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CISTICERCOSIS: IDENTIFICACION Y CARACTERIZACION INMUNOLOGICA DE EPITOPES PROTECTORES PARA EL DISEÑO DE UNA VACUNA POLIVALENTE

T E S I S

QUE PARA OBTENER EL GRADO ACADEMICO DE:

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P R E S E N T A

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P r e s e n t e

Por medio de la presente me permito informar a usted que en la reunión ordinaria del Comité Académico del Posgrado en Ciencias Biológicas, celebrada el día 16 de febrero del 2004, se acordó poner a su consideración el siguiente jurado para el examen de grado del Doctorado en Ciencias del alumno(a) **Andrea Alejandra Toledo Rojas**, con número de cuenta 88536460 y número de expediente 3961361, con la tesis titulada: **"Cisticercosis: Identificación y caracterización inmunológica de epítopes protectores para el diseño de una vacuna polivalente"**, bajo la dirección del (la) **Dra. Edda Lydia Sciutto Conde.**

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A t e n t a m e n t e
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El presente trabajo de tesis se realizó en el departamento de Inmunología del Instituto de Investigaciones Biomédicas, en la Universidad Nacional Autónoma de México bajo la dirección de la Dra. Edda Sciutto Conde.

El comité tutoral que evaluó el desarrollo del trabajo doctoral estuvo integrado por:

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Durante el desarrollo de los créditos de doctorado se contó con una beca otorgada por el CONACYT y con una beca complementaria otorgada por la DGEP (UNAM).

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porque con su amor, cariño y sonrisa hace
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RESUMEN

La cisticercosis es un problema que afecta principalmente a los países en vías de desarrollo. Diferentes estrategias se han considerado con el fin de prevenir esta parasitosis. Entre ellas figura el desarrollo de una vacuna contra la cisticercosis porcina con el propósito de interrumpir el ciclo de vida del parásito. En el presente trabajo de tesis se procedió a identificar dos epítopes protectores en contra de la cisticercosis murina (KETc1 y KETc12) causada por *Taenia crassiceps* así como caracterizar sus propiedades inmunogénicas.

En base a la secuencia de aminoácidos codificadas por las clonas recombinantes protectoras KETc1 y KETc12 identificadas en una biblioteca de cDNA de *T. crassiceps*, se sintetizaron los respectivos péptidos químicamente en forma lineal. La capacidad protectora de los mismos se evaluó utilizando el modelo de cisticercosis murina y los péptidos administrados individualmente en forma lineal utilizando saponina como adyuvante. La inmunización con KETc1 indujo desde un 67 a un 100% de protección en contra de cisticercosis murina, mientras la vacunación con KETc12 indujo niveles de protección del 53 al 88%. La evaluación de la respuesta inmune asociada a la protección inducida indicó que esta forma de administración de los péptidos induce bajos niveles de anticuerpos y una exacerbada respuesta inmune celular. La respuesta inmune inducida a través de la vacunación de ratones indica que ambos péptidos contienen al menos un epítope B y un epítope T.

En las células provenientes de ratones inmunizados se observó un alto porcentaje de células que expresaron principalmente citocinas de tipo inflamatorio lo que señala a estos péptidos como inductores de una respuesta preferentemente de tipo T1.

La capacidad protectora de estos péptidos aunado a su presencia en los tres estadios de desarrollo de *T. solium*, permiten proponer a estos dos péptidos como candidatos a considerar en el diseño de una vacuna sintética polivalente en contra de cisticercosis porcina.

ABSTRACT

Taenia solium cysticercosis is a major health problem mainly in undeveloped countries. Many and different efforts have been done in the attempt to prevent the parasitic disease. The indispensable role of the pigs as an obligatory intermediate host in the parasite life cycle offers the possibility of interfering with transmission through vaccination of pigs. In the present thesis the immunological properties of two protector epitopes KETc1 and KETc12, that protect against murine *Taenia crassiceps* cysticercosis, were identified.

Based on the complete sequence encoded by the recombinant clones KETc1 and KETc12, previously identified in a cDNA library of *T. crassiceps*, the respective peptides were synthesized in a linear form. The protective capacity of the peptides was evaluated using the murine model of cysticercosis and the peptides were administrated individually using saponine as adjuvant. The immunizations with KETc1 induced protective levels from 67% to 100%, whilst using KETc12 the levels of protection were from 53% to 88%. The evaluation of immune response associated to protection indicates that this form of administration of the peptides induces low level of antibodies but a strong cellular immune response. The immune response generated after immunization indicates that both peptides are B and T cell epitopes. In the cells from immunized mice we found an increment in the levels of the inflammatory cytokines point this peptides as inductor at T1 response.

Due to the protective capacity of these peptides and the presence of both peptides in all the stages of *T. solium* development we propose these peptides to be considered as candidates to be included in a mixed polyepitopic synthetic vaccine to be used against *T. solium* cysticercosis in pigs.

INTRODUCCION

La cisticercosis causada por la forma larvaria de *Taenia solium* es una parasitosis frecuente en humanos y en cerdos especialmente en países en vías de desarrollo donde prevalecen condiciones que favorecen su transmisión como son la falta de higiene, fecalismo al aire libre, la crianza rústica de cerdos y consumo de carne sin inspección sanitaria adecuada (Gemmell *et al.,* 1982). Dentro de las zonas más afectadas se encuentran América Latina, Asia y África (Sotelo, *et al.,* 1996) en donde esta parasitosis, causa serios problemas tanto de salud como económicos (Larralde, *et al.,* 1992; Del Brutto, *et al.*, 1998). Aunque la cisticercosis ha sido prácticamente erradicada en los países desarrollados, esta enfermedad ha comenzado a reportarse en países como Estados Unidos, Canadá, Australia y algunos países de Europa debido al incremento de inmigrantes provenientes de zonas endémicas los cuales son portadores del estadio adulto del parásito, reportándose cientos de casos en años recientes (McCormick., 1982; Richards, *et al.,* 1991; Guerra L, 1993; Shandera., 1994; Sotelo and Del Brutto, 2000; White., 2000).

La localización del parásito en el sistema nervioso central de humanos es causa de neurocisticercosis, enfermedad grave y de consecuencias severas (Del Bruto, *et al.,* 1996, 2001; Rosenfeld, 2003). En México es un padecimiento frecuente, de alta endemicidad, las zonas de mayor seroprevalencia se encuentran en las regiones centro occidental y en el sureste del país (Larralde, 1992). La neurocisticercosis resulta así un problema de salud en países endémicos donde se reporta como la primer causa de epilepsia en adultos (30-50%) y constituye la tercera causa mas común de ingresos en el Hospital de Neurología en México (Fleury, *et al.,* 2003). En México, existen datos que estiman una prevalencia de neurocisticercosis del 1.3 % al 3.1 % en diferentes estudios de autopsias realizados en el Hospital General de la ciudad de México (Villagrán and Olvera 1988; Del Brutto, *et al.,* 1998).

Dada la importancia de la cisticercosis en la salud humana y en la porcicultura en México, ésta ha sido objeto de numerosos estudios relacionados con aspectos clínicos (Schantz *et al.,* 1994; Morales, et al 2000; Del Brutto, *et al.,* 2001), patológicos (Restrepo, *et al.,* 1998; Herrera *et al.,* 1999; 2000), así como con el diagnóstico (Schantz, *et al.,* 1994; Ito A, *et al.,* 1998; Sciutto, *et al.,* 1998; Hernández, *et al.,* 2000; Proaño-Narvaez, *et al.,* 2002; Kojic E, *et al.,* 2003), tratamiento (Del Bruto, *et al.,* 2000; García H, *et al.,* 2002) y prevención (Malagón-Valdez, 1999 ; White, et al ., 2000).

Respecto de la teniosis, ésta es una enfermedad exclusiva del hombre y a fin de poder controlarla se han considerado diferentes alternativas para su control como son campañas educativas a fin de informar la importancia de dicha enfermedad así como los riesgos que implica la falta de higiene y el consumo de carne de cerdo contaminada con cisticercos, también se ha utilizado el tratamiento cisticida masivo o selectivo de personas portadoras de gusanos adultos en las comunidades rurales. Actualmente se encuentran en evaluación nuevos métodos específicos para diagnosticar portadores sanos de *T. solium* por técnicas de ELISA y PCR en heces a fin de poder contar con nuevos métodos de detección (González LM *et al.,* 2002; Nunes CM *et al.,* 2003).

Sin embargo, pocos avances se han observado a este respecto, es por esto que la mayoría de los estudios se han enfocado a controlar la enfermedad en el cerdo. Debido a que el cerdo es un huésped intermediario indispensable en el ciclo de vida del parásito, la disminución de la incidencia de la cisticercosis en el cerdo a través de vacunación se ha considerado como una alternativa para prevenir esta enfermedad. Esta estrategia parece una medida realista que no implica modificar las condiciones socioeconómicas en las que subyace esta enfermedad. Además, es factible que la vacunación sea acogida con interés por parte de los propietarios de cerdos criados en forma rústica, considerando que frecuentemente resultan cisticercosos lo que implica pérdidas importantes para el productor por confiscación o venta a bajo costo sin inspección sanitaria, mas aún las posibilidades de éxito de una vacuna aumentan debido a que ésta estaría dirigida a

los cerdos que en su mayoría tienen una expectativa de vida corta (aprox. 1 año) lo cual no demanda una respuesta inmune de larga duración.

Adicionalmente, el desarrollo de la biotecnología ha contribuido en forma importante hacia el diseño de vacunas de nueva generación, más seguras, potentes, económicas y fáciles de aplicar lo que implicaría un control mas efectivo de la enfermedad a corto plazo (Sciutto *et al.,* 2000; Lightowlers, *et al.,* 2001, 2003.)

Existen diferentes especies de tenias que son capaces de infectar a distintas especies de mamíferos. Esto ha promovido la búsqueda de inmunógenos protectores en diferentes modelos de infección (roedores, ovinos y bovinos) (Cardenas F *et al.*, 1989; Harrison and Parkhouse 1989; Ito A 1997). A diferencia de otras parasitosis, en la prevención de la cisticercosis los resultados son alentadores ya que diferentes inmunógenos han demostrados ser efectivos, lo que señala la potencial vulnerabilidad del parásito a la respuesta inmune exacerbada. A este respecto durante los últimos veinte años numerosos estudios han sido realizados a fin de establecer una vacuna dirigida a interrumpir el ciclo de la transmisión de esta parasitosis (Sciutto, *et al.,* 1990, Nascimento, *et al.,* 1995, Molinari, *et al.,* 1997, Plancarte, *et al.,* 1999, Lightowlers, *et al.,* 2001). Algunos de estos estudios han utilizado extractos antigénicos de excreción/secreción obtenidos a partir de cultivos de oncosferas (Pathak and Gaur, 1990), antígenos obtenidos directamente de oncosferas (Molinari, *et al.,* 1988), desarrollos tempranos de cisticercos de *T. solium* (Nascimento *et al.,* 1995) o de *T. crassiceps* (Sciutto, *et al.,* 1995; Manoutcharian, et al, 1996), basándose en el argumento de que estos son los estadios más vulnerables a la respuesta inmune.

Otra de las estrategias por la que se ha optado es la identificación de antígenos protectores (independientemente de la localización que éstos tengan) basándose en la capacidad protectora en modelos experimentales (Sciutto *et al.,* 2000; Vázquez-Talavera, 2001, Vibanco-Pérez, 2002), obteniéndose diferentes grados de protección. Otra consideración para la búsqueda de antígenos de interés resulta la propuesta de candidatos a vacuna entre los antígenos expresados

tempranamente por el parásito en la fase de huevo o de cisticerco recién establecido. Se ha observado que tanto los antígenos de oncosferas como los de cisticercos de *T. solium* pueden conferir una buena protección contra especies homólogas y heterólogas de tenias, probablemente a consecuencia de la conservación de antígenos en las diferentes fases del parásito y entre diferentes especies de tenias (Lightowlers, *et al.,* 1996, Plancarte, *et al.,*1999).

La mayor parte de los inmunógenos de interés se han evaluado en condiciones experimentales obteniéndose en diferente magnitud resultados prometedores al respecto de su capacidad para disminuir la carga parasitaria esperada. Menos alentadores son los resultados obtenidos en la capacidad de vacunas para inducir inmunidad esterilizante, que con diferentes inmunógenos se ha reportado entre el 40 al 50 % (Sciutto, *et al*., 2000).

Si bien aún no se ha enfatizado en la importancia de los efectos terapéuticos asociados a la vacunación, recientemente hemos señalado su relevancia considerando la importancia en términos de transmisión de modificar la viabilidad de los cisticercosis e impedir su desarrollo a tenias (Garcia, *et al.,* 2001).

Al respecto de las formas de evaluación de los inmunógenos de interés identificados, la mayor información se ha obtenido en cerdos desafiados en forma experimental, y aunque estas condiciones de evaluación no necesariamente reflejan las circunstancias del desafío natural, resulta un método conveniente para seleccionar antígenos promisorios para el desarrollo de una vacuna. Debido al alto costo y las dificultades que implica el trabajar con cerdos naturalmente expuestos al parásito, existen pocos reportes de ensayos de vacunas en campo (Molinari, *et al.,* 1997, Huerta, *et al.,* 2000). Sin embargo, estas formas de evaluación resultan críticas y son las únicas que validan definitivamente el uso de inmunógenos para vacunación. En el estudio realizado por Molinari y colaboradores (1997) el inmunógeno evaluado consistió en un extracto antigénico total proveniente de cisticercos de *T. solium.* Este estudio fue realizado en una comunidad rural en donde se encontró una prevalencia de cisticercosis porcina del 2.4 %, la cual disminuyó hasta un 0.4 % después de la vacunación. El efecto de la vacunación

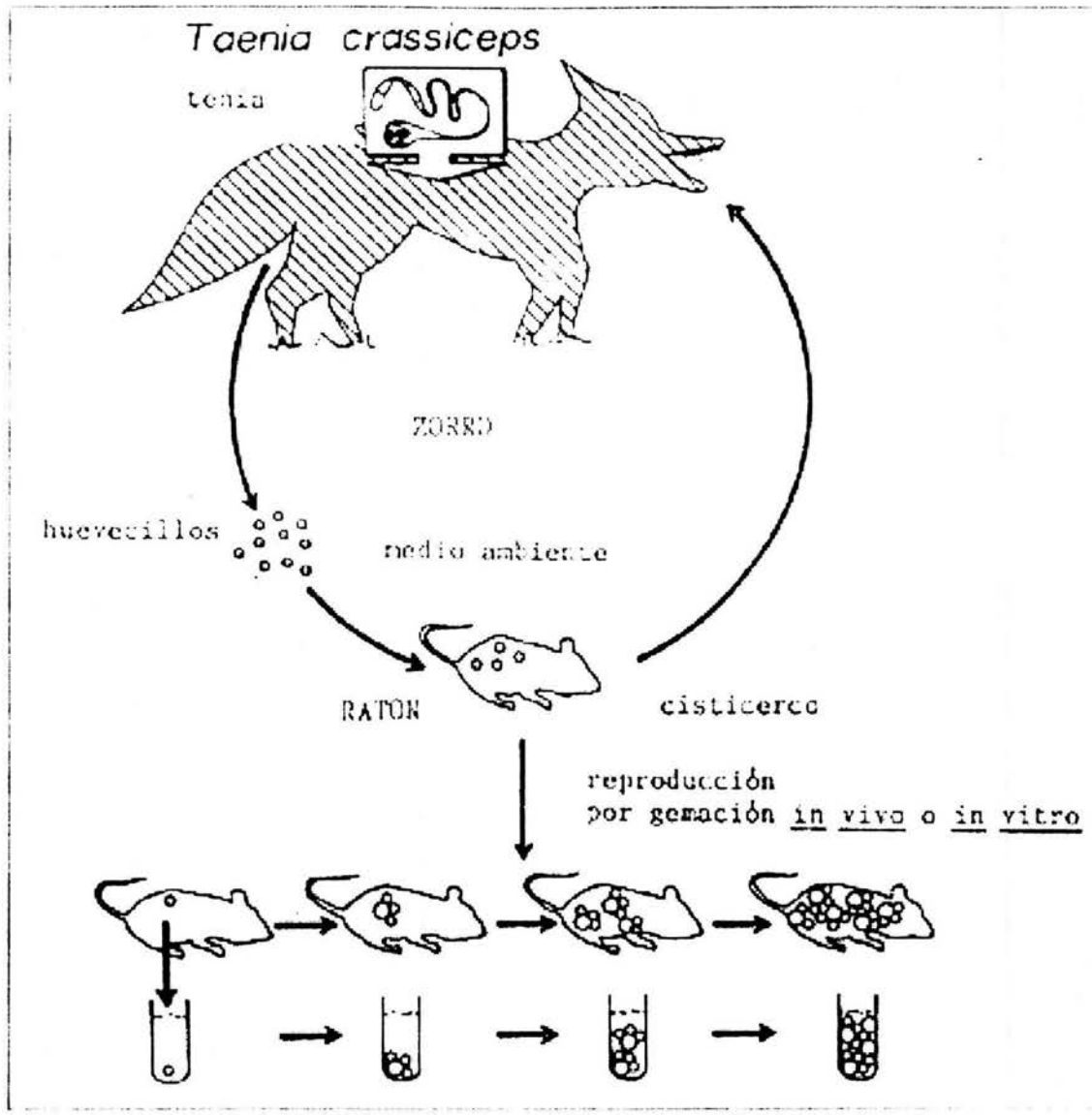

Figura 1. Ciclo de vida de *Taenia crassiceps*

fue evaluado mediante la inspección en lengua de los cerdos vacunados a lo largo de un año. Este trabajo señala por primera vez en la literatura la capacidad de antígenos del parásito de reducir la prevalencia de cisticercosis en condiciones naturales de transmisión con cerdos expuestos al desafío una o múltiples veces, mal nutridos, estresados y genéticamente heterogéneos. Cabe señalar que la detección de cisticercos en la lengua, aunque es muy específica si se practica por personal bien adiestrado, no es muy sensible, pudiendo así no detectarse aquellos cerdos que estuvieran infectados con pocos parásitos. Este aspecto es especialmente importante considerando la baja capacidad de las vacunas aplicadas experimentalmente en inducir inmunidad esterilizante.

