.n.* de los lactococos recombinantes. Para dicho propósito se evaluó la sobrevivencia a grupos de 3 ratones con un tumor desarrollado (18 días después del reto de con la línea TC-1), los cuales fueron inmunizados con las mismas combinaciones de cepas recombinantes mencionadas en el párrafo anterior. Las inmunizaciones se comenzaron el día 18 después de la inducción de tumor y repitiéndolas dos veces mas a intervalos de dos semanas. Los resultados obtenidos nos muestran que la administración *i.n* de *L. lactis*

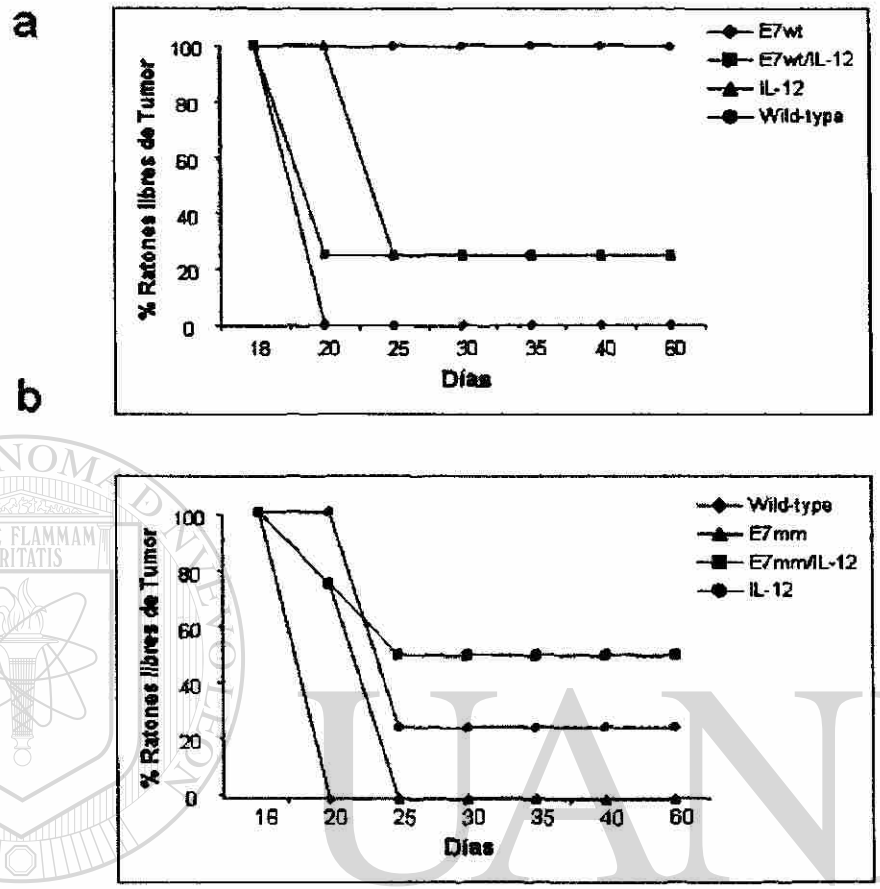

Fig. 5. Efecto profiláctico en ratones contra el desarrollo de tumores. Grupos de 3 ratones fueron inmunizados *i.n.* con los siguientes tratamientos, *L. lactis* wild-type: wild-type; NZ(pCWA:Usp-E7wt): E7wt, sola o en combinación con NZ(pSEC:scIL-12): E7wt/IL-12 o NZ(pSEC:scIL-12): IL-12, a los días 0, 14 y 28. Al día 35 se hizo un reto 50,000 células de la línea tumoral TC-1 y se monitoreo el desarrollo tumoral.

recombinante expresando la E7wt es capaz de regresar el tumor en un 25% al igual que la administración de *L. lactis* secretando la IL-12 sola; una vez mas la co-administración de E7wt e IL-12 no aumenta e incluso disminuye el efecto terapéutico observado con cualquiera de las dos cepas administradas de forma individual (Fig. 6a). Los resultados obtenidos con la cepa de *L. lactis* expresando la E7mm nos muestran al igual que los experimentos profilácticos que la co-administración con la IL-12 mejora significativamente el efecto terapéutico en comparación a la E7mm sola (75% *versus* 0%, Fig. 6b).

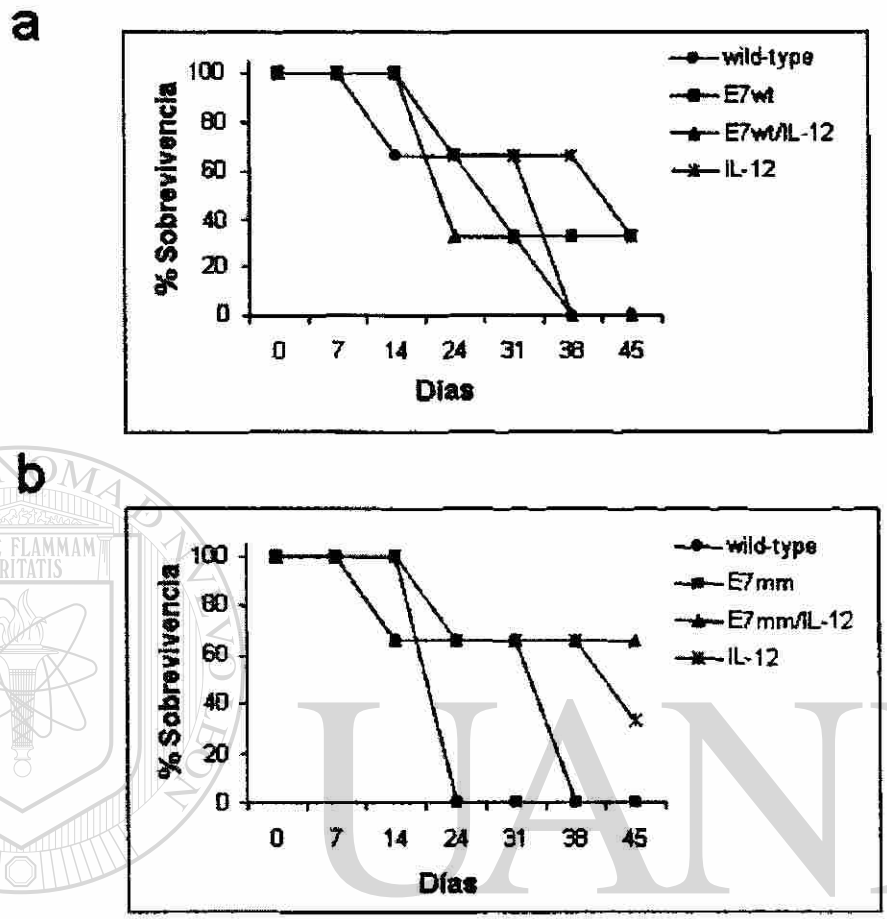

Fig. 6. Efecto terapéutico en ratones contra el desarrollo de tumores. Grupos de 3 ratones fueron inmunizados *i.n.* con los siguientes tratamientos, *L. lactis* wild-type: wild-type; NZ(pCWA:Usp-E7wt): E7wt, sola o en combinación con NZ(pSEC:scIL-12): E7wt/IL-12 o NZ(pSEC:scIL-12): IL-12, a los días 0, 14 y 28. Al día 35 se hizo un reto 50,000 células de la línea tumoral TC-1 y se monitoreo el desarrollo tumoral.

Tomando en cuenta el aumento significativo de la respuesta inmune observado con la co-administración de *L. lactis* expresando la E7wt y secretando la IL-12 (incremento en la producción de citocinas Th1: IL-2 e IFN-gamma, Fig. 3a y3b) y los observados en los experimentos profilácticos y terapéuticos, nos hacen hipotetizar que el impacto en la respuesta inmune de la co-administración de E7wt e IL-12 es tan alto, que en un modelo de tumor *in vivo* se presenta un caso de regulación negativa de la respuesta inmune. Esto se corrobora observando el efecto positivo de la co-administración de la IL-12 con la E7mm que aparentemente sola no posee el mismo efecto inmunogénico que su contraparte

wt. Sin embargo, el número de ratones utilizados en estos ensayos es muy bajo (tres) para poder concluir algo con certeza.

En este contexto procedimos a analizar mas a detalle la expresión de la E7mm en *L. lactis*. Primero se analizo por ensayos de inmunofluoresencia (ver estrategia experimental) si *L. lactis* expresa la proteína E7mm eficazmente en su superficie celular. Como se puede observar en los resultados de la Fig. 7, la proteína E7mm es producida en la superficie de *L. lactis* a niveles comparables a E7wt. Por lo tanto, una explicación probable de la diferencia vista en los ensayos profilácticos y terapéuticos entre la E7wt y la E7mm, es la cantidad de proteína, ya que como se observo en la Fig. 4, la cantidad total de E7wt producida por *L. lactis* es aproximadamente dos veces mayor que la E7mm, y esto puede influir en los niveles de inmunogenicidad de la proteína, tal y como observamos en los experimentos de la Fig. 1a y 2a. La producción de E7mm esta bajo el control de un promotor inducible (PnisA) igual que la E7wy, pero el cassette de expresión esta en un vector de bajo número de copias (pIL252) y la E7wt en uno de mediano número de copias (pGK) de ahí la diferencia en la cantidad de proteína producida entre las dos cepas.

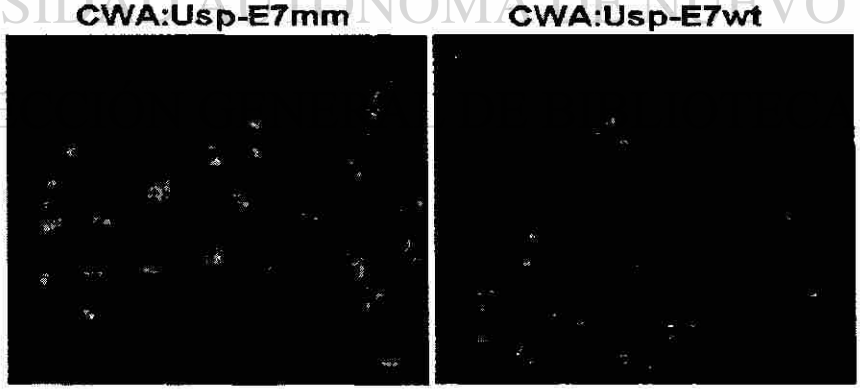

Fig. 7. Análisis de la producción de E7wt y E7mm en superficie en *Lactococcus lactis* por inmunofluorescencia. Se incubaron cultivos inducidos de las cepas recombinantes NZ(pCWA:Usp-E7wt) y NZ(pIL:Usp-E7mm-CWA) con anticuerpos monoclonales específicos de E7 (ver estrategia experimental) y después se marcaron con un conjugado Alexa Fluor 546 dye. Aumento, 1000X.

4.5 Nuevas construcciones para la expresión de una proteína E7 no fusionada. En un esfuerzo por producir iguales cantidades de proteínas E7wt y E7mm en *L. lactis* se construyeron dos nuevos vectores de expresión, basados en el mismo numero de copias, bajo el control del mismo promotor y esta vez se eliminaron 15 aa provenientes de la proteína Usp45 (ver capitulo 3) y que pueden influir a la hora de analizar la inmunogenicidad de las dos formas de proteína E7. Los dos plásmidos construidos para este fin: pCWA:E7wt y pCWA:E7mm fueron introducidos en la cepa NZ9000 de *L. lactis* y antes de los ensayos in vivo se analizo su producción por análisis tipo Western Blot. Como podemos observar en la Fig. 8, esta vez la producción de E7wt o E7mm es casi idéntica, por lo tanto se procedió a evaluar la respuesta inmune en ratones inmunizados. Sin embargo, dado que la proteína E7wt esta vez no esta fusionada a los 15 aa de la Usp45, primero se procedio a analizar el efecto profiláctico de esta nueva construcción comparandola con la proteína fusionada (Usp-E7wt). Además en este experimento se usaron grupos de 8 ratones para que el resultado sea más significativo que los primeros ensayos. Como se puede observar en la Fig. 9 todos los ratones no inmunizados mueren (o tienen que ser sacrificados) al día 35, mientras que los ratones inmunizados con *L. lactis* expresando la proteína E7 fusionada (Usp-

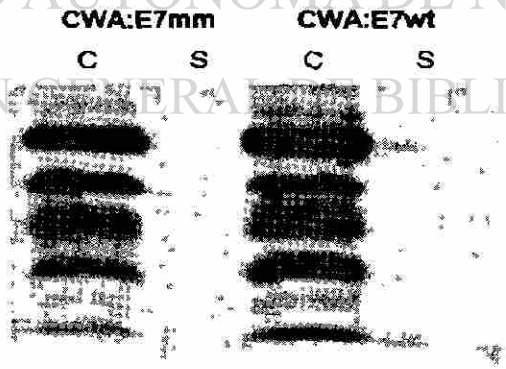

Fig. 8. Análisis de la producción de E7wt y E7mm en *Lactococcus lactis* bajo el control de un promotor inducible y en un vector de mediano numero de copias. Se analizó la producción de E7wt y E7mm por análisis tipo Western Blot de extractos proteicos de las cepas NZ(pCWA:E7wt) y NZ(pCWA:E7mm). C, fracción celular; S, muestras de sobrenadante.

E7wt) presentan un 40% de protección. Este porcentaje es inferior al obtenido en el primer experimento profiláctico (Fig. 5a), no obstante en este experimento se usaron más animales (8) por lo cual es estadisticamente más significativo. Por otro lado los ratones inmunizados con la E7 no fusionada a los 15 aa de la Usp45 (E7wt) presentan un 25% de protección (Fig. 9), inferior a la observada con su contraparte Usp-E7wt. la en el primer experimento profiláctico. Una vez más los resultados difieren mucho entre las dos proteínas, por lo tanto se decidió determinar con exactitud la localización de la proteína en las dos nuevas connstrucciones no fusionadas a los 15 aa de la Usp45 (pCWA:E7wt y pCWA:E7mm). Sorprendentemente como podemos observar en la Fig. 10 y comparandola con los resultados de la Fig. 7, estas dos nuevas cepas de *L. lactis* modificadas para expresar una E7wt ó E7mm, no expresan eficazmente en su superficie la proteína. Por lo tanto el peptido de 15 aa proveniente de la proteína Usp45 debe contribuir de algun modo a darle mayor estabilidad a la proteína cuando esta es anclada a la superficie de *L. lactis*, o simplemente la extensión de 15 aa le permite a la proteína salir lo

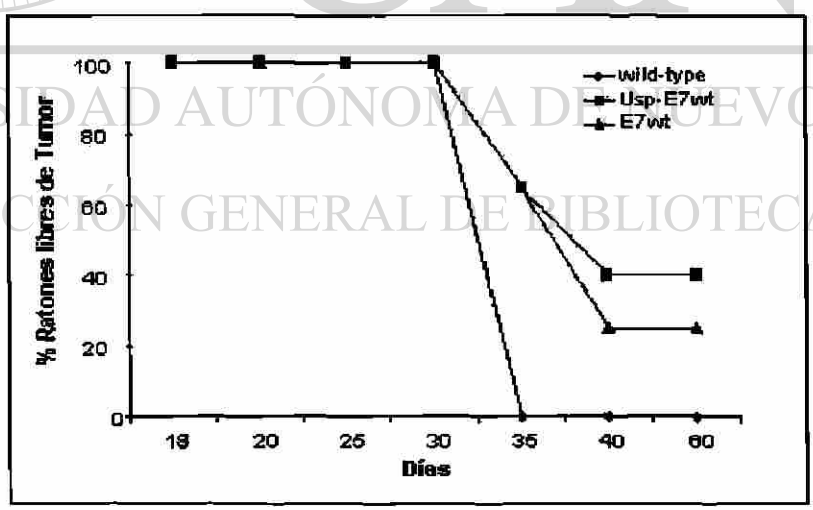

Fig. 9. Efecto profiláctico en ratones contra el desarrollo de tumores. Grupos de 8 ratones fueron inmunizados *i.n.* con los siguientes tratamientos, *L. lactis* wild-type: wild-type; NZ(pCWA:Usp-E7wt): Usp-E7wt ó NZ(pCWA:E7wt): E7wt, a los días 0, 14 y 28. Al día 35 se hizo un reto 50,000 células de la línea tumoral TC-1 y se monitoreo el desarrollo tumoral.

suficiente de la membrana y pared celular y ser expresada de forma eficaz en superficie. Como conclusion de este experimento y confirmando una hipotesis planteada en la Fig. 2 podemos decir que si es necesario la expresión en superficie de la proteína, al menos de forma parcial, para que esta sea altamente inmunogénica. Así pues, la construcción NZ(pCWA:Usp-E7wt) es la más apropiada para inmunizar ratones y protegerlos de forma parcial o incluso tratar tumores que expresan el antígeno E7 del HPV-16. No obstante nuevos experimentos deberan hacerse en estudios posteriores con más ratones y con la forma de E7mm, la cual hasta ahora ha sido reportada como más inmunogénica. Quiza una explicación de porque no vemos esa alta inmunogenicidad en nuestro modelo es que la proteína E7mm expresada en *L. lactis* no es tan frágil como la expresada en sistemas eucariotes (Shi *et al.*, 1999) tal y como lo demuestran los resultados de Western blot e inmunofluorescencia (Fig. 8 y 10).

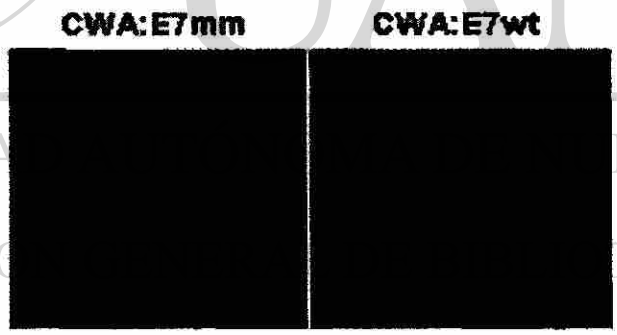

Fig. 10. Análisis de la producción de E7wt y E7mm en superficie en *Lactococcus lactis* por inmunofluorescencia. Se incubaron cultivos inducidos de las cepas recombinantes NZ(pCWA:E7wt) y NZ(pCWA:E7mm) con anticuerpos monoclonales específicos de E7 y después se marcaron con un conjugado Alexa Fluor 546 dye. Aumento, 1000X.

CAPITULO 5

DISCUSION Y CONCLUSIONES

En este trabajo, mostramos el potencial de *L. lactis*, una BL grado-alimenticio y no patógena, para ser usada como un vector vivo en el desarrollo de una vacuna profiláctica y/o terapeútica para prevenir y tratar el CaCu, respectivamente. Además, el desarrollo de una vacuna basada *L. lactis* representa ser un vector de bajo costo de producción. Es bien sabido que la proteína E7 del HPV-16 es constitutivamente producida por queratinocitos infectados en pacientes con CaCu, por lo tanto es considerada un blanco potencial para el desarrollo de vacunas terapéuticas contra este cáncer. Uno de los primeros objetivos de la presente tesis fue el de analizar la respuesta inmune en ratones, generada después de la administración *i.n.* de diferentes cepas recombinantes de *L. lactis* modificadas genéticamente para producir la proteína E7 del HPV-16 en tres localizaciones celulares diferentes: citoplasma, secretada al medio de cultivo y anclada a superficie celular. Los resultados obtenidos muestran que la presentación al sistema inmune de E7 anclada a la superficie de *L. lactis* es mas inmunogénica que una forma intracelular o secretada al medio de cultivo. Estos resultados coinciden con los anteriormente obtenidos por otros investigadores (Norton et al., 1996; Reveneau et al., 2002).

En un trabajo previo se encontró que *L. lactis* expresando la proteína E7 en superficie era capaz de generar anticuerpos anti-E7 cuando era adminiostrada de forma *i.n.* (Cortes-Perez et al., 2003). No obstante, aunque la respuesta inmune mediada por anticuerpos es considerada esencial cuando se trata de infecciones productivas, hasta el momento no se conoce con exactitud el papel que podría desempeñar en NIC y el cáncer invasor. En este trabajo es por eso que decidimos evaluar también la capacidad de este modelo para inducir una respuesta inmune de tipo celular. Dicho ensayo fue el análisis de la produccion de citocinas Th1: IL-2 e IFN-gamma, dos moléculas estrechamente relacionadas con la respuesta inmune celular. El INF-gamma es una citocina producida por linfocitos T y células asesinas

naturales (NK), su principal función es la activación de macrófagos en la respuesta inmune innata y adaptativa, su producción supone la presencia de CTL's activados, además sugiere la inducción de una respuesta inmune de tipo celular. Por lo tanto nuestros resultados indican que *L. lactis* expresando E7 es capaz de inducir la producción de INF-gamma cuando es administrada *i.n.* en ratones (Fig. 2 y Fig. 3). Tomando en cuenta estos resultados, nos llevan a concluir que el modelo diseñado es capaz de inducir una respuesta inmune específica de antígeno de tipo humoral y celular.

Además, los resultados preliminares observados en los experimentos profilácticos y terapéuticos, nos indican que *L. lactis* expresando E7 es capaz de combatir y prevenir el desarrollo de tumores que expresen la oncoproteína E7 del HPV-16 en un modelo murino. No obstante la co-administración de una cepa de *L. lactis* que expresan E7 con una cepa de *L. lactis* que secreta una IL-12 biológicamente activa parece suprimir el efecto positivo de la E7 sola, al contrario de lo esperado y reportado en numeradas ocasiones, un efecto adyuvante de la IL-12 cuando es co-administrada con un antígeno. Se sabe que la IL-2 es producida por células T activadas por antígeno, actúa estimulando la proliferación de CTL's, además de estimular las funciones efectoras de las células NK y células B. Un aspecto importante a considerar en nuestro modelo es que una forma de autorregulación de la respuesta inmune mediada por CTL's es por medio de la producción de IL-2, por lo cual una sobre-estimulación no resultaría satisfactorio para nuestros propósitos, el cual es quiza el caso de la respuesta observada con la co-administración de E7 e IL-12. De igual manera los resultados obtenidos con una forma mutante de la proteína E7 (E7mm, Fig. 5b y 6b), no fueron los esperados por lo tanto nuevas combinaciones deberan probarse (bajando la cantidad de bacterias que produzcan la IL-12 o viceversa) antes de validar nuestro modelo para su uso en ensayos clínicos en humanos.

Otro resultado interesante encontrado en nuestro trabajo es el efecto estabilizador y/o adyuvante del peptido de 15 aa proveniente de la proteína Usp45. Este peptido puede servir entonces en futuros estudios para estabilizar proteína heterólogas en la superficie de *L. lactis* o aumentar la inmunogenicidad.

En resumen este trabajo provee evidencia del gran potencial de *L. lactis* para expresar un antígeno de interés médico y de su eficacia para combatir una enfermedad tan grave como lo es el CaCu. En un futuro este vector puede representar una alternativa para producir toda una gama de vacunas dirigidas contra agentes infecciosos que infecten vía mucosa, principal entrada de la mayor parte de patógenos.

CAPITULO 6

BIBLIOGRAFIA

Alcocer-González JM. (1997). Expresión de citocinas Th1 y Th2 en cáncer cervical avanzado y su relación a la presencia y expresión de los oncogenes E6 y E7 de VPH-16; Tesis Doctoral. FCB, Universidad Autónoma de Nuevo León, México. pp. 33-39.

Alexander M, Salgaller ML, Celis E, Sette A, Barnes WA, Rosenberg SA, Steller MA. Generation of tumor-specific cytolytic T lymphocytes from peripheral blood of cervical cancer patients by in vitro stimulation with a synthetic human papillomavirus type 16 E7 epitope. Am J Obstet Gynecol. 1996 Dec;175(6):1586-93.

Arnau J, Hjerl-Hansen E, & Israelsen H. (1997). Heterologous gene expression of bovine plasmin in *Lactococcus lactis*. *Applied and Environmental Microbiology*. **48:**331-338.

Arulanandam BP, & Metzger DW. (1999). Modulation of mucosal and systemic immunity by intranasal interleukin 12 delivery. *Vaccine*. **17:**252-260.

Baker, C. C., W. C. Phelps, V. Lindgren, M. J. Braun, M. A. Gonda, and P. M. Howley. 1987. Structural and transcriptional analysis of human papillomavirus type 16 sequences in cervical carcinoma cell lines. J. Virol. **61**:962-971.

Barbosa MS, Lowy DR, & Schiller JT. (1989). Papillomavirus polypeptides E6 and E7 are zinc-binding proteins. *Journal of Virology*. **63:**1404-1407.

Barbosa, M. S., D. R. Lowy, and J. T. Schiller. 1989. Papillomavirus polypeptides E6 and E7 are zinc-binding proteins. J. Virol. **63:**1404–1407.

Barnard P & McMillan NA. 1999 The human papillomavirus E7 oncoprotein abrogates signaling mediated by interferon-alpha. Virology 1999; 259: 305-313.

Bedell, M. A., K. H. Jones, and L. A. Laimins. 1987. The E6-E7 region of human papillomavirus type 18 is sufficient for transformation of NIH 3T3 and rat-1 cells. J Virol. 1987 **61**:3635-40.

Berezutskaya, E., and S. Bagchi. 1997. The human papillomavirus E7 oncoprotein functionally interacts with the S4 subunit of the 26 S proteasome. J. Biol. Chem. **272:**30135–30140.

Bermúdez-Humarán LG. (2002). Desarrollo de una Vacuna Probiótica para Combatir el Cáncer Cérvico-Uterino: Construcción de Cepas Recombinantes de *Lactococcus lactis* que expresan la E7 del VPH-16 y la IL-12 murina. Tesis Doctoral. FCB, Universidad Autónoma de Nuevo León, México. pp. 1-124.

Bermúdez-Humarán, L. G. Langella, P., Miyoshi, A., Gruss, A., Taméz-Reyes, R. S., Montes de Oca-Luna, R., & Le Loir, Y. (2002). Production of human papillomavirus type 16 E7 protein in *Lactococcus lactis. Appl Environ Microbiol* **68**, 917-922.

Bermúdez-Humarán, L. G., Cortes-Perez, N. G., Le Loir, Y., Gruss, A., Rodríguez-Padilla, C., Saucedo-Cardenas, O., Langella, P., & Montes de Oca-Luna R. (2003a). Fusion to a carrier protein and a synthetic propeptide enhances E7 HPV-16 production and secretion in *Lactococcus lactis. Biotech Prog* 19, 1101-1104.

Bermúdez-Humarán, L. G., Langella, P., Commissaire, J., Gilbert, S., Le Loir, Y., L'Haridon, R., & Corthier, G. (2003c). Controlled intra- or extracellular production of staphylococcal nuclease and ovine omega interferon in *Lactococcus lactis. FEMS Microbiol Lett* **224**, 307-313.

Bermúdez-Humarán, L. G., Langella, P., Cortes-Perez, N. G., Gruss, A., Taméz-Guerra, R. S., Oliveira, S. C., Cardenas, O., Montes de Oca-Luna, R., & Le Loir, Y. (2003b). Intranasal immunization with recombinant *Lactococcus lactis* secreting murine interleukin-12 enhances antigen-specific Th1 cytokine production. *Infec Immun* **71**, 1887-1896.

Berumen J, Miranda EI, Zafra G, Casas L, Segura E, Ordones RM, Aguirre J, Martinez M, Rosas A, Ibarra V, Pedraza L, Saad A, Marroquin A, Gutierrez M, Martinez A, & Gariglio P. (1997). Molecular epidemiology of high-incidence cancers in Mexico. *Gaceta Médica Mexicana.* **1**:35-41.

Birnboim HC, Doly J. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. 1979 Nov 24;7(6):1513-23.

Bontkes HJ, de Gruijl TD, Walboomers JM, Schiller JT, Dillner J, Helmerhorst TJ, Verheijen RH, Scheper RJ, Meijer CJ.1999. Immune responses against human papillomavirus (HPV) type 16 virus-like particles in a cohort study of women with cervical intraepithelial neoplasia. II. Systemic but not local IgA responses correlate with clearance of HPV-16. J Gen Virol. 1999 Feb;80 (Pt 2):409-17.

Bontkes HJ,Walboomers JMM, Meijer CJLM. 1999. Specific HLA class I down-regulation is an early eventin cervical dysplasia associated with clinical progression. Lancet 1998; 351: 187-188.

Boshart M, Gissmann L, Ikenberg H, Kleinheinz A, Scheurlen W, & zur Hausen H. (1984). A new type of papillomavirus DNA, its presence in genital cancer biopsies and in cell lines derived from cervical cancer. *EMBO Journal.* **3**:1151-1157.

Breitburd F, Kirnbauer R, Hubbert NL, Nonnenmacher B, Trindinh-Deamarquel C, Orth G, Schiller JT, & Lowly DR. (1995). Immunization with virus-like particles from cottontail rabbit papillomavirus (CRVP) can protect against experimental CRVP infection. *Journal of Virology.* **69**:3959-3963.

Broker TR, & Botchan M. (1986). Papillomaviruses: Retrospectives and prospectives. *Cancer Cell.* **4:**17-35.

Cadoz M, Strady A, Meignier B, Taylor J, Tartaglia J, Paoletti, & Plotkin S. (1992). Immunization with canarypox virus rabies glycoprotein. *Lancet.* **339:**1429-1431.

Carter JJ, Yaegashi N, Jenison SA, Galloway DA. 1991. Expression of human papillomavirus proteins in yeast Saccharomyces cerevisiae. Virology. 1991 Jun;182(2):513-21.

Caussy D, Goedert JJ, Palefsky J, Gonzales J, Rabkin CS, DiGioia RA, Sanchez WC, Grossman RJ, Colclough G, Wiktor SZ, Kramer A, Biggar RJ, & Blatner WA. (1990). Interaction of human immunodeficiency and papilloma viruses: association with anal epithelial abnormality in homosexual men. *International Journal of Cancer.* **46:**214-219.

Chen L, Mizuno MT, Singhal MC, Hu S-L, Galloway DA, Hellström I & Hellström KE. (1992). Induction of cytotoxic T lymphocytes specific for a syngeneic tumor expressing the E6 oncoprotein of human papillomavirus type 16. *Journal of Immunology.* **148:**2617-2621.