El otro estudio de evaluación de vacunas en campo lo constituye el realizado por Huerta y col (2001). En este trabajo se reporta la evaluación de una vacuna basada en tres péptidos sintéticos, en una comunidad rural en el Estado de Puebla. Este estudio se realizó en cerdos rústicos que se distribuyeron en la Comunidad por pares (un vacunado y un control) y se adquirieron a los 8 y 12 meses después, para la inspección de cada canal por necropsias, contando en cada cerdo infectado el número total de cisticercos identificados. En este estudio se observó en un total de 240 cerdos inspeccionados que la vacunación disminuía el número total de cisticercos en un 98% y la prevalencia en un 52 % (Huerta, *et al.,* 2001).

Considerando las dificultades económicas y experimentales que se presentan en la experimentación con cerdos en la evaluación de inmunógenos de interés para el desarrollo de una vacuna, nuestro grupo de trabajo propuso utilizar la cisticercosis murina causada por otro cestodo, *Taenia crassiceps* (Freeman, 1962) como un modelo experimental sencillo donde realizar una primera evaluación de la capacidad protectora de diferentes inmunógenos. Este parásito tiene la particularidad de reproducirse rápidamente por gemación polar múltiple en la cavidad peritoneal de ratones, ofreciendo así una importante fuente de antígenos obtenidos en condiciones experimentales controladas (Figura 1) (Dorais, *et al.,* 1962, Freeman, 1962, Larralde, *et al.,* 1992). La cantidad de parásitos

recuperados puede utilizarse como un parámetro de susceptibilidad a ser modificado por vacunación. Diferentes observaciones nos permiten sostener que ambos cestodos presentan similitudes que permiten considerar adecuado a este modelo para identificar antígenos de potencial interés para el diseño de una vacuna, entre ellas cabe mencionar:

a) Las similitudes entre el ciclo de ambos céstodos incluyen un hospedero intermediario (roedores/cerdo) y un hospedero definitivo (cánidos-felinos/ hombre).

b) Los cisticercos como las tenias de ambas especies presentan una estructura macroscópica similar, con algunas diferencias: los cisticercos de *T. crassiceps* son más pequeños y además presentan la característica única que es su capacidad de dividirse por gemación. Esta característica nos permite mantenerlos en el laboratorio infectando ratones con solo algunos cisticercos recuperando en pocos meses de infección, una gran cantidad que permite obtener hasta gramos de antígeno a partir de cada ratón infectado.

c) Los antígenos de ambos céstodos comparten sus propiedades antigénicas. Así, los antígenos de *T. solium* se pueden reemplazar por los de *T. crassiceps* en el inmunodiagnóstico de la cisticercosis humana (Larralde et al, 1989; Ramos-Kuri et al, 1992) sin detrimento en sensibilidad ni especificidad.

d) Los antígenos de *T. crassiceps* y de *T. solium*, inducen inmunoprotección cruzada. Los antígenos del cisticerco murino son capaces de proteger contra la cisticercosis porcina y viceversa (Manoutcharian, et al, 1995).

e) Diferentes inmunógenos caracterizados como protectores en el modelo murino de cisticercosis fueron confirmados como tales en contra de la cisticercosis porcina (Manoutcharian, *et al.,* 1995) validando la capacidad predictiva de este modelo.

La identificación de antígenos de interés para el diseño de la vacuna actualmente disponible implicó diferentes etapas de evaluación. En una primera etapa se evaluaron los antígenos totales del cisticerco de *T. crassiceps* encontrando que protegen parcialmente en contra de la cisticercosis porcina y que

la capacidad protectora depende críticamente de la dosis de antígeno utilizada, pudiendo inducirse hasta facilitación de la parasitosis a altas dosis (Sciutto *et al.,* 1995).

Considerando estos hallazgos se identificaron en el conjunto de antígenos totales aquellos antígenos protectores presentes en este extracto (Manoutcharian, *et al.,* 1996). Con este propósito se obtuvieron fracciones antigénicas a partir de antígenos totales de cisticercos de *T. crassiceps*. Estas fueron separadas electroforéticamente obteniéndose 12 fracciones antigénicas (8kDa-220kDa) las cuales fueron evaluadas para su capacidad protectora en ratones. De estas 12 fracciones antigénicas sólo ocho fueron capaces de tener un efecto significativo en la reducción de la carga parasitaria de los animales inmunizados con respecto a los controles (Valdez *et al.,* 1994). De ellas se seleccionaron las tres fracciones antigénicas (56, 66 y 74 kDa) que indujeron los mayores niveles de protección a bajas y altas dosis confirmando su capacidad protectora (Manoutcharian, et al, 1995, Valdez, *et al.,* 1994) tanto en contra de la cisticercosis murina como porcina.

Con el objetivo de disponer de cantidades adecuadas de estas fracciones para aplicar una vacuna en forma masiva, se decidió producirlos por medio de técnicas de ADN recombinante. Para ello, se construyó una biblioteca de cADN en el vector Uni-ZapXR utilizando ARNm de cisticercos de *T. crassiceps*. A partir de esta biblioteca se seleccionaron trece clonas recombinantes las cuales fueron identificadas por medio de inmunodetección utilizando anticuerpos policlonales específicos en contra de las fracciones antigénicas protectoras de 56, 74 y 66 kDa. De las clonas identificadas se seleccionaron aquellas que también fueron reconocidas por sueros de cerdos infectados con cisticercos de *T. solium*, ya que de esta manera se podría asegurar que las clonas codificaban para antígenos compartidos por ambos céstodos. Las clonas seleccionadas se denominaron KETc1, KETc4, KETc7, KETc11 y KETc12. (Manoutcharian, *et al,* 1995; 1996). Los antígenos recombinantes codificados por estas clonas se evaluaron en el modelo de cisticercosis murina encontrándose diferentes niveles de protección en cuatro de las clonas utilizadas: KETc1, 4, 7, y 12 (Manoutcharian, *et al.,* 1996).

Con el propósito de identificar las regiones antigénicas protectoras en los antígenos recombinantes, en el presente trabajo de tesis se identificaron y caracterizaron inmunológicamente dos secuencias protectoras denominadas KETc1 y KETc12 en contra de la cisticercosis murina las cuales constituyen parte de la vacuna actualmente propuesta para el control de la cisticercosis.

OBJETIVOS

OBJETIVOS GENERALES

- Identificar secuencias que codifican para epítopes protectores contra la cisticercosis murina, a partir de los antígenos recombinantes protectores previamente reportados.
- Evaluar en el modelo de cisticercosis murina las propiedades inmunogénicas de los epítopes protectores identificados.

OBJETIVOS PARTICULARES

1.- Evaluar la capacidad protectora inducida por epítopes incluidos en los distintos antígenos recombinantes protectores identificados.

2.- Inmunolocalizar los diferentes epítopes en el cisticerco de *Taenia crassiceps* y en las distintas fases del desarrollo de la *Taenia solium* (huevo, cisticerco y adulto).

3.- Evaluar y caracterizar la respuesta inmune humoral y celular en ratones inmunizados con cada uno de estos péptidos.

RESULTADOS

Los resultados publicados de este trabajo de doctorado se presentan en el artículo: Two epitopes shared by *Taenia crassiceps* and *Taenia solium* confer protection against murine *T. crassiceps* cysticercosis along with a prominent T1 response. Andrea Toledo, Gladis Fragoso, Gabriela Rosas, Marisela Hernández, Goar Gevorkian, Fernando López-Casillas, Beatriz Hernández, Gonzalo Acero, Mirna Huerta, Carlos Larralde and Edda Sciutto. Infection and Immunity, 69 (3): 1766-1773, 2001, en el cual se describen los principales hallazgos encontrados relacionados con la protección y caracterización de la respuesta inmune asociada a los péptidos KETc1 y KETc12.

En un apéndice se adjuntan los artículos publicados y los trabajos realizados en colaboración en los que participé durante el periodo de desarrollo de mi trabajo doctoral.

RESULTADOS Y DISCUSION

Anexo en esta sección la descripción más extensa de los resultados obtenidos según los objetivos inicialmente planteados para este trabajo de tesis y su discusión. Esta sección describe detalladamente el proceso y las observaciones que condujeron a los resultados generados algunos de los cuales se incluyen en el artículo principal de mi tesis.

Identificación del efecto protector inducido por la inmunización con los péptidos KETc1 y KETc12

En estudios previamente realizados se identificaron y secuenciaron 4 clonas que codificaban para antígenos protectores en contra de cisticercosis murina. Estas clonas provenían de fracciones antigénicas que habían demostrado ser protectoras no solo en contra de la cisticercosis murina sino en contra de la cisticercosis experimental porcina por *Taenia solium* (Manoutcharian, *et al.,* 1996). Durante varios años se evalúo en nuestro laboratorio la posibilidad de expresar los antígenos recombinantes identificados en diferentes sistemas bacterianos sin lograrlo. Considerando estas dificultades se decidió identificar las secuencias immunogénicas protectoras en estos antígenos recombinantes en miras de producir una vacuna sintética.

Durante mi trabajo de maestría identifiqué una secuencia que codifica para el péptido que denominamos GK1. Este péptido se identificó analizando la secuencia del antígeno recombinante KETc7 (fragmento de 100 aa). Este fragmento antigénico corresponde a una proteína nativa de 56kDa, presenta un alto contenido de prolinas y había demostrado ser protector contra la cisticercosis murina (Manoutcharian, *et al.,* 1996). La capacidad protectora de KETc7 además de ser confirmada por procedimientos convencionales de inmunización lo fue también por vacunación con DNA (Rosas *et al.,* 1998; Cruz *et al.,* 2000). A partir de esta secuencia se realizó una predicción teórica de las regiones de mayor antigenicidad identificándose varias secuencias de potencial interés, entre ellos GK-

1. En un primer análisis se determinó la capacidad antigénica de GK1 evaluando su capacidad de reconocimiento en un panel de sueros provenientes de ratones infectados con cisticercos de *T. crassiceps* y de pacientes neurocisticercosos (Gevorkian, *et al.,* 1996).

Con estos antecedentes comencé mi proyecto de investigación determinando la capacidad inmunogénica de GK1. En los primeros experimentos observamos que la inmunización con este péptido producido en forma sintética indujo la reducción del 57% al 70 % de la carga parasitaria esperada, obteniéndose una inmunidad esterilizante en el 60% de los animales vacunados (Toledo, *et al.,* 1999), mientras que en los animales controles se observaron de 6.9 al 25.1 cisticercos/ratón en los ratones vacunados se recuperaron de 0.7-1.3 cisticercos/ ratón.

Estos resultados parecían alentadores. Sin embargo, considerando que el aumento del tamaño del inmunógeno pudiera aumentar sus propiedades inmunogénicas se consideró presentarlo al sistema inmune utilizando diferentes estrategias. Una estrategia consistió en asociarlo covalentemente utilizando la seroalbúmina bovina como proteína acarreadora. Otra estrategia fue utilizar el sistema multi-antigénico de péptidos (MAP), polímero de lisina que permite expresar el péptido polimerizado en dos, tres, cuatro y cinco unidades por molécula, pudiendo es esta forma aumentar su inmunogenicidad (Tam, 1996). La capacidad protectora del péptido libre, asociado a BSA y a MAP se evalúo utilizando el modelo de cisticercosis murina. No se observó ningún mejoramiento en la capacidad inmunogénica de GK1, de hecho la polimerización con MAP redujo de forma considerable la capacidad de protección. Considerando que en GK1 se destacan regiones altamente hidrofóbicas es probable que la preparación del polímero incluyendo 5 unidades por molécula promoviera interacciones entre las cadenas de los mismos péptidos reduciendo su capacidad de interaccionar con moléculas del sistema inmune.

Así, la identificación de GK1 constituyó un avance importante en la identificación de epítopes protectores. Sin embargo, considerando la complejidad

Table 1. Specificity and sensitivity of ELISA assays using different antigens and CSF from Panel 1 (28 neurological patients with clinically and imagenologically defined diagnosis)

Antigen used	NCC[a]		Other neurological disease[b]	Sensitivity[c]	Specificity
	Active	Inactive			
T. solium[d]	15	1	1	93.7	90
KETc1	11	1	0	68.7	100
KETc12	14	1	0	87.5	100
KETc410	11	1	0	68.7	100
KETc413	9	1	0	56.2	100
Total	16	2	10		

[a] Number of positive patients with active and inactive neurocysticercosis, respectively.
[b] Number of positive non-NCC patients.
[c] Sensitivity was calculated considering only active NCC patients; i.e.: 93.7% represent 15/16. Differences were not statistically significant.
[d] Vesicular fluid from *T. solium* cysticerci was used as a source of antigen.

Tomado de Hernández et al., 2000

de las parasitosis y del propio cisticerco, parece una visión extremadamente sencilla pretender prevenir la cisticercosis utilizando un solo epítope. Basados en las secuencias de antígenos recombinantes protectores adicionales a KETc7 se decidió continuar con la identificación de epítopes adicionales que pudiesen aumentar la eficiencia de vacunación y prevenir las posibles diferencias antigénicas entre parásitos.

Para este propósito se analizaron las secuencias de los antígenos KETc1 y KETc12 basándose en el método descrito por Hoop y Woods (1983), encontrándose que codificaban para dos polipéptidos los cuales se denominaron KETc1 (APMSTPSATSVR) y KETc12 (GNLLLSCL) de 12 y 8 aminoácidos, respectivamente.

En un primer análisis se evalúo la capacidad antigénica de los dos péptidos determinando si eran reconocidos por sueros de ratones y líquidos cefalorraquídeos de individuos neurocisticercosos detectando la presencia de anticuerpos mediante el método de ELISA. Al respecto de su reconocimiento por humanos con neurocisticercosis los resultados indicaron que el uso de ambos péptidos como antígeno para fines diagnósticos presenta una especificidad del 100% y que son capaces de reconocer el 87.5 % en el caso de KETc12 y de 68.7 % para KETc1 de los individuos infectados (Tabla 1, Hernández *et al.*, 2000) cuando los anticuerpos se detectan en LCR. Mientras tanto, en suero los péptidos presentan una muy baja especificidad, observación que aún no se ha explorado más profundamente.

Con el propósito de evaluar la inmunogenicidad de KETc1 y de KETc12 y considerando el tamaño reducido de los 2 péptidos decidimos producirlos en forma sintética incluyendo aminoácidos del vector con el fin de lograr una mayor estabilidad de las secuencias. Así, a las secuencias de KETc1 y de KETc12 se les adicionó 2 (AR) y 4 (NSAR) aminoácidos respectivamente evaluando las propiedades inmunogénicas generadas a través de determinar la capacidad

A: alanina; R: arginina; N:asparagina; S: serina.

protectora de estas secuencias. La incorporación de estos aminoácidos no aumentó la capacidad protectora. De hecho se observaron mayor número de animales totalmente protegidos cuando los epítopes incluían solo las secuencias que codifican para los antígenos recombinantes de 8 y 12 aminoácidos.

La capacidad protectora de estos péptidos fue extensamente evaluada inmunizando ratones machos BALBc/AnN (dos dosis de 10μg de cada uno de los péptidos), y saponina como adyuvante. Como puede observarse en la Tabla 1 la inmunización con cada uno de los péptidos indujo niveles de protección que variaron entre un 67 y 100% en el caso de los animales inmunizados con KETc1, y entre 53 % y 88% en los inmunizados con KETc12. Sólo en un 14-44% y 28-33% de los ratones se obtuvo inmunidad esterilizantes al vacunar con KETc1 y KETc12, respectivamente. Cabe mencionar que estos resultados de protección fueron repetidos en 4 experimentos independientes encontrando resultados muy similares para cada uno de los péptidos.