Chen L, Thomas EK, Hu S-L, Hellström I, & Hellström KE. (1991). Human Papillomavirus type 16 nucleoprotein E7 is a tumor rejection antigen. *Proceedings of the National Academy of Sciences, USA.* **88:**110-114.

Connor JP, Ferrer K, Kane JP, Goldberg JM. 1999 Evaluation of Langerhans' cells in the cervical epithelium of women with cervical intraepithelial neoplasia. Gynecol Oncol. 1999 Oct;75(1):130-5.

Cortes-Perez, N. C., Bermúdez-Humarán, L. G., Le Loir, Y., Rodríguez-Padilla, C., Gruss, A., Saucedo-Cardenas, O., Langella, P., & Montes de Oca-Luna, R. (2003). Mice immunization with recombinant lactococci displaying a surface HPV-16 E7 oncoprotein. *FEMS Microbiol Lett* **229**, 37-42.

Cristensen ND, Raad CA, Cladel NM, Han R, & Kreider JW. (1996). Immunization with virus like particles induces long-term protection of rabbits against challenge with cottontail rabbits papillomavirus. *Journal of Virology.* **70:**960-965.

de Ruyter PG, Kuipers OP, & de Vos WM. (1996). Controlled gene expression systems for *Lactococcus lactis* with the food- grade inducer nisin. *Applied and Environmental Microbiology.* **62:**3662-3667.

de Vos WM. (1999). Gene expression systems for lactic acid bacteria. *Current Opinion in Microbiology.* **2:**289-295.

Dieye, Y., Usai, S., Clier, F., Gruss, A., & Piard, J. C. (2001). Design of a protein targeting system for lactic acid bacteria. *J Bacteriol* **183**, 4157-4166.

Disis ML, Calenoff E, McLaughlin G, Murphy AE, Chen W, Groner B, Jeschke M, Lydon N, McGlynn, Livingston RB, Moe R, & Cheever MA. (1994a). Existent T cell and antibody immunity to HER-2/neu protein in patients with breast cancer. *Cancer Research*. **54:**16-20.

Disis ML, Smith JW, Murphy AE, Chen W, & Cheever M. (1994b). In vitro generation of human cytolytic T-cells specific for peptides derived from the HER-2/neu protooncogene protein. *Cancer Research*. **54:**1071-1076.

Donnelly JJ, Ulmer JB, Shiver JW, & Lui MA. (1998). DNA vaccines. *Annual Review of Immunology*. **15:**617-648.

Drouault, S., Corthier, G., Ehrlich, S. D., & Renault, P. (1999). Survival, physiology, and lysis of *Lactococcus lactis* in the digestive tract. *Appl Environ Microbiol* **65,** 4881-4886.

Durst M, Gissmann L, Ikenberg H, & zur Hausen H. (1983). A papillomavirus DNA from a cervical carcinoma and its prevalence in cancer biopsy samples from different geographic regions. *Proceedings of National Academic of Sciences, USA*. **80:**3812-3815.

Dyson, N., P. M. Howley, K. Mûnger, and Harlow E. 1989. The human papilloma virus-16 oncoprotein is able to bind to the retinoblastoma gene product. Science. **243**:934-937.

Edmonds, C., and K. H. Vousden. 1989. A point mutational analysis of human papillomavirus type 16 E7 protein. J. Virol. **63:**2650–2656.

Felkamp MCW, Vreugdenhil GR, Vierboom MPM, Ras E, van de Burg SH, Schegget J, Melief CJM, & Kast WM. (1995). Cytotoxic T lymphocytes raised against a subdominant epitope offered as a synthetic peptide eradicate human papillomavirus type 16-induced tumors. *European Journal of Immunology*. **25:**2638-2642.

Firzlaff JM, Luscher B, & Eisenman RN. (1991). Negative charge at the casein kinase II phosphorylation site is important for transformation but not for Rb protein binding by the E7 protein of human papillomavirus type 16. *Proceedings of National Academic of Sciences, USA*. **88:**5187-5191.

Furumoto H, Irahara M. 2002. Human papilloma virus (HPV) and cervical cancer. J Med Invest. 49(3-4):124-33.

Galloway D.A. 2003 Papillomavirus vaccines in clinical trials. Lancet Infect Dis. 2003 Aug;3(8):469-75.

Galloway DA. (1994). Human papillomavirus vaccines: a warty problem. *Infectious Agents and Disease*. **3:**187-193.

Garland SM. 2003. Can we really beat cervical cancer? Med J Aust. 2003. 178(12):647-9.

Gately MK, Wolitzky AG, Quinn PM, & Chizzonite R. (1992). Regulation of human cytolytic lymphocyte responses by interleukin-12. *Cell Immunology*. **143:**127-42.

Geoffroy MC, Guyard C, Quatannens B, Pavan S, Lange M, & Mercenier A. (2000). Use of green fluorescent protein to tag lactic acid bacterium strains under development as live vaccine vectors. *Applied and Environmental Microbiology*. **66:**383-391.

Ghim SJ, Young R, & Jenson AB. (1996). Antigenicity of bovine papillomavirus type 1 (BPV-1) virus-like particles compared with that of intact BPV-1 virions. *Journal of General Virology*. **77:**183-188.

Gibson, T. J. 1984. PhD thesis. University of Cambridge, Cambridge, England.

Gruzza M, Fons M, Ouriet MF, Duval-Iflah Y, & Ducluzeau R. (1994). Study of gene transfer in vitro and in the digestive tract of gnotobiotic mice from *Lactococcus lactis* strains to various strains belonging to human intestinal flora. *Microbiology Releases*. **2:**183-189.

Gunneriusson E, Samuelson P, Uhlen M, Nygren P, & Stahl S. (1996). Surface display of a functional single-chain Fv antibody to staphylococci. *Journal of Bacteriology*. **178:**1341-1346.

Hagensee ME. 1999.Human papillomavirus vaccune. Infect Urol 12(1):11-15, 18-19

Hansson M, Stahl S, Nguyen TN, Bachi T, Robert A, Binz H, Sjolander A, & Uhlen M. (1992). Expression of recombinants proteins on the surface of the coagulase-negative bacterium *Staphylococcus xylosus. Journal of Bacteriology*. **174:**4239-4245.

Hansson M, Stahl S, Nguyen TN, Bachi T, Robert A, Binz H, Sjolander A, & Uhlen M. (1992). Expression of recombinants proteins on the surface of the coagulase-negative bacterium *Staphylococcus xylosus. Journal of Bacteriology*. **174:**4239-4245.

Heino P, Dillner J & Schwartz S. (1995). Human papillomavirus type 16 capside protein produced from recombinant semliki forest virus assemble into virus-like particles. *Virology*. **214:**349-359.

Hernandez-Pando R, Orozcoe H, Sampieri A, Pavon L, Velasquillo C, Larriva-Sahd J, Alcocer JM, Madrid MV. (1996). Correlation between the kinetics of Th1, Th2 cells and pathology in a murine model of experimental pulmonary tuberculosis. *Immunology*. **89:**26-33.

Hofmann KJ, Neeper MP, Markus HZ, Braun DR, Muller M & Jansen KU. (1996). Sequence conservation within the major capside protein of human papillomavirus (VPH) type 18 and formation of HPV-18 virus-like particles in *Saccharomyces cerevisae. Journal of General Virology*. **77:**465-468.

Holly EA. 1996. Cervical intraepithelial neoplasia, cervical cancer, and hpv. AR. Public Healh 17(1):69-84

Howley PM, Munger K, Werness BA, Phelps WC, Schlegel R. 1989. Molecular mechanisms of transformation by the human papillomaviruses. Princess Takamatsu Symp. 1989;20:199-206.

Imai Y, Matsushima Y, Sugimura T, & Terada M. (1991). Purification and characterization of human papillomavirus type 16 E7 protein with preferential binding capacity to the underphosphorylated form of retinoblastoma gene product. *Journal of Virology.* **65:**4966-4972.

Iwaki M, Okahashi N, Takahashi I, Kanamoto T, Sugita-Konishi Y, Aibara K, & Koga T. (1990). Oral immunization with recombinant *Streptococcus lactis* carrying the *Streptococcus mutants* surface protein antigen gene. *Infection and Immunity.* **28:**2929-2934.

Jabbar, I. A., G. J. Fernando, N. Saunders, A. Aldovini, R. Young, K. Malcolm, and I. H. Frazer. 2000. Immune responses induced by BCG recombinant for human papillomavirus L1 and E7 proteins. Vaccine. 18: 2444-2453.

Jeon S, Allen-Hoffmann L, & Lambert PF. (1995). Integration of human papillomavirus type 16 into the human genome correlates with a selective growth advantage of cells. *Journal of Virology.* **69:**2989-2997.

Jochmus I, Wolfram O, Altman A, Buck G, Hofman B, Schneider A, Gissmann L, & Rammensee H-G. (1997). Specificity of human cytotoxic induced by a human papillomavirus type 16 E7-derived peptides. *Journal of General Virology.* **78:**1689-1695.

Jones DH, Corris S, McDonald S, Clegg JCS & Farrar GH. (1997b). Poly (DL-lactideco-glycolide) encapsulated plasmid DNA elicits systemic and mucosal antibody response to encoded protein after oral administration. *Vaccine.* **15:**814-817.

Jones DL, Thompson DA, & Munger K. (1997a). Destabilization of the RB tumor suppressor protein and stabilization of p53 contribute to VPH type 16 E7-induced apoptosis. *Virology.* **239:**97-107.

Jung S, & Schluesener HJ. (1991). Human T lymphocytes recognize a peptide of single point-mutated, oncogenic *ras* proteins. *Journal of Experimental Medicine.* **73:**273-276.

Kanda T, Furuno A, Yoshiike K. 1988. Human papillomavirus type 16 open reading frame E7 encodes a transforming gene for rat 3Y1 cells. J Virol. 1988 Feb;62(2):610-3.

Kanda T, Watanabe S, Yoshiike K. 1988. Immortalization of primary rat cells by human papillomavirus type 16 subgenomic DNA fragments controlled by the SV40 promoter. Virology. 1988 Jul;165(1):321-5

Keating PJ, Cromme FV, Duggan-Keen M. 1995. Frequency of down-regulation of individual HLA-A and-Balleles in cervical carcinomas in relation to TAP-1 expression. British Journal of Cancer 1995; 72: 405-411.

Kiniwa M, Gately M, Gubler U, Chizzonite R, Fargeas C & Delespesse G. (1992). Recombinant inteleukin-12 suppresses the synthesis of immunoglobulin E by interleukyn-4 stimulated human lymphocytes. *Journal of Clinical Investigation.* **90:**262-266.

Kirnbauer R, Booy F, Cheng N. 1992. Papillomavirus L1 major capsid protein self-assembles into virus-like particles that are highly immunogenic. Proceedings of the National Academy of Sciences of the USA 1992; 89:12180-12184.

Klavinskis LS, Gao L; Barnfield C, Lehener T, & parker S. (1997). Mucosal immunization with DNA-liposome complexes. *Vaccine*. **15:**818-820.

Klijn N, Weerkamp AH, & Vos WM. (1995). Genetic marking of *Lactococcus lactis* shows its survival in the human gastrointestinal tract. *Applied and Environmental Microbiology*. **61:**2771-2774.

Kok, J., van der Vossen, & J. M., Venema, G. (1984). Construction of plasmid cloning vectors for lactic streptococci which also replicate in *Bacillus subtilis* and *Escherichia coli, Appl Environ Microbiol* **48,** 726-731.

Koopman LA, Corver WE, van der Slik AR. 2000 Multiple genetic alterations cause frequent and heterogeneous human histocompatibility leukocyte antigen class I loss in cervical cancer. Journal of Experimental Medicine 2000; 191: 961-976.

Koutsky, L. A., Ault, K. A, Wheeler. C. M., Brown, D. R., Barr, E., Alvarez, F. B., Chiacchierini, L. M., Jansen, K. U., et al. 2002. A controlled trial of a human papillomavirus type 16 vaccine. *N Engl J Med*. **347,** 1645-1651.

Kuipers, O. P., P. G. de Ruyter, M. Kleerebezen, and W. M. de Vos. 1998. Quorum sensing-controlled gene expression in lactic acid bacteria. J. Biotechnol. **64**:15-21.

Labrecque S, Naor N, Thomson D, & Matlshewki G. (1993). Analysis of the anti-p53 antibody response in cancer patients. *Cancer Research*. **53:**34683471.

Langella, P., Y. Le Loir, S. D. Ehrlich, and A. Gruss. 1993. Efficient plasmid mobilization by plP501 in *Lactococcus lactis* subsp. *lactis*. J. Bacteriol. **175:**5806-5813.

Lau LL, Jamieson BD, Somasundaram T, & Ahmed R. (1994). Cytotoxic T cells memory without antigen. *Nature*. **369:**648-652.

Le Loir Y, Gruss A, Ehrlich SD, & Langella P. (1998). A nine-residue synthetic propeptide enhances secretion efficiency of heterologous proteins in *Lactococcus lactis*. *Journal of Bacteriology*. **180:**1895-1903.

Lee Cann P, Touze A, Enogat N, Leboutleoux D, Mougin C, Legrand MC, Calvet C, Afoutou JM, & Couraagef P. (1995). Detection of antibodies against human papillomavirus (VPH) type 16 virions by enzyme-linked immunosorbent assay using recombinant VPH 16 L1 capside produced by recombinant baculovirus. *Journal of Clinical Microbiology*. **33:**1380-1382.

Li S, Labrecque S, Gauzzi MC. 1999. The human papilloma virus (HPV)-18 E6 oncoprotein physically associates with Tyk2 and impairs Jak-STAT activation by interferon-alpha. Oncogene 1999; 18: 5727-5737.

Lin KY, Guarnieri FG, Staveley-O'Carroll KF, Levitsky HI, August JT, Pardoll DM, Wu TC. 1996. Treatment of established tumors with a novel vaccine that enhances major histocompatibility class II presentation of tumor antigen. Cancer Res. 1996 Jan 1;56(1):21-6.

Londoño LP, Chatfield S, Tindle RW, Herd K, Gao XM, Frazer I, & Dougan G. (1996). Immunization of mice using *Salmonella typhymurium* expressing human papillomavirus type 16 E7 epitopes inserted into hepatitis B virus core antigen. *Vaccine.* **14:**545-552.

Mansur CP, & Androphy EJ. (1993). Cellular transformation by papillomavirus oncoproteins. *Biochemical and Biophysical Acta.* **1155:**323-345.

McCance DJ. (1986). Human papillomaviruses and cancer. *Biochemical et Biophysical Acta.* **823:**195-205.

McIntyre MC, Ruesch MN, & Laimins LA. (1996). Human papillomavirus E7 oncoproteins bind a single form of cyclin E in a complex with cdk2 and p107. *Virology.* **215:**73-82.

Medaglini D, Pozzi G, King TP, & Fischetti VA. (1995). Mucosal and systemic immune responses to a recombinant protein expressed on the surface of the oral commensal bacterium *Streptococcus gordonii* after oral colonization. *Proceedings of the National Academy of Sciences, USA.* **92:**6868-6872.

Metzger DW, Buchanan JM, Collins JT, Lester TL, Murray KS, Van V, Cleave H, Vogel LA, & Dunnick WA. (1996). Enhancement of humoral immunity by interleukin-12. *Annals of New York Academic Sciences.* **795:**100-115.

Metzger DW, McNutt RM, Collins JT, Buchanan JM, Van V, Cleave H, & Dunnick WA. (1997). Interleukin-12 acts as an adjuvant for humoral immunity through interferon-gamma -dependent and -independent mechanisms. *European Journal of Immunology.* **27:**1958-1965.

Mohar A, Frías-Mendivil M, Suchil-Bernal L, Mora-Macías T, & de la Garza J. (1997). Descriptive epidemiology of cancer at the Instituto Nacional de Cancerología of México. *Salud Pública,México.***39:**253-258.

Mota F, Rayment N, Chong S. 1999. The antigen-presenting environment in normal and human papillomavirus (HPV)-related premalignant cervical epithelium. Clinical Experimental Immunology 1999;116: 33-40.

Mullbacher A. (1994). The long-term maintenance of cytotoxic T-cell memory does not require persistence of antigen. *Journal of Experimental Medicine.* **179:**317-321.

Münger K, Phelps WC, Bubb V, Howley PM, & Schlegel R. (1989). The E6 and E7 genes of the human papillomavirus type 16 together are necessary and sufficient for transformation of primary human keratinocytes. *Journal of Virology.* **63:**4417-4423.

Munoz N, Bosch FX, de Sanjose S, Herrero R, Castellsague X, Shah KV, Snijders PJ, Meijer CJ. 2003.Epidemiologic c lassification of human papillomavirus types associated with cervical cancer N Engl J Med. 6;348(6):518-27.

Nguyen TN, Hansson M, Stahl S, Bachi T, Robert A, Domzig W, Binz H, & Uhlen M. (1993). Cell-surface display of heterologous epitopes on *Staphylococcus xylosus* as a potential delivery system for oral vaccination. *Gene.* **128:**89-94.

Nijman HW, van der Burg SH, Vierboom MPM, Houbiers J GA, Kast WM, & Melief CJM. (1994). p53 a potential target for tumor-directed T cell. *Immunology Letters.* **40:**171-178.

Nonnenmacher B, Hubbert NL, Kirnbauer R. 1995. Serologic response to human papillomavirus type 16 (HPV-16) virus-like particles in HPV-16 DNA-positive invasive cervical cancer and cervical intraepithelialneoplasia grade III patients and controls from Colombia and Spain. Journal of Infectious Diseases 1995; 172:19-24.

Norton PM, Wells JM, Brown HW, Macpherson AM, & Le Page RW. (1997). Protection against tetanus toxin in mice nasally immunized with recombinant *Lactococcus lactis* expressing tetanus toxin fragment C. *Vaccine.* **15:**616-619.

Norton PM, Wells JM, Brown HW, Macpherson AM, & Le Page RW. (1997). Protection against tetanus toxin in mice nasally immunized with recombinant *Lactococcus lactis* expressing tetanus toxin fragment C. *Vaccine.* **15:**616-619.

Pahel G, Aulabaugh A, Short SA, Barnes JA, Painter GR, Ray P, & Phelps WC. (1993). Structural and functional characterization of the HPV16 E7 protein expressed in bacteria. *Journal of Biological Chemistry.* **268:**26018-26025.

Peace DJ, Smith JW, Chen W, You SG, Cosand WL, Blake J, & Cheever M. (1994). Lysis of *ras* oncogene-transformed cells by specific cytotoxic T lymphocytes elicited by primary immunization with mutated *ras* peptide. *Journal of Experimental Medicine.* **179:**473-479.

Perez-Martinez G, Kok J, Venema G, van Dijl JM, Smith H, & Bron S. (1992). Protein export elements from *Lactococcus lactis. Molecular and General Genetics.* **234:**401-411.

Piard JC, Jimenez-Diaz R, Ehrlich SD, Fischetti VA, & Gruss A. (1997). The M6 protein of *Streptococcus pyogenes* and its potential as a tool to anchor biologically active molecules at the surface of lactic acid bacteria. *Advances in Experimental Medicine and Biology.* p. 545-550.

Pilacinski WPGK, Reed DE, Lum MA. 1986. Immunization against bovine papilloma virus infection. In Papillomaviruses. Ciba Foundation Symposium II, pp 2136-2156. Chichester: John Wiley & Sons, 1986.

Pozzi G, Contorni M, Oggioni MR, manganelli R, Tommasino M, Cavalieri F, & Fischetti VA. (1992). Delivery and expression of a heterologous antigen on the surface of streptococci. *Infection and Immunity.* **60:**1902-1907.

Pozzi G, Oggioni MR, Manganelli R, Medaglini D, Fischetti VA, Fenoglio D, Valle MT, Kunkl A, & Manca F. (1994). Human T-helper cell recognition of an immunodominant epitope of HIV-1 gp120 expressed on the surface of *Streptococcus gordonii. Vaccine.* **12:**1071-1077.

Rawls JA, Pusztai R, & Green M. (1990). Chemical synthesis of human papillomavirus type 16 E7 oncoprotein: autonomous protein domains for induction of cellular DNA synthesis and for trans activation. *Journal of Virology.* **64:**6121-6129.

Robinson HL. (1997). Nucleic acid vaccines: an overview. *Vaccine.* **15:**785-787.

Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Samuelson P, Hansson M, Ahlborg N, Andreoni C, Gotz F, Bachi T, Nguyen TN, Binz H, Uhlen M, & Stahl S. (1995). Cell surface display of recombinant proteins on *Staphylococcus carnosus. Journal of Bacteriology.* **177:**1470-1476.

Samuelson P, Hansson M, Ahlborg N, Andreoni C, Gotz F, Bachi T, Nguyen TN, Binz H, Uhlen M, & Stahl S. (1995). Cell surface display of recombinant proteins on *Staphylococcus carnosus. Journal of Bacteriology.* **177:**1470-1476.

Sato H, Watanabe S, Furuno A, & Yoshiike K. (1989). Human papillomavirus type 16 E7 protein expressed in *Escherichia coli* and monkey COS-1 cells: immunofluorescence detection of the nuclear E7 protein. *Virology.* **170:**311-315.

Savijoki K, Kahala M, & Palva A. (1997). High-level heterologous protein production in *Lactococcus* and *Lactobacillus* using a new secretion system based on the *Lactobacillus brevis* S-layer signals. *Gene.* **186:**255-262.

Scheffner M, Romanczuk H, Münger K, Huibregste JM, Mietz JA, & Howley PM. (1994). Functions of human papillomavirus proteins. *Current Topics in Microbiology and Immunology.* **186:**83-99.

Scott P. (1993). IL-12: initiation cytokine for cell-mediated immunity. *Science.* **260:**496-507.

Seedorf K, Oltersdorf T, Krämmer G, & Röwenkan W. (1987). Identification of early proteins of the human papillomavirus type 16 (HPV-16) and type 18 (HPV-18) in cervical carcinoma cells. *EMBO Journal.* **6**:139-144.

Shi W, Bu P, Liu J, Polack A, Fisher S, Qiao L. 1999. Human papillomavirus type 16 E7 DNA vaccine: mutation in the open reading frame of E7 enhances specific cytotoxic T-lymphocyte induction and antitumor activity. J Virol. 1999 Sep;73(9):7877-81.

Shodell M. (1986). En: Banbury Report 21: Viral etiology of cervical cancer. Cold Spring Harbor Laboratory. *Cold Spring Harbor. New York. IX* . pp. 45-47.

Sillman F, Stanek A, Sedlis A, Rosenthal J, Lanks KW, Buchhagen D, Nicastri A, & Boyce J. (1984). The relationship between human papillomavirus and lower genital intraepithelial neoplasia in immunosuppressed women. *American Journal of Obstetrician Gynecology*. **150:**300-308.

Simonen M, & Palva I. (1993). Protein secretion in *Bacillus* species. *Microbiology Reviews*. **57:**109-137.

Simonen M, & Palva I. (1993). Protein secretion in *Bacillus* species. *Microbiology Reviews*. **57:**109-137.

Smotkin D, & Wettstein FO. (1986). Transcription of human papillomavirus type 16 early genes in a cervical cancer and a cancer-derived cell line and identification of the E7 protein. *Proceedings of National of Academic Sciences, USA.* **83**:4680-4684.

Staerz U, Zepp F, Schmid R, Hill M, & Rothbard J. (1989). Recruitment of alloreactive cytotoxic T lymphocytes by an antigenic peptide. *European Journal of Immunology*. **19:**2191-2196.

Steidler L, Robinson K, Chamberlain L, Schofield KM, Remaut E, Le Page RWF, & Wells JM. (1998). Mucosal delivery of murine Interleukin-2 (IL-2) and IL-6 by recombinant strains of *Lactococcus lactis* coexpressing antigen and cytokine. *Infection and Immunology*. **66:**3183-3189.

Steidler L, Wells JM, Raeymaekers A, Vandekerckhove J, Fiers W, & Remaut E. (1995). Secretion of biologically active murine interleukin-2 by *Lactococcus lactis* subsp. *lactis. Applied and Environmental Microbiology*. **61:**1627-1629.

Steller, M. Cervical cancer vaccines: progress and prospects. J. Soc. Gynecol. Investig. 2002 Sep-Oct; 9(5): 254-64.

Ster P., Faulkner R., Veranes E., Davidson E. 2001.The role of human papillomavirus vaccine in cervical neoplasia. Best Practice & Research Clinical Obstetrics and Gynaecology. 15(5):783-799

Storey A, Pim D, Murray A, Osborn K, Banks L, & Crawford L. (1988). Comparison of the in vitro transforming activities of human papillomavirus types. *EMBO Journal.* **7:**1815-1820.

Suzich JA, Ghim SJ, Palmer-Hill FJ, Withe WI, Tamura JK, Bell JA, Newsome JA, Jenson AB, & Schiegel R. (1995). Systemic immunization with papillomavirus L1 protein completely prevents the development of viral mucosal papillomas. *Proceedings of National of Academic Sciences, USA.* **92:**11553-11557.

Tacket CO, Hone DM, Curtiss R III, Kelly SM, Losonsky G, Guers L, Harris AM, Edelman R, & Levine MM. (1992). Comparison of the safety and immunogenicity of □-*aroC* □-*aroD* and □-*cya* □-*crp Salmonella typhi* strains in adult volunteers. *Infection and Immunity*. **60:**536-541.

Tanaka A, Noda T, Yajima H, Hatanaka M, & Ito Y. (1989). Identification of a transforming gene of human papillomavirus type 16. *Journal of Virology.* **63:**1465-1469.

Tarpey I, Stacey S, Hickling J, Birley DL, Renton A, McIndoe A, & Davies DH. (1994). Human cytotoxic T lymphocytes stimulated by endogenously processed human papillomavirus type 11 E7 recognize a peptide containing a HLA-A2 (A-0201) motif. *Immunology*. **81:**222-227.

Tartaglia J, Perkus ME, Taylor J, Norton EK, Adonnet J, Cox WI, Davis SW, Van der Hoeven J, Meignier B, & Riviere M. (1992). NYVAC: a highly attenuated of Vaccinia virus. *Virology*. **188:**217-232.

Thierry F, & Yaniv M. (1987). The BPV-E2 trans-acting protein can be either an activator or a repressor of the HPV-18 regulatory region. *EMBO Journal*. **6:**3391-3397.

Thoms WW, Unger ER, Johnson PR, Spann CO, Hunter SH, Smith R, Horowitz IR, Icenogle JP, Vernon SD, & Reeves WC. (1995). Cervical cancer survival in a high risk urban population. *Cancer*. **6:**2518-2523.

Tindle RW. (1996). Human papillomavirus vaccines for cervical cancer. *Current Opinion in Immunology*. **8:**643-650.

Tindle RW. (1996). Human papillomavirus vaccines for cervical cancer. *Current Opinion in Immunology*. **8:**643-650.

Tommasino M, Contorni M, Cavalieri F. 1990. HPV16 E7 phosphorylation in fission yeast: characterization and biological effects. Gene. 1992 Feb 1;111(1):93-8.

Ulmer JB, Donnelly JJ, Parker SE, Rhodes GH, Felgner PL, Dwarki VJ, Gromkowski SH, Ramdall Deck R, Dewitt CM, Friedman A, Hawe LA, Leander KR, Martinez D, Perry HC, Shilver JW, Montgomery DL, & Liu MA. (1993). Heterologous protection against influenza by injection of DNA encoding viral protection. *Science*. **259:**1745-1749.

van Asseldonk M, de Vos WM, & Simons G. (1993). Functional analysis of the *Lactococcus lactis* usp45 secretion signal in the secretion of a homologous proteinase and a heterologous alpha-amylase. *Molecular and General Genetics*. **240:**428-434.

Vousden KH, Doniger J, DiPaolo JA, Lowy DR. 1988. The E7 open reading frame of human papillomavirus type 16 encodes a transforming gene. Oncogene Res. 1988 Sep;3(2):167-75.

Wang B, Dang K, Agadjanyan MG, Srikantan V, Li F, Ugen KE, Boyer J, Merva M, Williams WV, & Weiner DB. (1997). Mucosal immunization with a DNA vaccine induces immune responses against HIV-1 at a mucosal site. *Vaccine*. **15:**821-825.

Wells JM, & Schofield KM. (1996). Cloning and expression vectors for lactococci. *NATO Asl Ser*. **98:**37-63.

Wells JM, Wilson PW, Norton PM, Gasson MJ, & Le Page RWF. (1993). *Lactococcus lactis*: high-level expression of tetanus toxin fragment C and protection against lethal challenge. *Molecular Microbiology*. **8:**1155-1162.

Yanuck M, Carbone DP, Pendlenton CD, Tsukui T, Winter SF, Minna JD, & Berzofsky JA. (1994). A mutant p53 tumor suppressor protein is a target for peptide induced CD8+ cytotoxic T-cells. *Cancer Research*. **53:**3257-3261.

Zerfass K, Schulze A, Spitkovsky D, Friedman V, Henglein B, & Jansen-Durr P. (1995). Sequential activation of cyclin E and cyclin A gene expression by human papillomavirus type 16 E7 through sequences necessary for transformation. *Journal of Virology*. **69:**6389-6399.

Zerfass-Thome K, Zwerschke W, Mannhardt B, Tindle R, Botz JW, & Jansen-Durr P. (1996). Inactivation of the cdk inhibitor p27KIP1 by the human papillomavirus type 16 E7 oncoprotein. *Oncogene*. **13:**2323-2330.

zur Hausen H. (1996). Papillomavirus infections- a major cause of human cancers. *Biochemical and Biophysical Acta*. **1288:**F55-F78.

zur Hausen, H. (1985). Genital papillomavirus infections. *Progress in Medical Virology*. **32:**15-21.

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Mice immunization with live lactococci displaying a surface anchored HPV-16 E7 oncoprotein

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Abstract

E7 oncoprotein of human papillomavirus-16 (HPV-16) is constitutively produced in cervical cancer (CxCa) and is a good candidate for the design of therapeutic vaccines. In this work, the nisin-controlled expression system was used to display the E7 protein at the cell surface of the food-grade Gram-positive bacterium *Lactococcus lactis*. An efficient cell wall anchoring of E7 was obtained. Intranasal administration of these recombinant lactococci in mice induced an HPV-16 E7-specific immune response. This is the first report of E7 cell wall anchoring in *L. lactis* and represents one more step towards the use of live food-grade bacteria to fight against CxCa.

Keywords: *Lactococcus lactis*; Cancer; Human papillomavirus; Vaccine; Immunogenic

1. Introduction

Cervical cancer (CxCa) is the second cause of death in women around the world and the human papillomavirus type 16 (HPV-16) has been identified as the main etiologic agent [1]. It has been demonstrated that E7 oncoprotein is produced constitutively in CxCa [2]; therefore it represents a potential target for the development of a vaccine against this neoplasia. However, the extreme lability of E7 renders difficult its production in potential bacterial hosts [3–5]. We have previously described that the native form of E7 protein is very labile and degraded in the cytoplasm when produced in the Gram-positive food-grade lactic acid bacterium (LAB) *Lactococcus lactis* [4,5]. Interestingly, E7 production is rescued when fused to either a lactococcal signal peptide (SP) or to a stable protein, staphylococcal nuclease (Nuc) [4,5].

L. lactis is widely used for the production of fermented milk products and more recently as a live vector for heterologous protein delivery ([4–12]; see [13] for a review). The use of *L. lactis* as a live oral vaccine to induce mucosal immune response is a very promising field of health applications [13]. These strategies are well illustrated by two recent studies which investigated whether genetically modified orally administered lactococci or lactobacilli (other LAB species) could be used as HIV vaccines [14,15]. In this study, we used the nisin-inducible system [16,17] to produce and target the HPV-16 E7 protein to the cell surface of *L. lactis*, and evaluate its ability to induce an immune response.

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Table 1
Bacterial strains and plasmids used

Strain	Genotype		Reference
E. coli TG1	*supE, hsd, Δ5, thi, Δ(lac-proAB), F'(traD36 proAB-lacZΔM15)*		[18]
L. lactis MG1363	Wild-type, plasmid-free		[19]
L. lactis NZ9000	MG1363 (*nisRK* genes into chromosome), plasmid-free		[17]
L. lactis NZE7-CWA	*L. lactis* strain expressing E7 protein on its surface		This work
Plasmids	**Replicon**	**Plasmid characteristics; cloned cassettes characteristics**	**Reference**
pSEC-E7	pWV01	Cm^r; gene, expressed under P_{nisA}, encodes SP_{Usp}-E7 precursor	[4]
pBS:E7	ColE1	Amp^r; PCR fragment encoding mature E7	This work
pVE5547	pIL252	Ery^r; gene, expressed under P_{nisA}, encodes the SP_{Usp}-Nuc-CWA_{M6} precursor	[20]
pIL:E7-CWA	pIL252	Ery^r; gene, expressed under P_{nisA}, encodes the SP_{Usp}-E7-CWA_{M6} precursor	This work

2. Materials and methods

2.1. Bacterial strains, plasmids and methods used

Bacterial strains and plasmids used in this work are listed in Table 1. *L. lactis* was grown in GM17 (M17 Difco, 1% glucose) at 30°C without shaking. *Escherichia coli* was grown in Luria–Bertani medium at 37°C, with shaking. Plasmid constructions were first established in *E. coli* and then transferred into *L. lactis* [21]. Clones were selected by the addition of antibiotics as follows: for *L. lactis*, chloramphenicol (Cm, 10 μg ml^{-1}) and erythromycin (Ery, 5 μg ml^{-1}), and for *E. coli*, chloramphenicol (10 μg ml^{-1}) and ampicillin (Amp, 100 μg ml^{-1}). DNA manipulations were essentially performed as described previously [22]. Polymerase chain reaction (PCR) (Perkin Elmer Cetus apparatus, Norwalk, CT, USA) was performed using Vent DNA polymerase (Promega) and PCR sequences were confirmed using the Dye-terminator sequencing kit (ABI Prism® BigDye™ Terminators, Applied Biosystems).

2.2. Conditions of nisin induction

Strains were grown until $OD_{600} = 0.4–0.6$ and induction was carried out with 10 ng ml^{-1} of nisin (Sigma) for 1 h. Protein extractions and immunoblotting assays were performed as previously described [4].

2.3. Construction of a vector for E7 cell surface displaying in L. lactis

To target E7 to the surface of *L. lactis*, E7 was fused to a cell wall anchor fragment of *Streptococcus pyogenes* M6 protein [23]. Briefly, the *E7* gene was PCR-amplified from the vector pSEC-E7 [4]. Primer sequences used are: 5'E7-CWA (5'-AGTCGACCCATTGCATGGAGATACACCTACATTG-3') for the coding strand and 3'E7-CWA (5'-CGATATCTCTGGTTTCTGAGAACAGATGGGGCA-3') for the complementary strand. The PCR product was subcloned into *Hin*cII-pVE8001 (kindly provided by I. Poquet, URLGA, INRA, Jouy en Josas, France) resulting in plasmid pBS:E7 (Table 1). The *E7* cassette was isolated

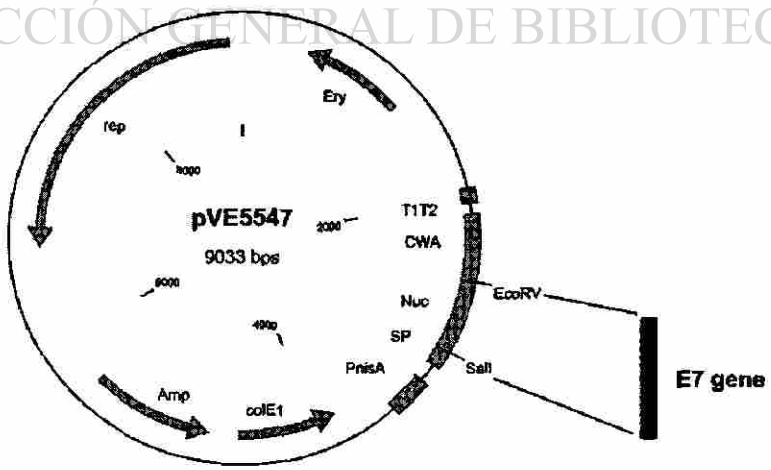

Fig. 1. Schematic of plasmid construction for E7-CWA expression in *L. lactis*. The rep and ColE1 replication origins are derived from pVE5547 plasmid where the *nuc* gene is fused downstream with a DNA fragment encoding the signal peptide (SP) of the Usp45 protein and upstream to the cell wall anchor (CWA) of the *S. pyogenes* M6 protein [20]. A DNA fragment encoding the mature moiety of E7 protein was obtained from a pBS:E7 plasmid (Table 1) with *SalI*/*Eco*RV and cloned in pVE5547 vector digested with the same enzymes and replacing the corresponding DNA fragment of *nuc*. The E7-CWA cassette is under the transcriptional control of the P_{nisA} promoter (*PnisA*). T1T2 indicates transcription terminator. The resulting plasmid was designated pIL:E7-CWA.

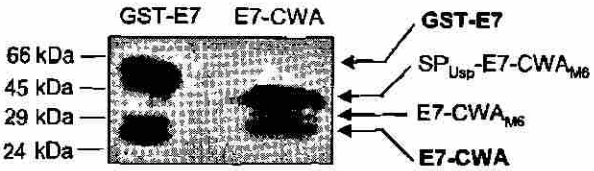

Fig. 2. Detection of E7 by Western blot assay. Cell extracts of induced cultures of recombinant *L. lactis* containing pIL:E7-CWA were analyzed by Western blot using monoclonal anti-E7 antibodies. Arrows indicate the positions of precursor SP$_{Usp}$-E7-CWA$_{M6}$, product of a first cleavage (E7-CWA$_{M6}$) and mature form (E7-CWA). Glutathione S-transferase (GST)-E7 represents cell extracts from an *E. coli* strain expressing E7 fused to GST used as positive control. Positions of molecular mass marker are indicated on the left.

with *Sal*I/*Eco*RV and cloned into *Sal*I/*Eco*RV-cut pVE5547 (Table 1) resulting in pIL:E7-CWA (Table 1, Fig. 1). In this vector, the *E7* gene was fused downstream of RBS$_{Usp45}$ and in frame with the signal peptide (SP$_{Usp45}$) of Usp45 (the predominant secreted protein in *L. lactis* [24]) at its amino-terminus and with a DNA fragment encoding the cell wall anchor (CWA$_{M6}$) of the *S. pyogenes* M6 protein at its carboxy-terminus (Fig. 1 [20]). This C-terminal CWA fragment is 190 amino acids long, thus, the expected size of the hybrid precursor is 38 kDa. Expression was controlled by the P$_{nisA}$ promoter [16]. This construct, pIL:E7-CWA plasmid, was introduced into *L. lactis* strain NZ9000, which carries regulatory genes *nisR* and *nisK* resulting in NZ9000 (pIL:E7-CWA) (Table 1; hereafter called NZE7-CWA).

2.4. Protein sample preparation and immunodetection

Protein sample preparation from recombinant *L. lactis* cultures, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), Western blots and immunodetection with anti-E7 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were performed as previously described [4].

2.5. Immunofluorescence microscopy

To confirm the E7-CWA surface expression, cultures of the recombinant strain NZE7-CWA were analyzed by immunofluorescence essentially as previously described [25] (Fig. 3). In this experiment, a *L. lactis* strain carrying the plasmid pVE5547 [20] and producing a cell wall anchored form of Nuc was used as a negative control. Briefly, 2 ml of NZE7-CWA-induced cultures were harvested at OD_{600} = 0.5–0.6 and suspended in 1 ml of sterile phosphate-buffered saline (PBS)–3% bovine serum albumin containing anti-E7 antibodies and incubated overnight at room temperature. After three washes with PBS–Tween 0.05%, the cell–antibody complex was incubated for 6 h at room temperature (avoiding light exposure) with a goat anti-mouse IgG Alexa Fluor conjugate (1:50 dilution). Cells were washed three times in PBS–Tween, laid on a glass slide, air-dried and heat-fixed. Analysis was performed on a confocal microscopy (Olympus Fluoview IX70).

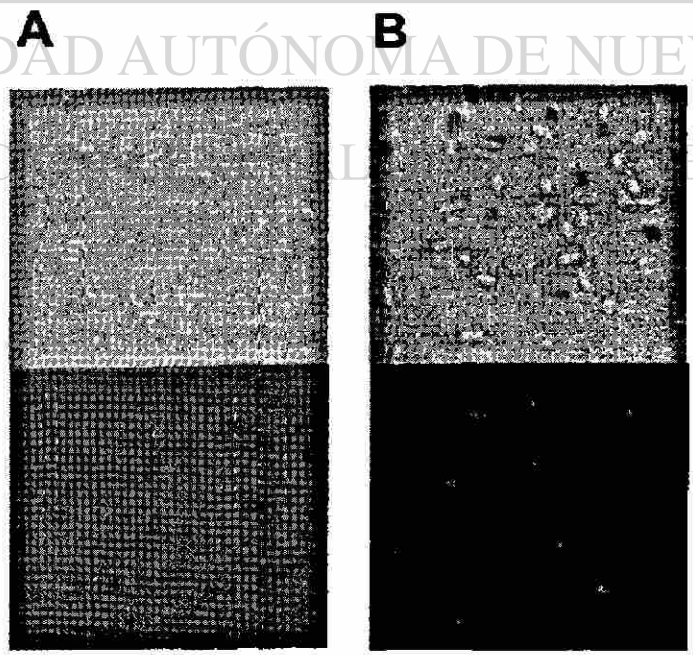

Fig. 3. Detection of E7 at the cell surface of *L. lactis* by immunofluorescence. A: *L. lactis* harboring the control plasmid pVE5547. B: Recombinant *L. lactis* expressing E7-CWA$_{M6}$. Bacterial samples were treated with specific anti-E7 monoclonal antibodies and then fluorescence-stained with goat anti-IgG conjugate Alexa Fluor (lower panels). Visual light micrographs are included for identification of bacterial cells (upper panels). Magnification, ×1000.

2.6. Animals

C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Mice were housed in the animal facility at the Immunology and Virology Laboratory at the University of Nuevo León, San Nicolás de los Garza, N.L., México. Experiments were performed according to protocols approved by the International Animal Studies Committee.

2.7. Preparation of live bacterial inoculum and immunization protocol

Bacterial cultures were induced as described above and cell pellets were harvested by centrifugation at $3000 \times g$, 4°C and washed three times with sterile PBS. The pellets were suspended in 10 μl of PBS to obtain a final concentration of 1×10^9 colony-forming units (CFU). Three mice (6–8 weeks) were immunized intranasally with 1×10^9 CFU of induced recombinant *L. lactis* strain (5 μl were administered with a micropipette into each nostril) on days 0, 14 and 28. Mice were partially anesthetized by i.p. injection of a combination of xylazine and Ketamine (0.40 ml for 10 kg of weight, Cheminova de México, S.A. de C.V.). Plate counts were performed to check the amount of CFU administered and E7 production was controlled by immunodetection with E7 antibodies. Control mice ($n = 3$) received identical quantities of wild-type *L. lactis* strain.

2.8. Sample collection and detection of E7 antibodies

Samples were collected from anesthetized animals. Seven days after the last *L. lactis* administration (day 35) mice were bled and serum pooled. The mouse antiserum was tested by immunoblotting against a GST-E7 hybrid protein (*E7* gene fused to glutathione *S*-transferase, kindly provided by V. Bermúdez) produced in *E. coli*.

3. Results and discussion

3.1. Production of E7-CWA in L. lactis

Production of E7-CWA hybrid protein was analyzed by Western blot analysis on total cell extracts using anti-E7 antibodies. In cell extracts of the recombinant *L. lactis* carrying the E7-CWA cassette (NZE7-CWA; Fig. 2), one major band was detected at about 38 kDa, the expected size for the SP_{Usp}-E7-CWA_{M6} precursor. Two other bands of lower molecular mass were detected corresponding to (i) the 32-kDa E7-CWA_{M6} form, which results from the cleavage of SP_{Usp}, and (ii) the mature 27-kDa E7-CWA_{M6} generated after processing of CWA_{M6} (i.e. cleavage of CWA_{M6} and covalent link between E7 and the cell wall; Fig. 2). These results show that E7-CWA was efficiently produced and processed in *L. lactis* and are consistent with previous published results where one other heterologous protein (Nuc), was cell wall anchored in *L. lactis* using CWA. [20]. As a positive control, a GST-E7 hybrid protein produced in and purified from *E. coli* was included in Western blot analysis.

3.2. E7 is efficiently cell wall anchored in L. lactis

To confirm that E7-CWA fusion protein was properly addressed to the cell wall, cultures of NZE7-CWA strain were analyzed by immunofluorescence. Analysis by confocal microscopy revealed fluorescent cells only in the recombinant bacteria NZE7-CWA but not in the control strain (Fig. 3). About 90% of the recombinant bacteria were stained. These results show that E7 was properly and efficiently displayed at the cell surface of *L. lactis* strain NZE7-CWA.

3.3. Immunogenicity of E7-CWA in mice

Immunogenicity of NZE7-CWA strain against E7 was tested in mice. Groups of three mice were intranasally immunized with 1×10^9 CFU of live recombinant or a plasmid-free *L. lactis* strain and mouse sera (pooled) were tested for E7 antibodies by Western blot analysis using a GST-E7 protein as antigen. The pooled antiserum from mice immunized with wild-type *L. lactis* did not react with the blotted GST-E7 hybrid protein (Fig. 4A), whereas the antiserum from mice immunized with NZE7-CWA strain clearly reacted with a protein of the expected size for GST-E7 (Fig. 4B). The specificity of this band was confirmed by reprobing the blot against an anti-E7 monoclonal antibody (Santa Cruz Biotechnology). The same band was recognized by the monoclonal antibody (Fig. 4C). These results demonstrate that E7 protein displayed at the cell surface of *L. lactis* was able to evoke an antigen-specific immune response in mice.

3.4. Perspectives

We previously reported that HPV-16 E7 protein is an extremely labile protein and is intracellularly degraded when expressed in its native form in *L. lactis*; in contrast, secretion of E7 in *L. lactis* results in a good yield without degradation [4,5]. Furthermore, we reported that E7 protein could be rescued from intracellular proteolysis by fusion of a compact and resistant protein, Nuc, at either the N- or the C-terminus. In this work, the expression levels and stability of E7-CWA hybrid protein were similar to those reported for Nuc-E7 fusion (data not shown) suggesting that fusions to CWA_{M6} or Nuc have similar positive effects on E7 stability. This also suggests that cell wall anchoring, like secretion, could be a way to escape intracellular proteolysis.

This study demonstrates the potential of *L. lactis*, a

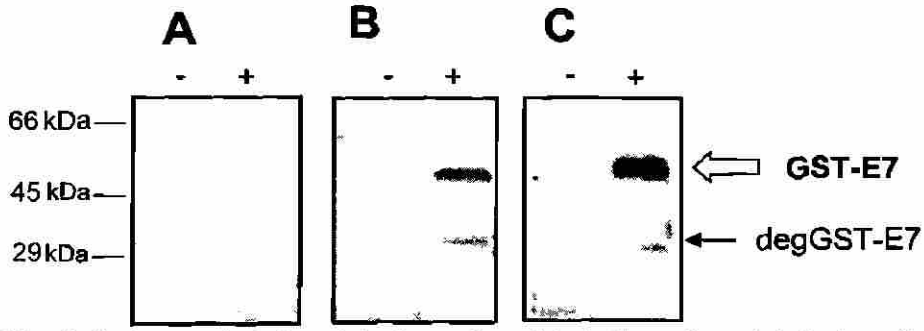

Fig. 4. Detection of E7 antibodies in serum of mice immunized with recombinant *L. lactis*. Western blot analysis showing antibodies against HPV-16 E7 protein after intranasal immunization with recombinant *L. lactis* NZE7-CWA. Cell extracts of *E. coli* expressing GST-E7 protein, used as antigen (+), were resolved in 12% SDS–PAGE gels, transferred onto polyvinylidene difluoride membrane and reacted with pooled sera from immunized mice. An *E. coli* strain harboring a GST plasmid was used as a negative control (–). Panels A and B were probed with sera of mice immunized with wild-type and recombinant *L. lactis* NZE7-CWA strain, respectively. In panel C the membrane of panel B was stripped and reprobed with anti-E7 monoclonal antibody. The arrow indicates the GST-E7 protein. Positions of molecular mass markers are indicated on the left.

food-grade, non-pathogenic and non-invasive LAB, to be used as a live vector to design a prophylactic and/or therapeutic vaccine to prevent and treat respectively CxCa. The fact that antibodies against E7 were found in the serum means that *L. lactis* expressing E7 at the surface was able to induce a systemic response. We recently showed that an E7 antigen-specific cellular response (i.e. secretion of Th1 cytokines, interleukin-2 and interferon-γ) was elicited in mice by a recombinant *L. lactis* strain displaying a cell wall anchored E7 [11]. Furthermore, expression of E7 at the surface of *L. lactis* appears to evoke a stronger immune response than cytoplasmic or secreted forms: splenocytes obtained from mice immunized by intranasal administration with NZE7CWA and restimulated in vitro produced higher Th1 cytokines levels than those obtained in mice immunized with recombinant lactococcal strains producing a cytoplasmic or secreted E7 antigen (Bermúdez-Humarán et al., submitted).

Immune response against cell wall anchored E7 has also been achieved using *Streptococcus gordonii* [26] in combination with complete Freund's adjuvant. In this work, an antigen-specific immune response was obtained after intranasal administration of recombinant *L. lactis* alone. Furthermore, *S. gordonii* is a commensal (persistent microorganisms that can induce immunotolerance) and generally regarded as safe but not a food-grade bacterium. In contrast, *L. lactis* is totally innocuous and non-persistent and can lead to the development of live vaccines, with a reduced risk of colonization and thus reduced risks of immune tolerance. In addition, LAB are good candidates for vaccination purposes because the route of immunization (intranasal, oral or vaginal) makes this model of easy administration and low costs.

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References

[1] zur Hausen, H. (1991) Human papillomaviruses in the pathogenesis of anogenital cancer. Virology 184, 9–13.
[2] Smotkin, D. and Wettstein, F.O. (1987) The major human papillomavirus protein in cervical cancers is a cytoplasmic phosphoprotein. J. Virol. 61, 1686–1689.
[3] Reinstein, E., Scheffner, M., Oren, M., Ciechanover, A. and Schwartz, A. (2000) Degradation of the E7 human papillomavirus oncoprotein by the ubiquitin-proteasome system: targeting via ubiquitination of the N-terminal residue. Oncogene 19, 5944–5950.
[4] Bermúdez-Humarán, L.G., Langella, P., Miyoshi, A., Gruss, A., Taméz-Reyes, R.S., Montes de Oca-Luna, R. and Le Loir, Y. (2002) Production of human papillomavirus type 16 E7 protein in *Lactococcus lactis*. Appl. Environ. Microbiol. 68, 917–922.
[5] Bermúdez-Humarán, L.G., Cortes-Perez, N.G., Le Loir, Y., Gruss, A., Rodriguez-Padilla, C., Saucedo-Cardenas, O., Langella, P. and Montes de Oca-Luna, R. (2003) Fusion to a carrier protein and a synthetic propeptide enhances E7 HPV-16 production and secretion in *Lactococcus lactis*. Biotechnol. Prog. 19, 1101–1104.
[6] Steidler, L., Robinson, K., Chamberlain, L., Schofield, M., Remaut, E., Le Page, R.W.F. and Wells, J.M. (1998) Mucosal delivery of murine interleukin-2 (IL-2) and IL-6 by recombinant strains of *Lactococcus lactis* coexpressing antigen and cytokine. Infect. Immun. 66, 3183–3189.
[7] Steidler, L., Hans, W., Schotte, L., Neirynck, S., Obermeier, F., Falk, W., Fiers, W. and Remaut, E. (2000) Treatment of murine colitis by *Lactococcus lactis* secreting interleukin-10. Science 289, 1352–1355.
[8] Gaeng, S., Scherer, S., Neve, H. and Loessner, M.J. (2000) Gene cloning and expression of *Listeria monocytogenes* bacteriophage-lytic enzymes in *Lactococcus lactis*. Appl. Environ. Microbiol. 66, 2951–2958.
[9] Chatel, J.M., Langella, P., Adel-Patient, K., Commissaire, J., Wal, J.M. and Corthier, G. (2001) Induction of mucosal immune response after intranasal or oral inoculation of mice with *Lactococcus lactis* producing bovine beta-lactoglobulin. Clin. Diagn. Lab. Immunol. 8, 545–551.
[10] Enouf, V., Langella, P., Commissaire, J., Cohen, J. and Corthier, G.

(2001) Bovine rotavirus nonstructural protein 4 produced by *Lactococcus lactis* is antigenic and immunogenic. Appl. Environ. Microbiol. 67, 1423–1428.

[11] Bermúdez-Humarán, L.G., Langella, P., Cortes-Perez, N.C., Gruss, A., Tamèz-Guerra, R.S., Oliveira, S.C., Cardenas, O., Montes de Oca-Luna, R. and Le Loir, Y. (2003) Intranasal immunization with recombinant *Lactococcus lactis* secreting murine interleukin-12 enhances antigen-specific Th1 cytokine production. Infect. Immun. 71, 1887–1896.

[12] Bermúdez-Humarán, L.G., Langella, P., Commissaire, J., Gilbert, S., Le Loir, Y., L'Haridon, R. and Corthier, G. (2003) Controlled intra- or extracellular production of staphylococcal nuclease and ovine omega interferon in *Lactococcus lactis*. FEMS Microbiol. Lett. 224, 307–313.

[13] Nouaille, S., Ribeiro, L.A., Pontes, D., Miyoshi, A., Le Loir, Y., Oliveira, S., Langella, P. and Azevedo, V. (2003) Heterologous proteins production and delivery systems for *Lactococcus lactis*. Gen. Mol. Res. 2, 7–28.

[14] Xin, K.Q., Hoshino, Y., Toda, Y., Igimi, S., Kojima, Y., Jounai, N., Ohba, K., Kushiro, A., Kiwaki, M., Hamajima, K., Klinman, D. and Okuda, K. (2003) Immunogenicity and protective efficacy of orally administered recombinant *Lactococcus lactis* expressing surface-bound HIV Env. Blood 102, 223–228.

[15] Chang, T.L., Chang, C.H., Simpson, D.A., Xu, Q., Martin, P.K., Lagenaur, L.A., Schoolnik, G.K., Ho, D.D., Hillier, S.L., Holodniy, M., Lewicki, J.A. and Lee, P.P. (2003) Inhibition of HIV infectivity by a natural human isolate of *Lactobacillus jensenii* engineered to express functional two-domain CD4. Proc. Natl. Acad. Sci. USA 100, 11672–11677.

[16] de Ruyter, P.G.G.A., Kuipers, O.P. and de Vos, W.M. (1996) Controlled gene expression systems for *Lactococcus lactis* with the food-grade inducer nisin. Appl. Environ. Microbiol. 62, 3662–3667.

[17] Kuipers, O.P. (1998) Quorum sensing-controlled gene expression in lactic acid bacteria. J. Biotechnol. 64, 15–21.

[18] Gibson, T.J. (1984) PhD Thesis. University of Cambridge, Cambridge.

[19] Gasson, M.J. (1983) Plasmid complements of *Streptococcus lactis* NCDO 712 and other lactic acid streptococci after protoplast-induced curing. J. Bacteriol. 154, 1–9.

[20] Dieye, Y., Usai, S., Clier, F., Gruss, A. and Piard, J.C. (2001) Design of a protein targeting system for lactic acid bacteria. J. Bacteriol. 183, 4157–4166.

[21] Langella, P., Le Loir, Y., Ehrlich, S.D. and Gruss, A. (1993) Efficient plasmid mobilization by pIP501 in *Lactococcus lactis* subsp. *lactis*. J. Bacteriol. 175, 5806–5813.

[22] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd ed.n Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

[23] Piard, J.C., Jimenez-Diaz, R., Ehrlich, S.D., Fischetti, V.A. and Gruss, A. (1997) The M6 protein of *Streptococcus pyogenes* and its potential as a tool to anchor biologically active molecules at the surface of lactic acid bacteria. Adv. Exp. Med. Biol. 418, 545–550.

[24] van Asseldonk, M., Rutten, G., Oteman, M., Siezen, R.J., de Vos, W.M. and Simons, G. (1990) Cloning, expression in *Escherichia coli* and characterization of *usp45*, a gene encoding a highly secreted protein from *Lactococcus lactis* MG1363. Gene 95, 155–160.

[25] Kwak, Y.D., Yoo, S.K. and Kim, E.J. (1999) Cell surface display of human immunodeficiency virus type 1 gp120 on *Escherichia coli* by using ice nucleation protein. Clin. Diagn. Lab. Immunol. 6, 499–503.

[26] Pozzi, G., Contorni, M., Oggioni, M.R., Manganelli, R., Tommasino, M., Cavalieri, F. and Fischetti, V.A. (1992) Delivery and expression of a heterologous antigen on the surface of streptococci. Infect. Immun. 60, 1902–1907.

Fusion to a Carrier Protein and a Synthetic Propeptide Enhances E7 HPV-16 Production and Secretion in *Lactococcus lactis*

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An inducible system to improve and stabilize the production of an extremely labile protein (E7 antigen of human papillomavirus type 16) was developed in the food-grade bacterium *Lactococcus lactis*. A protein carrier, the staphylococcal nuclease Nuc, was fused either to N- or C-termini of E7 protein, and the resulting hybrid proteins were rescued from intracellular proteolysis but poorly secreted by *L. lactis*. A synthetic propeptide (LEISSTCDA) was then fused and significantly improved the secretion efficiency of the hybrid protein Nuc-E7 by *L. lactis*.

Introduction

The lactic acid bacteria (LAB) are a group of Gram-positive microorganisms including members of the genera *Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Pediococcus*, and *Streptococcus*. LAB are widely used in the food industry, and research works on LAB were first oriented toward better understanding for improved control of the fermentation process. Many efforts are now focusing on the production of heterologous proteins in these Generally Regarded As Safe (GRAS) microorganisms for commercial uses (Mercenier et al., 2000; Seegers, 2002). To date, the best characterized LAB is *Lactococcus lactis*, for which efficient genetic tools have been developed and whose genome sequence is now completed (Bolotin et al., 1999). *Lactococcus lactis* is a food-grade, nonpathogenic, and noncolonizing bacterium that represents a good organism to produce heterologous proteins and could be exploited to produce enzymes, metabolites, and antigens of medical interest. Moreover, recent works demonstrated the potential of *L. lactis* for antigen delivery in live vaccine development (for review, see Mercenier et al., 2000; Mielcarek et al., 2001).

There is a considerable interest in the overproduction of heterologous proteins in *L. lactis*. Several strategies have been developed for this purpose using constitutive and inducible gene expression systems (Wells et al., 1995; de Vos, 1999). We recently used the NICE system (based on the nisin-inducible promoter P_{nisA}; de Ruyter et al., 1996) to produce the E7 oncoprotein from human papillomavirus type 16 (HPV-16) in *Lactococcus lactis* (Bermúdez-Humarán et al., 2002). The E7 oncoprotein is a very labile nuclear phosphoprotein (98 amino acids) with limited production in both eukaryotic and prokaryotic systems. Thus, it represents a good model to analyze heterologous production and stabilization. We previously observed that E7 is degraded in the cell even in a *L. lactis* strain deficient for ClpP, the main intracellular protease (Bermúdez-Humarán et al., 2002). Interestingly, E7 instability was rescued by fusion of the staphylococcal nuclease (Nuc) protein to its amino N-terminal end, but secretion efficiency (the proportion of total protein that is present in mature secreted form; SE) of this hybrid protein was very low (~5%). Moreover, Reinstein et al. (2000) showed that E7 protein is rescued from ubiquitin proteolysis, in an eukaryotic system, by Myc-tagging to its N- but not C-terminus. Thus, to improve both Nuc-E7 production and secretion by *L. lactis*, several parameters were analyzed and modified: (i) amounts of nisin used to induce the production of hybrid Nuc-E7, (ii) use of a synthetic propeptide LEISSTCDA (hereafter called LEISS) to improve SE (Le Loir et al., 1998 and 2001); (iii) fusion of Nuc to E7 C-terminus. Our results show that Nuc-E7 production and SE by *L. lactis* depend on the quantity of nisin used. Moreover, SE was improved by LEISS fused to the hybrid protein E7-Nuc. Interestingly, Nuc can protect E7 protein from intracellular degradation in *L. lactis* when fused to Nuc at both extremities (N- or C-terminus). This work has generated useful tools for in vivo production of E7 antigen by fusion to a stable and compact protein (Nuc) and provides alternative means of stabilizing heterologous fragile proteins in *L. lactis*.