Un aspecto que llama la atención es la gran variabilidad intra e inter experimento al respecto del número de parásitos recuperados por ratón. En esta variabilidad pudieran estar participando diferencias individuales en la capacidad infectiva de los parásitos, diferencias en la capacidad infectiva de diferentes lotes de parásitos de ratones infectados para este propósito así como diferencias en el hospedero. Entre estas últimas pudiéramos considerar diferencias en el nivel de stress de los animales infectados, diferencias en factores asociados a hormonas sexuales de los ratones, elementos que se han encontrado participan en la susceptibilidad a la cisticercosis murina. Estos aspectos están siendo evaluados en nuestro laboratorio por otros estudiantes participantes en el proyecto.

Respuesta inmune humoral inducida por la inmunización con los péptidos KETc1 y KETc12

Con el objetivo de identificar la presencia de epítopes B en los péptidos KETc1 y KETc12 determinamos si los sueros de ratones inmunizados con cada uno de los péptidos eran capaces de reconocerlos utilizando la técnica de ELISA. Como

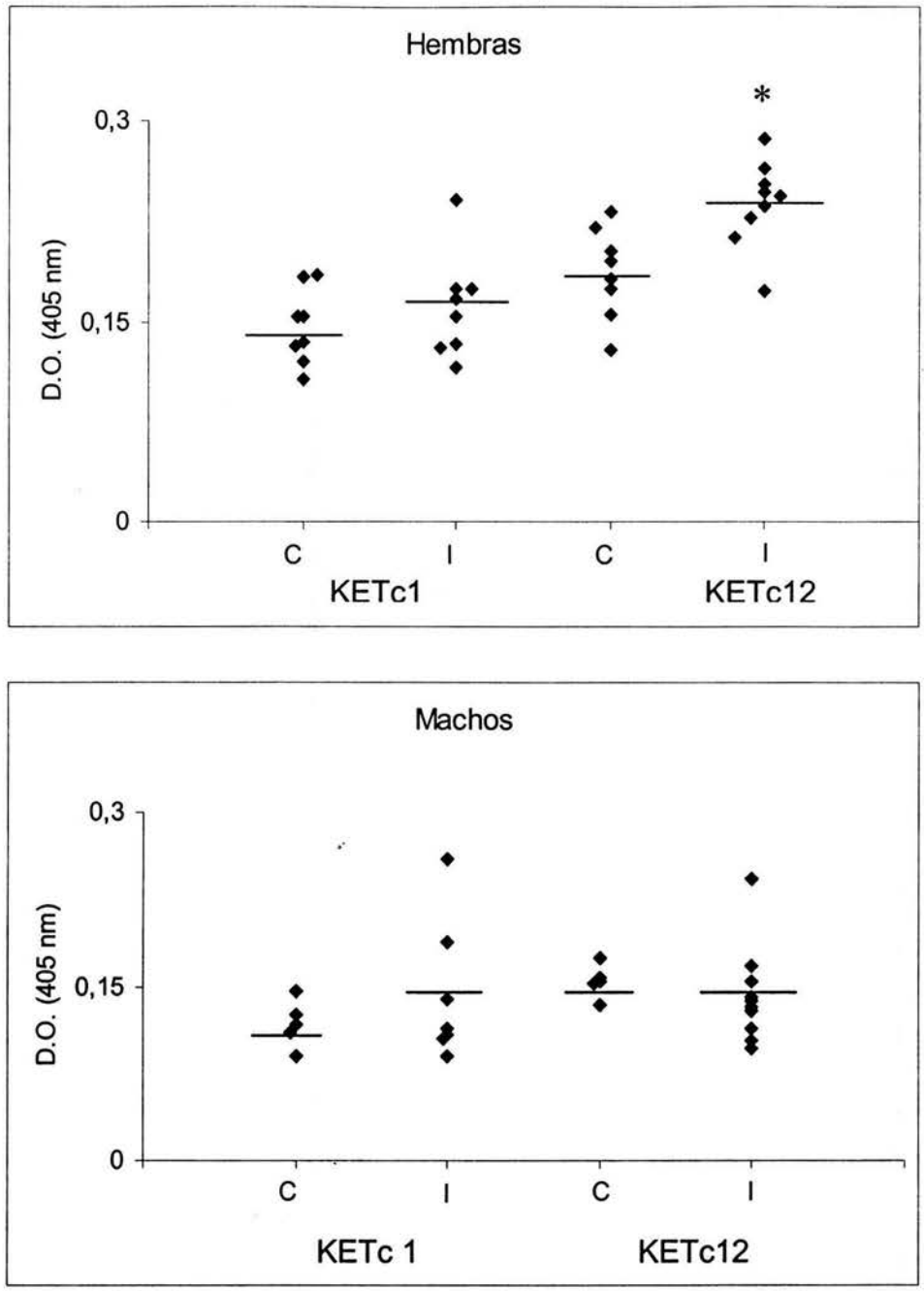

Figura A. Niveles de anticuerpos detectados por ELISA en ratones hembras y machos controles (C) e Inmunizados (I) con los péptidos KETc1 y KETc12, reaccionando con los respectivos péptidos fijados a la placa. *Diferencias significativas ($P \leq 0.004$) Test de Mann Whitney

puede observarse la inmunización indujo, aunque detectables, muy bajos niveles de anticuerpos contra los propios péptidos (Figura A). En algunos ratones no se detectaron anticuerpos en suero después de las dos inmunizaciones realizadas. Como se observa en la figura se detectaron niveles superiores de anticuerpos en hembras que en machos, resultado que contrasta con la mayor eficiencia de protección observada en machos que en hembras. Estas observaciones nos permiten sostener la presencia de dimorfismo sexual tanto en los niveles de anticuerpos como en la eficiencia de protección.

Con el propósito de determinar si en extractos que incluyeran los antígenos solubles del cisticerco se podían detectar la presencia de los péptidos, se realizaron ensayos de ELISA similares, utilizando los sueros provenientes de ratones inmunizados con cada uno de los péptidos y los antígenos solubles tanto de *Taenia solium* como de *Taenia crassiceps*. Los niveles de anticuerpos detectados en contra de los propios péptidos fueron menores que los detectados utilizando antígeno total tanto proveniente de *Taenia solium* como de *Taenia crassiceps*. Es factible que estas diferencias sean consecuencia de la pérdida de reactividad de los péptidos una vez que estos se asocian a la placa de ELISA.

En el intento de confirmar el tamaño de los antígenos nativos se utilizaron los anticuerpos producidos en inmunoblots evaluando su capacidad de reconocimiento de los antígenos provenientes de extractos totales tanto de *Taenia crassiceps* como de *Taenia solium*. Aunque realicé numerosos intentos promoviendo las condiciones experimentales para favorecer el reconocimiento de los antígenos nativos, utilizando diferentes concentraciones de antígeno en el gel, distintos tiempos de incubación del primer y del segundo anticuerpo en ningún caso se detectó reacción positiva. Este hallazgo aunque negativo posiblemente podría ser consecuencia de la modificación de la capacidad antigénica de los respectivos epítopes una vez fijados a la nitrocelulosa.

Inmunolocalización de los péptidos KETc1 y KETc12 en T. crassiceps y T. solium

Los anticuerpos anti-KETc1 y anti-KETc12 inducidos por inmunización se utilizaron con resultados satisfactorios para identificar y localizar a la proteína nativa en el cisticerco de *T. crassiceps*, así como en las distintas fases de desarrollo del parásito de *T. solium*. Para este propósito establecimos una colaboración con la Biol. Beatriz Hernández de la Fac. de Medicina de la UNAM quien maneja y tiene establecidas estas metodologías en su laboratorio.

Como puede observarse en la Figura 2, tanto KETc1 como KETc12 se detectaron en cortes de cisticerco, aunque con una distribución diferencial en el tegumento del cisticerco (Figura 2E y 2G). KETc1 se expresó predominantemente en la parte más externa del tegumento y en los pliegues cuticulares del canal espiral de los cisticercos de *T. solium* (Figura 2F) y solamente en el tegumento de *T. crassiceps* (Figura 2E). KETc12 fue mucho más abundante en ambos cisticercos, sin embargo se observó un patrón de fluorescencia más intenso en el tegumento, parénquima y alrededor de los corpúsculos calcáreos de cisticercos de *T. crassiceps* (Figura 2G), al igual que en tegumento, parénquima y células en flama de cisticercos de *T. solium* (Figura 2H). También fue detectado KETc12 en numerosas partes de la oncosfera (Figura 3G), no así KETc1 que solo se expresa como puntos muy localizados (Figura 3E). Ambos epítopes se detectaron en el gusano adulto, siendo KETc1 abundante en la parte más externa del tegumento (Figura 3F) y KETc12 distribuido hacia el interior del mismo (Figura 3H).

Evaluación de la respuesta inmune celular inducida por la inmunización con KETc1 y KETc12

El estudio de la respuesta inmune celular nos reveló que las células provenientes de los esplenocitos de ratones vacunados tanto con KETc1 y KETc12 presentan capacidad de proliferar ante su inmunógeno respectivo así como cuando son estimuladas con antígenos solubles de *T. crassiceps* (Tabla 2) capacidad proliferativa que no se observa en ausencia de los antígenos

mencionados ni en los animales no inmunizados cuyas células son estimuladas in vitro.

Con el propósito de optimizar las condiciones de proliferación celular se utilizaron diferentes estrategias. Este procedimiento se estandarizó empleando el péptido GK1, por disponer de cantidades adecuadas del mismo y haber caracterizado previamente sus propiedades inmunogénicas. Una de las estrategias empleadas fue la adición a nuestros cultivos de células de bazo, 10^5 células peritoneales provenientes de los mismos ratones inmunizados, encontrando que los niveles de proliferación aumentaban considerablemente. En experimentos posteriores probamos simultáneamente la adición de células peritoneales y β-mercaptoetanol a nuestro medio de cultivo, obteniendo resultados de proliferación muy similares en ambos ensayos. En base a estos experimentos actualmente adicionamos a nuestros medios de cultivo β-mercaptoetanol por ser este un sistema más controlado y más sencillo de manejar con el cual obtenemos resultados de proliferación muy similares a los observados en los experimentos iniciales.

Por medio de citometría de flujo se determinó el incremento de las células proliferadas in vitro tanto con antígeno de *T. crassiceps* (el cual fue de un 3.5 o 4.5% a un 8 o 11%), como con los respectivos péptidos encontrando un incremento del 12.6 % y de 16.3 % para KETc1 y KETc12, respectivamente (Figura 4). Si bien la composición de la población de linfocitos estimulados in vitro resultó estar enriquecida tanto en células CD4+ como en CD8+, encontramos en proporción un menor aumento de las células CD4+ que de células CD8+ al ser estimuladas con KETc1 o KETc12 in vitro (Figura 4).

Para determinar si existía alguna correlación entre la capacidad proliferativa de estas células y un patrón de citocinas en particular, se procedió a la determinación de citocinas intracelulares por medio de citometría de flujo en células previamente estimuladas in vitro (Tabla 3). Observamos un incremento en el nivel de la citocina de tipo inflamatorio INF-γ y de IL-2 producidas por células CD3+ mientras que los niveles del IL-4 e IL-10 también aumentaron pero en una

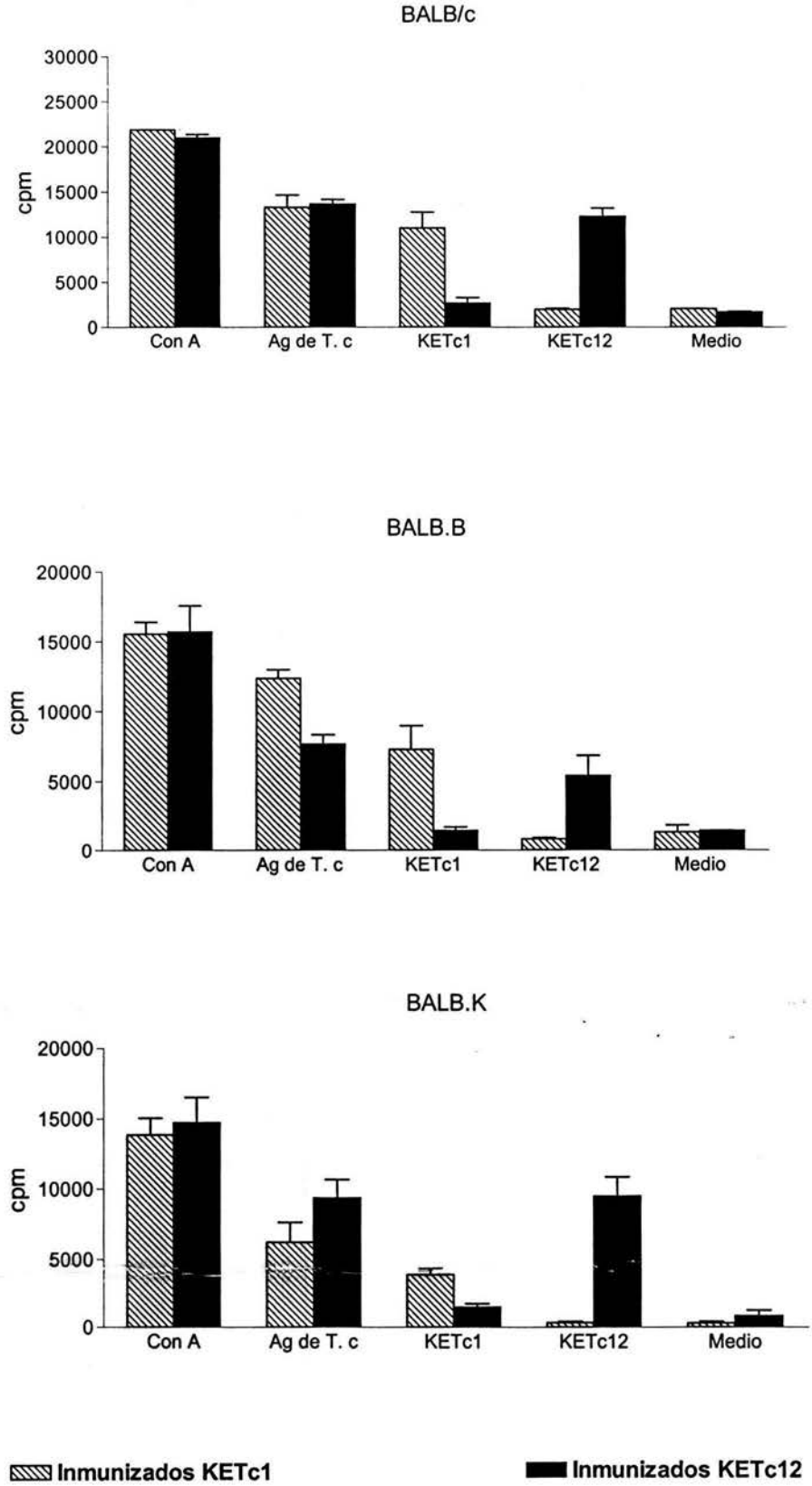

Figura B. Respuesta proliferativa de células T en células de bazo provenientes de ratones inmunizados con KETc1 y KETc12, en diferentes cepas de ratones congénicos.

menor magnitud. Este tipo de perfil de citocinas encontradas en células proliferadas específicamente in vitro fue compatible con el bajo nivel de anticuerpos previamente detectado, en este estudio solo se determinaron las IgG totales, seria interesante poder evaluar si existe la presencia de inmunoglobulinas de tipo inflamatorio como la IgG2a

Con el propósito de determinar las características de restricción por H-2 en la presentación de los péptidos KETc1 y KETc12, realicé una estancia de investigación durante el desarrollo de mi doctorado en el laboratorio del Dr. John Robinson en la Universidad de Newcastle, Reino Unido. A través de esta colaboración se evalúo la capacidad inmunogénica de los péptidos en cepas congénicas de fondo BALB en un bioterio libre de patógenos utilizando lotes de péptidos sintetizados por la propia Universidad de Newcastle.

Para este estudio utilizamos diferentes cepas de ratones congénicos

Cepa de ratón	Haplotipo
BALBc/AnN	d
BALB.B	k
BALB.K	b

Ratones hembras de las distintas cepas fueron inmunizados con los péptidos KETc1 y KETc12, utilizando saponina como adyuvante. Los resultados obtenidos se describen en la Figura B como puede observarse pudimos detectar proliferación celular específica contra cada uno de los péptidos en las cepas BALBc/AnN, BALB.B y BALB.K. Este resultado señala que los péptidos no se encuentran restringidos por MHC. La ausencia de restricción por MHC es compatible con una alta promiscuidad de los péptidos que pudieran ser reconocidos por diferentes haplotipos y también pudiera señalar que su presentación ocurre en el contexto de otras moléculas presentadoras de antígenos más conservadas. En cualquier caso esta observación es promisoria para los fines de vacunación y aumenta la posibilidad de la capacidad inmunogénica de los péptidos en otra especie. De hecho la capacidad protectora de los tres péptidos administrados en conjunto ha

demostrado inducir protección en contra de la cisticercosis porcina. Actualmente, estamos realizando estudios para ahondar en la respuesta inmune inducida por los péptidos a través de diferentes estrategias (i.e. clonación de células T específicas, activación de mecanismos de inmunidad innata, distribución de los péptidos en el organismo, caracterización de las células proliferativas).