Material and Methods

Bacterial Strains, Plasmids, and Antibiotics. *Escherichia coli* was grown in LB at 37 °C and *L. lactis* NZ9000 (NZ; *L. lactis* strain that carries chromosomal copies of regulatory genes *nisR* and *nisK*, Kuipers et al., 1998) and *L. lactis* NZ(pSEC:Nuc-E7) (Bermúdez-Humarán et al., 2002) strains in M17 medium (DIFCO) supplemented with 1% (w/v) glucose (GM17) at 30 °C without agitation. The plasmids pSEC:Nuc-E7, pBS:Nuc,

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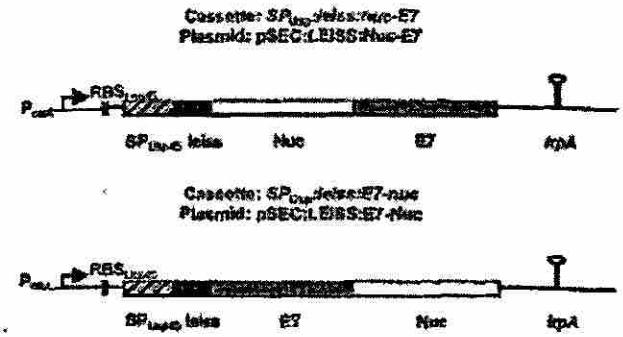

Figure 1. Design of cassettes to improve E7 production and secretion by *L. lactis*. Schematic structures of LEISS:Nuc-E7 and LEISS:E7-Nuc fusions expressed under the lactococcal P_{nisA} promoter and carried by the indicated plasmids. For details of plasmid constructions, see text. Legend: (—) *L. lactis* promoter sequences of nisin-inducible promoter (P_{nisA}); (■) ribosome binding site of the *usp45* gene; (diagonal pattern) signal peptide of the *usp45* gene; (dotted pattern), fragment encoding LEISSTCDA propeptide; (white) NucB coding sequence, (grey) E7 coding region; (♀) transcription terminator (not to scale).

pSEC:LEISS:Nuc, and pBS:E7 have been previously described (Bermúdez-Humarán et al., 2002). Unless otherwise indicated, plasmid constructions were first established in *E. coli* and then transferred to *L. lactis* by electrotransformation and selected by the addition of antibiotics as follows: for *E. coli*, ampicillin (100 μg/mL) and chloramphenicol (10 μg/mL), and for *L. lactis*, chloramphenicol (10 μg/mL).

DNA Manipulations. General procedures for DNA isolations and manipulations were essentially performed as previously described (Bermúdez-Humarán et al., 2002). PCR (Perkin-Elmer Cetus apparatus, Norwalk, CT) was performed using Vent DNA Polymerase (Promega), and PCR sequences were confirmed using dye-terminator sequencing kit (ABI PRISM BigDye Terminators, Appl. Biosystems).

Nisin-Inducible System. For nisin promoter induction, NZ strain and its derivative strains were all grown to an OD_{600} of 0.4−0.6 and induced for 1 h with 0.05, 0.1, 1, 5, 10, and 15 nisin ng/mL.

Protein Samples Preparation and Immunodetection. Protein samples preparation from recombinant *L. lactis* cultures, SDS−PAGE, Western blots, and immunodetection with anti-Nuc or anti-E7 antibodies were performed as described (Le Loir et al., 1998; Bermúdez-Humarán et al., 2002).

Fusion of LEISSTCDA Synthetic Propeptide to Nuc-E7 Hybrid Protein. To improve the SE of the hybrid protein Nuc-E7 (Bermúdez-Humarán et al., 2002), a *RBS-SP$_{Usp}$-leiss-nuc-E7* cassette was constructed (Figure 1), where *RBS* corresponds to a DNA fragment containing the ribosome binding site of the homologous *usp45 L. lactis* gene; the *SP$_{Usp}$*, which encodes the signal peptide (SP, essential signal for protein secretion) of Usp45 protein, the main secreted protein by *L. lactis* (van Asseldonk et al., 1990); and *leiss*, which encodes LEISSTCDA (LEISS), a synthetic propeptide previously described as a secretion enhancer in *L. lactis* (Le Loir et al., 1998). Briefly, an *E7* cassette was purified from a *NsiI/EcoRV*-cut pBS:E7 and cloned into a pSEC:LEISS backbone purified from *EcoRI*-Klenow/*NsiI*-cut pSEC:LEISS:Nuc, resulting in pSEC:LEISS:E7. A *nuc* cassette, isolated from *NsiI*-cut pBS:Nuc was then cloned into *NsiI*-cut pSEC:LEISS:E7 to generate pSEC:LEISS:Nuc-E7. This plasmid was introduced into *L. lactis* NZ.

Figure 2. Kinetics of nisin concentration necessary to obtain efficient Nuc-E7 production and secretion by *L. lactis*. We tested different concentrations of nisin (0.05, 0.1, 1, 5, 10, 15 ng/mL) to determine the best one to produce and secrete Nuc-E7 hybrid by *L. lactis*. Proteins were extracted from an induced culture of NZ(pSEC:Nuc-E7) strain and then analyzed by Western blot with anti-Nuc antibodies. The arrows indicate the positions of mature protein, the precursor SP$_{Usp}$:Nuc-E7 (preNuc-E7): (C) cell lysates; (S) supernatant fraction. Positions of molecular weight markers are shown on the left.

Fusion of Nuc to E7 C-Terminus. To test the effect of a Nuc fusion to E7 C-terminus, *SP$_{Usp}$-leiss-E7-nuc* cassette was constructed as follows (Figure 1). An *E7* cassette was PCR amplified from pBS:E7 with two oligonucleotides having *NsiI* sites in their 5′ extremities (5′-GATGCATCACAACATGGAGATACACCTACATTG-CAT-3′ for coding strand and 5′-GGATGCATGTGGTT-TCTGAGAACAGAT-3′ for complementary strand). The PCR fragment was subcloned into pCR-TOPO kit (Invitrogen, Carlsbad, CA), resulting in pCR-TOPO-*Nsi*IE7. The *E7* cassette purified from a *NsiI*-cut pCR-TOPO-*Nsi*IE7 was cloned into a *NsiI*-cut pSEC:LEISS:Nuc, resulting in pSEC:LEISS:E7-Nuc. The pSEC:LEISS:E7-Nuc plasmid was then introduced into *L. lactis* NZ.

Results and Discussion

Effects of Nisin Concentrations on Production of Nuc-E7 in *L. lactis*. Since the expression of genes transcribed under the control of P_{nisA} depends on nisin concentration (de Ruyter et al., 1996), different nisin concentrations were tested. Immunodetection of the hybrid Nuc-E7 protein by anti-Nuc antibodies indicates that Nuc-E7 expression is induced with a minimal concentration of 0.05 ng of nisin/mL (Figure 2). At this concentration, a maximum SE is observed as no precursor is detected in the cell fraction. The highest level of Nuc-E7 secreted in the medium was reached at 10 ng of nisin/mL. Higher concentration of nisin (i.e., 15 ng/mL) caused cell lysis since the SP$_{Usp}$-Nuc-E7 precursor form (preNuc-E7) was detected in the medium (Figure 2). Thus, further inductions were performed using 10 nisin ng/mL.

LEISSTCDA Improves Secretion Efficiency and Yield of Nuc-E7 Hybrid Protein in *L. lactis*. We previously reported the secretion of the Nuc-E7 hybrid protein by *L. lactis* (Bermúdez-Humarán et al., 2002) with a very low SE (~5%), whereas E7 native SE was high (~95%). Previous studies showed that LEISS enhanced SE of heterologous proteins by *L. lactis* (Le Loir et al., 1998). To increase the SE of Nuc-E7, LEISS was fused to this hybrid protein, resulting in LEISS:Nuc-E7 (encoded by the plasmid pSEC:LEISS:Nuc-E7), and then protein secretion was evaluated by immunodetection with anti-Nuc antibodies (Figure 3A). The results show a SE of 5% for Nuc-E7 protein versus 15% for LEISS:Nuc-E7 (as determined by comparison of Nuc-E7 signals with Image Quant Program). Moreover, the total amount of LEISS:Nuc-E7 protein was considerably enhanced. The molecular weights of the detected LEISS:Nuc-E7 forms were higher than that of Nuc-E7 as a result of the addition of nine amino acids of LEISS. The upper band corresponds to the LEISS:Nuc-E7 precursor, the middle band to the mature form, and the lower band to a degradation product. In the supernatant, the SE of

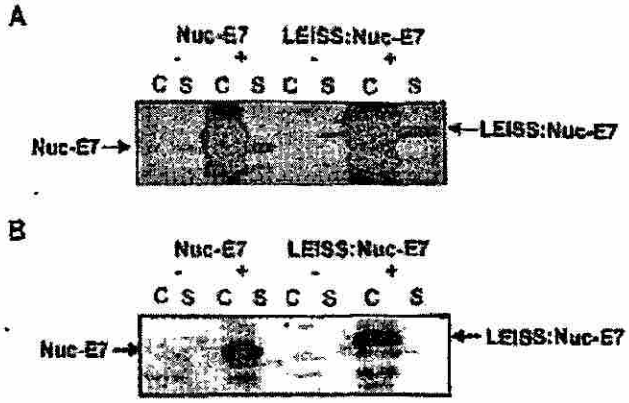

Figure 3. LEISSTCDA synthetic propeptide enhances SE and improves Nuc-E7 production yield in *L. lactis*. Nuc-E7 and LEISS:Nuc-E7 production were analyzed by Western blot with antibodies anti-Nuc (A) and anti-E7 (B). The induction was carried out with 10 ng/mL of nisin. Noninduced (−) and induced (+) cultures of indicated strains. preLEISS:Nuc-E7, preNuc.E7, and Nuc-E7 forms are indicated by arrows: (C) cell lysates; (S) supernatant fraction. Positions of molecular weight marker are shown on the left.

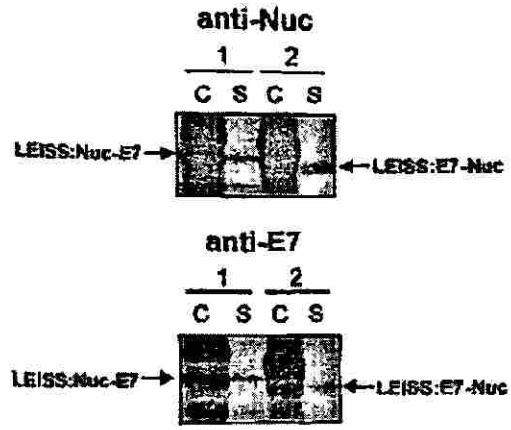

Figure 4. Fusion of Nuc at either N- or C-terminus can rescue E7 production. LEISS-Nuc-E7 and LEISS-E7-Nuc were analyzed by Western blot experiment on induced cultures of wt *L. lactis* strains containing pSEC-LEISS-Nuc-E7 (lane 1) or pSEC-LEISS-E7-Nuc (lane 2) using anti-Nuc or anti-E7 antibodies. Migration positions of precursor forms (prec), LEISS-Nuc-E7 mature form (LEISS-Nuc-E7), and LEISS-E7-Nuc mature form (LEISS-E7-Nuc) are indicated by arrows: (C) cell lysates; (S) supernatant fraction.

LEISS:Nuc-E7 was reproducibly higher (~15%) compared to that observed for Nuc-E7 (~5%).

The membrane of Figure 3A was then stripped and probed with anti-E7 monoclonal antibodies (Figure 3B). The pattern of proteins was similar to that observed with anti-Nuc antibodies with the difference of a lower percentage of mature protein in the medium (Figure 3B). Here, we suggest that supernatant band detection with anti-E7 antibodies is not markedly visible as anti-Nuc detection because degradation may preferentially target the E7 moiety of the fusion.

In addition, several bands of lower molecular weight are detected in cell fractions that correspond to degradation products. Although the migration was performed in denaturing conditions (SDS−PAGE), several bands of high molecular weight are detected above the precursor form in the cell fraction. These bands might be due to precursor aggregates that are not completely dissociated by the mild conditions of protein samples preparation (due to E7 fragility, Bermúdez-Humarán et al., 2002) and are indeed reported insensitive to intracellular housekeeping proteolysis (Wild et al., 1996).

Nuc Protects E7 from Degradation When Fused to Either N- or to C-Terminus. Here, we observed that the fusion of both LEISS and Nuc improves dramatically E7 production in *L. lactis*. To test whether C-terminus fusion of Nuc could protect E7 from degradation, pSEC:LEISS:E7-Nuc was constructed and introduced into NZ9000, resulting in NZ(pSEC:LEISS:E7-Nuc). E7 protein products from both strains NZ(pSEC:LEISS:E7-Nuc) and NZ(pSEC:LEISS: Nuc-E7) were analyzed by Western blot assays. However, in this experiment we overexposed the PVDF membrane in the autoradiographic film for better detection of the supernatant band with anti-E7 antibodies. Anti-Nuc and anti-E7 antibodies revealed (i) in the cell fractions of NZ(pSEC:LEISS:Nuc-E7) and NZ(pSEC:LEISS: E7-Nuc) two major bands that correspond to the precursor preLEISS:E7-Nuc and mature protein LEISS:E7-Nuc (Figure 4); (ii) in the supernatants, one major band at the expected size for LEISS:Nuc-E7 and LEISS:E7-Nuc (mature protein). The E7-Nuc hybrid protein products run slightly smaller than Nuc-E7 ones; such aberrant migration properties had been already observed in fusion at E7 C-terminus (Reisntein et al., 2000). These results show that a fusion to Nuc at either N- or C-terminus of E7 can rescue E7 production.

Perspectives in the use of *L. lactis* as Host Vector. The use of *L. lactis* to produce and export proteins offers several advantages that makes it an attractive host for heterologous protein production: (i) some strains do not secrete proteases or other proteins at high levels, which simplifies purification and analyses of secreted proteins of interest (Le Loir et al., 1998); (ii) *L. lactis* is widely used in the food industry, a feature that may facilitate its acceptance as a vector for heterologous proteins production; (iii) their GRAS status represents an important advantage to be used as a live vaccine vector; (iv) some strains exhibit antitumor activity (Kelkar et al., 1998). Moreover, *L. lactis* is a noncommensal and transient bacterium in the digestive tract. This could be an advantage to avoid any tolerance response to a given antigen.

Conclusions

Recent studies in our laboratory suggested that low production and inefficient SE of heterologous proteins by *L. lactis* could be enhanced by the fusion of Nuc protein at the N-terminus (Ribeiro et al., 2002; Bermúdez-Humarán et al., 2002) and/or LEISSTCDA propeptide to the protein of interest (Le Loir et al., 1998, 2001; Ribeiro et al., 2002). Here, we found that Nuc fusion to E7 C-terminus acts in a way similar to that of the N-terminus fusion in *L. lactis*. In contrast, N- but not C-terminus tagging of E7 rescues it from ubiquitin proteolysis in an eukaryotic system (Reinstein et al., 2000). In the other hand, the synthetic propeptide LEISSTCDA adds two negative charges at positions +2 and +8 of the mature moiety of the fusion Nuc-E7 (LEISS:Nuc-E7) secreted protein, which indeed improve the SE (5 vs 15%). These results are totally in agreement with other reports of the enhancer properties of this nine-residue synthetic propeptide (Le Loir et al., 1998, 2001; Ribeiro et al., 2002). Finally, the nisin-inducible system used in this work allows the expression of foreign proteins that can be toxic to the bacterium when produced at high levels (de Ruyter et al., 1996).

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References and Notes

Bermúdez-Humarán, L. G.; Langella, P.; Miyoshi, A.; Gruss, A.; Tamez-Guerra, R.; Montes de Oca-Luna, R.; Le Loir, Y. Production of human papillomavirus type 16 E7 protein in *Lactococcus lactis*. *Appl. Environ. Microbiol.* **2002**, *68*, 917–922.

Bolotin, A.; Mauger, S.; Malarme, K.; Ehrlich, S. D. Sorokin, A. Low-redundancy sequencing of the entire *Lactococcus lactis* IL1403 genome. *Antonie Van Leeuwenhoek.* **1999**, *76*, 27–76.

de Ruyter, P. G.; Kuipers, O. P.; de Vos, W. M. Controlled gene expression systems for *Lactococcus lactis* with the food-grade inducer nisin. *Appl. Environ. Microbiol.* **1996**, *62*, 3662–3667.

de Vos, W. M. Gene expression systems for lactic acid bacteria. *Curr. Opin. Microbiol.* **1999**, *2*, 289–295.

Kelkar, S. M.; Shenoy, M. A.; Kaklij, G. S. Antitumor activity of lactic acid bacteria on a solid fibrosarcoma, sarcoma-180, and Ehrlich ascites carcinoma. *Cancer Lett.* **1988**, *42*, 73–77.

Kuipers, O. P.; de Ruyter, P. G.; Kleerebezem M.; de Vos, W. M. Quorum sensing-controlled gene expression in lactic acid bacteria. *J. Biotechnol.* **1998**, *64*, 15–21.

Le Loir, Y.; Gruss, A.; Ehrlich, S. D.; Langella, P. A nine-residue synthetic propeptide enhances secretion efficiency of heterologous proteins in *Lactococcus lactis*. *J. Bacteriol.* **1998**, *180*, 1895–1903.

Le Loir, Y.; Nouaille, S.; Commissaire, J.; Brétigny, L.; Gruss A.; Langella, P. Signal peptide and propeptide optimisatio for protein secretion in *Lactococcus lactis*. *Appl. Enviror Microbiol.* 2001, *67* (9): 4119–4127.

Mercenier A.; Muller-Alouf, H.; Grangette, C. Lactic aci bacteria as live vaccines. *Curr. Issues Mol. Biol.* 2000, *2*, 17–25.

Mielcarek, N.; Alonso, S.; Locht, C. Nasal vaccination using liv bacterial vectors. *Adv. Drug. Delivery Rev.* 2001, *51*, 55–6

Ribeiro, L. A.; Azevedo, V.; Le Loir, Y.; Oliveira, S. C.; Dieu Y.; Piard, J. C.; Gruss, A.; Langella, P. Efficient targeting the *Brucella abortus* immunodominant antigen L7/L12 b *Lactococcus lactis*: a first step towards new live vaccine against brucellosis. *Appl. Environ. Microbiol.* **2002**, *68*, 910–916.

Reinstein, E.; Scheffner, M.; Oren, M.; Ciechanover, A.; Schwart A. Degradation of the E7 human papillomavirus oncoprotei by the ubiquitin-proteasome system: targeting via ubiquit nation of the N-terminal residue. *Oncogene* **2000**, *19*, 5944–5950.

Seegers, J. F. Lactobacilli as live vaccine delivery vector progress and prospects. *Trends Biotechnol.* **2002**, *20*, 508–515.

van Asseldonk, M.; Rutten, G.; Oteman, M.; Siezen, R. J.; d Vos, W. M.; Simons, G. Cloning, expression in *Escherichi coli*, and characterization of *usp45*, a gene encoding a highl secreted protein from *Lactococcus lactis* MG1363. *Gene* 199 *95*, 155–160.

Wells, J. M.; Norton, P. M.; Le Page, R. W. F. Progress in th development of mucosal vaccines based on *Lactococcus lactis* *Int. Dairy J.* 1995, *40*, 225–230.

Wild, J.; Rossmeissl, P.; Walter, W. A. Gross, C. A. Involvemen of the DnaK-DnaJ-GrpE chaperone team in protein secretio in *Escherichia coli*. *J. Bacteriol.* **1996**, *178*, 3608–3613.

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Intranasal Immunization with Recombinant *Lactococcus lactis* Secreting Murine Interleukin-12 Enhances Antigen-Specific Th1 Cytokine Production

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Interleukin-12 (IL-12), a heterodimeric cytokine, plays an important role in cellular immunity to several bacterial, viral, and parasitic infections and has adjuvant activity when it is codelivered with DNA vaccines. IL-12 has also been used with success in cancer immunotherapy treatments. However, systemic IL-12 therapy has been limited by high levels of toxicity. We describe here inducible expression and secretion of IL-12 in the food-grade lactic acid bacterium *Lactococcus lactis*. IL-12 was expressed as two separate polypeptides (p35-p40) or as a single recombinant polypeptide (scIL-12). The biological activity of IL-12 produced by the recombinant *L. lactis* strain was confirmed in vitro by its ability to induce gamma interferon (IFN-γ) production by mouse splenocytes. Local administration of IL-12-producing strains at the intranasal mucosal surface resulted in IFN-γ production in mice. The activity was greater with the single polypeptide scIL-12. An antigen-specific cellular response (i.e., secretion of Th1 cytokines, IL-2, and IFN-γ) elicited by a recombinant *L. lactis* strain displaying a cell wall-anchored human papillomavirus type 16 E7 antigen was dramatically increased by coadministration with an *L. lactis* strain secreting IL-12 protein. Our data show that IL-12 is produced and secreted in an active form by *L. lactis* and that the strategy which we describe can be used to enhance an antigen-specific immune response and to stimulate local mucosal immunity.

Interleukin-12 (IL-12) is a multifunctional cytokine that was originally described as a maturation factor for cytotoxic (T) lymphocytes and a cell stimulatory factor for natural killer cells (8, 23, 28). IL-12 is a heterodimeric glycoprotein composed of two disulfide-linked chains (p35 and p40) that has numerous effects on T and natural killer cells, resulting in enhancement of cytotoxic activity and induction of gamma interferon (IFN-γ) production. In experimental models, IL-12 has been shown to be involved in protection against several bacterial, viral, and parasitic infections (4, 25, 45). This cytokine has also been shown to block angiogenesis (34, 60). Finally, the immunomodulatory effects of IL-12 are reportedly beneficial in AIDS treatment (26). The immunostimulatory properties of IL-12 have led to experimentation with its use as a vaccine adjuvant (1, 3). In addition, IL-12 stimulates serum immunoglobulin G antibody responses and helps during differentiation of Th0 cells into Th1 cells (17, 21, 41). This is particularly interesting for vaccine development for antigens that are poorly immunogenic.

Local administration of IL-12 confers antitumor activity in vivo that results in regression of established tumors and reduction of metastasis in animal models (7, 43, 49). Nevertheless, systemic IL-12 therapy can have toxic effects in animals and humans (36, 38, 39) and has been a cause of mortality in clinical trials (10, 36). For example, intratumoral treatment of mice with a vaccinia virus expressing IL-12 resulted in significant tumor growth inhibition but also induced clear signs of toxicity (9).

Il-12 has also been considered for use as an adjuvant in vaccine therapies. Current therapies involving mucosal routes are limited by a lack of suitable adjuvants that can be safely given to humans. Cholera toxin and *Escherichia coli* enterotoxin are potent mucosal adjuvants but frequently cause secondary effects, such as severe diarrhea and induction of Th2 responses that can lead to undesirable immune responses (21). Although various IL-12 delivery systems based on retroviral vectors and gene gun techniques have been described (38, 40, 49), an efficient and cost-effective means of delivery remains to be developed.

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TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Replicon	Genotype or characteristics	Reference or source
Strains			
E. coli TG1		*supE hsdΔ5 thi Δ(lac-proAB) F'(traD36 proAB$^+$ lacI lacZΔM15)*	19
L. lactis MG1363		Wild type, plasmid free	14
L. lactis NZ9000		MG1363 (*nisRK* genes in chromosome), plasmid free	30
Plasmids			
pWRG3169	ColE1	Ap^r, pBS derivative containing coding sequences for p35 and p40 subunits	49
pCR:TOPO	ori pUC	Ap^r	Invitrogen
pCR-TOPO:Δp35exon1	ori pUC	Ap^r, DNA fragment encoding first exon of p35 subunit devoid of its SP	This study
pCR-TOPO:p35exon2	ori pUC	Ap^r, DNA fragment encoding second exon of p35 subunit	This study
pCR-TOPO:Δp35	ori pUC	Ap^r, DNA fragment encoding p35 mature moiety devoid of intron	This study
pVE8001	ColE1	Ap^r, pBS derivative containing *trpA* transcription terminator	48
pBS:Δp40:trpA	ColE1	Ap^r, DNA fragment encoding p40 mature moiety devoid of its SP plus *trpA*	This study
pVE8022	ColE1/pAMβ	Ap^r Em^r, pFUN derivative containing Exp4-ΔSP_{Nuc} fusion	48
pBS:SP_{Exp4}	ColE1	Ap^r, PCR fragment encoding SP_{Exp4} SP	This study
pBS:SP_{Exp4}:p40:trpA	ColE1	Ap^r, gene expressed from P_{nisA} encodes SP_{Usp}-*p40:trpA* p40 subunit precursor	This study
pSEC:E7	pWV01	Cm^r, gene expressed from P_{nisA} encodes SP_{Usp}-*E7* precursor	5
pSEC:p35	pWV01	Cm^r, gene expressed from P_{nisA} encodes SP_{Usp}-*p35* p35 subunit precursor	This study
pSEC:p35-p40	pWV01	Cm^r, gene expressed from P_{nisA} encodes SP_{Usp}-*p35-p40* p35 and p40 subunit precursors	This study
pCDNA3:IL-12	ColE1	Ap^r, PCR fragment encoding *IL-12* single chain	P. Melby[a]
pCR:TOPO:scIL-12	ori pUC	Ap^r, PCR fragment encoding *scIL-12* single chain devoid of its SP	This study
pSEC:scIL-12	pWV01	Cm^r, gene expressed from P_{nisA} encodes SP_{Usp}-*IL-12* single-chain precursor	This study

[a] The University of Texas Health Science Center, San Antonio.

The gram-positive and nonpathogenic lactic acid bacteria are considered promising candidates for the development of oral live vaccines. *Lactococcus lactis*, the model lactic acid bacterium, has been extensively engineered for the production of heterologous proteins (5, 13, 16, 35), including some antigens of bacterial or viral origin (5, 13, 33). *L. lactis* is of particular interest for oral delivery of functional proteins since it is a noncommensal, food bacterium that does not survive in the digestive tracts of animal models and humans (12, 16). In the case of IL-12 cytokine delivery, these properties could ensure transient expression of the protein, thereby limiting the risks of toxicity.

IL-12 production requires assembly of two subunits involving two disulfide bonds (DSB) (47). In gram-negative bacteria, DSB mediate protein folding during export; however, the final destination of exported proteins is the periplasm. In contrast, DSB formation is poorly documented in gram-positive bacteria. Only a few extracytoplasmic proteins with DSB have been identified, and to our knowledge, exported heterologous proteins containing DSB have been reported only in *Bacillus subtilis* (44, 46). However, in contrast to gram-negative bacteria, protein secretion in gram-positive bacteria leads to release of the protein into the medium, which provides an immediate advantage for a delivery system. Until now, secretion of a heterologous protein containing DSB by *L. lactis* has not been reported. In this study we demonstrated the capacity of *L. lactis* to produce and secrete a biologically active form of IL-12, a complex two-subunit cytokine with two DSB that are essential for its activity (47). We found that IL-12-producing recombinant *L. lactis* strains induce IFN-γ production in splenocyte cultures and after intranasal administration in mice.

Additionally, the potential adjuvant properties of an *L. lactis* strain secreting IL-12 were examined in combination with the human papillomavirus type 16 (HPV-16) E7 antigen. This antigen, which has been implicated in the progression of cervical cancer, is considered a potential candidate antigen for anticancer vaccine development. One drawback for its use is its poor induction of a cellular immune response (42). Administration of an *L. lactis* strain displaying a cell wall-anchored HPV-16 E7 antigen was significantly enhanced after coadministration of an *L. lactis* strain secreting IL-12 protein, corroborating the hypothesis that the recombinant strain described here is a promising candidate for mucosal codelivery of proteins of medical interest. This work marks a new step in the development of live protein presentation systems for nasal and/or oral administration.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this work are listed in Table 1. *L. lactis* was grown in M17 medium supplemented with 1% glucose or in brain heart infusion at 30°C without agitation. *Escherichia coli* was grown in Luria-Bertani medium at 37°C. Unless otherwise indicated, plasmid constructs were first established in *E. coli* and then transferred to *L. lactis* by electrotransformation (32, 51). Plasmids were selected by addition of antibiotics, as follows: 5 μg of chloramphenicol per ml for *L. lactis*, 10 μg of chloramphenicol per ml for *E. coli*, and 100 μg of ampicillin per ml for *E. coli*.

DNA manipulation and methods used. Plasmid DNA isolation and general procedures for DNA manipulation were performed essentially as described previously (51). PCR amplification was performed with a Perkin-Elmer Cetus (Norwalk, Conn.) apparatus by using Vent DNA polymerase (Promega). DNA sequences were confirmed by using a Dye terminator sequencing kit.

Deletion of an intron in the p35 subunit. The genes encoding p35 and p40 of murine IL-12 were isolated from pWRG3169, a vector previously described as functional in a eukaryotic system (kindly provided by Alexander Rakhmilevich) (Table 1) (49). Sequence analysis of pWRG3169 revealed an intron in the p35 subunit (data not shown). As introns are spliced in mammalian cells but not in prokaryotes, directed mutagenesis by PCR was performed to remove the p35 intron (Fig. 1). Briefly, the two exons were PCR amplified, subcloned into a pCR-TOPO kit (Invitrogen, Carlsbad, Calif.), and ligated to generate a DNA segment encoding the p35 mature moiety devoid of the intron and of its signal peptide (SP) (Δp35). The first exon was amplified by using primer 5'-p35-start (5'-GATGCATCAGAGAGGGTCATTCCAGTCTCTGGA-3') for the coding

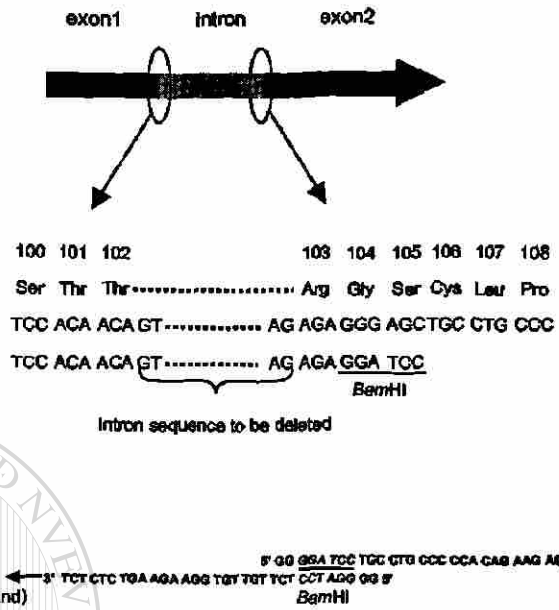

FIG. 1. Site-specific mutagenesis. The p35 intron was eliminated by site-directed mutagenesis. Briefly, we inserted a *Bam*HI restriction site (underlined) in the three codons (encoding Arg, Gly, Ser) to the right of the intron. Primers were designed to PCR amplify two fragments of p35 that were joined at the *Bam*HI site, such that p35 was reconstituted but intron free.

strand and primer 5'-p35 (5'-GGGGATCCTCTTGTTGTGGAAGAAGTCTC TCT-3') for the complementary strand (Fig. 1). The second exon was amplified by using primer 3'-p35 (5'-GGGGATCCTGCCTGCCCCCACAGAAGACG-3') for the coding strand and primer 3'-p35-stop (5'-GGAATTCTCAGGCGG AGCTCAGATAGCCCA-3') for the complementary strand (Fig. 1). The primers flanking the intron sequence were designed so that a *Bam*HI site was introduced without modifying the amino acid sequence. The two exons were cloned into pCR-TOPO, resulting in pCR-TOPO:Δp35exon1 and pCR-TOPO: p35exon2, respectively. The fragment encoding p35exon2 was then isolated from *Bam*HI/*Xba*I-blunted pCR-TOPO:p35exon2 and ligated to *Bam*HI/*Sma*I-cut pCR-TOPO:Δp35exon1, resulting in pCR-TOPO:Δp35. This final plasmid encodes the unmodified p35 subunit of IL-12.

Construction of the *L. lactis* strain producing the p35 and p40 subunits. A fragment encoding the p40 mature sequence devoid of its SP (p40) was PCR amplified from plasmid pWRG3169 and subcloned into *Hinc*II-cut pVE8001 (Table 1) (kindly provided by Isabelle Poquet, Unité de Recherches Laitières et de Génétique Appliquée, INRA, Jouy en Josas, France), resulting in pBS:Δp40: trpA. The pVE8001 vector has a transcriptional terminator (*trpA*) and has been used previously to express heterologous proteins in *L. lactis* (5, 48). The primers used were primer 5'-p40 (5'-GATGCATCAGAGATGTGGGAGCTGGAGAA AGAC-3') for the coding strand and primer 3'-p40 (5'-GGAGCTCCTAGGA TCGGACCCTGCAGGGAA-3') for the complementary strand. Subsequently, different constructs were made in order to fuse prokaryotic lactococcal SPs to p35 and p40 subunits. For the p40 subunit, a DNA fragment was PCR amplified from pVE8022 (a derivative of plasmid pFUN, in which the ΔSP_{Nuc} reporter is fused to Exp4, a putative *L. lactis* secreted protein of unknown function [48]). This fragment contains the ribosome binding site (RBS) and the signal peptide of Exp4 (SP_{Exp4}) (48). The primers used were primer 5'-Exp4 (5'-GGGTACC TTAAGGAGATATAAAAATGAA-3') for the coding strand and primer 3'-Exp4 (5'-GATGCATCATCAGCAAATACAACGGC-3') for the complementary strand. The PCR product was cloned into *Hinc*II-cut pVE8001, resulting in pBS:SP_{Exp4}. pBS:SP_{Exp4}:p40:trpA was obtained by insertion of the *NsiI*/*Kpn*I fragment (containing Δp40:*trpA*) obtained from pBS:Δp40:trpA into *NsiI*/*Kpn*I-cut pBS:SP_{Exp4}. For the p35 subunit, the *NsiI*/*Eco*RI fragment (containing Δp35) obtained from pCR-TOPO:Δp35 was cloned into a pSEC backbone purified from *NsiI*/*Eco*RI-cut pSEC:E7 (7) (Table 1), resulting in pSEC:p35. In this vector, the p35 gene is fused to the RBS and SP_{Usp45} of *usp45*, the gene encoding Usp45, the main secreted protein in *L. lactis* (59). Expression is controlled by the P_{nisA} inducible promoter, whose expression depends on the nisin concentration used (11, 30). Finally, to obtain the plasmid that expressed both the p35 subunit and the p40 subunit, a *Kpn*I/*Bam*HI-Klenow cassette encoding SP_{Exp4}:p40:*trpA* was isolated from the pBS:SP_{Exp4}:p40:trpA vector and cloned into the *Kpn*I/*Sma*I-cut pSEC:p35 backbone, resulting in pSEC:p35-p40 (Table 1) (Fig. 2). This vector, in which the two subunits were transcribed from the P_{nisA} promoter, was established in *L. lactis* NZ9000 carrying the regulatory genes *nisR* and *nisK* (30). The resulting strain is referred to below as NZ(pSEC:p35-p40).

Construction of an *L. lactis* strain with an scIL-12 gene. A single-chain IL-12 (scIL-12) gene (*scIL-12*) was amplified by PCR from plasmid pCDNA3:IL-12 (kindly provided by Peter Melby, The University of Texas Health Science Center, San Antonio). The primers used were primer 5'-IL-12 (5'-GATGCATCAGAG ATGTGGGAGCTG GAGAAAGAC-3') for the coding strand and primer 3'-IL-12 (5'-GGAATTCTCAGGCGGAGCTCAGATAGCCCA-3') for the complementary strand. Primer 5'-IL-12 was designed to delete the first 22 codons in the *scIL-12* coding sequence. These codons encode the p40 eukaryotic SP that is replaced by the lactococcal SP_{Usp45}. The PCR product was cloned into pCR:

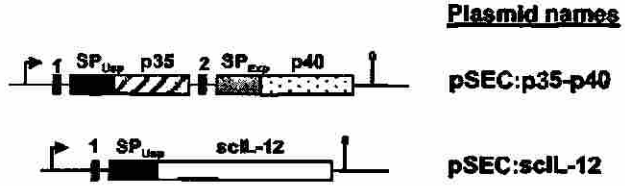

FIG. 2. Expression cassettes to produce and secrete IL-12 in *L. lactis*: schematic structures of p35 and p40 subunits and scIL-12 cassette expressed under the lactococcal P_{nisA} promoter and carried by the plasmids indicated. For details concerning plasmid construction, see the text and Table 1. The arrows indicate the presence of the nisin-inducible promoter (P_{nisA}); the solid vertical bars indicate the RBS of the *usp45* gene (bar 1 for p35) or of the *exp4* gene (bar 2 for p40); the dark gray bars indicate the SP of the *usp45* gene; the light gray bar indicates the SP of the *exp4* gene (48); the cross-hatched bar indicates the p35 mature coding sequence; the dotted bar indicates the p40 mature coding sequence; the open bar indicates the scIL-12 coding sequence; and the stem-loop symbols indicate *trpA* transcription terminators (not to scale).

TOPO (Invitrogen), resulting in pCR:TOPO:scIL-12 (Table 1). An *scIL-12* cassette was purified from *NsiI*/*Not*I-cut pCR:TOPO:scIL-12 and cloned into a pSEC backbone purified from *NsiI*/*Not*I-cut pSEC:E7. In the resulting plasmid, pSEC:scIL12 (Table 1 and Fig. 2), the *scIL-12* mature moiety was fused in frame with a DNA fragment encoding the RBS and SP_{Usp45} of *usp45*. Expression was controlled by a P_{nisA} promoter. This plasmid was established in *L. lactis* strain NZ9000 to obtain NZ(pSEC:scIL12).

IL-12 expression and detection. For induction of the nisin promoter, strains were grown until the optical density at 600 nm was ~0.6, and this was followed by induction with 10 ng of nisin (Sigma) per ml for 1 h. These parameters (amount of nisin and time of induction) were previously determined to be optimal (Bermúdez-Humarán, unpublished data). *L. lactis* culture extraction and immunoblotting assays were performed as previously described (5, 35); mouse IL-12 antibodies (R&D Systems, Minneapolis, Minn.) were used for immunodetection. The concentration of IL-12 secreted in a culture was estimated by using an enzyme-linked immunosorbent assay (ELISA) kit that recognized the IL-12 heterodimer but not the individual subunits (R&D Systems).

Preparation of bacterial supernatants for IL-12 nondenaturing analyses. Supernatant samples from induced cultures were concentrated 50-fold by using an Ultrafree Biomax NMWL membrane. After centrifugation, 10 μl of nondenaturing loading buffer (i.e., buffer lacking dithiothreitol and sodium dodecyl sulfate [SDS]) was added to 10 μl of supernatant concentrate. Electrophoresis was performed as described by Laemmli (31), except that SDS was omitted from all solutions.

Animals. C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, Maine). The mice were housed in the animal facility at the Immunology and Virology Laboratory at the University of Nuevo León, San Nicolás de los Garza, Mexico. Experiments were performed by using protocols approved by the animal studies committee.

Preparation of bacterial cells for IL-12 biological activity assays. Twenty milliliters of an induced culture was centrifuged, and the pellet and supernatant were separated. The bacterial cells were washed three times with sterile phosphate-buffered saline (PBS) and resuspended in 200 μl of PBS. The supernatant was concentrated 100-fold by using an Ultrafree Biomax NMWL membrane in 200 μl (final volume) of PBS.

In vitro IFN-γ detection. Spleens were obtained from four mice that were 6 to 8 weeks old, and splenocytes were separated on a Ficoll-Hypaque (Sigma) density gradient. A preparation containing 2×10^6 cells/ml in AIM-V medium (GIBCO) was plated on a 24-well plate (2 ml per well) at 37°C under 5% CO_2. As a positive control, mouse splenocytes were incubated with 50 pg of recombinant IL-12 (rIL-12) (R&D Systems). Splenocytes were incubated with 10 μl of recombinant *L. lactis* cells or with culture supernatant samples that were adjusted beforehand to provide ~50 pg per sample, as determined by an ELISA. Supernatants from the treated splenocytes were harvested after 48 h and tested for the presence of IFN-γ by ELISA (R&D Systems) according to the manufacturer's directions. All samples were prepared in triplicate.

Intranasal administration of recombinant *L. lactis* strains encoding IL-12. Groups of three mice that were 6 to 8 weeks old were inoculated intranasally with recombinant or wild-type *L. lactis* or with PBS. Prior to treatment, the mice were partially anesthetized intraperitoneally with a combination of Xilacyne and Ketamine (0.40 ml per 20 lb; Cheminova de México, Mexico). A total of 5×10^8 CFU (prepared as described above) of each induced *L. lactis* strain was resuspended in 10 μl of PBS, and 5 μl was administered with a micropipette into each nostril on days 0, 14, and 28.

IFN-γ induction assay. Animals treated with recombinant *L. lactis* strains were sacrificed on day 35. Splenocytes were separated and cultured as described above. Mouse cells were stimulated in vitro with 50 μl of phytohemagglutinin (5 μg/ml; M form, a polyclonal activator; GIBCO) to increase the proliferative response and to mimic a situation in which IFN-γ is induced. Supernatants from the treated splenocytes were harvested after 24 h and tested for the presence of IFN-γ by ELISA (R&D Systems). All samples were prepared in triplicate.

Coadministration of *L. lactis* strains expressing HPV-16 E7 and IL-12. In order to confirm the ability of an *L. lactis* strain to secrete IL-12 and to enhance an antigen-specific T-cell response, we used the HPV-16 E7 protein as an antigen. This protein was successfully expressed previously in *L. lactis* (5). Groups of three mice were immunized (as described above) with 5×10^8 CFU of an *L. lactis* strain displaying a cell wall-anchored E7 antigen [NZ(pCWA-E7) (Cortes-Perez, unpublished data)] alone or in combination with 5×10^8 CFU of strain NZ (pSEC:scIL12). A control group received the wild-type *L. lactis* strain. Splenocytes from immunized animals were used for detection of IL-2 and IFN-γ, cytokines characteristic of a Th1 type of immune response (17, 24, 53).

Determination of Th1 cytokine production in splenocytes. Mice immunized with *L. lactis* strains were sacrificed on day 35. Splenocytes were separated and cultured as described above. Cell suspensions from each different treatment were cultured with 2 μg of a synthetic E7 peptide (positions 49 to 57; RAHYNIVTF) to determine whether in vitro restimulation induced a peptide-specific (i.e., antigen-specific) cellular response or with PBS alone as a control. After 24 h, cell suspensions were filtered, and supernatants were tested for the presence of IL-2 and IFN-γ by ELISA (R&D Systems).

Statistics. Student's *t* test was performed by using MINITAB, a computer software package (Minitab Inc., State College, Pa.).

RESULTS

p35 and p40 subunits are secreted and correctly processed by *L. lactis*. The ability of *L. lactis* to secrete the two IL-12 subunits was tested by using strain NZ(pSEC:p35-p40). Cultures of NZ(pSEC:p35-p40) were harvested after induction (final optical density at 600 nm, ~1). Expression and secretion of the p40 and p35 subunits were analyzed by Western blotting by using anti-IL-12 antiserum (Fig. 3). Protein samples were prepared for cell and supernatant fractions. A pattern of diverse molecular weight forms was obtained for the cell fraction of induced cells, suggesting that there was accumulation of p40 and p35 precursors, as well as proteolysis in the cytoplasm or at the cell surface (Fig. 3A). In contrast, the supernatant fraction produced two distinct bands that migrated at the sizes expected for p35 and p40 (Fig. 3A). For both subunits, the secretion efficiency (i.e., the proportion of the mature form secreted into the supernatant) was low (<15%). Western analyses were performed with noninduced recombinant *L. lactis* strains, and no IL-12 production was detected (data not shown). The approximately equal intensities of the two subunits suggest that the subunits are produced in the proper stoichiometry to form an active IL-12 heterodimer. These results demonstrate that *L. lactis* is able to produce and secrete both IL-12 subunits.

We tested the capacity of *L. lactis* to produce and secrete IL-12p35-p40 in its assembled heterodimeric form. Protein samples were prepared from induced NZ(pSEC:p35-p40) cultures and analyzed by immunoblotting by using anti-IL-12 antiserum after polyacrylamide gel electrophoresis (PAGE) under nondenaturing conditions. A band that comigrated with the IL-12 control was detected in NZ(pSEC:p35-p40) supernatant (data not shown). This band could have corresponded to IL-12 or to a p40 homodimer. A p40 subunit may indeed associate with another p40 subunit to form a homodimer ($p40_2$) with a molecular mass of 80 kDa. A $p40_2$ form reportedly is an antagonist of IL-12 in vitro (37). To determine whether this high-molecular-weight form corresponds to the assembled form of IL-12, culture supernatants of NZ(pSEC:p35-p40) induced for IL-12 synthesis were analyzed by an ELISA that is specific for quantification of native murine IL-12p35-p40 (61). The concentration of secreted IL-12p35-p40 was estimated to be 25 pg/ml (Fig. 3B), compared to 80 pg/ml for an rIL-12 standard. ELISA were also performed with noninduced recombinant *L. lactis* strains, and no IL-12 production was detected (data not shown). Altogether, these results show that (i) *L. lactis* is able to secrete both p35 and p40 subunits and (ii) at least a proportion of these subunits is properly assembled in the supernatant.

Production of IL-12 as a single-chain polypeptide in *L. lactis*. One way to favor proper assembly of the p35 and p40 subunits is to synthesize a fusion protein comprising the two polypeptides. This strategy has already been proven to be ef-

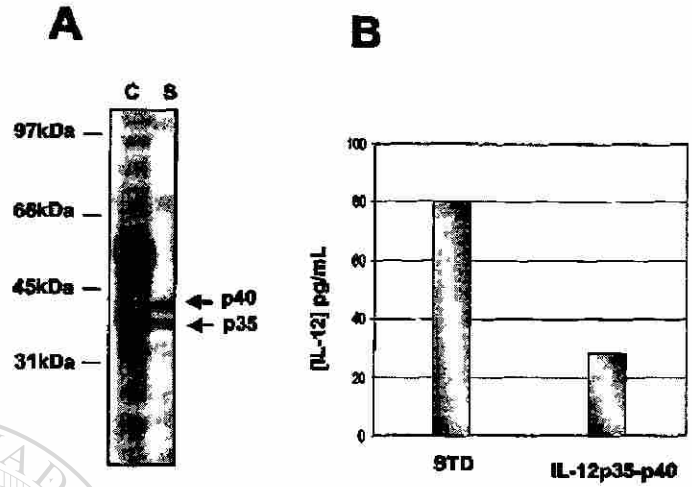

FIG. 3. Production of IL-12 by recombinant *L. lactis*. IL-12 production was analyzed by immunoblotting by using anti-IL-12 antibodies. Protein samples were prepared from induced recombinant *L. lactis* cultures. (A) Immunodetection after SDS-PAGE. Lane C, cell fraction of the NZ(pSEC:p35-p40) strain encoding the p35 and p40 subunits (IL-12p35-p40); lane S, supernatant samples. The positions and sizes of molecular weight markers are indicated on the left. (B) Quantification of IL-12p35-p40 by ELISA (R&D Systems). STD, 80 pg of commercial murine rIL-12 per ml; IL-12p35-p40, supernatant sample of NZ(pSEC:p35-p40) culture.

ficient in eukaryotic systems (15). Expression of scIL-12 was tested in *L. lactis* by using strain NZ(pSEC:scIL-12) and was compared to expression in NZ(pSEC:p35-p40) (Fig. 4A). Induced culture samples were prepared as described above. After induction, Western blot analysis with anti-IL-12 antibody revealed a clear band in the supernatant at the expected size for scIL-12 (70 kDa). The amount of secreted scIL-12 was found to be two- to threefold larger than the amounts of the separate p35 and p40 subunits (Fig. 4A). We also measured the scIL-12 concentration in the supernatant using the ELISA that recognized IL-12 only in the native conformation. The amount of scIL-12 was found to be twofold larger than the amounts of the p35 and p40 subunits (~65 pg/ml for scIL-12 and ~25 pg/ml for IL-12p35-p40) (Fig. 4B). Western blotting and

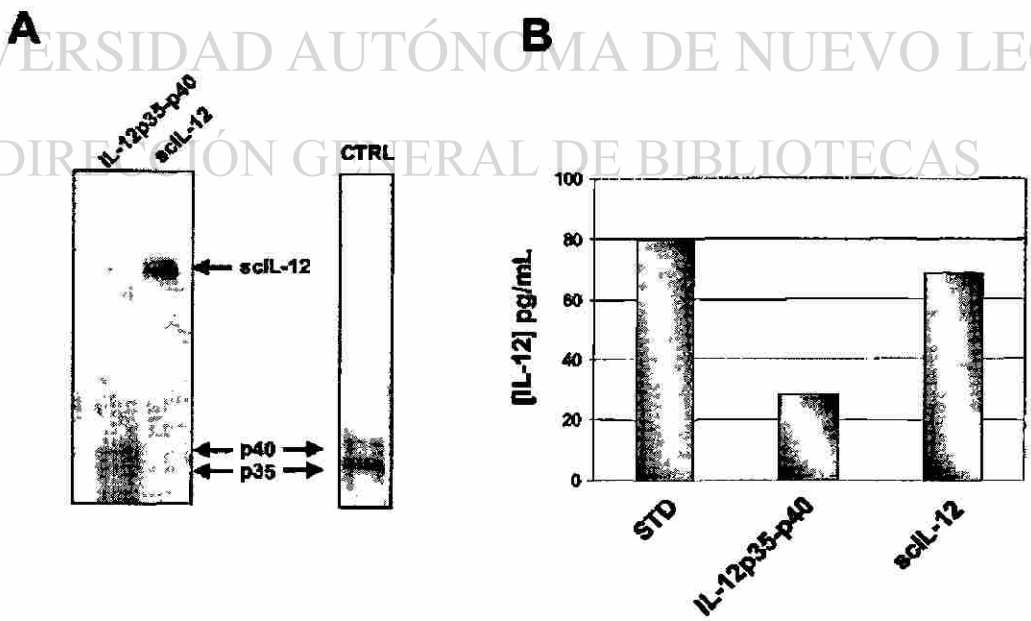

FIG. 4. Secretion analysis of the two IL-12 forms produced by *L. lactis*. (A) rIL-12 production compared by SDS-PAGE and Western blot analysis of supernatant samples prepared from induced cultures of NZ(pSEC:p35-p40) and NZ(pSEC:scIL-12) encoding IL-12p35-p40 and scIL-12, respectively. Lane CTRL contained rIL-12 (R&D Systems) as a control. (B) Quantification of IL-12 forms produced by *L. lactis* by ELISA by using supernatants of induced cultures of NZ(pSEC:p35-p40) and NZ(pSEC:scIL-12) encoding IL-12p35-p40 and scIL-12. STD, 80 pg of commercial rIL-12 per ml.

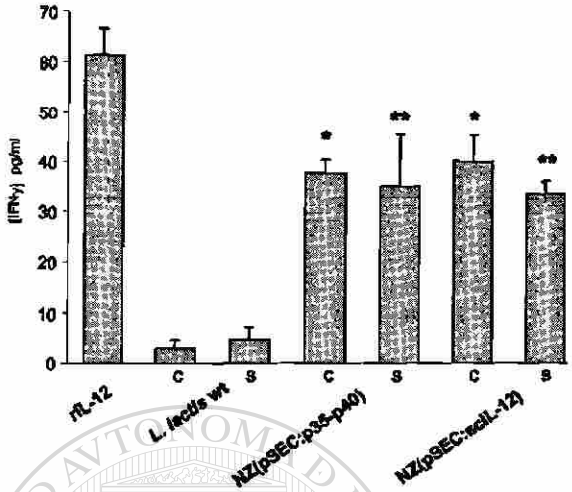

FIG. 5. In vitro induction of IFN-γ in mouse splenocytes by recombinant *L. lactis*. Mouse splenocytes were incubated in the presence of 50 μg of rIL-12 per ml as a control and with cells (C) and supernatant (S) of wild-type *L. lactis* (*L. lactis* wt), *L. lactis* NZ(pSEC:p35-p40) IL-12p35-p40, or *L. lactis* NZ(pSEC:scIL-12) encoding scIL-12. The concentrations of the induced culture samples were adjusted to ~50 pg/ul as determined by ELISA. The IFN-γ concentrations are the means and standard deviations determined in three independent experiments. Significant differences compared to the data obtained for recombinant *L. lactis* cells and supernatant samples are indicated by one asterisk and two asterisks, respectively ($P < 0.05$).

ELISA were performed with noninduced recombinant *L. lactis* strains, and no IL-12 production was detected (data not shown). This result shows that scIL-12 is efficiently secreted in *L. lactis* and is folded into a native conformation. The results described above show that both IL-12p35-p40 and scIL-12 are expressed and secreted in *L. lactis* and suggest that at least portions of these products assume a native and thus potentially active form.

Biological activity of IL-12 produced by recombinant *L. lactis*. Recombinant *L. lactis* strains producing IL-12p35-p40 and scIL-12 were evaluated for the ability to induce IFN-γ production in mouse splenocytes. Splenocytes were cultured for 48 h with 50 pg of commercial rIL-12 per ml or with supernatant or a total culture of recombinant *L. lactis*. The amounts of bacterially produced IL-12 added to splenocytes were adjusted to ~50 pg/ml as determined by the quantitative ELISA. After in vitro IL-12 stimulation, splenocyte culture supernatants were collected to measure the concentrations of IFN-γ (Fig. 5). The results show that 50 pg of commercial rIL-12 per ml induced production of ~60 pg of IFN-γ per ml. The scIL-12 produced by 5×10^8 CFU of *L. lactis* induced production of ~40 pg/ml, and supernatants of the same cells induced production of ~33 pg/ml. The concentrations of IFN-γ induced by an *L. lactis* strain expressing p35-p40 were ~37 pg/ml for 1×10^9 CFU and ~34 pg/ml for the supernatant. Splenocytes in the presence of a wild-type *L. lactis* strain did not produce significant amounts of IFN-γ, as expected (Fig. 5). These results suggest that both scIL-12 and IL-12p35-p40 are biologically active and stimulate IFN-γ production by mouse splenocytes.

Intranasal administration of *L. lactis* expressing IL-12 induces IFN-γ production in mouse splenocytes. The biological activities of the IL-12-producing *L. lactis* strains were also tested in vivo after intranasal administration of induced recombinant *L. lactis* strains in mice. It has been shown previously that a regimen consisting of intranasal administration of IL-12 on days 0, 1, 2, and 3 with booster doses on days 14 and 28 (and repeating the schedule for four inoculations) and sacrifice of the animals on day 35 results in an absence of cytokine toxicity in a murine model (3). However, although this treatment schedule is very productive in mice, its use in human vaccination is limited due to the consecutive IL-12 inoculations during the treatment. To avoid this problem, in this experiment we tested single doses of IL-12-expressing *L. lactis* strains administered on days 0, 14, and 28. We administered 5×10^8 CFU of NZ(pSEC:p35-p40) or NZ(pSEC:scIL-12), which corresponds to quantities used previously for oral administration of recombinant *L. lactis* (50).

IFN-γ expression was significantly enhanced in mice that received *L. lactis* strains expressing IL-12p35-p40 or scIL-12 compared to IFN-γ expression in the placebo control groups (Fig. 6). Mice treated with the scIL-12-producing strain produced the largest amounts of IFN-γ in spleen cells. In contrast, the amounts of IFN-γ in mice treated with the p35-p40-producing strain were fourfold smaller. The differences in the degree of stimulation were probably due to the quantity of

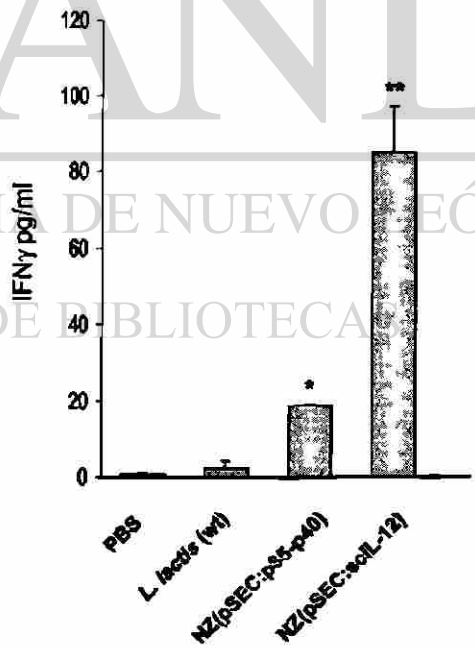

FIG. 6. Production of IFN-γ in mouse splenocytes after intranasal inoculation of recombinant *L. lactis*. Levels of IFN-γ were determined following sacrifice on day 35 for mice that received 5×10^8 CFU of wild-type *L. lactis* [*L. lactis* (wt)], NZ(pSEC:p35-p40), or NZ(pSEC:scIL-12) or PBS alone. The data are representative of one of three separate experiments in which similar results were obtained. The values are the means and standard deviations for three mice per treatment group. Statistically significant differences ($P < 0.05$) compared with the wild-type *L. lactis* and PBS control groups are indicated by one asterisk for the NZ(pSEC:p35-p40) group and by two asterisks for NZ(pSEC:scIL-12) group.

native IL-12 produced under the conditions used (Fig. 3 and 4). This experiment was repeated three times, and similar results were obtained in all cases. The results demonstrated that IL-12 can be effectively administered to mice in vivo by using recombinant *L. lactis*, which results in clear induction of the IFN-γ response. Furthermore, after intranasal administration of the *L. lactis* IL-12-producing strains, IFN-γ production was induced without apparent toxicity, and mice remained healthy after 24 weeks of treatment.

Because the concentrations of functional IL-12 measured in both in vitro and in vivo assays were greater for scIL-12 than for the two-subunit IL-12 form, we chose the single-chain form for the next in vivo experiment.

Intranasal coadministration of recombinant *L. lactis* strains expressing active IL-12 and HPV-16 E7 enhanced IFN-γ production. In order to examine the adjuvant properties of the recombinant *L. lactis* strain producing IL-12, the immune response to a coadministered antigen was analyzed. The antigen that was coexpressed with IL-12 was the HPV-16 E7 protein, the major worldwide etiological agent of cervical cancer. Groups of five C57BL/6 mice were immunized intranasally with three doses (on days 0, 14, and 28) of 5×10^8 CFU of NZ(pCWA-E7) alone or in combination with 5×10^8 CFU of NZ(pSEC:scIL12). The production of IL-2 and IFN-γ was then determined (Fig. 7). Spleen cells that were obtained 1 week after the last inoculation with recombinant *L. lactis* (day 35) and were restimulated in vitro with a synthetic E7 peptide (RAHYNIVTF) produced significant levels of IL-2 (Fig. 7A) and IFN-γ (Fig. 7B). As an in vitro control, spleen cells were restimulated with PBS alone. The responses were greater in mice immunized with an *L. lactis* strain displaying a cell wall-anchored E7 antigen than in animals immunized with a wild-type *L. lactis* strain. Strikingly, the antigen-specific cellular response measured by secretion of Th1 cytokines elicited by *L. lactis* expressing E7 antigen alone was dramatically increased by coadministration with an *L. lactis* strain secreting IL-12 protein (Fig. 7).

DISCUSSION

In this study, we produced bioactive forms of IL-12 in the food-grade gram-positive bacterium *L. lactis*, and we showed that the recombinant bacterium has a stimulatory effect on IFN-γ production in both in vitro and in vivo assays. Previous reports of IL-12 production involved the use of eukaryotic systems (15, 29, 52, 57), which may have limitations in broad-scale or in vivo applications. Recently, Steidler et al. demonstrated that *L. lactis* could be used to produce and secrete biologically active murine monomeric cytokines (55, 56). The production of a more complex molecule (i.e., a heterodimer that contains several DSB) further extends the potential of *L. lactis* to deliver therapeutic molecules in vivo.

IL-12 is a heterodimer composed of two distinct subunits (p35 and p40) encoded by separate genes that are coordinately expressed. Previous studies have demonstrated that p40 overexpression can have an inhibitory effect on IL-12 activity (37). We used two approaches to overcome this potential problem. First, we developed a bicistronic cassette for coexpression of p35 and p40 subunits in *L. lactis*. Second, we designed a vector that expressed IL-12 as a single-chain polypeptide, thus allowing stoichiometric formation of this cytokine. This strategy also overcomes problems with inefficient association of independently produced subunits or formation of homodimers (20, 37). Consistent with this hypothesis, under equivalent induction conditions, the concentrations of functional IL-12 were greater for scIL-12 than for the two-subunit IL-12 form. In view of the lower activities of p35 and p40, the single-chain form of IL-12 may be preferred for in vivo applications.