CONCLUSIONES

Los resultados obtenidos durante el desarrollo de esta tesis nos permitieron generar las siguientes conclusiones:

1 - Los péptidos sintéticos KETc1 y KETc12 protegen en contra de la cisticercosis murina por *Taenia crassiceps*. La inmunización con péptido KETc1 reduce la cantidad de parásitos esperada de un 67 a un 100% y la vacunación con KETc12 de un 53 a un 81%.

2 - Se detectaron anticuerpos específicos contra los péptidos KETc1 y KETc12 en ratones inmunizados con los respectivos péptidos utilizados en forma lineal

3- Se localizaron los antígenos nativos por inmunofluorescencia en el cisticercos de *T. crassiceps*, así como en huevo, cisticerco y tenia de *T. solium*. Se encontró que ambos péptidos se encuentran ampliamente distribuidos en forma distinta en todos los estadios de desarrollo del parásito *T. solium*

4.- La inmunización con KETc1 y KETc12 indujo una respuesta inmune celular observando proliferación celular, con una población de linfocitos estimulados enriquecidos en células T $CD4^+$ y T $CD8^+$, y un alto porcentaje de células proliferadas que específicamente expresaron IL-2 e INF-γ. Estos resultados indican una respuesta preferencialmente de tipo T1.

5.- Los péptidos KETc1 y KETc12 se presentan en contexto de H-2d, H-2b y H-2k de acuerdo a los resultados observados inmunizando las diferentes cepas congénicas BALB.

APENDICE I

Trabajos en colaboración:

Durante mi proyecto de doctorado participé en otros proyectos relacionados con el estudio de la vacuna contra la cisticercosis porcina. En los distintos trabajos mi colaboración consistió desde el asesoramiento de algunos de los estudiantes de maestría y licenciatura participantes como en la realización de algunas de las partes experimentales de los estudios realizados así como el análisis de los resultados obtenidos y su interpretación.

Algunos de los trabajos realizados durante este periodo se encuentran publicados o sometidos para su publicación, y otros estamos preparando los respectivos manuscritos para ser sometidos a su publicación en un futuro cercano.

Resultados incluidos en artículos publicados o sometidos

I.- Inhibitory role of antibodies in the development of *Taenia solium* and *Taenia crassiceps* towards reproductive and pathogenic stages. Gemma García, Edda Sciutto, Gladis Fragoso, Carmen Cruz Revilla, **Andrea Toledo**, Nelly Villalobos, Iván flores, Aline Aluja, Marco V. José and Carlos Larralde. J. of Parasitology. vol 87 (3) 582-586. 2001

II.- Huerta M., De Aluja A.S., Fragoso G., **Toledo A.**, Villalobos N., Hernández M., Gevorkian G., Acero G., Díaz A., Alvarez I., Avila R., Beltrán C., García G., Martínez J.J., Larralde C., Sciutto E. Synthetic peptide vaccine against *Taenia solium* pig cysticercosis: successful vaccination in a controlled field trial in rural Mexico. Vaccine 20, 262-266, 2002.

III.- Edda Sciutto, **Andrea Toledo** , Carmen Cruz-Revilla, Gabriela Rosas , Gabriela Meneses, Diego Laplagne , Natalia Ainciart, Gladis Fragoso, Fernando Goldbaum . 2004. *Brucella spp.* lumazine synthase: A novel antigen delivery system. Articulo sometido para su publicación en Vaccine.

IV.- En agradecimiento.

Diaz MA, Villalobos N, de Aluja A, Rosas G, Gomez-Conde E, Hernandez P, Larralde C, Sciutto E, Fragoso G. Th1 and Th2 indices of the immune response in pigs vaccinated against *Taenia solium* cysticercosis suggest various host immune strategies against the parasite. Vet Immunol Immunopathol, 20;93(3-4):81-90, 2003

Resultados adicionales no publicados

Con el fin de optimizar la producción de los péptidos en gran escala así como aumentar su capacidad inmunogénica se ha ahondado en la búsqueda de nuevas alternativas para la expresión y presentación de los antígenos vacunales. Entre ellas he participado evaluando la capacidad inmunogénica y protectora de los péptidos expresados en el fago filamentoso M13 y en forma quimérica asociado a lumasina sintetaza. A continuación se presentan los principales resultados utilizando estas estrategias que señalan el potencial interés de las mismas.

Expresión de epítopes en fagos filamentosos

La técnica de despliegue en fagos (Phage Display) se refiere a la fusión de péptidos/proteínas a proteínas de cubierta de bacteriófagos con la consecuente expresión de los mismos en la superficie de los fagos. M13 es uno de los bacteriófagos filamentosos más comúnmente utilizado y está constituido por una hebra de DNA genómico circular de cadena sencilla empaquetado en una estructura de forma tubular. La proteína principal pVIII del fago se expresa en 2,700 copias y cubre la mayor parte de su superficie. Adicionalmente el fago expresa otras cuatro proteínas menores denominadas pIII, pVI, pVII y p IX, localizadas a los extremos del fago como puede observarse en la Figura C (Marvin et al., 1998). Las bibliotecas de fagos son capaces de mostrar un gran repertorio ($> 10^{11}$) de péptidos, fragmentos de anticuerpos o cDNA en fusión con pIII o p VIII, lo que permite caracterizar *in vitro* e *in vivo* una gran cantidad de

interacciones sean estas proteína-proteína, enzimas–substratos, receptores-ligandos, proteínas-ADN y hasta interacciones proteicas-no proteicas mediante procedimientos de bioselección (Manoutcharian et al., 2004). La amplificación de estos fagos se lleva a cabo mediante la infección de bacterias, y debido a que el fago es no-lítico, estos se recuperan en el medio de cultivo.

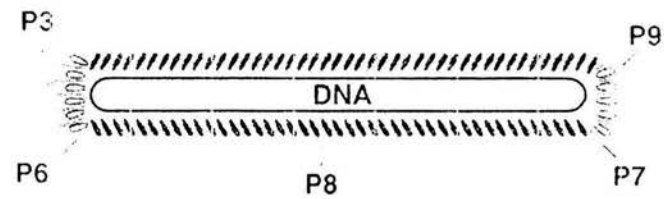

Figura C. Diagrama del bacteriófago mostrando la hebra de DNA (core) y las 5 proteínas de cubierta.

Numerosos estudios han demostrado que las partículas de fagos son altamente inmunogénicas y capaces de inducir anticuerpos antígeno específicos en ratones y conejos aún sin necesidad de uso de adyuvantes (Willis et al., 1993; Meola *et al.*, 1995) debido a la capacidad del fago para reclutar células T cooperadoras. También se ha descrito la capacidad de los fagos para inducir una respuesta inmune con la participación de linfocitos T citotóxicos. Al respecto de la cisticercosis se ha reportado la generación de una repuesta protectora de tipo celular en contra de la cisticercosis experimental murina utilizando como inmunógeno partículas de fagos recombinantes que expresaban en su superficie epítopes para células T (Manoutcharian *et al.*, 2001).

Como se mencionó anteriormente, con el propósito de disponer de grandes cantidades de los péptidos que constituyen actualmente la vacuna sintética y reducir los costos de producción, se procedió a expresarlos en la superficie del fago filamentoso M13.

Los péptidos vacunales GK-1, KETc1 y KETc12 y el antígeno recombinante KETc7 previamente identificados como protectores en contra de cisticercosis experimental murina se expresaron como proteínas N-terminales fusionadas a las

proteínas de cubierta del fago: KETc1 se fusionó a la proteína pIII, la cual se presenta en 3-5 copias por partícula viral, mientras que GK-1, KETc12 y KETc7 se fusionaron en un fago híbrido M13 a PVIII obteniéndose entre 300-900 copias por fago. Estas clonas se designaron como FKETc7, FKETc12, FGK-1 y FKETc1 y se purificaron individualmente. Como inmunógeno se utilizó una mezcla de las 4 clonas (1:1:1:30) a la cual se le denominó CPhV (cysticercal phage vaccine; Manoutcharian K. *et al.*, 2004). Este inmunógeno fue evaluado en ratones y cerdos experimentalmente encontrando que los péptidos mantenían su capacidad protectora expresándolos en M13.

Con el propósito de evaluar más extensamente la capacidad protectora de este nuevo inmunógeno se utilizaron diferentes concentraciones y condiciones de inmunización utilizando el modelo de cisticercosis experimental murino (Tabla I). Un primer hallazgo fue la gran capacidad protectora del fago per se que reduce la cantidad de parásitos promedio esperados de 483 a 89 o 35 según la cantidad de fago utilizada. Este hecho podría ser factible debido a que el propio fago genere un proceso inflamatorio reclutando células inmunes en forma no-específica que interrumpa la instalación del parásito. Esta respuesta podría estar mediada por su capacidad de incluir secuencias que puedan ser reconocidas por receptores tipo "Toll-like", sin embargo esta posibilidad habría que explorarla ya que no se ha descrito previamente en la literatura.

Además de la respuesta protectora asociada al propio fago, la incorporación de las secuencias vacunales aumenta la capacidad protectora en contra de la cisticercosis murina desde un 88% a un 96% (respecto a los animales no vacunados) y de 82% a 92 % respecto a los animales inmunizados solamente con el fago M13 de acuerdo a la dosis de inmunógeno utilizada, induciendo porcentajes similares a los observados por la inmunización con la vacuna sintética. Interesantemente encontramos que muchos de los animales se encontraron totalmente protegidos (5 ratones totalmente protegidos cuando se utilizó la dosis baja y 2 cuando utilizamos la dosis alta).

Tabla I. Efecto de la inmunización con fagos en la carga parasitaria en contra de cisticercosis experimental murina.

No inmunizados	483,781,299,370,483[b] 483 ± 212 [a]
Ratones inmunizados con:	
5×10^{10} Fago M13	5,17,51,82,154,156,164 89 ± 68
Fago CPhV	0,0,0,0,0, 3,7,144,143,288 58 ± 99
5×10^{11} Fago M13	1,7,7,8,8,19,36,79,80,110 35 ± 39
Fago CPhV	0,0,1,2,3,3,3,5,8,60,106 17 ± 34

Ratones hembras fueron inmunizados con el fago M13 o CPhV (cysticercosis phage vaccine) en dos dosis ($5x10^{10}$ fagos/ratón ó $5x10^{11}$ fagos/ratón) en intervalos de 15 días. Quince días posteriores a la última dosis los ratones se infectaron con 10 cisticercos inoculados con aguja de insulina en 0.5 ml de solución salina. Los ratones fueron sacrificados 45 días más tarde y se determinó la cantidad total de parásitos en cada uno de ellos. [a] promedio ± DS., [b] Número individual de cisticercos por ratón.

Considerando que los fagos se cultivan creciendo bacterias en medios de cultivo convencionales, las partículas de fagos se recuperan a partir de los sobrenadantes de los medios de cultivo y posteriormente se realiza el proceso de purificación por medio de precipitación con polietielenglicol, fase de purificación que aumenta considerablemente el costo de su producción masiva. Con el propósito de reducir los costos de la vacuna se consideró omitir el proceso de purificación e inactivar productos bacterianos como se procede para la preparación de otras vacunas. Se evalúo la capacidad de dos compuestos, formaldehído y

cresol en diferentes condiciones experimentales para inactivar posibles productos patogénicos y/o tóxicos provenientes de las propias bacterias. Adicionalmente, y con el propósito de no permitir la replicación del fago en el organismo vacunado se evaluó el uso de luz ultravioleta para su inactivación.

Los resultados de protección obtenidos con los fagos sometidos a los distintos tratamientos se presentan en la Tabla II. Como puede observarse las mejores condiciones de inocuidad e inmunogenicidad de la vacuna se obtuvieron utilizando formaldehído al 0.05%. En estas condiciones se redujo significativamente la carga parasitaria, induciendo protección esterilizante en 7 de los 10 ratones vacunados. También se observó una reducción significativa de la carga parasitaria utilizando fago inactivado con cresol al 0.05%, sin embargo, los animales presentaron síntomas de un estado de salud alterado (pelo erizado, necrosis en el sitio de inoculación). Al respecto de los demás tratamientos si bien se obtuvieron algunos ratones totalmente protegidos no se observó una reducción significativa de la carga parasitaria y en algunas de las condiciones utilizadas las condiciones de inocuidad no fueron satisfactorias.

El uso de saponina como adyuvante si bien redujo la carga parasitaria en los ratones vacunados y protegió en forma esterilizante del 50 %, indujo alteraciones en el estado de salud de los ratones. Los efectos colaterales observados podrían resultar consecuencia de la capacidad de la saponina de exacerbar la inflamación induciendo hemólisis. El uso de luz UV tanto con el fago M13 purificado como en la vacuna purificada (CPhV P) si bien disminuyó importantemente el título de fagos no modificó la carga parasitaria esperada. Sin embargo, como puede observarse en el grupo control M13, todos los ratones murieron. Este efecto no creemos se asocie al tratamiento con UV sino a una posible contaminación con polietilenglicol remanente utilizado para la purificación. Actualmente, se está continuando esta investigación con el propósito de acceder a la evaluación en campo de esta nueva versión de la vacuna expresada en fagos recombinantes inactivados.

Tabla II. Efecto de la inmunización con fagos filamentosos utilizando diferentes métodos de inactivación

	Promedio del número de parásitos ± D.S. (ratones con Np>0)	Número de ratones totalmente protegidos	Estado de salud
Naive	219 ± 139	1/10	Sin modificaciones
Tratamiento			
M13 P + UV	-	-	Todos muertos
CPhV P + UV	143 ± 154	2/7	3 muertos. 7 pelo erizado, necrosis en sitio de inoculación
M13 NP + UV	126 ± 106	4/9	Sin modificaciones
CPhV NP + 0.05% CH_2O	34 ± 53[a]	7/10	Sin modificaciones
CPhV NP + 0.05% Cresol	75 ± 53[b]	5/9	1 muerto. 9 Sin modificaciones
CPhV NP +0.5% CH_2O	200 ± 165	2/9	Sin modificaciones
CPhV NP + 0.5% CH_2O + SAP	94 ± 107	4/8	2 muertos. 8 sin modificaciones

Ratones hembras Balb/cAnN fueron inmunizados con el fago M13 o CPhV (cysticercosis phage vaccine) inactivado de diferentes formas, en dos dosis ($5x10^{10}$/ratón) en intervalos de 15 días. Quince días posteriores a la última dosis los ratones se infectaron con 10 cisticercos inoculados con aguja de insulina en 0.5 ml de solución salina. Los ratones fueron sacrificados 40 días más tarde y se determinó la cantidad total de parásitos en cada uno de ellos. NP: No purificada. CH_2O: Formaldehído. [a]. p=0.019, [b]. p= 0.057 estadísticamente significativo Fisher's exact test.

Uso de proteínas bacterianas como acarreadores. Lumazina Sintetasa de Brucella spp.

La enzima lumazina sintetasa de *Brucella spp.* es una proteína altamente inmunogénica (18 kDa en su forma monomérica) aún en la ausencia de adyuvantes. Tiene una estructura pentamérica (90 kDa) en su forma nativa y presenta su sitio activo en la interfase formada por dos monómeros adyacentes. La estructura tridimensional muestra que los últimos 8-10 residuos amino terminales de cada monómero no son esenciales para el plegamiento general del pentámero (Goldbaum *et al.*, 1998). Debido a esta característica es posible crear proteínas quiméricas insertando péptidos en los residuos N-terminales sin dañar el plegamiento general, funcionando así como proteína acarreadora. Alternativamente, se ha demostrado su capacidad como molécula activadora de la respuesta inmune celular, activando células T CD8+ vía MHC I, provocando una buena y muy prolongada respuesta inmune de tipo humoral y celular debido al rearreglo polimérico cuaternario que presenta su estructura (Velikovsky *et al.*, 2000) (Figura D).

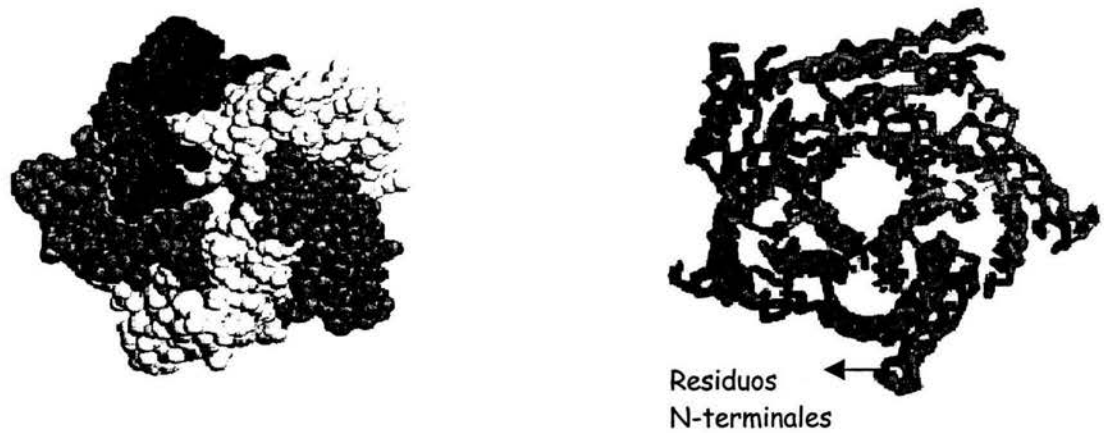

Figura D.

Modelo espacial de la enzima pentamérica lumazina sintetasa de *Brucella*.

Base estructural de la lumazina sintetasa como molécula acarreadora de otros péptidos inmunogénicos.

En base a las características inmunogénicas y estructurales que presenta la proteína lumazina sintetasa se procedió a la construcción y expresión de las quimeras utilizando la estructura pentamérica de la lumazina (BLS) como un acarreador. Actualmente se han producido satisfactoriamente dos construcciones quiméricas: BLS-KETc1 y BLS-KETc12.

A la fecha hemos avanzado evaluando la capacidad inmunogénica de la quimera BLS-KETc1. En el trabajo anexo se presentan los resultados obtenidos en forma de artículo el cual ha sido sometido para su publicación en una revista internacional, los cuales claramente señalan la capacidad de estas construcciones para promover la respuesta inmune humoral.

Brucella spp. lumazine synthase: A novel antigen delivery system

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Abstract

Lumazine synthase from *Brucella spp.* (BLS) was evaluated as a protein carrier to improve antigen delivery of KETc1, one of the peptides of the anti-cysticercosis vaccine. KETc1 fused to BLS improves its antigenicity and immunogenicity. KETc1 and BLS-KETc1 were not MHC haplotype-restricted albeit KETc1 is preferentially presented in *H-2*^{b} haplotype. KETc1 is not class I-restricted since no differences in the proliferative response were found between C57BL/6J.B2m^{tm1Unx} and BALB.B. The higher proliferative response in BALB.B indicates epitopes class I MHC restricted in BLS. These findings support that BLS is a potent new delivery system for the improvement of subunit vaccines.

Keywords: *Taenia solium*, antigen delivery, lumazine synthase

Running Headline: antigen delivery

1. Introduction

Over recent years new types of vaccines have been developed based on important advances in biotechnology [1, 2]. Subunit vaccines involving peptides that contained epitopes capable of inducing protective immunity against infectious organisms have been identified [3, 4]. However, frequently, subunit vaccines are weak immunogens, require the use of adjuvant and need to be given in more than one dose [5]. Different novel approaches are being used to improve vaccine efficacy avoiding side effects and reducing the cost of immunization. Among them, several protein carriers have been tested to improve vaccine delivery [6, 7, 8, 9]. A recently structurally described bacterial enzyme identified from *Brucella spp.* has several properties that make it a promising antigen carrier for vaccine preparations. It has been proved that BLS assembles as a remarkable stable dimer of pentamers, representing a new quaternary order between the lumazine synthases family [10]. The presence of ten sites of linkage and the conformation of its N-termini linked to its high stability indicate the possibility to insert small epitopes without disturbing its conformation. In addition, this protein seems to be useful as a carrier molecule since it possesses immunostimulatory properties, even in the absence of any adjuvant. It can activate the cellular responses by delivering CD8+ T-cell epitopes into the MHC class I pathway [11], and elicits a strong and long-lasting humoral and cellular responses [12]. Considering its multivalence and the possibility of applying different protein engineering strategies on BLS [13] it is also possible to consider this carrier to design multi-epitope-vaccines.

On the other hand, a vaccine against porcine cysticercosis due to *Taenia solium*, a parasite that seriously affects human health in non-developing countries has been recently developed [14, 15]. Considering that pigs are the only intermediate host of the parasite, this vaccine is proposed as a tool to interrupt transmission and prevent the human disease [16]. The vaccine is composed by three well-defined peptides of 8 (KETc12), 12 (KETc1) and 17 (GK1) amino acids originally identified in *Taenia crassiceps* [17] a parasite closely related to *T. solium*. These peptides have been shown to induce high level of protection against experimental murine cysticercosis, an experimental model successfully used to test

candidate antigens for vaccination against porcine *T. solium* cysticercosis and were identified in all the developmental stages of the parasite [18, 19]. More recently the vaccine was successfully tested in a field trial against pigs cysticercosis importantly reducing the number of established parasites and their viability [16]. Thus, it has been considered a useful tool to effectively interrupt transmission. For this purpose, the main limitation is that the vaccine is synthetically produced at a very high price, particularly when considering that its intended target is a population of pigs belonging to people of scarce means [20]. Thus, it is an urgent need to improve the delivery of the developed vaccine to be extensively used as a tool to control cysticercosis in developing countries where cysticercosis is endemic.

The goal of the present study was to evaluate the effect of *Brucella spp.* lumazine synthase (BLS) as a vaccine carrier, which is known to possess convenient physiochemical properties for this purpose, and to study the possibility of improving the magnitude of the immune response induced by one of the peptides (KETc1) of the anti-cysticercosis vaccine described above. The antigenicity and immunogenicity of the chimeric protein named, BLS-KETc1, created by fusing the peptide KETc1 to the 10 N-termini of BLS and expressed in *E. coli* was evaluated and compared with the linear peptide KETc1.

2. Materials and Methods

2.1. Mice

Six to 8-week-old female mice of the GDM strain (C57BL/6J-B2m^{tm1Unx}) on a C57BL/6J genetic background, and the *H-2* congenic BALB.B and BALB. K strains mice were obtained from The Jackson Laboratory (Bar Harbor, ME.). Female BALB/cAnN mice were bred at the Instituto de Investigaciones Biomédicas animal facilities by brother-sister mating. All mice were housed in our animal facilities in microisolators supplied with sterile food and water under pathogen-free conditions. The experiments reported herein were conducted according to the principles set

forth in the Guide for the Care and Use of Laboratory Animals, Institute of Laboratory Animals Resources, National Research Council, Washington, D.C.

2.2. Peptide

A *Taenia crassiceps* derived peptide that is shared by *Taenia solium* [19], KETc1 (APMSTPSATSVR(G)) was synthesized by stepwise solid-phase synthesis with N^{α}-*tert-butyloxycarbonyl* (BOC) derivatives of L-amino acids on PAM (phenyl-acetamidomethyl) resin (Sigma Chemical Co, St. Louis, MO.). The peptide was 95% pure as judged by HPLC in an analytical C_{18} reversed-phase column (3.9 x 150 mm; Delta Pak, Waters). The correct amino acid sequence was confirmed by protein sequencing on a pulsed-liquid-phase protein sequencer (Applied Biosystems, Foster City, CA.) at the National Institute of Cardiology, Mexico City.

2.3. Expression of the BLS Protein

The *Brucella spp.* BLS gene was cloned in pET11a vector (Novagen, EMD Biosciences, USA) as reported previously [10]. The plasmid was used to transform BL21 (DE3) strain *Escherichia coli* competent cells (Stratagene, La Jolla, CA.). Ampicillin resistant colonies were grown until OD_{600}= 1.0 in LB medium containing 100 µg/ml of ampicillin, at 37°C with agitation (300 rpm). Five ml of this culture were diluted to 500 ml and grown to reach an OD_{600} of 1.0. At this point the culture was induced adding 1 mM IPTG and incubated for 4 hours at 37°C with agitation (300 rpm). The bacteria were centrifuged at 15,000 X g during 20 min at 4°C.

2.4. Protein Purification and Refolding

BLS protein was successfully expressed as inclusion bodies by transformation of strain BL21 (DE3) *E. coli* competent cells. The inclusion bodies

were solubilized in 50 mM Tris, 5 mM EDTA, 8 M urea pH 8.0 at room temperature overnight with agitation. The solubilized material was refolded by dialysis during 72 hours against PBS containing 1mM dithiothreitol. This preparation was purified in a MonoQ column in a FPLC apparatus (Pharmacia, Uppsala, Sweden) using a linear gradient of buffer B (50 mM Tris, 1 M NaCl, pH 8.5). The peak enriched with BLS was further purified on a Superdex-200 column with buffer PBS, 1 mM DTT. The purity of the BLS preparation was determined on SDS- 15% (w/v) polyacrylamide gels. Purified BLS was concentrated (10 mg/ml), frozen in liquid N2 and stored at –20°C.

2.5. Preparation and expression of the chimeric protein BLS- KETc1

A pET11a plasmid containing the open reading frame of *Brucella spp.* lumazine synthase was digested with the restriction enzymes BamH I and Xba I and subcloned in the vector pALTER-Ex1 (Promega, Madison, WI.). Using the altered Sites II (Promega) site-directed mutagenesis kit, two new restriction sites were introduced: a Nsi I site in the two first codons of the 5' extreme and a Afl II site in the two codons comprising residues 8 and 9 of the wild type amino acid sequence. The resulting sequence contains a His residue instead of Ala at position 1, and a Leu residue instead of Asn at position 8 of the coding sequence of the wild type protein. After checking the mutations by sequencing, the construction was subcloned again in the pET11a vector. The mutated pET11a plasmid was digested with the restriction enzymes Nsi I and Afl II, removing the coding sequence of the first 8 residues. The wild type sequence was exchanged for the sequences pertaining to the peptide KETc1. To insert this sequence, two oligonucleotides were synthesized (Integrated DNA Technologies, Coralville, IA.) in a way that, after annealing, they contain the coding sequence APMSTPSATSVR followed by two additional residues for spacing (GS) plus the corresponding overhangs for ligation at the Nsi I (5´) and Afl II (3´) ends. The generated double stranded oligonucleotide was ligated with the previously digested mutated pET11a by overnight incubation at 16 C with T4 DNA ligase (Promega). The ligation mix

was used to transform competent *E. coli* DH5α cells. The insertion was verified by colony-PCR using as primers one of the peptide specific oligonucleotides and the T7 terminator primer. The construction was finally checked by automated sequencing.

2.6. Immunization of mice

To evaluate the humoral response, groups of eight female BALB/cAnN mice each were subcutaneously (s.c.) immunized with 10 μg/mouse or 2.5 μg/mouse of KETc1 in saponin (100 μg/mouse) (Sigma Chemical Co.) as described elsewhere [19] or 50 μg/mouse of BLS-KETc1 plus saponin (50 μg/mouse). Immunization was performed twice at 10-day intervals and the animals were bled by retro orbital plexus puncture 10 days after the second immunization. Sera were stored at 70 °C until assayed.

To evaluate the cellular immune response, groups of 3 to 5 mice of different strains were s.c. immunized with two doses of 50 μg/mouse of BLS-KETc1 plus saponin (50 μg/mouse) or KETc1 (10 μg/mouse) plus saponin (50 μg/mouse). For the inhibition experiment, no adjuvant was used.

2.7. ELISA for antibodies

Microtitre plates (Costar, Corning Incorporated, Corning, NY, USA) were coated with 2.0 μg per ml of the synthetic peptide, or BLS wild type or BLS-KETc1 at room temperature. To determine the antibody levels, plates were incubated with the diluted sera (1:100) followed by the addition of alkaline phosphatase-conjugated anti mouse IgG, following the previously described procedure [19]. Color was developed at RT with p-nitrophenyl phosphate disodium (Sigma Diagnostics, Poole Dorset, UK.) and absorbance was measured at 405 nm in a Humareader ELISA processor (Human Gessellschaft für Biochemica und Diagnostica, Taunnusstein, Germany).

An inhibition ELISA experiment was performed using the same procedure as described above diluting the sera 1:200 and previously incubated or not with soluble BLS-WT at 10 or 20 μg/ml during 20 min at room temperature before being added to the plate.

2.8. Proliferation assay

Spleens from non-immunized and immunized BALB/cAnN (*H-2*^{d}), BALB.K (*H-2*^{k}), BALB.B (*H-2*^{b}), and C57BL/6J-B2m ^{tm1Unx} mice were collected under aseptic conditions 3 days after the last immunization. The cells were washed and cultured in RPMI 1640 medium (Gibco, InVitrogen Corporation, Grand Island, N.Y.), supplemented with L-glutamine (0.2mM), 2-mercaptoethanol (0.05mM), non-essential amino acids (0.01mM), penicillin (100 U/ml), streptomycin (100 μg/ml) and 10 % fetal bovine serum (FBS). Cells were cultured with media, or Con A (5 μg/ml) or KETc1 or BLS-KETc1 (10 μg/ml) as described elsewhere [21]. Briefly, cells were incubated at 37°C in a 5% CO_2 humidified atmosphere, in flat-bottomed microtiter plates, at a concentration of 2 x 10^5 cells per 200 μl final volume. After 72 hr, the cultured cells were pulsed (1 μCi per well) for a further 18 hr with [methyl-^{3}H] thymidine (Amersham Biosciences, UK.). Then, cells were harvested and the amount of incorporated label was measured by counting in a 1205 betaplate^{TM} liquid scintillation counter (Wallac Oy, Turku, Finland). All assays were performed in triplicate cell samples from each of 3 to 5 individual mice per group. Data are expressed as the mean counts per minute (cpm) for each experimental group.

2.9. Statistical analysis

Data were analyzed by one-way ANOVA Tukey´s test. All analyses were performed using the Instat Software Program (GraphPad).

3. Results and Discussion

This study revealed that *Brucella spp.* lumazine synthase is an adequate carrier to present foreign epitopes to the immune system. It tolerates the insertion of a 12 amino acid peptide encoding one of the peptides of the vaccine against *Taenia solium* cysticercosis, increasing the length of the inserted epitope, as well as inserting multicopies of this epitope, improving both its antigenicity and immunogenicity.

As Table 1 shows, KETc1-immunized mice elicited detectable levels of IgG antibodies (Abs) against KETc1 only detectable when BLS-KETc1 was used as the source of antigen in the ELISA plate. No differences were observed between the two doses tested (2.5 or 10 μg of KETc1/mouse). Considering the small size of this peptide (12 amino acids), its conformation is probably modified when bound to the ELISA plate with the consequent loss of its capacity for antibody recognition. The possibility that KETc1 was not bound to the plate was discarded considering that sera from *T. crassiceps*-infected mice recognized the peptide in ELISA.

Moreover, BLS also improves the immunogenicity of KETc1. As shown in Table 2, one dose of 50 μg of BLS-KETc1/mouse (containing 2.5 μg of KETc1) elicited IgG antibodies that recognized BLS-WT and more importantly BLS-KETc1. Levels of IgG Abs are effectively increased after boosting in both cases. The increased level of Ab determined when BLS-KETc1 is used as antigen is specific against KETc1 as shown in Figure 1. Previous incubation of anti-BLS-KETc1 sera with 20 μg of BLS-WT importantly reduced the level of antibodies generated by the BLS epitopes of the chimera (Fig 1A) remaining an important reactivity against BLS-KETc1 (Fig. 1B), confirming the presence of specific antibodies against KETc1. These results are not unexpected since it was previously reported that BLS per se, is able to induce a vigorous IgG response, also in the absence of any adjuvant [11]. The polymeric nature of BLS (i.e., that of a repetitive and spatially ordered array of the same epitopes) could explain this strong B-cell response producing a strong signal transduction mediated by B-cell receptors, as described previously in a study using haptens as model antigens [22]. I n this sense, the

three-dimensional structure of BLS shows that any given epitope would be inserted at ten different points located at the vertices of two planar pentagons, separated by a regular distance of around 40 Å and would be displayed to the solvent in a regular array, increasing their intrinsic immunogenicity [13, 23]. Based on the previous analysis, BLS-KETc1 could behave as a very stable decamer as compared with the wild-type, suggesting that, in this setting, KETc1 would have the stability conferred by the carrier protein, increasing its *in vivo* half-life by several-fold. Thus, the use of BLS as a protein carrier has the benefit of improving the immunogenicity of peptides by increasing immunogen size as well as providing T-helper epitopes.

Furthermore, BLS may also be able to provide better processing of KETc1 for antigen presentation. The ability of splenic antigen-presenting cells (APC) to present KETc1 in the linear form or on the surface of the recombinant BLS protein to *in vivo* primed T cells was determined using proliferation assays. As Table 3 shows BLS-KETc1 was a better recall antigen than the linear KETc1 peptide to splenocytes from mice immunized with the synthetic KETc1 peptide.