Remarkably, mouse IL-12 contains 7 and 13 cysteines in its p35 and p40 subunits, respectively, and two DSB that are essential for proper IL-12 assembly (47). The secretion of biologically active IL-12 suggests that DSB are formed after the protein is exported from *L. lactis*. DSB formation is often a major bottleneck in heterologous protein production in prokaryotic systems and particularly in gram-positive bacteria, which themselves encode very few secreted proteins that contain DSB (44, 46). Possibly, the lower pH of *L. lactis* during fermentative growth favors formation of DSB in secreted proteins. Although the mechanism remains to be proven, this system may be promising for expression of other proteins containing DSB.

The main biological effect of IL-12 is stimulation of IFN-γ production. This cytokine has both adjuvant and antitumor activities. Because a number of subunit vaccines are poorly immunogenic, the use of adjuvants is of particular interest for new formulations of vaccines against infectious diseases. To enhance the mucosal immune response, adjuvants such as cholera toxin and *E. coli* enterotoxin have been used, and they indeed induce potent Th1 and Th2 cell responses. However, these adjuvants cause severe diarrhea and are not suitable for use as mucosal adjuvants in humans. Strikingly, IL-12 has proven adjuvant activity when it is coexpressed with an antigen in targeted vaccines (1, 6). It may also prevent the development of immunological tolerance to a given antigen (54). Finally, IL-12 has potent antitumor effects and may be an attractive agent for cancer immunotherapy.

Despite the efficacy of IL-12 therapy for cancer and infectious diseases, experimental models in clinical trials with systemic IL-12 showed unacceptable levels of toxicity related to elevated IFN-γ production (9, 36). The limitations of IL-12 treatment include the need for daily administration (27). Here, to circumvent this problem, we explored mucosal (intranasal) delivery of active IL-12 by using the safe vector *L. lactis*, which repeatedly has been reported to be noninvasive and noncolonizing in a murine model (12, 16, 22). Recently, a recombinant *L. lactis* strain delivering IL-10 via an oral route exhibited positive effects during treatment of murine colitis. The dose of IL-10 given orally was estimated to be 10-fold lower than the dose required for systemic administration (56). Targeted administration of other interleukins, such as IL-12, to the intestinal tract by food-grade *L. lactis* may also reduce toxicity and have advantages compared to treatment by the systemic route, and it may even maximize the response (39).

There is continual interest in developing mucosally based vaccines for a variety of different pathogens, including HPV. The use of live oral delivery systems for tumor therapy or vaccine delivery may thus reduce toxic side effects resulting from systemic administration. In this study, we showed the adjuvant effect of a recombinant *L. lactis* strain producing IL-12 protein which enhanced the mucosal immune responses

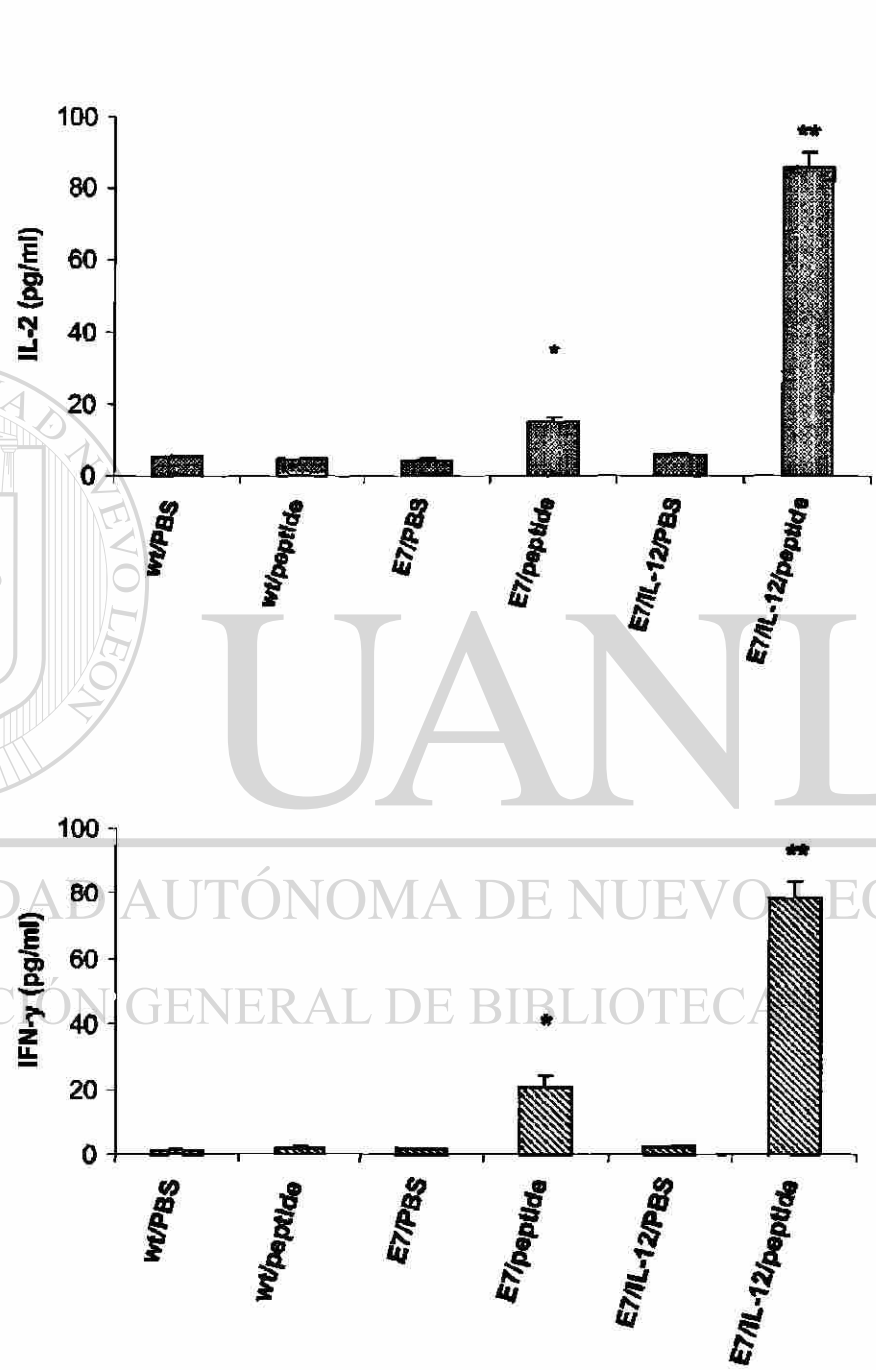

FIG. 7. Production of Th1 cytokines by splenocytes of mice immunized with recombinant *L. lactis*. Levels of Th1 cytokines were determined following sacrifice on day 35 for mice immunized with 5×10^8 CFU of wild-type *L. lactis* (wt) or recombinant *L. lactis* displaying E7 antigen (E7) and for mice coimmunized with *L. lactis* displaying E7 and an *L. lactis* strain secreting active murine IL-12 (E7/IL-12). Spleen cells were cultured for 24 h with 2 μg of E7 peptide (RAHYNIVTF) (peptide) or PBS, and the levels of the Th1 cytokines IL-2 (A) and IFN-γ (B) in the culture supernatants were determined by ELISA. The values are the means and standard deviations for three mice per treatment group. Statistically significant differences ($P < 0.05$) compared to the E7/PBS group are indicated by one asterisk and by two asterisks for the E7/peptide and E7/IL-12/peptide groups, respectively.

against a coadministered antigen. The IL-2 and IFN-γ production elicited by a recombinant *L. lactis* strain displaying a cell wall-anchored HPV-16 E7 antigen was dramatically increased by coadministration with an *L. lactis* strain secreting IL-12 protein.

It is well established that IL-12 plays an essential role in switching of the immune response, inducing Th1 cells and suppressing Th2 responses (58). On the other hand, the elevated density of Th2 cells during the pathogenesis of advanced cervical cancer is well known, while the level of Th1 cells is dramatically diminished (2, 18). We believe that successful immunotherapeutic treatments of cervical cancer patients will use a vaccine that will be able to switch the immune response from the Th2 class to the Th1 class. Therefore, on the basis of this belief, an *L. lactis* strain modified to secrete IL-12 together with a specific antigen is a good candidate for cervical cancer therapy.

In summary, for vaccine applications, oral or nasal delivery may provoke local immune responses at the portal of entry of most pathogens. The use of *L. lactis* to deliver IL-12 to a mucosal surface (e.g., the intranasal surface, gut, or vaginal mucosa) may have clear advantages over a systemic therapy approach because it reduces toxic side effects and provides a low-cost, simple method of administration.

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REFERENCES

1. **Afonso, L. C., T. M. Scharton, L. Q. Vieira, M. Wysocka, G. Trinchieri, and P. Scott.** 1994. The adjuvant effect of interleukin-12 in a vaccine against *Leishmania major*. Science 263:235–237.
2. **al-Saleh, W., S. L. Giannini, N. Jacobs, M. Moutschen, J. Doyen, J. Boniver, and P. Delvenne.** 1998. Correlation of T-helper secretory differentiation and types of antigen-presenting cells in squamous intraepithelial lesions of the uterine cervix. J. Pathol. 184:283–290.
3. **Arulanandam, B. P., M. O'Toole, and D. W. Metzger.** 1999. Intranasal interleukin-12 is a powerful adjuvant for protective mucosal immunity. J. Infect. Dis. 180:940–994.
4. **Barth, H., R. Klein, P. A. Berg, B. Wiedenmann, U. Hopf, and T. Berg.** 2001. Induction of T helper cell type 1 response and elimination of HBeAg during treatment with IL-12 in a patient with therapy-refractory chronic hepatitis B. Hepatogastroenterology 48:553–555.
5. **Bermúdez-Humarán, L. G., P. Langella, A. Miyoshi, A. Gruss, R. S. Tamez-Reyes, R. Montes de Oca-Luna, and Y. Le Loir.** 2002. Production of human papillomavirus type 16 E7 protein in *Lactococcus lactis*. Appl. Environ. Microbiol. 68:917–922.
6. **Boyaka, P. N., and J. R. McGhee.** 2001. Cytokines as adjuvants for the induction of mucosal immunity. Adv. Drug Deliv. Rev. 51:71–79.
7. **Brunda, M., L. Luistro, R. Warrier, R. Wright, B. Hubbard, M. Murphy, S. Wolf, and M. K. Gately.** 1993. Antitumor and antimetastatic activity of interleukin 12 against murine tumors. J. Exp. Med. 178:1223–1230.
8. **Chan, S. H., B. Perussia, J. W. Gupta, M. Kobayashi, M. Pospisil, H. A. Young, S. G. Wolf, D. Young, S. C. Clark, and G. Trinchieri.** 1991. Induction of interferon gamma production by natural killer cell stimulatory factor: characterization of the responder cells and synergy with other inducers. J. Exp. Med. 173:869–879.
9. **Chen, B., T. M. Timiryasova, D. S. Gridley, M. L. Andres, R. Dutta-Roy, and I. Fodor.** 2001. Evaluation of cytokine toxicity induced by vaccinia virus-mediated IL-2 and IL-12 antitumour immunotherapy. Cytokine 15:305–334.
10. **Cohen, J.** 1995. IL-12 deaths: explanation and a puzzle. Science 270:908.
11. **de Ruyter, P. G., O. P. Kuipers, and W. M. de Vos.** 1996. Controlled gene expression systems for *Lactococcus lactis* with the food-grade inducer nisin. Appl. Environ. Microbiol. 62:3662–3667.
12. **Dronault, S., G. Corthier, S. D. Ehrlich, and P. Renault.** 1999. Survival, physiology, and lysis of *Lactococcus lactis* in the digestive tract. Appl. Environ. Microbiol. 65:4881–4886.
13. **Enouf, V., P. Langella, J. Commissaire, J. Cohen, and G. Corthier.** 2001. Bovine rotavirus nonstructural protein 4 produced by *Lactococcus lactis* is antigenic and immunogenic. Appl. Environ. Microbiol. 67:1423–1428.
14. **Gasson, M. J.** 1983. Plasmid complements of *Streptococcus lactis* NCDO 712 and other lactic acid streptococci after protoplast-induced curing. J. Bacteriol. 154:1–9.
15. **Gaucher, D., and K. Chadee.** 2001. Molecular cloning of gerbil interleukin 12 and its expression as a bioactive single-chain protein. Cytokine 14:177–183.
16. **Geoffroy, M. C., C. Guyard, B. Quatannens, S. Pavan, M. Lange, and A. Mercenier.** 2000. Use of green fluorescent protein to tag lactic acid bacterium strains under development as live vaccine vectors. Appl. Environ. Microbiol. 66:383–391.
17. **Gherardi, M. M., J. C. Ramirez, and M. Esteban.** 2000. Interleukin-12 (IL-12) enhancement of the cellular immune response against human immunodeficiency virus type 1 Env antigen in a DNA prime/vaccinia virus boost vaccine regimen is time and dose dependent: suppressive effects of IL-12 boost are mediated by nitric oxide. J. Virol. 74:6278–6286.
18. **Ghim, S. J., J. Sundberg, G. Delgado, and A. B. Jenson.** 2001. The pathogenesis of advanced cervical cancer provides the basis for an empirical therapeutic vaccine. Exp. Mol. Pathol. 71:181–185.
19. **Gibson, T. J.** 1984. Ph.D. thesis. University of Cambridge, Cambridge, England.
20. **Gillessen, S., D. Carvajal, P. Ling, F. J. Podlaski, D. L. Stremlo, P. C. Familletti, U. Gubler, D. H. Presky, A. S. Stern, and M. K. Gately.** 1995. Mouse interleukin-12 (IL-12) p40 homodimer: a potent IL-12 antagonist. Eur. J. Immunol. 25:200–206.
21. **Graham, B. S., G. S. Henderson, Y. W. Tang, X. Lu, K. M. Neuzil, and D. G. Colley.** 1993. Priming immunization determines T helper cytokine mRNA expression patterns in lungs of mice challenged with respiratory syncytial virus. J. Immunol. 151:2032–2040.
22. **Grangette, C., H. Muller-Alouf, M. Geoffroy, D. Goudercourt, M. Turneer, and A. Mercenier.** 2002. Protection against tetanus toxin after intragastic administration of two recombinant lactic acid bacteria: impact of strain variability and in vivo persistence. Vaccine 20:3304–3309.
23. **Gubler, U., A. O. Chua, D. S. Schoenhaut, C. M. Dwyer, W. McComas, R. Motyka, N. Nabavi, A. G. Wolitzky, P. M. Quinn, P. C. Familletti, and M. K. Gately.** 1991. Coexpression of two distinct genes is required to generate secreted bioactive cytotoxic lymphocyte maturation factor. Proc. Natl. Acad. Sci. USA 88:4143–4147.
24. **Hoover, D. L., R. M. Crawford, L. L. Van De Verg, M. J. Izadjoo, A. K. Bhattacharjee, C. M. Paranavitana, R. L. Warren, M. P. Nikolich, and T. L. Hadfield.** 1999. Protection of mice against brucellosis by vaccination with *Brucella melitensis* WR201(16MDeltapurEK). Infect. Immun. 67:5877–5884.
25. **Hultgren, O. H., M. Stenson, and A. Tarkowski.** 2001. Role of IL-12 in *Staphylococcus aureus*-triggered arthritis and sepsis. Arthritis Res. 3:41–47.
26. **Jacobson, M. A., D. Hardy, E. Connick, J. Watson, and M. DeBruin.** 2000. Phase 1 trial of a single dose of recombinant human interleukin-12 in human immunodeficiency virus-infected patients with 100–500 CD4 cells/microl. J. Infect. Dis. 182:1070–1076.
27. **Jiang, C., D. M. Magee, and R. A. Cox.** 1999. Construction of a single-chain interleukin-12-expressing retroviral vector and its application in cytokine gene therapy against experimental coccidioidomycosis. Infect. Immun. 67:2996–3001.
28. **Kobayashi, M., L. Fitz, M. Ryan, R. M. Hewick, S. C. Clark, S. Chan, R. Loudon, F. Sherman, B. Perussia, and G. Trinchieri.** 1989. Identification and purification of natural killer cell stimulatory factor (NKSF), a cytokine with multiple biologic effects on human lymphocytes. J. Exp. Med. 170:827–845.
29. **Kokuho, T., S. Watanabe, Y. Yokomizo, and S. Inumaru.** 1999. Production of biologically active, heterodimeric porcine interleukin-12 using a monocistronic baculoviral expression system. Vet. Immunol. Immunopathol. 72:289–302.
30. **Kuipers, O. P., P. G. de Ruyter, M. Kleerebezem, and W. M. de Vos.** 1998. Quorum sensing-controlled gene expression in lactic acid bacteria. J. Biotechnol. 64:15–21.
31. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685.
32. **Langella, P., Y. Le Loir, S. D. Ehrlich, and A. Gruss.** 1993. Efficient plasmid mobilization by pIP501 in *Lactococcus lactis* subsp. *lactis*. J. Bacteriol. 175:5806–5813.
33. **Lee, M. H., Y. Roussel, M. Wilks, and S. Tabaqchali.** 2001. Expression of *Helicobacter pylori* urease subunit B gene in *Lactococcus lactis* MG1363 and its use as a vaccine delivery system against *H. pylori* infection in mice. Vaccine 19:3927–3935.
34. **Lee, S., M. Zheng, S. Deshpande, S. K. Eo, T. A. Hamilton, and B. T. Rouse.** 2002. IL-12 suppresses the expression of ocular immunoinflammatory lesions by effects on angiogenesis. J. Leukoc. Biol. 71:469–476.
35. **Le Loir, Y., A. Gruss, S. D. Ehrlich, and P. Langella.** 1998. A nine-residue

synthetic propeptide enhances secretion efficiency of heterologous proteins in *Lactococcus lactis*. J. Bacteriol. **180**:1895–1903.
36. **Leonard, J. P., M. L. Sherman, G. L. Fisher, L. J. Buchanan, G. Larsen, M. B. Atkins, J. A. Sosman, J. P. Dutcher, N. J. Vogelzang, and J. L. Ryan.** 1997. Effects of single-dose interleukin-12 exposure on interleukin-12-associated toxicity and interferon-gamma production. Blood **90**:2541–2548.
37. **Ling, P., M. K. Gately, U. Gubler, A. S. Stern, P. Lin, K. Hollfelder, C. Su, Y.-C. E. Pan, and J. Hakimi.** 1995. Human IL-12 p40 homodimer binds to the IL-12 receptor but does not mediate biologic activity. J. Immunol. **154**: 116–127.
38. **Lui, V. W., L. D. Falo, and L. Huang.** 2001. Systemic production of IL-12 by naked DNA mediated gene transfer: toxicity and attenuation of transgene expression in vivo. J. Gene Med. **3**:384–393.
39. **Magee, D. M., and R. A. Cox.** 1996. Interleukin-12 regulation of host defenses against *Coccidioides immitis*. Infect. Immun. **64**:3609–3613.
40. **Mazzolini, G., I. Narvaiza, A. Perez-Diez, M. Rodriguez-Calvillo, C. Qian, B. Sangro, J. Ruiz, J. Prieto, and I. Melero.** 2001. Genetic heterogeneity in the toxicity to systemic adenoviral gene transfer of interleukin-12. Gene Ther. **8**:259–267.
41. **Metzger, D. W., R. M. McNutt, J. T. Collins, J. M. Buchanan, V. H. Van Cleave, and W. A. Dunnick.** 1997. Interleukin-12 acts as an adjuvant for humoral immunity through interferon-gamma-dependent and -independent mechanisms. Eur. J. Immunol. **27**:1958–1965.
42. **Michel, N., W. Osen, L. Gissmann, T. N. Schumacher, H. Zentgraf, and M. Muller.** 2002. Enhanced immunogenicity of HPV 16 E7 fusion proteins in DNA vaccination. Virology **294**:47–59.
43. **Nastala, C., H. Edington, T. McKinney, H. Tahara, M. Nalesnik, M. Brunda, M. K. Gately, S. Wolf, R. Schreiber, W. Storkus, and M. Lotze.** 1994. Recombinant IL-12 administration induces tumor regression in association with IFN-gamma production. J. Immunol. **153**:1697–1703.
44. **Paik, S. H., A. Chakicherla, and J. N. Hansen.** 1998. Identification and characterization of the structural and transporter genes for, and the chemical and biological properties of, sublancin 168, a novel lantibiotic produced by *Bacillus subtilis* 168. J. Biol. Chem. **273**:23134–23142.
45. **Park, A. Y., B. Hondowicz, M. Kopf, and P. Scott.** 2002. The role of IL-12 in maintaining resistance to *Leishmania major*. J. Immunol. **168**:5771–5777.
46. **Payne, M. S., and E. N. Jackson.** 1991. Use of alkaline phosphatase fusions to study protein secretion in *Bacillus subtilis*. J. Bacteriol. **173**:2278–2282.
47. **Podlaski, F. J., Y. B. Nanduri, J. D. Hulmes, Y. C. Pan, W. Levin, W. Danho, R. Chizzonite, M. K. Gately, and A. S. Stern.** 1992. Molecular characterization of interleukin 12. Arch. Biochem. Biophys. **294**:230–237.
48. **Poquet, I., S. D. Ehrlich, and A. Gruss.** 1998. An export-specific reporter designed for gram-positive bacteria: application to *Lactococcus lactis*. J. Bacteriol. **180**:1904–1912.
49. **Rakhmilevich, A. L., J. Turner, M. J. Ford, D. McCabe, W. H. Sun, P. M. Sondel, K. Grota, and N.-Y. Yang.** 1996. Gene gun-mediated skin transfection with interleukin 12 gene results in regression of established and metastatic murine tumors. Proc. Natl. Acad. Sci. USA **93**:6291–6296.
50. **Robinson, K., L. M. Chamberlain K. M. Schofield, J. M. Wells, and R. W.** 1997. Oral vaccination of mice against tetanus with recombinant *Lactococcus lactis*. Nat. Biotechnol. **15**:653–657.
51. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
52. **Shen, H., C. Li, M. Lin, Z. Zhang, and Q. Shen.** 1998. Expression of human interleukin 12 (hIL-12) in insect cells. Chin. J. Biotechnol. **14**:205–212.
53. **Sin, J. I., J. J. Kim, R. L. Arnold, K. E. Shroff, D. McCallus, C. Pachuk, S. P. McElhiney, M. W. Wolf, S. J. Pompa-de Bruin, T. J. Higgins, R. B. Ciccarelli, and D. B. Weiner.** 1999. IL-12 gene as a DNA vaccine adjuvant in a herpes mouse model: IL-12 enhances Th1-type $CD4^+$ T cell-mediated protective immunity against herpes simplex virus-2 challenge. J. Immunol. **162**:2912–2921.
54. **Sinkovics, J. G., and J. C. Horvath.** 2000. Vaccination against human cancers. Int. J. Oncol. **16**:81–96.
55. **Steidler, L., K. Robinson, L. Chamberlain, K. M. Schofield, E. Remaut, R. W. Le Page, and J. M. Wells.** 1998. Mucosal delivery of murine interleukin-2 (IL-2) and IL-6 by recombinant strains of *Lactococcus lactis* coexpressing antigen and cytokine. Infect. Immun. **66**:3183–3189.
56. **Steidler, L., W. Hans, L. Schotte, S. Neirynck, F. Obermeier, W. Falk, W. Fiers, and E. Remaut.** 2000. Treatment of murine colitis by *Lactococcus lactis* secreting interleukin-10. Science **289**:1352–1355.
57. **Takehara, K., T. Nagata, R. Kikuta, T. Takanashi, S. Yoshiya, and A. Yamaga.** 2000. Expression of a bioactive interleukin-12 using baculovirus. Vet. Immunol. Immunopathol. **77**:15–25.
58. **Trinchieri, G.** 1995. Interleukin-12: a proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity. Annu. Rev. Immunol. **13**:251–276.
59. **van Asseldonk, M., G. Rutten, M. Oteman, R. J. Siezen, W. M. de Vos, and G. Simons.** 1990. Cloning, expression in *Escherichia coli* and characterization of *usp45*, a gene encoding a highly secreted protein from *Lactococcus lactis* MG1363. Gene **95**:155–160.
60. **Voest, E. E., B. M. Kenyon, M. S. O'Reilly, G. Truitt, R. J. D'Amato, and J. Folkman.** 1995. Inhibition of angiogenesis *in vivo* by interleukin 12. J. Natl. Cancer Inst. **19**:581–586.
61. **Wilkinson, V. L., R. R. Warrier, T. P. Truitt, P. Nunes, M. K. Gately, and D. H. Presky.** 1996. Characterization of anti-mouse IL-12 monoclonal antibodies and measurement of mouse IL-12 by ELISA. J. Immunol. Methods **189**:15–24.

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Intranasal Immunization with Recombinant *Lactococcus lactis* Secreting Murine Interleukin-12 Enhances Antigen-Specific Th1 Cytokine Production

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Interleukin-12 (IL-12), a heterodimeric cytokine, plays an important role in cellular immunity to several bacterial, viral, and parasitic infections and has adjuvant activity when it is codelivered with DNA vaccines. IL-12 has also been used with success in cancer immunotherapy treatments. However, systemic IL-12 therapy has been limited by high levels of toxicity. We describe here inducible expression and secretion of IL-12 in the food-grade lactic acid bacterium *Lactococcus lactis*. IL-12 was expressed as two separate polypeptides (p35-p40) or as a single recombinant polypeptide (scIL-12). The biological activity of IL-12 produced by the recombinant *L. lactis* strain was confirmed in vitro by its ability to induce gamma interferon (IFN-γ) production by mouse splenocytes. Local administration of IL-12-producing strains at the intranasal mucosal surface resulted in IFN-γ production in mice. The activity was greater with the single polypeptide scIL-12. An antigen-specific cellular response (i.e., secretion of Th1 cytokines, IL-2, and IFN-γ) elicited by a recombinant *L. lactis* strain displaying a cell wall-anchored human papillomavirus type 16 E7 antigen was dramatically increased by coadministration with an *L. lactis* strain secreting IL-12 protein. Our data show that IL-12 is produced and secreted in an active form by *L. lactis* and that the strategy which we describe can be used to enhance an antigen-specific immune response and to stimulate local mucosal immunity.

Interleukin-12 (IL-12) is a multifunctional cytokine that was originally described as a maturation factor for cytotoxic (T) lymphocytes and a cell stimulatory factor for natural killer cells (8, 23, 28). IL-12 is a heterodimeric glycoprotein composed of two disulfide-linked chains (p35 and p40) that has numerous effects on T and natural killer cells, resulting in enhancement of cytotoxic activity and induction of gamma interferon (IFN-γ) production. In experimental models, IL-12 has been shown to be involved in protection against several bacterial, viral, and parasitic infections (4, 25, 45). This cytokine has also been shown to block angiogenesis (34, 60). Finally, the immunomodulatory effects of IL-12 are reportedly beneficial in AIDS treatment (26). The immunostimulatory properties of IL-12 have led to experimentation with its use as a vaccine adjuvant (1, 3). In addition, IL-12 stimulates serum immunoglobulin G antibody responses and helps during differentiation of Th0 cells into Th1 cells (17, 21, 41). This is particularly interesting for vaccine development for antigens that are poorly immunogenic.

Local administration of IL-12 confers antitumor activity in vivo that results in regression of established tumors and reduction of metastasis in animal models (7, 43, 49). Nevertheless, systemic IL-12 therapy can have toxic effects in animals and humans (36, 38, 39) and has been a cause of mortality in clinical trials (10, 36). For example, intratumoral treatment of mice with a vaccinia virus expressing IL-12 resulted in significant tumor growth inhibition but also induced clear signs of toxicity (9).

Il-12 has also been considered for use as an adjuvant in vaccine therapies. Current therapies involving mucosal routes are limited by a lack of suitable adjuvants that can be safely given to humans. Cholera toxin and *Escherichia coli* enterotoxin are potent mucosal adjuvants but frequently cause secondary effects, such as severe diarrhea and induction of Th2 responses that can lead to undesirable immune responses (21). Although various IL-12 delivery systems based on retroviral vectors and gene gun techniques have been described (38, 40, 49), an efficient and cost-effective means of delivery remains to be developed.

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TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Replicon	Genotype or characteristics	Reference or source
Strains			
E. coli TG1		*supE hsdΔ5 thi Δ(lac-proAB) F'(traD36 proAB$^+$ lacI lacZΔM15)*	19
L. lactis MG1363		Wild type, plasmid free	14
L. lactis NZ9000		MG1363 (*nisRK* genes in chromosome), plasmid free	30
Plasmids			
pWRG3169	ColE1	Ap^r, pBS derivative containing coding sequences for p35 and p40 subunits	49
pCR:TOPO	ori pUC	Ap^r	Invitrogen
pCR-TOPO:Δp35exon1	ori pUC	Ap^r, DNA fragment encoding first exon of p35 subunit devoid of its SP	This study
pCR-TOPO:p35exon2	ori pUC	Ap^r, DNA fragment encoding second exon of p35 subunit	This study
pCR-TOPO:Δp35	ori pUC	Ap^r, DNA fragment encoding p35 mature moiety devoid of intron	This study
pVE8001	ColE1	Ap^r, pBS derivative containing *trpA* transcription terminator	48
pBS:Δp40:trpA	ColE1	Ap^r, DNA fragment encoding p40 mature moiety devoid of its SP plus *trpA*	This study
pVE8022	ColE1/pAMβ	Ap^r Em^r, pFUN derivative containing Exp4-ΔSP_{Nuc} fusion	48
pBS:SP_{Exp4}	ColE1	Ap^r, PCR fragment encoding SP_{Exp4} SP	This study
pBS:SP_{Exp4}:p40:trpA	ColE1	Ap^r, gene expressed from P_{nisA} encodes SP_{Usp}-*p40:trpA* p40 subunit precursor	This study
pSEC:E7	pWV01	Cm^r, gene expressed from P_{nisA} encodes SP_{Usp}-*E7* precursor	5
pSEC:p35	pWV01	Cm^r, gene expressed from P_{nisA} encodes SP_{Usp}-*p35* p35 subunit precursor	This study
pSEC:p35-p40	pWV01	Cm^r, gene expressed from P_{nisA} encodes SP_{Usp}-*p35-p40* p35 and p40 subunit precursors	This study
pCDNA3:IL-12	ColE1	Ap^r, PCR fragment encoding *IL-12* single chain	P. Melby[a]
pCR:TOPO:scIL-12	ori pUC	Ap^r, PCR fragment encoding *scIL-12* single chain devoid of its SP	This study
pSEC:scIL-12	pWV01	Cm^r, gene expressed from P_{nisA} encodes SP_{Usp}-*IL-12* single-chain precursor	This study

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The gram-positive and nonpathogenic lactic acid bacteria are considered promising candidates for the development of oral live vaccines. *Lactococcus lactis*, the model lactic acid bacterium, has been extensively engineered for the production of heterologous proteins (5, 13, 16, 35), including some antigens of bacterial or viral origin (5, 13, 33). *L. lactis* is of particular interest for oral delivery of functional proteins since it is a noncommensal, food bacterium that does not survive in the digestive tracts of animal models and humans (12, 16). In the case of IL-12 cytokine delivery, these properties could ensure transient expression of the protein, thereby limiting the risks of toxicity.