To determine if this proliferative capacity is MHC haplotype-restricted three independent mouse major histocompatibility complex haplotypes (*H-2^d, H-2^b and H-2^k*) were tested in a BALB background [24]. As Table 4 shows, the magnitude of the response was similar for the three haplotypes when mice were immunized with KETc1 and *in vitro* stimulated with BLS-KETc1 with limited variation between certain haplotypes which can be ranked as follows: $H2^d = H2^k < H2^b$. Thus, KETc1 seems to be preferentially presented in the *H-2^b* haplotype. Furthermore, in the genetically deficient MHC class I C57BL/6J-B2m^{tm1Unx} strain, not significant differences in the proliferative response were found compared with the response observed in BALB.B which implies that KETc1 is not class I-restricted.

As expected, a higher proliferative response was observed in mice immunized with BLS-KETc1 and primed *in vitro* with the same chimera. In these conditions, neither MHC-haplotype restriction nor preferential presentation was observed probably due to the presence of a higher number of epitopes that override the differences observed for KETc1 presentation. In addition, using KO

mice, which fail to express class I MHC antigens, a clearly diminished response was obtained respect to that obtained in BALB.B which demonstrates that BLS could contain epitopes class I MHC restricted. These results agree with a previous report which shows that spleen cells from BLS-immunized mice *in vitro* primed with BLS, proliferated and produced IL-2, IFN-γ, IL-10, and IL-4, suggesting the induction of a mixed Th1-Th2 response probably involving antigen presentation in the context of MHC class I and class II antigens [11]. In addition, the similar level of proliferative response found in the KO mice either immunized with the linear peptide or the chimera suggest that the peptide can maintain its immunogenic properties when it is presented in a class II MHC context, regardless its delivery. Overall, these results show that BLS-KETc1 include epitopes capable of being presented in the class I and class II context increasing their potential to elicit T cell helper and cytotoxic immune response.

Thus, results obtained herein let us propose BLS as a novel and effective delivery system for the improvement of different subunit vaccines. Numerous proteins have been proposed and tested for antigen delivery [25, 26, 27, 28]. Unfortunately, many of them present properties that limit their use. Widely used vaccines such as tetanus toxoid (TT) have not been uniformly effective as carrier proteins because of the phenomenon of epitope-specific suppression in which induction of an immune response against a synthetic peptide, conjugated to TT, is prevented by preexisting immunity to TT [29]. Another protein extensively used is the Cholera toxin, which induces both mucosal and systemic immune responses via a Th2 cell-dependent pathway [27]. However, and in addition to potential type-I hypersensitivity, a major concern for using mucosal adjuvants such as CT is that this molecule is not suitable for use in humans because of its toxicity for the central nervous system [30]. In the case of BLS none of the mice employed exhibited symptoms of discomfort. However, considering the potential use of BLS as a carrier, its safety must be thoroughly studied.

Another point that merits comments is that the increased immunogenicity of KETc1 is also an important advance in the improvement of the vaccine against cysticercosis. It is now possible to express KETc1 as a recombinant chimera in

bacteria at more accessible production costs and with an improved immunogenicity, which could be reflected as an increase in its protective capacity. Based on these results it will be convenient to express the other two peptides that constitute the vaccine and confirm their protective capacity against pig cysticercosis.

In summary, results presented here offer a novel antigen delivering protein that open new possibilities for controlling pathogens for which vaccines are not yet available.

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Table 1.

Increased antigenicity of KETc1 expressed as a recombinant chimeric protein using *Brucella spp* lumazine synthase

	Before Immunization	After immunization with KETc1			
		2.5 μ g/mouse		10 μ g/mouse	
Abs against:		1st dose	2nd dose	1st dose	2nd dose
KETc1	0.09 ± 0.02	0.08 ± 0.01[a]	0.093±0.06[a]	0.095 ± 0.02[a]	0.086± 0.03[a]
BLS-KETc1	0.06 ± 0.01	0.38 ± 0.05[b]	0.58 ±0.17[c]	0.41 ± 0.02[b]	0.52 ± 0.12[c]

Mean ± SD of IgG antibody level (OD_{405}) of groups of eight BALB/cAnN female mice each KETc1-immunized reacting with KETc1 or BLS-KETc1. Literals indicate the significant differences ($P < 0.01$).

Table 2

Immunogenicity of KETc1 expressed as a recombinant chimeric protein using *Brucella spp* lumazine synthase

	Before Immunization	After BLS-KETc1 immunization	
Abs against:		1st dose	2nd dose
KETc1	0.09 ± 0.02 [a]	0.13 ± 0.03 [a]	0.14 ± 0.03[a]
BLS-WT	0.06 ± 0.02 [a]	0.30 ± 0.14[b]	0.76 ± 0.14[c]
BLS-KETc1	0.04 ± 0.01 [a]	0.72 ± 0.32[c]	1.42 ±0.26[d]

Mean ± SD of IgG antibody level (OD_{405}) of groups of eight BALB/cAnN female mice each BLS-KETc1-immunized reacting with KETc1, BLS-WT or BLS-KETc1. Literals indicate the significant differences ($P < 0.01$).

Table 3

Specific *in vitro* splenocyte proliferation induced by KETc1 expressed as a recombinant chimeric protein using *Brucella spp* lumazine synthase

	In vitro treatment:				
Immunized with:	Media	KETc1	BLS-WT	BLS-KETc1	ConA
Saponin	152±33	696± 344	212±111	1012± 604	190590±96826
KETc1	123±23	1176±1145	589± 94	6711±2936	109457±51629

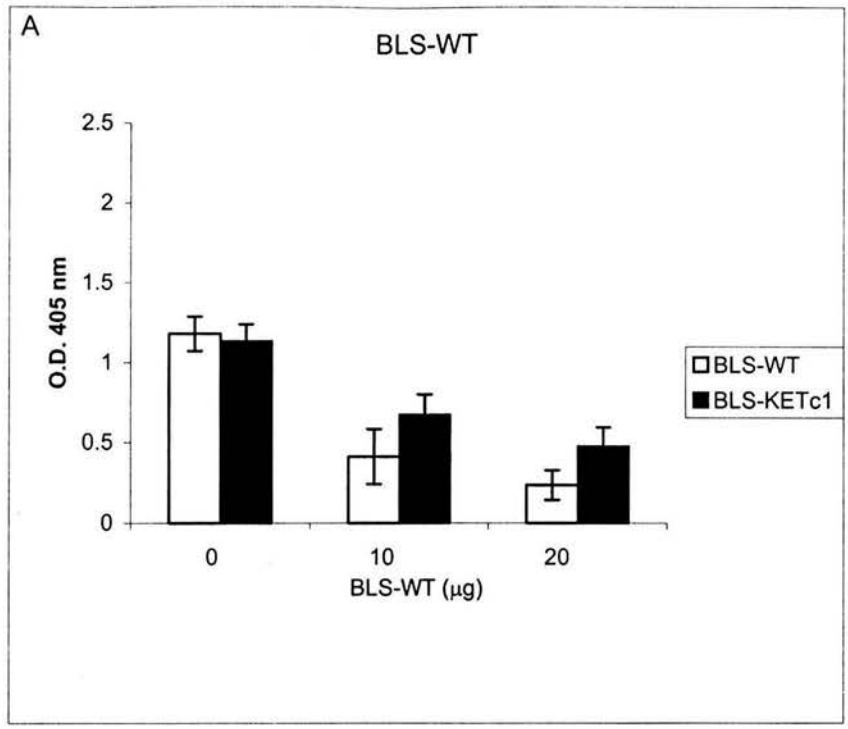
Mean ± SD of [3H] TdR incorporation (cpm) after *in vitro* stimulation of splenocytes from BALB/cAnN female mice immunized with KETc1 synthetically produced in the linear form plus saponin or saponin alone. Data are representative of three different experiments separately performed.

Table 4

Specific *in vitro* splenocyte proliferation induced by KETc1 expressed as a recombinant chimeric protein using *Brucella spp* lumazine synthase in different mice strains

In vitro treatment	Cells from mice immunized with: KETc1	BLS-KETc1
BALB		
(H-2^d)		
Media	179 ± 119	153 ± 54
KETc1	114 ± 25	272 ± 220
BLS-KETc1	3144 ± 81*	8922 ± 4759*
(H-2^k)		
Media	94 ± 22	175 ± 132
KETc1	43 ± 9	146 ± 80
BLS-KETc1	2375 ± 709*	11510 ± 7798*
(H-2^b)		
Media	189 ± 110	102 ± 82
KETc1	379 ± 294	1736 ± 421*
BLS-KETc1	5686 ± 2030*	15170 ± 2537*
KOI C57BI6J		
(H-2^b)		
Media	365 ± 97	828 ± 296
KETc1	372 ± 216	1401 ± 721
BLS-KETc1	4930 ± 1903*	5673 ± 1254*

Mean ± SD of [3H] TdR incorporation (cpm) after *in vitro* stimulation of splenocytes from different strains of mice immunized with KETc1 synthetically produced or BLS-KETc1 plus saponin or saponin alone. *Significant increase in the cpm respect to splenocytes incubated with media alone ($P<0.05$)



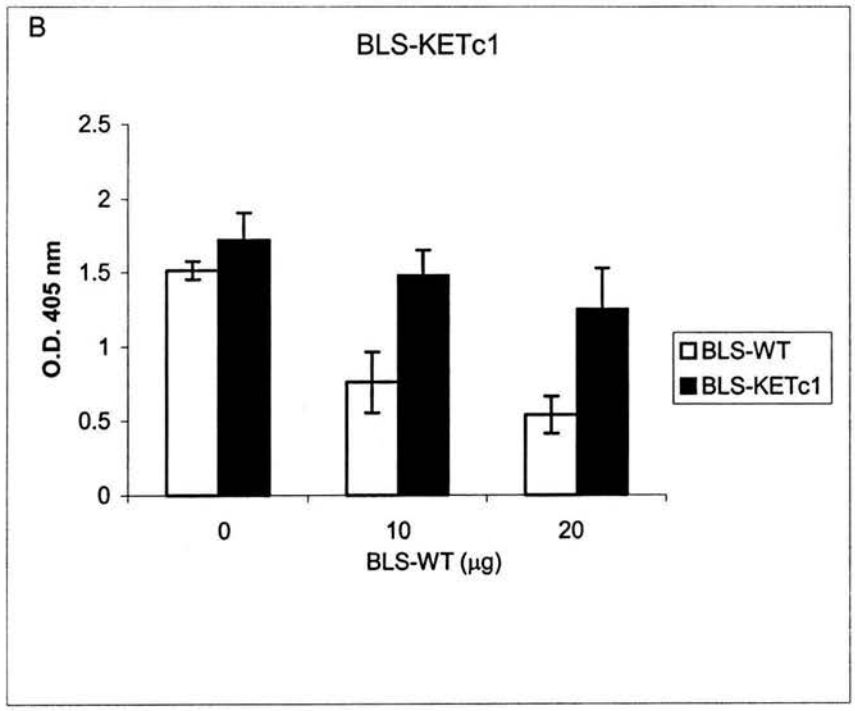


Figure 1. Inhibition of the binding capacity of anti-BLS antibodies to BLS-WT (A) and BLS-KETc1 (B) in the ELISA plate after sera incubation without or with two different concentrations of soluble BLS-WT. Antibodies against BLS-WT (□) or BLS-KETc1 (■).

DISCUSIÓN GENERAL

La cisticercosis es una enfermedad que afecta tanto al humano como al cerdo y es causada por la larva de *Taenia solium*, siendo esta una enfermedad endémica en países en desarrollo. En diferentes estudios epidemiológicos realizados en México se han reportado prevalencias del 5 al 30 % de cisticercosis porcina en cerdos criados en forma rústica, dato que señalan la alta tasa de transmisión de esta parasitosis. Es por esto la urgente necesidad de contar con nuevas herramientas para un control efectivo de la enfermedad y su posible erradicación.

Dentro de las numerosas acciones que se han tomado en cuenta para la erradicación de la enfermedad, la vacunación en cerdos parece ser una buena alternativa para el control de la misma. A este respecto actualmente existen muchos estudios que abordan aspectos relacionados con la inmunidad específica generada contra el parásito, conocimiento que ha contribuido a conocer los mecanismos que median la resistencia a la infección los cuales son de interés en cuanto al conocimiento para el desarrollo de una vacuna capaz de proteger en contra del parásito.

En el presente trabajo de tesis se describe la identificación de dos epítopes protectores KETc1 y KETc12, así como la respuesta inmune asociada a la protección en contra de cisticercosis murina. Estos péptidos constituyen parte de la vacuna actualmente propuesta para el control de la cisticercosis porcina.

En estudios previos se identificaron y secuenciaron clonas que codificaban para antígenos protectores en contra de cisticercosis murina que provenían de fracciones antigénicas protectoras en contra de cisticercosis porcina, se identificaron las regiones inmunogénicas en el antígeno recombinante KETc7.A partir de la secuencia de este antígeno recombinante se realizó una predicción teórica de las regiones de mayor antigenicidad identificándose varios péptidos de interés, entre ellos GK-1. Este péptido indujo la reducción del 57% al 70 % de la carga parasitaria esperada, obteniéndose una inmunidad esterilizante en el 60% de los animales vacunados.

Considerando que GK-1 es un péptido de sólo 17 aa. se evaluó si su acoplamiento a acarreadores (MAP o BSA) pudiese mejorar esta protección, sin embargo, esto no aumentó considerablemente su capacidad inmunogénica.

La respuesta inmune generada a partir de la inmunización con el péptido GK-1 fue principalmente del tipo Th1, encontrándose un incremento en las poblaciones celulares provenientes de los ratones inmunizados, las cuales se encontraron enriquecidas principalmente en linfocitos T CD8+, con un incremento en las citocinas IL-2 e INF- γ. Debido a estas propiedades es que consideró a GK-1 como un buen candidato para formar parte de la vacuna actualmente propuesta para controlar la cisticercosis porcina.

Con el objetivo de encontrar epítopes adicionales que pudiesen aumentar la eficiencia de la vacuna y prevenir las posibles diferencias antigénicas entre parásitos se procedió a identificar epítopes protectores en los adicionales antígenos recombinantes previamente identificados KETc1 y KETc12.

La inmunización con el péptido KETc1 indujo niveles de protección que fueron entre 67% a 100% mientras que con KETc12 estos fueron entre el 53 % y el 88%. Adicionalmente se indujo inmunidad esterilizante en algunos ratones.

Un aspecto que cabe señalar con especial interés en los resultados obtenidos es la variación importante en la cantidad de parásitos. Como puede observarse en cada experimento existe una gran variación individual aún en los ratones no tratados y adicionalmente se observa importante variación entre experimentos. Estas variaciones implican que los experimentos se realizaron siempre incluyendo los grupos controles correspondientes con un alto número de ratones por grupo y se repitieron al menos en tres ocasiones. Si bien la variación observada es un hallazgo usualmente obtenido en esta forma de infección, las razones no están aún totalmente identificadas. Una posibilidad que se está explorando son posibles diferencias en la capacidad infectiva de cada parásito inoculado. Resultados previos nos han indicado que la sola presencia de un parásito que haya sido capaz de instalarse en el hospedero, es eventualmente capaz de reproducirse y modificar considerablemente la intensidad de la parasitosis.

Con el propósito de detectar si dentro de las secuencias de nuestros péptidos se incluía al menos un epitope B, procedimos a evaluar la respuesta de anticuerpos específicos tanto en contra de antígenos totales de *T. crassiceps* como en contra de KETc1 y KETc12. Los niveles de anticuerpos detectados en contra de los propios péptidos fueron menores que en contra del antígeno total, lo que podría deberse a la pérdida de reactividad de los péptidos una vez que estos se asocian a la placa de ELISA. Si bien estos niveles fueron bajos nos permitieron detectar a la proteína nativa tanto en *T. crassiceps* como en *T. solium.*

Es importante señalar que estos 2 epítopes protectores que constituyen parte de la vacuna sintética junto con el epítope GK-1, se encuentran distribuidos en forma distinta en todos los estadios de desarrollo del parásito *T. solium* (Toledo et al., 1999; 2001), y es de especial interés su localización en la oncosfera la cual ha sido reportada para otros cestodos como la fase del parásito más susceptible al daño por parte del sistema inmunológico (Rickard and Williams, 1982)

Una de las mayores limitaciones de la investigación a nivel molecular en la teniosis/cisticercosis es la necesidad de contar con modelos de laboratorio ya sean in vivo y/o in vitro en los cuales poder evaluar la vulnerabilidad de los diferentes estadios de desarrollo del parásito frente a los diferentes componentes del sistema inmunológico. Si bien existen diferentes modelos reportados previamente para observar el efecto de los anticuerpos o células inmunes frente a huevos, oncosferas de *T. solium* (Rickard and Williams, 1982; Molinari et al., 1993), estos son frecuentemente limitados debido a la dificultad de obtener los huevos, el tiempo y manera de obtención así como el número necesario para experimentación. En la cisticercosis por *T. solium* se ha demostrado la capacidad de los anticuerpos de bloquear la instalación de las oncosferas utilizando un modelo murino de infección (Molinari et al., 1988). Estudios en la morfología en oncosferas de *Hymenolepis nana* que han sido capaces de penetrar el epitelio intestinal de animales inmunizados sugieren el daño que provocan los anticuerpos localizados en la membrana externa del parásito (Rickard, 1982). En otras enfermedades infecciosas (Furuya, 1991) particularmente en Schistosomiasis, el

uso de cultivos primarios o líneas celulares (Bayne, 1998) ha permitido avanzar considerablemente en la fisiología del parásito así como en los mecanismos que emplea el mismo para evadir a la respuesta inmune del huésped (Ivanchenko, M., et al, 1999). En base a esta experiencia en nuestro laboratorio se desarrollaron cultivos celulares primarios a partir de cisticercos de *T. crassiceps* (Toledo, et al, 1997), estas células fueron capaces de infectar ratones naive y desarrollarse posteriormente en cisticercos completos. Contando con este tipo de sistema in vitro se procedió a evaluar la relevancia de la respuesta inmune humoral desarrollada por la vacunación con nuestros péptidos a fin de poder conocer y caracterizar de cierta forma los mecanismos asociados a la protección.