IL-12 production requires assembly of two subunits involving two disulfide bonds (DSB) (47). In gram-negative bacteria, DSB mediate protein folding during export; however, the final destination of exported proteins is the periplasm. In contrast, DSB formation is poorly documented in gram-positive bacteria. Only a few extracytoplasmic proteins with DSB have been identified, and to our knowledge, exported heterologous proteins containing DSB have been reported only in *Bacillus subtilis* (44, 46). However, in contrast to gram-negative bacteria, protein secretion in gram-positive bacteria leads to release of the protein into the medium, which provides an immediate advantage for a delivery system. Until now, secretion of a heterologous protein containing DSB by *L. lactis* has not been reported. In this study we demonstrated the capacity of *L. lactis* to produce and secrete a biologically active form of IL-12, a complex two-subunit cytokine with two DSB that are essential for its activity (47). We found that IL-12-producing recombinant *L. lactis* strains induce IFN-γ production in splenocyte cultures and after intranasal administration in mice.

Additionally, the potential adjuvant properties of an *L. lactis* strain secreting IL-12 were examined in combination with the human papillomavirus type 16 (HPV-16) E7 antigen. This antigen, which has been implicated in the progression of cervical cancer, is considered a potential candidate antigen for anticancer vaccine development. One drawback for its use is its poor induction of a cellular immune response (42). Administration of an *L. lactis* strain displaying a cell wall-anchored HPV-16 E7 antigen was significantly enhanced after coadministration of an *L. lactis* strain secreting IL-12 protein, corroborating the hypothesis that the recombinant strain described here is a promising candidate for mucosal codelivery of proteins of medical interest. This work marks a new step in the development of live protein presentation systems for nasal and/or oral administration.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this work are listed in Table 1. *L. lactis* was grown in M17 medium supplemented with 1% glucose or in brain heart infusion at 30°C without agitation. *Escherichia coli* was grown in Luria-Bertani medium at 37°C. Unless otherwise indicated, plasmid constructs were first established in *E. coli* and then transferred to *L. lactis* by electrotransformation (32, 51). Plasmids were selected by addition of antibiotics, as follows: 5 μg of chloramphenicol per ml for *L. lactis*, 10 μg of chloramphenicol per ml for *E. coli*, and 100 μg of ampicillin per ml for *E. coli*.

DNA manipulation and methods used. Plasmid DNA isolation and general procedures for DNA manipulation were performed essentially as described previously (51). PCR amplification was performed with a Perkin-Elmer Cetus (Norwalk, Conn.) apparatus by using Vent DNA polymerase (Promega). DNA sequences were confirmed by using a Dye terminator sequencing kit.

Deletion of an intron in the p35 subunit. The genes encoding p35 and p40 of murine IL-12 were isolated from pWRG3169, a vector previously described as functional in a eukaryotic system (kindly provided by Alexander Rakhmilevich) (Table 1) (49). Sequence analysis of pWRG3169 revealed an intron in the p35 subunit (data not shown). As introns are spliced in mammalian cells but not in prokaryotes, directed mutagenesis by PCR was performed to remove the p35 intron (Fig. 1). Briefly, the two exons were PCR amplified, subcloned into a pCR-TOPO kit (Invitrogen, Carlsbad, Calif.), and ligated to generate a DNA segment encoding the p35 mature moiety devoid of the intron and of its signal peptide (SP) (Δp35). The first exon was amplified by using primer 5'-p35-start (5'-GATGCATCAGAGAGGGTCATTCCAGTCTCTGGA-3') for the coding

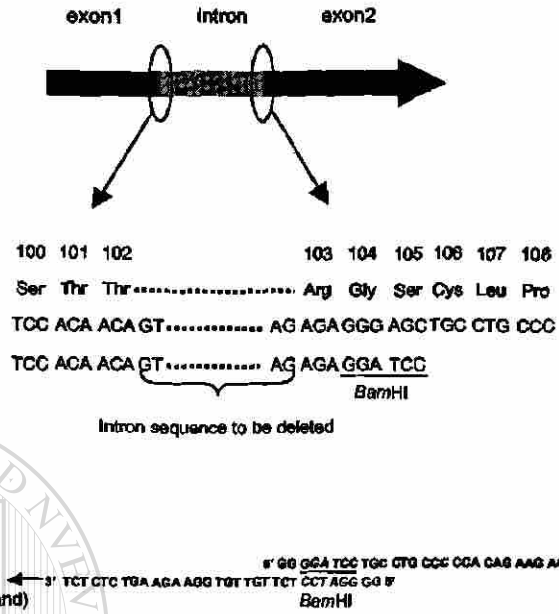

FIG. 1. Site-specific mutagenesis. The p35 intron was eliminated by site-directed mutagenesis. Briefly, we inserted a *Bam*HI restriction site (underlined) in the three codons (encoding Arg, Gly, Ser) to the right of the intron. Primers were designed to PCR amplify two fragments of p35 that were joined at the *Bam*HI site, such that p35 was reconstituted but intron free.

strand and primer 5'-p35 (5'-GGGGATCCTCTTGTTGTGGAAGAAGTCTC TCT-3') for the complementary strand (Fig. 1). The second exon was amplified by using primer 3'-p35 (5'-GGGGATCCTGCCTGCCCCCACAGAAGACG-3') for the coding strand and primer 3'-p35-stop (5'-GGAATTCTCAGGCGG AGCTCAGATAGCCCA-3') for the complementary strand (Fig. 1). The primers flanking the intron sequence were designed so that a *Bam*HI site was introduced without modifying the amino acid sequence. The two exons were cloned into pCR-TOPO, resulting in pCR-TOPO:Δp35exon1 and pCR-TOPO: p35exon2, respectively. The fragment encoding p35exon2 was then isolated from *Bam*HI/*Xba*I-blunted pCR-TOPO:p35exon2 and ligated to *Bam*HI/*Sma*I-cut pCR-TOPO:Δp35exon1, resulting in pCR-TOPO:Δp35. This final plasmid encodes the unmodified p35 subunit of IL-12.

Construction of the *L. lactis* strain producing the p35 and p40 subunits. A fragment encoding the p40 mature sequence devoid of its SP (p40) was PCR amplified from plasmid pWRG3169 and subcloned into *Hin*cII-cut pVE8001 (Table 1) (kindly provided by Isabelle Poquet, Unité de Recherches Laitières et de Génétique Appliquée, INRA, Jouy en Josas, France), resulting in pBS:Δp40: trpA. The pVE8001 vector has a transcriptional terminator (*trpA*) and has been used previously to express heterologous proteins in *L. lactis* (5, 48). The primers used were primer 5'-p40 (5'-GATGCATCAGAGATGTGGGAGCTGGAGAA AGAC-3') for the coding strand and primer 3'-p40 (5'-GGAGCTCCTAGGA TCGGACCCTGCAGGGAA-3') for the complementary strand. Subsequently, different constructs were made in order to fuse prokaryotic lactococcal SPs to p35 and p40 subunits. For the p40 subunit, a DNA fragment was PCR amplified from pVE8022 (a derivative of plasmid pFUN, in which the ΔSP_{Nuc} reporter is fused to Exp4, a putative *L. lactis* secreted protein of unknown function [48]). This fragment contains the ribosome binding site (RBS) and the signal peptide of Exp4 (SP_{Exp4}) (48). The primers used were primer 5'-Exp4 (5'-GGGTACC TTAAGGAGATATAAAAATGAA-3') for the coding strand and primer 3'-Exp4 (5'-GATGCATCATCAGCAAATACAACGGC-3') for the complementary strand. The PCR product was cloned into *Hin*cII-cut pVE8001, resulting in pBS:SP_{Exp4}. pBS:SP_{Exp4}:p40:trpA was obtained by insertion of the *NsiI*/*KpnI* fragment (containing Δp40:*trpA*) obtained from pBS:Δp40:trpA into *NsiI*/*KpnI*-cut pBS:SP_{Exp4}. For the p35 subunit, the *NsiI*/*Eco*RI fragment (containing Δp35) obtained from pCR-TOPO:Δp35 was cloned into a pSEC backbone purified from *NsiI*/*Eco*RI-cut pSEC:E7 (7) (Table 1), resulting in pSEC:p35. In this vector, the p35 gene is fused to the RBS and SP_{Usp45} of *usp45*, the gene encoding Usp45, the main secreted protein in *L. lactis* (59). Expression is controlled by the P_{nisA} inducible promoter, whose expression depends on the nisin concentration used (11, 30). Finally, to obtain the plasmid that expressed both the p35 subunit and the p40 subunit, a *Kpn*I/*Bam*HI-Klenow cassette encoding SP_{Exp4}:p40:*trpA* was isolated from the pBS:SP_{Exp4}:p40:trpA vector and cloned into the *Kpn*I/*Sma*I-cut pSEC:p35 backbone, resulting in pSEC:p35-p40 (Table 1) (Fig. 2). This vector, in which the two subunits were transcribed from the P_{nisA} promoter, was established in *L. lactis* NZ9000 carrying the regulatory genes *nisR* and *nisK* (30). The resulting strain is referred to below as NZ(pSEC:p35-p40).

Construction of an *L. lactis* strain with an scIL-12 gene. A single-chain IL-12 (scIL-12) gene (*scIL-12*) was amplified by PCR from plasmid pCDNA3:IL-12 (kindly provided by Peter Melby, The University of Texas Health Science Center, San Antonio). The primers used were primer 5'-IL-12 (5'-GATGCATCAGAG ATGTGGGAGCTG GAGAAAGAC-3') for the coding strand and primer 3'-IL-12 (5'-GGAATTCTCAGGCGGAGCTCAGATAGCCCA-3') for the complementary strand. Primer 5'-IL-12 was designed to delete the first 22 codons in the *scIL-12* coding sequence. These codons encode the p40 eukaryotic SP that is replaced by the lactococcal SP_{Usp45}. The PCR product was cloned into pCR:

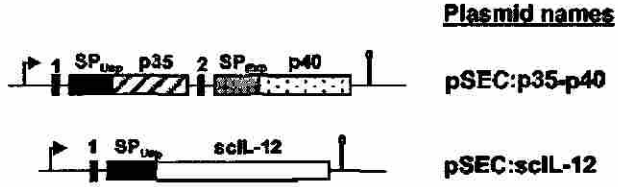

FIG. 2. Expression cassettes to produce and secrete IL-12 in *L. lactis*: schematic structures of p35 and p40 subunits and scIL-12 cassette expressed under the lactococcal P_{nisA} promoter and carried by the plasmids indicated. For details concerning plasmid construction, see the text and Table 1. The arrows indicate the presence of the nisin-inducible promoter (P_{nisA}); the solid vertical bars indicate the RBS of the *usp45* gene (bar 1 for p35) or of the *exp4* gene (bar 2 for p40); the dark gray bars indicate the SP of the *usp45* gene; the light gray bar indicates the SP of the *exp4* gene (48); the cross-hatched bar indicates the p35 mature coding sequence; the dotted bar indicates the p40 mature coding sequence; the open bar indicates the scIL-12 coding sequence; and the stem-loop symbols indicate *trpA* transcription terminators (not to scale).

TOPO (Invitrogen), resulting in pCR:TOPO:scIL-12 (Table 1). An *scIL-12* cassette was purified from *NsiI*/*Not*I-cut pCR:TOPO:scIL-12 and cloned into a pSEC backbone purified from *NsiI*/*Not*I-cut pSEC:E7. In the resulting plasmid, pSEC:scIL12 (Table 1 and Fig. 2), the *scIL-12* mature moiety was fused in frame with a DNA fragment encoding the RBS and SP_{Usp45} of *usp45*. Expression was controlled by a P_{nisA} promoter. This plasmid was established in *L. lactis* strain NZ9000 to obtain NZ(pSEC:scIL12).

IL-12 expression and detection. For induction of the nisin promoter, strains were grown until the optical density at 600 nm was ~0.6, and this was followed by induction with 10 ng of nisin (Sigma) per ml for 1 h. These parameters (amount of nisin and time of induction) were previously determined to be optimal (Bermúdez-Humarán, unpublished data). *L. lactis* culture extraction and immunoblotting assays were performed as previously described (5, 35); mouse IL-12 antibodies (R&D Systems, Minneapolis, Minn.) were used for immunodetection. The concentration of IL-12 secreted in a culture was estimated by using an enzyme-linked immunosorbent assay (ELISA) kit that recognized the IL-12 heterodimer but not the individual subunits (R&D Systems).

Preparation of bacterial supernatants for IL-12 nondenaturing analyses. Supernatant samples from induced cultures were concentrated 50-fold by using an Ultrafree Biomax NMWL membrane. After centrifugation, 10 μl of nondenaturing loading buffer (i.e., buffer lacking dithiothreitol and sodium dodecyl sulfate [SDS]) was added to 10 μl of supernatant concentrate. Electrophoresis was performed as described by Laemmli (31), except that SDS was omitted from all solutions.

Animals. C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, Maine). The mice were housed in the animal facility at the Immunology and Virology Laboratory at the University of Nuevo León, San Nicolás de los Garza, Mexico. Experiments were performed by using protocols approved by the animal studies committee.

Preparation of bacterial cells for IL-12 biological activity assays. Twenty milliliters of an induced culture was centrifuged, and the pellet and supernatant were separated. The bacterial cells were washed three times with sterile phosphate-buffered saline (PBS) and resuspended in 200 μl of PBS. The supernatant was concentrated 100-fold by using an Ultrafree Biomax NMWL membrane in 200 μl (final volume) of PBS.

In vitro IFN-γ detection. Spleens were obtained from four mice that were 6 to 8 weeks old, and splenocytes were separated on a Ficoll-Hypaque (Sigma) density gradient. A preparation containing 2×10^6 cells/ml in AIM-V medium (GIBCO) was plated on a 24-well plate (2 ml per well) at 37°C under 5% CO_2. As a positive control, mouse splenocytes were incubated with 50 pg of recombinant IL-12 (rIL-12) (R&D Systems). Splenocytes were incubated with 10 μl of recombinant *L. lactis* cells or with culture supernatant samples that were adjusted beforehand to provide ~50 pg per sample, as determined by an ELISA. Supernatants from the treated splenocytes were harvested after 48 h and tested for the presence of IFN-γ by ELISA (R&D Systems) according to the manufacturer's directions. All samples were prepared in triplicate.

Intranasal administration of recombinant *L. lactis* strains encoding IL-12. Groups of three mice that were 6 to 8 weeks old were inoculated intranasally with recombinant or wild-type *L. lactis* or with PBS. Prior to treatment, the mice were partially anesthetized intraperitoneally with a combination of Xilacyne and Ketamine (0.40 ml per 20 lb; Cheminova de México, Mexico). A total of 5×10^8 CFU (prepared as described above) of each induced *L. lactis* strain was resuspended in 10 μl of PBS, and 5 μl was administered with a micropipette into each nostril on days 0, 14, and 28.

IFN-γ induction assay. Animals treated with recombinant *L. lactis* strains were sacrificed on day 35. Splenocytes were separated and cultured as described above. Mouse cells were stimulated in vitro with 50 μl of phytohemagglutinin (5 μg/ml; M form, a polyclonal activator; GIBCO) to increase the proliferative response and to mimic a situation in which IFN-γ is induced. Supernatants from the treated splenocytes were harvested after 24 h and tested for the presence of IFN-γ by ELISA (R&D Systems). All samples were prepared in triplicate.

Coadministration of *L. lactis* strains expressing HPV-16 E7 and IL-12. In order to confirm the ability of an *L. lactis* strain to secrete IL-12 and to enhance an antigen-specific T-cell response, we used the HPV-16 E7 protein as an antigen. This protein was successfully expressed previously in *L. lactis* (5). Groups of three mice were immunized (as described above) with 5×10^8 CFU of an *L. lactis* strain displaying a cell wall-anchored E7 antigen [NZ(pCWA-E7) (Cortes-Perez, unpublished data)] alone or in combination with 5×10^8 CFU of strain NZ (pSEC:scIL12). A control group received the wild-type *L. lactis* strain. Splenocytes from immunized animals were used for detection of IL-2 and IFN-γ, cytokines characteristic of a Th1 type of immune response (17, 24, 53).

Determination of Th1 cytokine production in splenocytes. Mice immunized with *L. lactis* strains were sacrificed on day 35. Splenocytes were separated and cultured as described above. Cell suspensions from each different treatment were cultured with 2 μg of a synthetic E7 peptide (positions 49 to 57; RAHYNIVTF) to determine whether in vitro restimulation induced a peptide-specific (i.e., antigen-specific) cellular response or with PBS alone as a control. After 24 h, cell suspensions were filtered, and supernatants were tested for the presence of IL-2 and IFN-γ by ELISA (R&D Systems).

Statistics. Student's *t* test was performed by using MINITAB, a computer software package (Minitab Inc., State College, Pa.).

RESULTS

p35 and p40 subunits are secreted and correctly processed by *L. lactis*. The ability of *L. lactis* to secrete the two IL-12 subunits was tested by using strain NZ(pSEC:p35-p40). Cultures of NZ(pSEC:p35-p40) were harvested after induction (final optical density at 600 nm, ~1). Expression and secretion of the p40 and p35 subunits were analyzed by Western blotting by using anti-IL-12 antiserum (Fig. 3). Protein samples were prepared for cell and supernatant fractions. A pattern of diverse molecular weight forms was obtained for the cell fraction of induced cells, suggesting that there was accumulation of p40 and p35 precursors, as well as proteolysis in the cytoplasm or at the cell surface (Fig. 3A). In contrast, the supernatant fraction produced two distinct bands that migrated at the sizes expected for p35 and p40 (Fig. 3A). For both subunits, the secretion efficiency (i.e., the proportion of the mature form secreted into the supernatant) was low (<15%). Western analyses were performed with noninduced recombinant *L. lactis* strains, and no IL-12 production was detected (data not shown). The approximately equal intensities of the two subunits suggest that the subunits are produced in the proper stoichiometry to form an active IL-12 heterodimer. These results demonstrate that *L. lactis* is able to produce and secrete both IL-12 subunits.

We tested the capacity of *L. lactis* to produce and secrete IL-12p35-p40 in its assembled heterodimeric form. Protein samples were prepared from induced NZ(pSEC:p35-p40) cultures and analyzed by immunoblotting by using anti-IL-12 antiserum after polyacrylamide gel electrophoresis (PAGE) under nondenaturing conditions. A band that comigrated with the IL-12 control was detected in NZ(pSEC:p35-p40) supernatant (data not shown). This band could have corresponded to IL-12 or to a p40 homodimer. A p40 subunit may indeed associate with another p40 subunit to form a homodimer ($p40_2$) with a molecular mass of 80 kDa. A $p40_2$ form reportedly is an antagonist of IL-12 in vitro (37). To determine whether this high-molecular-weight form corresponds to the assembled form of IL-12, culture supernatants of NZ(pSEC:p35-p40) induced for IL-12 synthesis were analyzed by an ELISA that is specific for quantification of native murine IL-12p35-p40 (61). The concentration of secreted IL-12p35-p40 was estimated to be 25 pg/ml (Fig. 3B), compared to 80 pg/ml for an rIL-12 standard. ELISA were also performed with noninduced recombinant *L. lactis* strains, and no IL-12 production was detected (data not shown). Altogether, these results show that (i) *L. lactis* is able to secrete both p35 and p40 subunits and (ii) at least a proportion of these subunits is properly assembled in the supernatant.

Production of IL-12 as a single-chain polypeptide in *L. lactis*. One way to favor proper assembly of the p35 and p40 subunits is to synthesize a fusion protein comprising the two polypeptides. This strategy has already been proven to be ef-

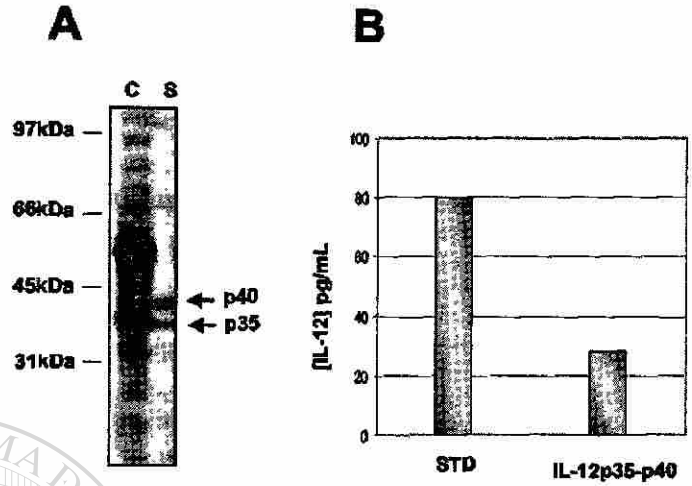

FIG. 3. Production of IL-12 by recombinant *L. lactis*. IL-12 production was analyzed by immunoblotting by using anti-IL-12 antibodies. Protein samples were prepared from induced recombinant *L. lactis* cultures. (A) Immunodetection after SDS-PAGE. Lane C, cell fraction of the NZ(pSEC:p35-p40) strain encoding the p35 and p40 subunits (IL-12p35-p40); lane S, supernatant samples. The positions and sizes of molecular weight markers are indicated on the left. (B) Quantification of IL-12p35-p40 by ELISA (R&D Systems). STD, 80 pg of commercial murine rIL-12 per ml; IL-12p35-p40, supernatant sample of NZ(pSEC:p35-p40) culture.

ficient in eukaryotic systems (15). Expression of scIL-12 was tested in *L. lactis* by using strain NZ(pSEC:scIL-12) and was compared to expression in NZ(pSEC:p35-p40) (Fig. 4A). Induced culture samples were prepared as described above. After induction, Western blot analysis with anti-IL-12 antibody revealed a clear band in the supernatant at the expected size for scIL-12 (70 kDa). The amount of secreted scIL-12 was found to be two- to threefold larger than the amounts of the separate p35 and p40 subunits (Fig. 4A). We also measured the scIL-12 concentration in the supernatant using the ELISA that recognized IL-12 only in the native conformation. The amount of scIL-12 was found to be twofold larger than the amounts of the p35 and p40 subunits (~65 pg/ml for scIL-12 and ~25 pg/ml for IL-12p35-p40) (Fig. 4B). Western blotting and

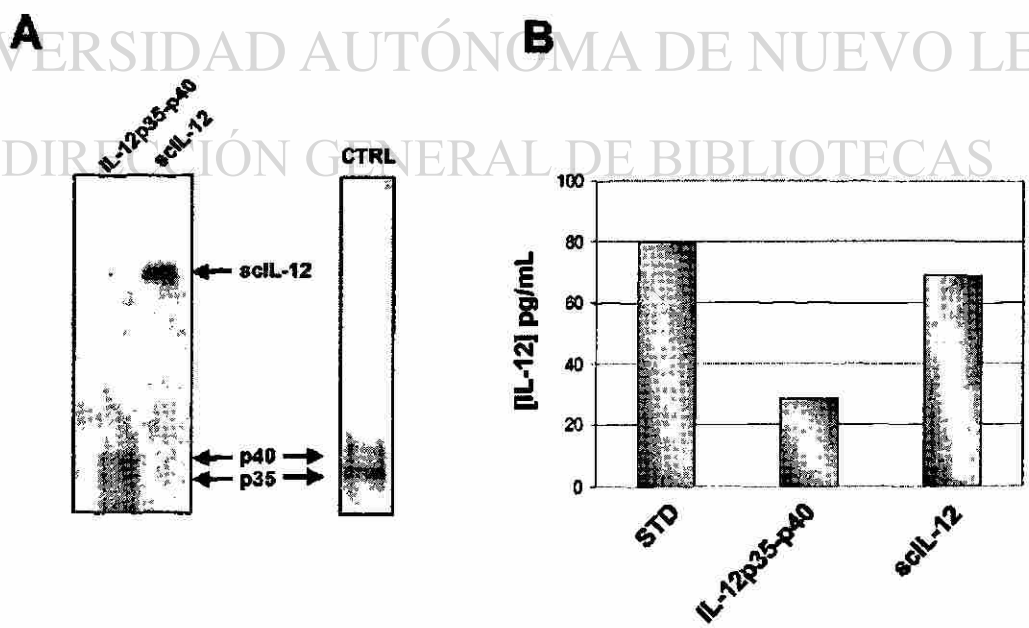

FIG. 4. Secretion analysis of the two IL-12 forms produced by *L. lactis*. (A) rIL-12 production compared by SDS-PAGE and Western blot analysis of supernatant samples prepared from induced cultures of NZ(pSEC:p35-p40) and NZ(pSEC:scIL-12) encoding IL-12p35-p40 and scIL-12, respectively. Lane CTRL contained rIL-12 (R&D Systems) as a control. (B) Quantification of IL-12 forms produced by *L. lactis* by ELISA by using supernatants of induced cultures of NZ(pSEC:p35-p40) and NZ(pSEC:scIL-12) encoding IL-12p35-p40 and scIL-12. STD, 80 pg of commercial rIL-12 per ml.

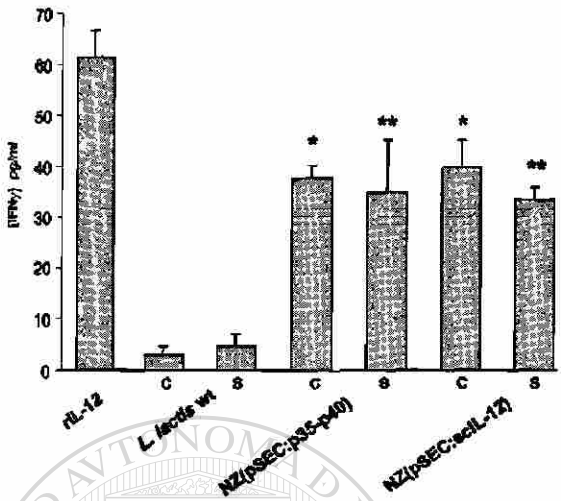

FIG. 5. In vitro induction of IFN-γ in mouse splenocytes by recombinant *L. lactis*. Mouse splenocytes were incubated in the presence of 50 μg of rIL-12 per ml as a control and with cells (C) and supernatant (S) of wild-type *L. lactis* (*L. lactis* wt), *L. lactis* NZ(pSEC:p35-p40) IL-12p35-p40, or *L. lactis* NZ(pSEC:scIL-12) encoding scIL-12. The concentrations of the induced culture samples were adjusted to ~50 pg/ml as determined by ELISA. The IFN-γ concentrations are the means and standard deviations determined in three independent experiments. Significant differences compared to the data obtained for recombinant *L. lactis* cells and supernatant samples are indicated by one asterisk and two asterisks, respectively ($P < 0.05$).

ELISA were performed with noninduced recombinant *L. lactis* strains, and no IL-12 production was detected (data not shown). This result shows that scIL-12 is efficiently secreted in *L. lactis* and is folded into a native conformation. The results described above show that both IL-12p35-p40 and scIL-12 are expressed and secreted in *L. lactis* and suggest that at least portions of these products assume a native and thus potentially active form.

Biological activity of IL-12 produced by recombinant *L. lactis*. Recombinant *L. lactis* strains producing IL-12p35-p40 and scIL-12 were evaluated for the ability to induce IFN-γ production in mouse splenocytes. Splenocytes were cultured for 48 h with 50 pg of commercial rIL-12 per ml or with supernatant or a total culture of recombinant *L. lactis*. The amounts of bacterially produced IL-12 added to splenocytes were adjusted to ~50 pg/ml as determined by the quantitative ELISA. After in vitro IL-12 stimulation, splenocyte culture supernatants were collected to measure the concentrations of IFN-γ (Fig. 5). The results show that 50 pg of commercial rIL-12 per ml induced production of ~60 pg of IFN-γ per ml. The scIL-12 produced by 5×10^8 CFU of *L. lactis* induced production of ~40 pg/ml, and supernatants of the same cells induced production of ~33 pg/ml. The concentrations of IFN-γ induced by an *L. lactis* strain expressing p35-p40 were ~37 pg/ml for 1×10^9 CFU and ~34 pg/ml for the supernatant. Splenocytes in the presence of a wild-type *L. lactis* strain did not produce significant amounts of IFN-γ, as expected (Fig. 5). These results suggest that both scIL-12 and IL-12p35-p40 are biologically active and stimulate IFN-γ production by mouse splenocytes.

Intranasal administration of *L. lactis* expressing IL-12 induces IFN-γ production in mouse splenocytes. The biological activities of the IL-12-producing *L. lactis* strains were also tested in vivo after intranasal administration of induced recombinant *L. lactis* strains in mice. It has been shown previously that a regimen consisting of intranasal administration of IL-12 on days 0, 1, 2, and 3 with booster doses on days 14 and 28 (and repeating the schedule for four inoculations) and sacrifice of the animals on day 35 results in an absence of cytokine toxicity in a murine model (3). However, although this treatment schedule is very productive in mice, its use in human vaccination is limited due to the consecutive IL-12 inoculations during the treatment. To avoid this problem, in this experiment we tested single doses of IL-12-expressing *L. lactis* strains administered on days 0, 14, and 28. We administered 5×10^8 CFU of NZ(pSEC:p35-p40) or NZ(pSEC:scIL-12), which corresponds to quantities used previously for oral administration of recombinant *L. lactis* (50).