Con el propósito de evaluar la relevancia de los anticuerpos específicos inducidos por vacunación se utilizaron células de *T. crassiceps* demostrándose que los anticuerpos anti-GK-1 pueden bloquear la regeneración de células provenientes de cisticercos de *T. crassiceps* a cisticercos (articulo en colaboración I, García et al., 2001).

Alternativamente se adaptó el modelo de teniasis por *T. solium* en hamsters inmunodeprimidos (Verset, 1974), y utilizando este modelo se observó que anticuerpos anti-GK-1 fueron capaces de bloquear e impedir la conversión y el desarrollo de cisticercos de *T. solium* a tenias (articulo en colaboración I, García et al., 2001). Dichos experimentos demostraron que las inmunoglobulinas anti-GK-1 reducen la habilidad de los cisticercos de transformarse en tenias. Aunque no hemos estudiado el posible mecanismo que media esta actividad cisticida, considerando la localización del péptido GK1, es posible que los anticuerpos sean capaces de penetrar y localizarse en el canal espiral de los cisticercos, impidiendo de esta forma su evaginación y desarrollo. En el estudio realizado por Díaz y cols. (2003) se demostró que cerdos inmunizados con los péptidos GK-1, KETc1 y KETc12 fueron capaces de seroconvertirse a los 7 días postinmunización, estos niveles de IgG permanecieron altos hasta el término del experimento. Sin embargo, los anticuerpos anti-KETc1 y anti-KETc12 no fueron capaces de bloquear el desarrollo de los cisticercos de *T. solium* a tenias. Estos resultados sugieren

diferente capacidad de la respuesta inmune inducida por los tres péptidos para dañar al parásito. Es posible que estas diferencias radiquen en las diferentes localizaciones de los péptidos en el cisticerco y/o a diferencias en sus propiedades fisicoquímicas. GK1 presenta una dualidad hidrofóbica-hidrofílica la cual puede permitir interacciones agua-lípido que pudiesen favorecer al péptido en su interacción con receptores B y T unidos a membrana, no así KETc1 y KETc12 que son péptidos con aminoácidos principalmente hidrofóbicos que pudieran reducir la probabilidad de interaccionar con receptores linfocitarios. Estas diferencias entre los tres péptidos sustentan su utilización conjunta para el diseño de una vacuna que pudiera estar dirigida a diferentes blancos en el parásito y sumar diferentes actividades inmunológicas potenciando la posible capacidad protectora.

El estudio de la respuesta inmune celular indicó que las células provenientes de ratones vacunados presentan gran capacidad proliferativa específica tanto en contra de los propios péptidos como en contra de extracto total de *T. crassiceps*. Esta capacidad proliferativa se vio favorecida por el marcado incremento en los niveles de citocinas IL-2 e INF-γ, mientras que los niveles del IL-4 e IL-10 también aumentaron pero en menor magnitud (Toledo et al., 2001). Cabe mencionar que este tipo de perfil de citocinas encontradas en células proliferadas específicamente in vitro es compatible con el bajo nivel de anticuerpos detectado. La composición de la población de linfocitos estimulados in vitro resultó estar enriquecida tanto en células CD4+ como en CD8+.

Cabe señalar que recientemente hemos observado diferencias en la capacidad proliferativa inducida por los péptidos sintéticos in vitro. Utilizando péptidos sintetizados en otras casas comerciales y de diferentes lotes que los empleados durante los primeros años no hemos obtenido capacidad proliferativa in vitro. Las causas de estas diferencias se han comenzado a explorar. Considerando la posibilidad de diferencias en procesos asociados a la síntesis se utilizaron péptidos provenientes de tres casas comerciales diferentes, no obteniendo en ninguno de los casos proliferación in vitro. Otro aspecto que evaluamos fueron los posibles cambios en la saponina empleada como adyuvante, para lo que se

utilizaron tres lotes diferentes de saponina lo que tampoco modificó la capacidad proliferativa in vitro. Un aspecto que cabe considerar son las modificaciones en las condiciones de mantenimiento de los ratones en el bioterio. Los experimentos publicados se realizaron en condiciones controladas, recientemente las condiciones del bioterio se han optimizado disponiendo actualmente de ratones libres de patógenos. Es así factible, que en los cultivos incluyéramos macrófagos con cierto grado de activación que favoreciera la actividad proliferativa in vitro. Actualmente, estamos explorando esta posibilidad agregando diferentes cantidades de macrófagos no activados y previamente activados in vitro. Esta posibilidad parece factible considerando que utilizando in vitro un péptido expresado en forma quimérica con proteína acarreadora (BLS) es capaz de inducir proliferación linfocitaria específica.

La protección inducida por la inmunización por los tres péptidos protectores y el patrón de citocinas observado sugiere una polarización de la respuesta inmune hacia un perfil inmunológico de tipo T1. Este tipo de respuesta se había reportado previamente asociada a fases tempranas de la parasitosis (1 a 2 semanas de infección) restrictivas al desarrollo del parásito, en donde se observaron una respuesta de tipo Th1, compuesta por niveles altos de IL-2 e INF-γ y anticuerpos IgG2a, sin embargo a medida que la infección se hace crónica se da una respuesta de tipo Th2 (Terrazas et al., 1998). Utilizando anticuerpos anti-citocinas como tratamiento en las primeras fases de la infección, los ratones que recibieron anti-IL10 desarrollaron una respuesta de tipo Th1 encontrándose una menor carga parasitaria que los que recibieron tratamiento con anti-INFγ los cuales desarrollaron una respuesta de tipo Th2 incrementándose significativamente la carga parasitaria (Terrazas et al., 1998). Utilizando citocinas recombinantes como tratamiento se observaron efectos similares ya que los ratones que recibieron INF-γ e IL-2 desarrollaron cargas parasitarias menores que aquellos que recibieron tratamiento con IL-10 (Terrazas et al., 1999). Se sabe que IL-12 participa en el inicio y mantenimiento de una respuesta de tipo Th1 (Afonso et al., 1994; Park y Scott, 2001) y es un inductor de la producción de INF-γ promoviendo una

respuesta de tipo Th1 la cual se ha observado que es esencial para controlar parásitos intracelulares como *Leishmania major* y *Toxoplasma gondii* (Cella et al., 1996; Gazzinelli et al., 1996; Manetti et al., 1993; Mattner et al., 1996). Sin embargo en parasitosis extracelulares recientemente se comprobó que utilizando ratones deficientes en IL-12 se observó un incremento del 100% en la susceptibilidad a la infección por *T. crassiceps* (Rodríguez M. et al., 2003), es decir la presencia de un ambiente Th1 es capaz de eliminar al parásito.

Es por todo lo anterior que podríamos pensar que este mismo caso puede ser aplicado a la vacunación, ya que en la cisticercosis experimental murina en etapas muy tempranas de infección o en fases de establecimiento del parásito, estos se inoculan a través de una aguja de 0.40 mm con la consecuente destrucción de los mismos acción que podría facilitar el efecto de la vacunación impidiendo la regeneración del parásito. Así, una vez en la cavidad peritoneal los parásitos deben instalarse desde formas microscópicas incluyendo células parasitarias para una vez desarrollados comenzar a reproducirse por gemación. Es nuestra propuesta que en esta fase de instalación la respuesta inflamatoria inducida por vacunación en los ratones pueda reducir la instalación de los parásitos y en una segunda etapa los anticuerpos puedan dañar los cisticercos establecidos y reducir su capacidad de reproducción.

Resultados similares se observaron en la respuesta inmune inducida por la vacunación en cerdos en donde los animales vacunados presentaron un patrón muy claro de citocinas involucradas en una respuesta de tipo inflamatorio (IL-2 e INF-γ) en los sobrenadantes recuperados después de estimulaciones específicas (Díaz, et al., 2003).

Es factible que la presencia de citocinas de tipo T1 exacerbe la respuesta inflamatoria inducida por la vacunación y pueda dañar a formas tempranas del parásito incluyendo oncosferas de *T. solium* y reduciendo la capacidad de establecerse de cisticercos en caso del modelo murino. Así, es probable que formas tempranas del parásito sean más susceptibles a radicales libres

superóxidos, óxido nitroso, componentes que se ha descrito tienen capacidad de dañar aún endotelios vasculares durante un proceso inflamatorio.

Si bien un patrón de tipo Th1 se describió inicialmente asociado a respuestas en contra de parásitos intracelulares, existen reportes para parásitos extracelulares como *Schistosoma mansoni*, en donde la inmunoprotección requiere de células activadas para INF-γ (Wynn, et al ., 1994; Jankovic, et al., 1999). En este caso la presencia de citocinas de tipo Th2 inhiben la activación y expansión de las células Th1 productoras de IL-2 e INF-γ, favoreciendo la infección. Así mismo, se ha descrito el papel crítico que juegan los macrófagos en la inmunidad frente a parásitos intracelulares por su habilidad de secretar citocinas de tipo Th1, así como óxido nítrico, el cual se ha demostrado que no sólo actúa como microbicida, sino como citotóxico frente a larvas de esquistosoma (Ahmed, et al.,1997).

Al respecto de las nuevas alternativas que se han comenzado a evaluar para la producción de la vacuna como es la expresión de los péptidos en la superficie de fagos filamentosos, así como la unión de los péptidos a la proteína acarreadora de lumazina sintetasa hemos obtenido resultados preliminares muy alentadores. Estos nos han permitido, en el caso de la producción de la vacuna a través de fagos filamentosos, tener resultados de protección muy similares a los que se obtuvieron utilizando la vacuna sintética. El propósito de producir la vacuna por este medio es disminuir los costos de producción sin alterar la capacidad protectora del inmunógeno, actualmente nos encontramos en fase de confirmación de resultados a fin de poder encontrar las mejores condiciones de inocuidad de la vacuna. Con respecto al uso de la lumazina sintetasa como molécula acarreadora, actualmente contamos con dos quimeras BLS-KETc1 y BLS-KETc12. Los primeros ensayos que se han realizado han sido enfocados hacia la evaluación de la respuesta inmune humoral.

Actualmente el diagnóstico y tratamiento de la neurocisticercosis se realiza en base a estudios imagenológicos de alto costo, mismos que pudieran ser reemplazados, por lo menos en parte si se pudiesen disponer de ensayos que tuviesen alta capacidad predictiva, es por esto que resultaría de interés si estas

dos proteínas pudieran ser detectadas por sueros y LCR de pacientes neurocisticercosos a fin de poder evaluar si tienen capacidad diagnostica utilizando para ello, ensayos convencionales de ELISA. Paralelamente se están realizando ensayos preliminares de evaluación de la capacidad inmunogénica de estas proteínas en el modelo murino de cisticercosis.

En resumen, en esta tesis se incluyen los estudios que justifican la evaluación en campo contra la cisticercosis porcina de una vacuna sintética compuesta por los tres mencionados péptidos. La composición química de estos péptidos ofrece la oportunidad de profundizar en los estudios de presentación antigénica así como el de evaluar diferentes formas de presentación de estos epítopes al sistema inmune.

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Two Epitopes Shared by *Taenia crassiceps* and *Taenia solium* Confer Protection against Murine *T. crassiceps* Cysticercosis along with a Prominent T1 Response

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***Taenia crassiceps* recombinant antigens KETc1 and KETc12 have been shown to induce high level of protection against experimental murine *T. crassiceps* cysticercosis, an experimental model successfully used to test candidate antigens for use in vaccination against porcine *Taenia solium* cysticercosis. Based on the deduced amino acid sequence, KETc1 and KETc12 were chemically synthesized in linear form. Immunization with KETc1 induced 66.7 to 100% protection against murine cysticercosis, and immunization with KETc12 induced 52.7 to 88.1% protection. The elicited immune response indicated that both peptides contain at least one B-cell epitope (as demonstrated by their ability to induce specific antibodies) and one T-cell epitope that strongly stimulated the proliferation of T cells primed with either the free peptide or total cysticercal *T. crassiceps* antigens. The high percentage of spleen cells expressing inflammatory cytokines points to the likelihood of a T1 response being involved in protection. The protective capacity of the peptides and their presence in all developmental stages of *T. solium* point to these two epitopes as strong candidates for inclusion in a polyepitopic synthetic vaccine against *T. solium* pig cysticercosis.**

Taenia solium cysticercosis is a common parasitic disease of the central nervous system of humans in several countries in Latin America, Africa, and Asia, where it represents a major health and economic problem (2, 28). The life cycle of this parasite includes a larval phase (cysticercus) that affects both pigs and humans after the ingestion of *T. solium* eggs. The parasite's life cycle is completed when humans consume improperly cooked cysticercotic pork and the adult intestinal tapeworm develops and, in turn, produces millions of eggs that are shed in human feces. In regions of endemic infection, transmission is clearly related to prevailing low standards of personal hygiene and environmental sanitation control (i.e., open air fecalism) in areas where rustic rearing of pigs is practiced by the rural population (pigs roaming about freely in search of edibles and/or deliberately fed with human feces [11]). Regrettably, control of transmission by general improvement of the social, economical, and educational status in developing countries or by proper and strict meat inspection programs is not within reach in the near future. However, since the pig is an indispensable intermediate host, transmission could be hindered by lowering the prevalence of pig cysticercosis through vaccination. Development of an effective vaccine to be used in pigs is being pursued by a number of scientists, with promising results (9, 15–17).

Because of the high costs of experimentation in pigs, murine cysticercosis caused by *Taenia crassiceps* has been used to test and select promising antigens before they are tested in pigs (13, 21). Thus, it has been shown that total *T. crassiceps* antigens can cross-protect pigs against *T. solium* cysticerosis. However, the effects of vaccination with whole-antigen extracts were strongly dose dependent; besides, some antigens were found to be protective while others led to facilitation of the infection (22). Such complications with the use of whole-antigen extracts led us to redirect our research to the identification of individual protective antigens (14, 26). Using recombinant DNA technology, several vaccine candidates were identified in murine *T. crassiceps* cysticercosis with crude lysates of the respective clones as the immunogen (13, 14). One of them, KETc7, which has a protective capacity confirmed by DNA immunization (1, 20), includes at least one protective epitope of 17 amino acids (GK1). GK1 is also expressed in *T. solium* oncospheres (25), the parasite's developmental stage most vulnerable to immunological attack (19). Two additional protective clones, KETc1 and KETc12 (14), were also identified. Herein we report the protective capacity against *T. crassiceps* murine cysticerosis of the peptides deduced from these last two clones. Furthermore, we describe the localization of the peptides in each parasite stage of *T. solium* and *T. crassiceps*, the immune response they elicit in immunized mice—where T1 is most prominent—and propose them as additional components for a synthetic vaccine to be tested in pigs in an attempt to block *T. solium* transmission.

MATERIALS AND METHODS

Peptides. Two *T. crassiceps*-derived peptides (14) that are shared by *T. solium* (24), KETc1 [APMSTPSATSVR(G)] and KETc12 [GNLLLSCL(G)], were synthesized by stepwise solid-phase synthesis with N^{α}-*tert*-butyloxycarbonyl derivatives of L-amino acids on phenyl-acetamidomethyl resin (Sigma Chemical Co., St. Louis, Mo.). The peptides were 95% pure as judged by high-pressure liquid

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FIG. 1. Antibody levels determined by ELISA in individual control (C) and immunized (I) mice against *Tc*Ag. The mean level of antibodies was significantly higher in immunized mice than in controls. O.D., optical density.

chromatography in an analytical C_{18} reversed-phase column (3.9 by 150 mm; Delta Pak [Waters]). The correct amino acid sequence of each peptide was confirmed by protein sequencing on a pulsed-liquid-phase protein sequencer (Applied Biosystems) at the National Institute of Cardiology, Mexico City.