IFN-γ expression was significantly enhanced in mice that received *L. lactis* strains expressing IL-12p35-p40 or scIL-12 compared to IFN-γ expression in the placebo control groups (Fig. 6). Mice treated with the scIL-12-producing strain produced the largest amounts of IFN-γ in spleen cells. In contrast, the amounts of IFN-γ in mice treated with the p35-p40-producing strain were fourfold smaller. The differences in the degree of stimulation were probably due to the quantity of

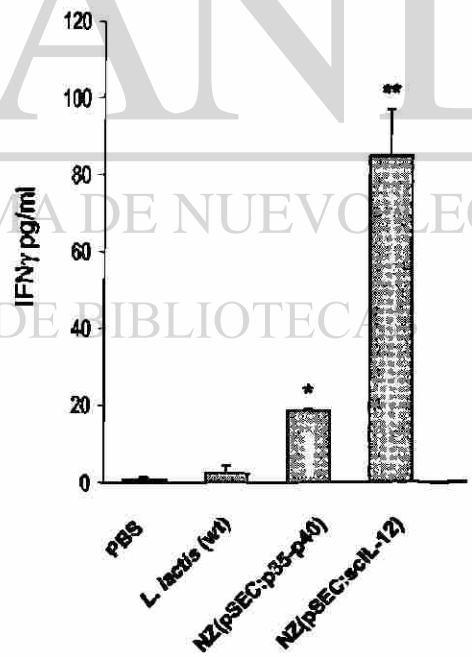

FIG. 6. Production of IFN-γ in mouse splenocytes after intranasal inoculation of recombinant *L. lactis*. Levels of IFN-γ were determined following sacrifice on day 35 for mice that received 5×10^8 CFU of wild-type *L. lactis* [*L. lactis* (wt)], NZ(pSEC:p35-p40), or NZ(pSEC:scIL-12) or PBS alone. The data are representative of one of three separate experiments in which similar results were obtained. The values are the means and standard deviations for three mice per treatment group. Statistically significant differences ($P < 0.05$) compared with the wild-type *L. lactis* and PBS control groups are indicated by one asterisk for the NZ(pSEC:p35-p40) group and by two asterisks for NZ(pSEC:scIL-12) group.

native IL-12 produced under the conditions used (Fig. 3 and 4). This experiment was repeated three times, and similar results were obtained in all cases. The results demonstrated that IL-12 can be effectively administered to mice in vivo by using recombinant *L. lactis*, which results in clear induction of the IFN-γ response. Furthermore, after intranasal administration of the *L. lactis* IL-12-producing strains, IFN-γ production was induced without apparent toxicity, and mice remained healthy after 24 weeks of treatment.

Because the concentrations of functional IL-12 measured in both in vitro and in vivo assays were greater for scIL-12 than for the two-subunit IL-12 form, we chose the single-chain form for the next in vivo experiment.

Intranasal coadministration of recombinant *L. lactis* strains expressing active IL-12 and HPV-16 E7 enhanced IFN-γ production. In order to examine the adjuvant properties of the recombinant *L. lactis* strain producing IL-12, the immune response to a coadministered antigen was analyzed. The antigen that was coexpressed with IL-12 was the HPV-16 E7 protein, the major worldwide etiological agent of cervical cancer. Groups of five C57BL/6 mice were immunized intranasally with three doses (on days 0, 14, and 28) of 5×10^8 CFU of NZ(pCWA-E7) alone or in combination with 5×10^8 CFU of NZ(pSEC:scIL12). The production of IL-2 and IFN-γ was then determined (Fig. 7). Spleen cells that were obtained 1 week after the last inoculation with recombinant *L. lactis* (day 35) and were restimulated in vitro with a synthetic E7 peptide (RAHYNIVTF) produced significant levels of IL-2 (Fig. 7A) and IFN-γ (Fig. 7B). As an in vitro control, spleen cells were restimulated with PBS alone. The responses were greater in mice immunized with an *L. lactis* strain displaying a cell wall-anchored E7 antigen than in animals immunized with a wild-type *L. lactis* strain. Strikingly, the antigen-specific cellular response measured by secretion of Th1 cytokines elicited by *L. lactis* expressing E7 antigen alone was dramatically increased by coadministration with an *L. lactis* strain secreting IL-12 protein (Fig. 7).

DISCUSSION

In this study, we produced bioactive forms of IL-12 in the food-grade gram-positive bacterium *L. lactis*, and we showed that the recombinant bacterium has a stimulatory effect on IFN-γ production in both in vitro and in vivo assays. Previous reports of IL-12 production involved the use of eukaryotic systems (15, 29, 52, 57), which may have limitations in broad-scale or in vivo applications. Recently, Steidler et al. demonstrated that *L. lactis* could be used to produce and secrete biologically active murine monomeric cytokines (55, 56). The production of a more complex molecule (i.e., a heterodimer that contains several DSB) further extends the potential of *L. lactis* to deliver therapeutic molecules in vivo.

IL-12 is a heterodimer composed of two distinct subunits (p35 and p40) encoded by separate genes that are coordinately expressed. Previous studies have demonstrated that p40 overexpression can have an inhibitory effect on IL-12 activity (37). We used two approaches to overcome this potential problem. First, we developed a bicistronic cassette for coexpression of p35 and p40 subunits in *L. lactis*. Second, we designed a vector that expressed IL-12 as a single-chain polypeptide, thus allowing stoichiometric formation of this cytokine. This strategy also overcomes problems with inefficient association of independently produced subunits or formation of homodimers (20, 37). Consistent with this hypothesis, under equivalent induction conditions, the concentrations of functional IL-12 were greater for scIL-12 than for the two-subunit IL-12 form. In view of the lower activities of p35 and p40, the single-chain form of IL-12 may be preferred for in vivo applications.

Remarkably, mouse IL-12 contains 7 and 13 cysteines in its p35 and p40 subunits, respectively, and two DSB that are essential for proper IL-12 assembly (47). The secretion of biologically active IL-12 suggests that DSB are formed after the protein is exported from *L. lactis*. DSB formation is often a major bottleneck in heterologous protein production in prokaryotic systems and particularly in gram-positive bacteria, which themselves encode very few secreted proteins that contain DSB (44, 46). Possibly, the lower pH of *L. lactis* during fermentative growth favors formation of DSB in secreted proteins. Although the mechanism remains to be proven, this system may be promising for expression of other proteins containing DSB.

The main biological effect of IL-12 is stimulation of IFN-γ production. This cytokine has both adjuvant and antitumor activities. Because a number of subunit vaccines are poorly immunogenic, the use of adjuvants is of particular interest for new formulations of vaccines against infectious diseases. To enhance the mucosal immune response, adjuvants such as cholera toxin and *E. coli* enterotoxin have been used, and they indeed induce potent Th1 and Th2 cell responses. However, these adjuvants cause severe diarrhea and are not suitable for use as mucosal adjuvants in humans. Strikingly, IL-12 has proven adjuvant activity when it is coexpressed with an antigen in targeted vaccines (1, 6). It may also prevent the development of immunological tolerance to a given antigen (54). Finally, IL-12 has potent antitumor effects and may be an attractive agent for cancer immunotherapy.

Despite the efficacy of IL-12 therapy for cancer and infectious diseases, experimental models in clinical trials with systemic IL-12 showed unacceptable levels of toxicity related to elevated IFN-γ production (9, 36). The limitations of IL-12 treatment include the need for daily administration (27). Here, to circumvent this problem, we explored mucosal (intranasal) delivery of active IL-12 by using the safe vector *L. lactis*, which repeatedly has been reported to be noninvasive and noncolonizing in a murine model (12, 16, 22). Recently, a recombinant *L. lactis* strain delivering IL-10 via an oral route exhibited positive effects during treatment of murine colitis. The dose of IL-10 given orally was estimated to be 10-fold lower than the dose required for systemic administration (56). Targeted administration of other interleukins, such as IL-12, to the intestinal tract by food-grade *L. lactis* may also reduce toxicity and have advantages compared to treatment by the systemic route, and it may even maximize the response (39).

There is continual interest in developing mucosally based vaccines for a variety of different pathogens, including HPV. The use of live oral delivery systems for tumor therapy or vaccine delivery may thus reduce toxic side effects resulting from systemic administration. In this study, we showed the adjuvant effect of a recombinant *L. lactis* strain producing IL-12 protein which enhanced the mucosal immune responses

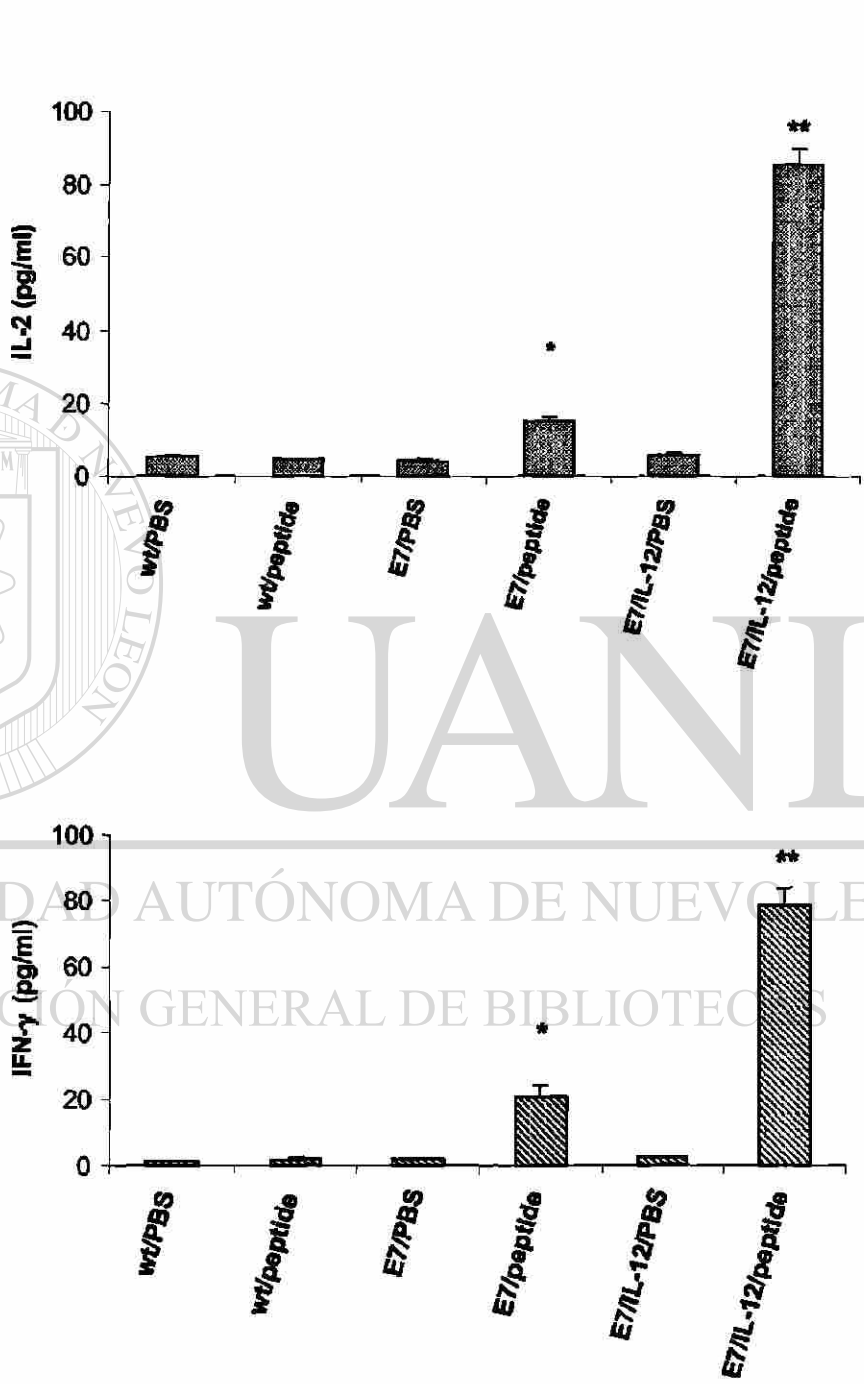

FIG. 7. Production of Th1 cytokines by splenocytes of mice immunized with recombinant *L. lactis*. Levels of Th1 cytokines were determined following sacrifice on day 35 for mice immunized with 5×10^8 CFU of wild-type *L. lactis* (wt) or recombinant *L. lactis* displaying E7 antigen (E7) and for mice coimmunized with *L. lactis* displaying E7 and an *L. lactis* strain secreting active murine IL-12 (E7/IL-12). Spleen cells were cultured for 24 h with 2 μg of E7 peptide (RAHYNIVTF) (peptide) or PBS, and the levels of the Th1 cytokines IL-2 (A) and IFN-γ (B) in the culture supernatants were determined by ELISA. The values are the means and standard deviations for three mice per treatment group. Statistically significant differences ($P < 0.05$) compared to the E7/PBS group are indicated by one asterisk and by two asterisks for the E7/peptide and E7/IL-12/peptide groups, respectively.

against a coadministered antigen. The IL-2 and IFN-γ production elicited by a recombinant *L. lactis* strain displaying a cell wall-anchored HPV-16 E7 antigen was dramatically increased by coadministration with an *L. lactis* strain secreting IL-12 protein.

It is well established that IL-12 plays an essential role in switching of the immune response, inducing Th1 cells and suppressing Th2 responses (58). On the other hand, the elevated density of Th2 cells during the pathogenesis of advanced cervical cancer is well known, while the level of Th1 cells is dramatically diminished (2, 18). We believe that successful immunotherapeutic treatments of cervical cancer patients will use a vaccine that will be able to switch the immune response from the Th2 class to the Th1 class. Therefore, on the basis of this belief, an *L. lactis* strain modified to secrete IL-12 together with a specific antigen is a good candidate for cervical cancer therapy.

In summary, for vaccine applications, oral or nasal delivery may provoke local immune responses at the portal of entry of most pathogens. The use of *L. lactis* to deliver IL-12 to a mucosal surface (e.g., the intranasal surface, gut, or vaginal mucosa) may have clear advantages over a systemic therapy approach because it reduces toxic side effects and provides a low-cost, simple method of administration.

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REFERENCES

1. **Afonso, L. C., T. M. Scharton, L. Q. Vieira, M. Wysocka, G. Trinchieri, and P. Scott.** 1994. The adjuvant effect of interleukin-12 in a vaccine against *Leishmania major*. Science 263:235–237.
2. **al-Saleh, W., S. L. Giannini, N. Jacobs, M. Moutschen, J. Doyen, J. Boniver, and P. Delvenne.** 1998. Correlation of T-helper secretory differentiation and types of antigen-presenting cells in squamous intraepithelial lesions of the uterine cervix. J. Pathol. 184:283–290.
3. **Arulanandam, B. P., M. O'Toole, and D. W. Metzger.** 1999. Intranasal interleukin-12 is a powerful adjuvant for protective mucosal immunity. J. Infect. Dis. 180:940–994.
4. **Barth, H., R. Klein, P. A. Berg, B. Wiedenmann, U. Hopf, and T. Berg.** 2001. Induction of T helper cell type 1 response and elimination of HBeAg during treatment with IL-12 in a patient with therapy-refractory chronic hepatitis B. Hepatogastroenterology 48:553–555.
5. **Bermúdez-Humarán, L. G., P. Langella, A. Miyoshi, A. Gruss, R. S. Tamez-Reyes, R. Montes de Oca-Luna, and Y. Le Loir.** 2002. Production of human papillomavirus type 16 E7 protein in *Lactococcus lactis*. Appl. Environ. Microbiol. 68:917–922.
6. **Boyaka, P. N., and J. R. McGhee.** 2001. Cytokines as adjuvants for the induction of mucosal immunity. Adv. Drug Deliv. Rev. 51:71–79.
7. **Brunda, M., L. Luistro, R. Wartier, R. Wright, B. Hubbard, M. Murphy, S. Wolf, and M. K. Gately.** 1993. Antitumor and antimetastatic activity of interleukin 12 against murine tumors. J. Exp. Med. 178:1223–1230.
8. **Chan, S. H., B. Perussia, J. W. Grupta, M. Kobayashi, M. Pospisil, H. A. Young, S. G. Wolf, D. Young, S. C. Clark, and G. Trinchieri.** 1991. Induction of interferon gamma production by natural killer cell stimulatory factor: characterization of the responder cells and synergy with other inducers. J. Exp. Med. 173:869–879.
9. **Chen, B., T. M. Timiryasova, D. S. Gridley, M. L. Andres, R. Dutta-Roy, and I. Fodor.** 2001. Evaluation of cytokine toxicity induced by vaccinia virus-mediated IL-2 and IL-12 antitumour immunotherapy. Cytokine 15:305–334.
10. **Cohen, J.** 1995. IL-12 deaths: explanation and a puzzle. Science 270:908.
11. **de Ruyter, P. G., O. P. Kuipers, and W. M. de Vos.** 1996. Controlled gene expression systems for *Lactococcus lactis* with the food-grade inducer nisin. Appl. Environ. Microbiol. 62:3662–3667.
12. **Drouault, S., G. Corthier, S. D. Ehrlich, and P. Renault.** 1999. Survival, physiology, and lysis of *Lactococcus lactis* in the digestive tract. Appl. Environ. Microbiol. 65:4881–4886.
13. **Enouf, V., P. Langella, J. Commissaire, J. Cohen, and G. Corthier.** 2001. Bovine rotavirus nonstructural protein 4 produced by *Lactococcus lactis* is antigenic and immunogenic. Appl. Environ. Microbiol. 67:1423–1428.
14. **Gasson, M. J.** 1983. Plasmid complements of *Streptococcus lactis* NCDO 712 and other lactic acid streptococci after protoplast-induced curing. J. Bacteriol. 154:1–9.
15. **Gaucher, D., and K. Chadee.** 2001. Molecular cloning of gerbil interleukin 12 and its expression as a bioactive single-chain protein. Cytokine 14:177–183.
16. **Geoffroy, M. C., C. Guyard, B. Quatannens, S. Pavan, M. Lange, and A. Mercenier.** 2000. Use of green fluorescent protein to tag lactic acid bacterium strains under development as live vaccine vectors. Appl. Environ. Microbiol. 66:383–391.
17. **Gherardi, M. M., J. C. Ramirez, and M. Esteban.** 2000. Interleukin-12 (IL-12) enhancement of the cellular immune response against human immunodeficiency virus type 1 Env antigen in a DNA prime/vaccinia virus boost vaccine regimen is time and dose dependent: suppressive effects of IL-12 boost are mediated by nitric oxide. J. Virol. 74:6278–6286.
18. **Ghim, S. J., J. Sundberg, G. Delgado, and A. B. Jenson.** 2001. The pathogenesis of advanced cervical cancer provides the basis for an empirical therapeutic vaccine. Exp. Mol. Pathol. 71:181–185.
19. **Gibson, T. J.** 1984. Ph.D. thesis. University of Cambridge, Cambridge, England.
20. **Gillessen, S., D. Carvajal, P. Ling, F. J. Podlaski, D. L. Stremlo, P. C. Familletti, U. Gubler, D. H. Presky, A. S. Stern, and M. K. Gately.** 1995. Mouse interleukin-12 (IL-12) p40 homodimer: a potent IL-12 antagonist. Eur. J. Immunol. 25:200–206.
21. **Graham, B. S., G. S. Henderson, Y. W. Tang, X. Lu, K. M. Neuzil, and D. G. Colley.** 1993. Priming immunization determines T helper cytokine mRNA expression patterns in lungs of mice challenged with respiratory syncytial virus. J. Immunol. 151:2032–2040.
22. **Grangette, C., H. Muller-Alouf, M. Geoffroy, D. Goudercourt, M. Turneer, and A. Mercenier.** 2002. Protection against tetanus toxin after intragastic administration of two recombinant lactic acid bacteria: impact of strain variability and in vivo persistence. Vaccine 20:3304–3309.
23. **Gubler, U., A. O. Chua, D. S. Schoenhaut, C. M. Dwyer, W. McComas, R. Motyka, N. Nabavi, A. G. Wolitzky, P. M. Quinn, P. C. Familletti, and M. K. Gately.** 1991. Coexpression of two distinct genes is required to generate secreted bioactive cytotoxic lymphocyte maturation factor. Proc. Natl. Acad. Sci. USA 88:4143–4147.
24. **Hoover, D. L., R. M. Crawford, L. L. Van De Verg, M. J. Izadjoo, A. K. Bhattacharjee, C. M. Paranavitana, R. L. Warren, M. P. Nikolich, and T. L. Hadfield.** 1999. Protection of mice against brucellosis by vaccination with *Brucella melitensis* WR201(16MDeltapurEK). Infect. Immun. 67:5877–5884.
25. **Hultgren, O. H., M. Stenson, and A. Tarkowski.** 2001. Role of IL-12 in *Staphylococcus aureus*-triggered arthritis and sepsis. Arthritis Res. 3:41–47.
26. **Jacobson, M. A., D. Hardy, E. Comick, J. Watson, and M. DeBruin.** 2000. Phase 1 trial of a single dose of recombinant human interleukin-12 in human immunodeficiency virus-infected patients with 100–500 CD4 cells/microl. J. Infect. Dis. 182:1070–1076.
27. **Jiang, C., D. M. Magee, and R. A. Cox.** 1999. Construction of a single-chain interleukin-12-expressing retroviral vector and its application in cytokine gene therapy against experimental coccidioidomycosis. Infect. Immun. 67: 2996–3001.
28. **Kobayashi, M., L. Fitz, M. Ryan, R. M. Hewick, S. C. Clark, S. Chan, R. Loudon, F. Sherman, B. Perussia, and G. Trinchieri.** 1989. Identification and purification of natural killer cell stimulatory factor (NKSF), a cytokine with multiple biologic effects on human lymphocytes. J. Exp. Med. 170:827–845.
29. **Kokuho, T., S. Watanabe, Y. Yokomizo, and S. Inumaru.** 1999. Production of biologically active, heterodimeric porcine interleukin-12 using a monocistronic baculoviral expression system. Vet. Immunol. Immunopathol. 72:289–302.
30. **Kuipers, O. P., P. G. de Ruyter, M. Kleerebezen, and W. M. de Vos.** 1998. Quorum sensing-controlled gene expression in lactic acid bacteria. J. Biotechnol. 64:15–21.
31. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685.
32. **Langella, P., Y. Le Loir, S. D. Ehrlich, and A. Gruss.** 1993. Efficient plasmid mobilization by pIP501 in *Lactococcus lactis* subsp. *lactis*. J. Bacteriol. 175: 5806–5813.
33. **Lee, M. H., Y. Roussel, M. Wilks, and S. Tabaqchali.** 2001. Expression of *Helicobacter pylori* urease subunit B gene in *Lactococcus lactis* MG1363 and its use as a vaccine delivery system against *H. pylori* infection in mice. Vaccine 19:3927–3935.
34. **Lee, S., M. Zheng, S. Deshpande, S. K. Eo, T. A. Hamilton, and B. T. Rouse.** 2002. IL-12 suppresses the expression of ocular immunoinflammatory lesions by effects on angiogenesis. J. Leukoc. Biol. 71:469–476.
35. **Le Loir, Y., A. Gruss, S. D. Ehrlich, and P. Langella.** 1998. A nine-residue

synthetic propeptide enhances secretion efficiency of heterologous proteins in *Lactococcus lactis*. J. Bacteriol. **180**:1895–1903.
36. **Leonard, J. P., M. L. Sherman, G. L. Fisher, L. J. Buchanan, G. Larsen, M. B. Atkins, J. A. Sosman, J. P. Dutcher, N. J. Vogelzang, and J. L. Ryan.** 1997. Effects of single-dose interleukin-12 exposure on interleukin-12-associated toxicity and interferon-gamma production. Blood **90**:2541–2548.
37. **Ling, P., M. K. Gately, U. Gubler, A. S. Stern, P. Lin, K. Hollfelder, C. Su, Y.-C. E. Pan, and J. Hakimi.** 1995. Human IL-12 p40 homodimer binds to the IL-12 receptor but does not mediate biologic activity. J. Immunol. **154**: 116-127.
38. **Lui, V. W., L. D. Falo, and L. Huang.** 2001. Systemic production of IL-12 by naked DNA mediated gene transfer: toxicity and attenuation of transgene expression in vivo. J. Gene Med. **3**:384–393.
39. **Magee, D. M., and R. A. Cox.** 1996. Interleukin-12 regulation of host defenses against *Coccidioides immitis*. Infect. Immun. **64**:3609–3613.
40. **Mazzolini, G., I. Narvaiza, A. Perez-Diez, M. Rodriguez-Calvillo, C. Qian, B. Sangro, J. Ruiz, J. Prieto, and I. Melero.** 2001. Genetic heterogeneity in the toxicity to systemic adenoviral gene transfer of interleukin-12. Gene Ther. **8**:259–267.
41. **Metzger, D. W., R. M. McNutt, J. T. Collins, J. M. Buchanan, V. H. Van Cleave, and W. A. Dunnick.** 1997. Interleukin-12 acts as an adjuvant for humoral immunity through interferon-gamma-dependent and -independent mechanisms. Eur. J. Immunol. **27**:1958–1965.
42. **Michel, N., W. Osen, L. Gissmann, T. N. Schumacher, H. Zentgraf, and M. Muller.** 2002. Enhanced immunogenicity of HPV 16 E7 fusion proteins in DNA vaccination. Virology **294**:47–59.
43. **Nastala, C., H. Edington, T. McKinney, H. Tahara, M. Nalesnik, M. Brunda, M. K. Gately, S. Wolf, R. Schreiber, W. Storkus, and M. Lotze.** 1994. Recombinant IL-12 administration induces tumor regression in association with IFN-gamma production. J. Immunol. **153**:1697–1703.
44. **Paik, S. H., A. Chakicherla, and J. N. Hansen.** 1998. Identification and characterization of the structural and transporter genes for, and the chemical and biological properties of, sublancin 168, a novel lantibiotic produced by *Bacillus subtilis* 168. J. Biol. Chem. **273**:23134–23142.
45. **Park, A. Y., B. Hondowicz, M. Kopf, and P. Scott.** 2002. The role of IL-12 in maintaining resistance to *Leishmania major*. J. Immunol. **168**:5771–5777.
46. **Payne, M. S., and E. N. Jackson.** 1991. Use of alkaline phosphatase fusions to study protein secretion in *Bacillus subtilis*. J. Bacteriol. **173**:2278–2282.
47. **Podlaski, F. J., V. B. Nanduri, J. D. Hulmes, Y. C. Pan, W. Levin, W. Danbo, R. Chizzonite, M. K. Gately, and A. S. Stern.** 1992. Molecular characterization of interleukin 12. Arch. Biochem. Biophys. **294**:230–237.
48. **Poquet, I., S. D. Ehrlich, and A. Gruss.** 1998. An export-specific reporter designed for gram-positive bacteria: application to *Lactococcus lactis*. J. Bacteriol. **180**:1904–1912.
49. **Rakhmilevich, A. L., J. Turner, M. J. Ford, D. McCabe, W. H. Sun, P. M. Sondel, K. Grota, and N.-Y. Yang.** 1996. Gene gun-mediated skin transfection with interleukin 12 gene results in regression of established and metastatic murine tumors. Proc. Natl. Acad. Sci. USA **93**:6291–6296.
50. **Robinson, K., L. M. Chamberlain K. M. Schofield, J. M. Wells, and R. W.** 1997. Oral vaccination of mice against tetanus with recombinant *Lactococcus lactis*. Nat. Biotechnol. **15**:653–657.
51. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
52. **Shen, H., C. Li, M. Lin, Z. Zhang, and Q. Shen.** 1998. Expression of human interleukin 12 (hIL-12) in insect cells. Chin. J. Biotechnol. **14**:205–212.
53. **Sin, J. I., J. J. Kim, R. L. Arnold, K. E. Shroff, D. McCallus, C. Pachuk, S. P. McElhiney, M. W. Wolf, S. J. Pompa-de Bruin, T. J. Higgins, R. B. Ciccarelli, and D. B. Weiner.** 1999. IL-12 gene as a DNA vaccine adjuvant in a herpes mouse model: IL-12 enhances Th1-type $CD4^+$ T cell-mediated protective immunity against herpes simplex virus-2 challenge. J. Immunol. **162**:2912–2921.
54. **Sinkovics, J. G., and J. C. Horvath.** 2000. Vaccination against human cancers. Int. J. Oncol. **16**:81–96.
55. **Steidler, L., K. Robinson, L. Chamberlain, K. M. Schofield, E. Remaut, R. W. Le Page, and J. M. Wells.** 1998. Mucosal delivery of murine interleukin-2 (IL-2) and IL-6 by recombinant strains of *Lactococcus lactis* coexpressing antigen and cytokine. Infect. Immun. **66**:3183–3189.
56. **Steidler, L., W. Hans, L. Schotte, S. Neirynck, F. Obermeier, W. Falk, W. Fiers, and E. Remaut.** 2000. Treatment of murine colitis by *Lactococcus lactis* secreting interleukin-10. Science **289**:1352–1355.
57. **Takehara, K., T. Nagata, R. Kikura, T. Takanashi, S. Yoshiya, and A. Yamaga.** 2000. Expression of a bioactive interleukin-12 using baculovirus. Vet. Immunol. Immunopathol. **77**:15–25.
58. **Trinchieri, G.** 1995. Interleukin-12: a proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity. Annu. Rev. Immunol. **13**:251–276.
59. **van Asseldonk, M., G. Rutten, M. Oteman, R. J. Siezen, W. M. de Vos, and G. Simons.** 1990. Cloning, expression in *Escherichia coli* and characterization of *usp45*, a gene encoding a highly secreted protein from *Lactococcus lactis* MG1363. Gene **95**:155–160.
60. **Voest, E. E., B. M. Kenyon, M. S. O'Reilly, G. Truitt, R. J. D'Amato, and J. Folkman.** 1995. Inhibition of angiogenesis *in vivo* by interleukin 12. J. Natl. Cancer Inst. **19**:581–586.
61. **Wilkinson, V. L., R. R. Warrier, T. P. Truitt, P. Nunes, M. K. Gately, and D. H. Presky.** 1996. Characterization of anti-mouse IL-12 monoclonal antibodies and measurement of mouse IL-12 by ELISA. J. Immunol. Methods **189**:15–24.

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