Mice. BALB/cAnN mice, previously characterized as susceptible to cysticercosis (3), were used in vaccine trials. The original murine stock was purchased from M. Bevan (University of Washington) and then bred and kept in our animal facilities by the "single-line breeding" system for more than 30 generations. All mice used were males that were 5 to 7 weeks of age at the beginning of the experiments. The experiments reported herein were conducted according to the principles set forth in the *Guide for the Care and Use of Laboratory Animals*, Institute of Laboratory Animals Resources, National Research Council, Washington, D.C.

Immunization of mice and serum collection. Groups of six to nine BALB/cAnN mice were subcutaneously immunized with two doses of 10 μg of each individual peptide in saponin (Sigma Chemical Co.) per mouse at a concentration of 100 μg/mouse as described elsewhere (25). This dose was determined as optimal in collateral experiments (data not shown). Ten days later, the mice were given a booster with the same immunizing dose of the same peptide in the same adjuvant as used before. Immune sera were obtained from each individual mouse before and after each immunization and stored at −70°C until individually tested for the presence of specific antibodies.

Parasites and cysticercal antigens. The ORF strain of *T. crassiceps* (4) has been maintained by serial passage in BALB/cAnN female mice for 15 years in our animal facilities. Cysticerci for infection were harvested from the peritoneal cavity of mice 1 to 3 months after inoculation of 10 nonbudding small cysticerci (2 to 3 mm in diameter) per animal. The soluble antigens were recovered from similar cysticerci by a previously described procedure (18). Whole *T. solium* cysticerci were dissected from skeletal muscle of highly infected pork carcasses 2 to 4 h after slaughter in an abattoir in Zacatepec, Morelos, Mexico; embedded in optimun-cutting-temperature compound (Miles, Inc.), and frozen at −70°C until used in immunofluorescence assays (see below). Segments from *T. solium* tapeworm and eggs were obtained from the feces of an infected man in Puebla, Mexico. The tapeworm was recovered after treatment with a single oral dose (2 g) of niclosamide (Yomesan; kindly supplied by Bayer). After being washed in saline plus antibiotics (100 U of penicillin per ml plus 100 μg of streptomycin per ml), several gravid proglottids were separated for immunofluorescence assays.

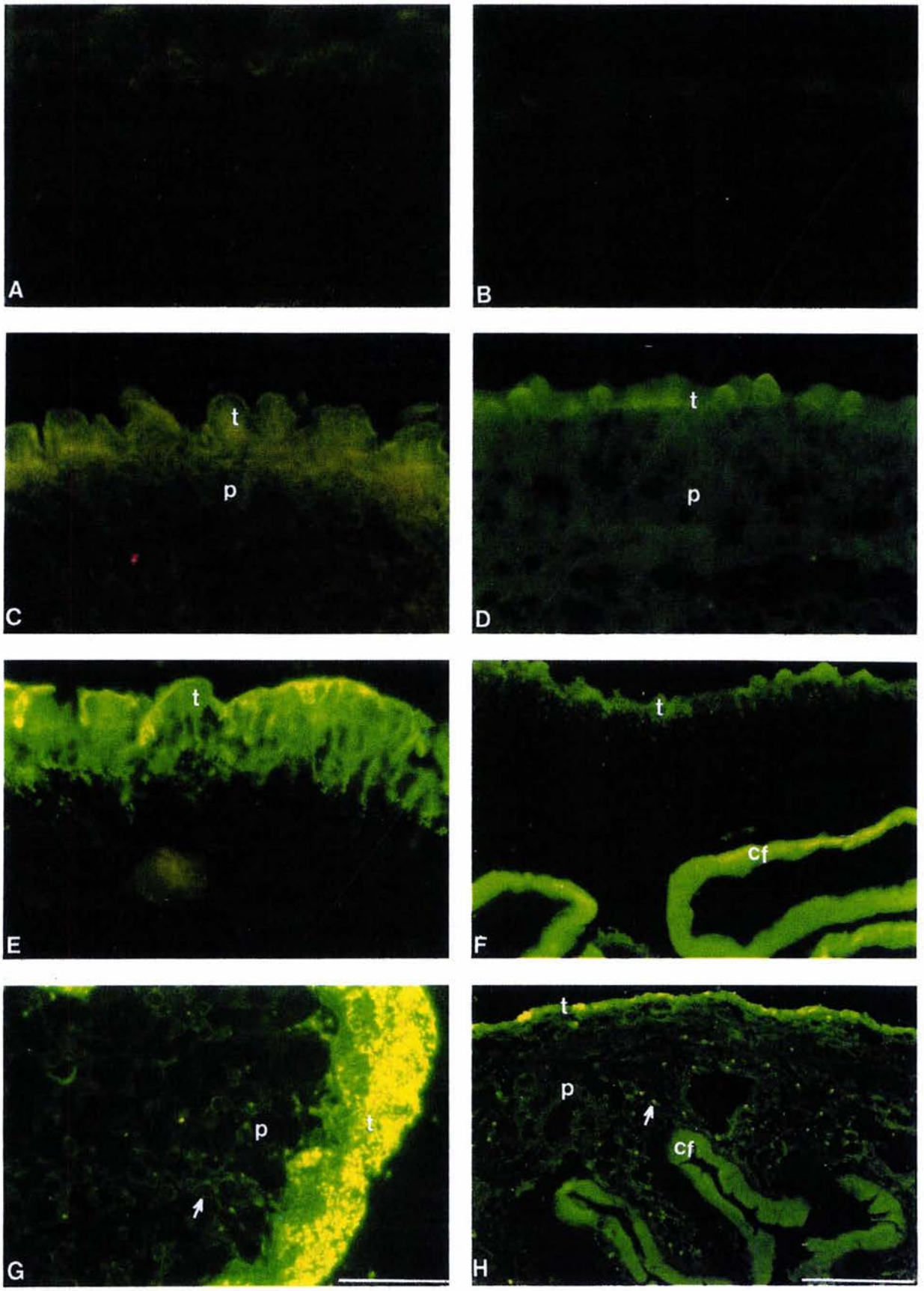
ELISA for antibody measurements. *T. crassiceps* whole soluble antigens (*Tc*Ag) were obtained as previously described (18) and used as the source of antigens in an enzyme-linked immunosorbent assay (ELISA) to measure the antibody response induced by peptide immunization by using a procedure described elsewhere (25).

Proliferation assay. Spleen cells from control and KETc1- and KETc12-immunized mice were harvested 15 days after the second immunization and cultured in RPMI 1640 medium supplemented with L-glutamine (0.2 mM), nonessential amino acids (0.01 mM), penicillin (100 U/ml), streptomycin (100 μg/ml) and fetal bovine serum FBS (10%). The cells were cultured with the appropriate concentration of concanavalin A (ConA) (5 μg/ml), KETc1 (50 μg/ml), KETc12 (10 μg/ml), or *Tc*Ag (10 μg/ml) and incubated at 37°C in a 5% CO_2 humidified atmosphere in flat-bottomed microtiter plates at a cell concentration of 2×10^5 cells per 200 μl of final volume. Then 10^5 peritoneal cells recovered from the

TABLE 1. Protective immunity against murine cysticercosis by immunization with KETc1 and KETc12 peptides

Group of experimental mice	Result for trial no.:									
	1			2			3			
	No. of cysticerci[a]	Parasite intensity[b]	% Protection[c]	No. of cysticerci	Parasite intensity	% Protection	No. of cysticerci	Parasite intensity	% Protection
Controls	55, 48, 40, 43, 47, 46	46.5 (41.1–51.8)		27, 24, 20, 21, 29, 20, 24, 27, 34	25.1 (21.5–28.7)		63, 55, 35, 73, 68, 97, 64	64.0 (47.7–82.3)	
KETc1 immunized	0, 0, 10, 36, 10, 19, 13, 18	11.5 (3.5–22.9)**	75.3	7, 10, 3, 4, 0, 0, 0, 0	0 (−0.2–5.5)**	100	23, 39, 29, 18, 0, 19, 21	21.3 (21.0–32.3)*	66.7
KETc12 immunized	16, 0, 35, 40, 25, 22, 0	22.0 (5.2–34.2)*	52.7	3, 0, 1, 0, 17, 21, 0, 21, 8	3 (0.8–15)**	88.1	30, 14, 27, 21, 17, 13, 15	17.0 (13.4–25.7)**	73.4

[a] Individual number of cysticerci recovered 30 days after infection from each mouse.
[b] Median (95% confidence interval) of individual parasite intensities in control mice injected with adjuvant alone or together with an immunizing peptide. Statistically significant differences between control and immunized mice at the 95% (*) or 99% (**).
[c] Percentage of protection with respect to controls.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same mice were added to each well in a volume of 50 μl. Peritoneal cells were obtained by ex vivo lavage with 5 ml of RPMI 1640 medium. After 72 h, the cultured cells were pulsed (1 μCi per well) for a further 18 h with [*methyl*-^{3}H] thymidine (Amersham Life Science, Little Chalfont, United Kingdom). Then all the cells were harvested and the amount of incorporated label was measured by counting in a 1205 β-plate spectrometer (Wallac).

Spleen cell phenotype analysis. After 3 days of in vitro culture with medium, *Tc*Ag, or peptides, splenocytes were harvested and CD8 and CD4 expression was determined by staining with fluorescein isothiocyanate (FITC)-conjugated anti-CD8 (Pharmingen, San Diego, Calif.) and phycoerythrin-conjugated anti-CD4 (Pharmingen), respectively, by a previously reported procedure (25). Parallel samples of the cells were stained with the corresponding isotype control to account for nonspecific staining of the cells. Briefly, the cells were washed with phosphate-buffered saline (PBS) containing 10% gamma globulin-depleted FBS plus 0.02% NaN_3 and incubated with the indicated antibodies at 4°C for 30 min. After being washed, the splenocytes were resuspended in cold 1% formaldehyde in isotonic solution and analyzed with a FACScan instrument (Becton Dickinson, Palo Alto, Calif.). The results are expressed as a percentage of positive cells.

Cytokine measurements. For detection of intracellular cytokines, spleen cells were treated with medium, KETc1, KETc12, or *Tc*Ag and cultured for 60 h. To inhibit cytokine secretion, brefeldin A (2 μM) was added to the cell cultures 10 h before the assay. At harvest, the cells were centrifuged at 500 × *g* for 10 min and washed twice in ice-cold PBS containing 10% gamma globulin-depleted FBS plus 0.02% NaN_3. CD3 and interleukin (IL) expression were determined by two-color fluorescence-activated cell sorting (FACS) as previously described (25). Briefly, the cells were stained with biotin anti-CD3 (Pharmingen) and then streptavidin-FITC (Sigma) was added. Intracellular cytokines were assayed by using a cyto-Stain TM kit (Pharmingen) to fix and permeabilize the cells. To stain intracellular cytokines, fixed and permeabilized cells were incubated with phycoerythrin-conjugated monoclonal rat anti-IL-2, anti-IL-4, anti-IL-10, or anti-gamma interferon (INF-γ) (all from Pharmingen). Parallel samples of the cells were stained with isotype control to account for nonspecific cell staining. Then 10^5 cells were analyzed with a CD3$^+$ lymphocyte gate as defined by light scatter in a FACScan instrument. The results are expressed as a percentage of positive cells.

Experimental challenge. Metacestodes used in challenge infections were harvested from the peritoneal cavity of BALB/cAnN female mice carrying the ORF strain of *T. crassiceps* cysticerci. Ten small (diameter, ca. 2 mm), nonbudding larvae were suspended in 0.5 M NaCl–0.01 M sodium phosphate buffer (pH 7.2) and intraperitoneally injected into each challenged mouse using a 27-gauge needle (this procedure disrupts the cysticerci upon entry, but the fragments reorganize into cystic structures in a matter of a few days [24]). Mice were killed 30 days after infection, and the cysts found inside the peritoneal cavity were counted. In this form of infection, the parasites do not migrate to another location in the host. The variation in individual parasite intensities within groups of vaccinated and control mice was attributed to differences in the infectivity of each parasite inoculum. In consequence, each experiment measuring levels of immunity by parasite intensity always included a group of nonimmunized mice to assess the infectivity of each inoculum. Thus, the effects of immunization measured in each experiment were contrasted with the control group.

Immunolocalization of KETc1 and KETc12 protein. *T. crassiceps* cysticerci and *T. solium* specimens (cysticerci and gravid proglottids) were placed on ice in a 50-ml conical plastic-bottom centrifuge tube containing ice-cold PBS. All tissues were treated to prepare slides as previously reported (20). The slides were rehydrated and blocked with 1% bovine serum albumin (BSA) in PBS plus 0.1% Triton X-100 (pH 7.2) (PAT) for 1 h. A second blocking in cysticercus-infected tissue sections was performed with sheep anti-mouse IgG (whole antibody; Amersham) diluted 1:100 in PBS plus 0.1% BSA, and then the samples were incubated for 1 h at 4°C. Slides of *T. solium* tapeworm and eggs were incubated 1 h at 4°C with horse serum diluted 1:100 in PBS plus 0.1% BSA as a second blocking agent. The solutions were removed, and the slides were overlaid with the appropriate sera from noninfected (negative control), infected (positive control), or anti-KETc1- or anti-KETc12-immunized mice diluted 1:10,000 in PBS plus 0.1% BSA, incubated overnight at 4°C, and then washed twice in PBS (pH 7.2). Finally, sections were incubated with FITC-labeled goat anti-mouse immunoglobulin G (Zymed) diluted 1:50 for 1 h at room temperature. The slides were washed twice and mounted with aqueous mounting solution (Zymed). Preparations were observed with an epifluorescence microscope Olympus BH2-RFCA.

Statistical analysis. Statistical comparison of individual parasite intensities between groups was performed by the Kruskal-Wallis nonparametric analysis of variance ANOVA test because many mice contained zero parasites in the immunized groups and because parasite intensity is a discontinuous variable (i.e., 0, 1, 2, . . . *n* parasites). Data were considered statistically significant at $P < 0.05$. A Student-Newman-Keuls multiple-comparison test was used to measure the statistical significance between the immune response elicited in vaccinated and control mice.

RESULTS

Protective effect of peptide immunization against *T. crassiceps* cysticercosis. The effect of peptide immunization on the number of cysticerci recovered from mice immunized with KETc1 and KETc12 or adjuvant alone (controls) is shown in Table 1: 66.7, 75.3 or 100% protection was induced using KETc1 as immunogen, and 52.7, 73.4, or 88.1% protection was induced using KETc12 as immunogen. Some mice were completely protected (no parasites) by immunization with either KETc1 or KETc12.

Antibody response induced by KETc1 and KETc12 immunization. To test for the presence of a B-cell epitope(s) within the two peptides, the levels of induced anti-KETc1 and anti-KETc12 specific antibodies were assessed. *T. crassiceps* cysticercal antigens (Fig. 1) as well as each of the peptides were used as antigens in ELISAs (data not shown). Figure 1 shows low but detectable levels of serum antibodies in both KETc1- and KETc12-immunized mice.

Immunolocalization of KETc1 and KETc12 in the parasite. Pooled sera with the highest antibody levels induced by KETc1 and KETc12 immunizations were used to immunolocalize the native antigen in both *T. crassiceps* and *T. solium* (Fig. 2 and 3). KETc1 and KETc12 were expressed in the tegument of *T. crassiceps* cysticerci, albeit with different distributions. KETc1 was restricted to the tegument of both cysticerci (Fig. 2E and F), while in *T. solium* it was found in the most external part of the tegument and also in the cuticular folds of the spiral canal (Fig. 2F). KETc12 (Fig. 2G and H) was very abundant in both metacestodes. Nevertheless, the *T. crassiceps* tegument showed an intensely positive wall surface and parenchyma, especially around the calcareous corpuscules (Fig. 2G). KETc12 was also detected in the oncosphere of the egg as numerous points (Fig. 3G), in contrast to KETc1, which was almost negative. Both epitopes were present in tapeworm tissue: KETc1 was very abundant on the most external side of the tegument (Fig. 3F), and KETc12 was distributed along the tegument's depth (Fig. 3H). When sera from infected mice were used, all structures were fluorescent (Fig. 2C and D and 3C and D). The specificity of these antibody reactions was demonstrated by the lack of reactivity of normal mouse serum with the used tissues (Fig. 2A and B and 3A and B).

FIG. 2. Immunofluorescent staining of *T. crassiceps* (A, C, E, and G) and *T. solium* (B, D, F, and H) cysticerci. Sections of 6 μm were processed and incubated with pooled sera from noninfected mice (A and B), *T. crassiceps*-infected mice (C and D), and KETc1-immunized (E and F) and KETc12-immunized (G and H) mice. The tegument (t) and the parenchyma (p) are evident in both cysticerci (C and D). In *T. crassiceps* cysticerci (E), KETc1 antigen shows a protruding and intensely positive signal in the tegument, while in *T. solium* cysticerci (F) it is clearly evident in the cuticular folds of the spiral canal (cf). KETc12 is quite abundant in both metacestodes; it is evident in the tegument and in the parenchyma of *T. crassiceps* (G) as well as in the tegument, parenchyma, and flame cells (arrows) of *T. solium* (H). Bar, 40 μm.

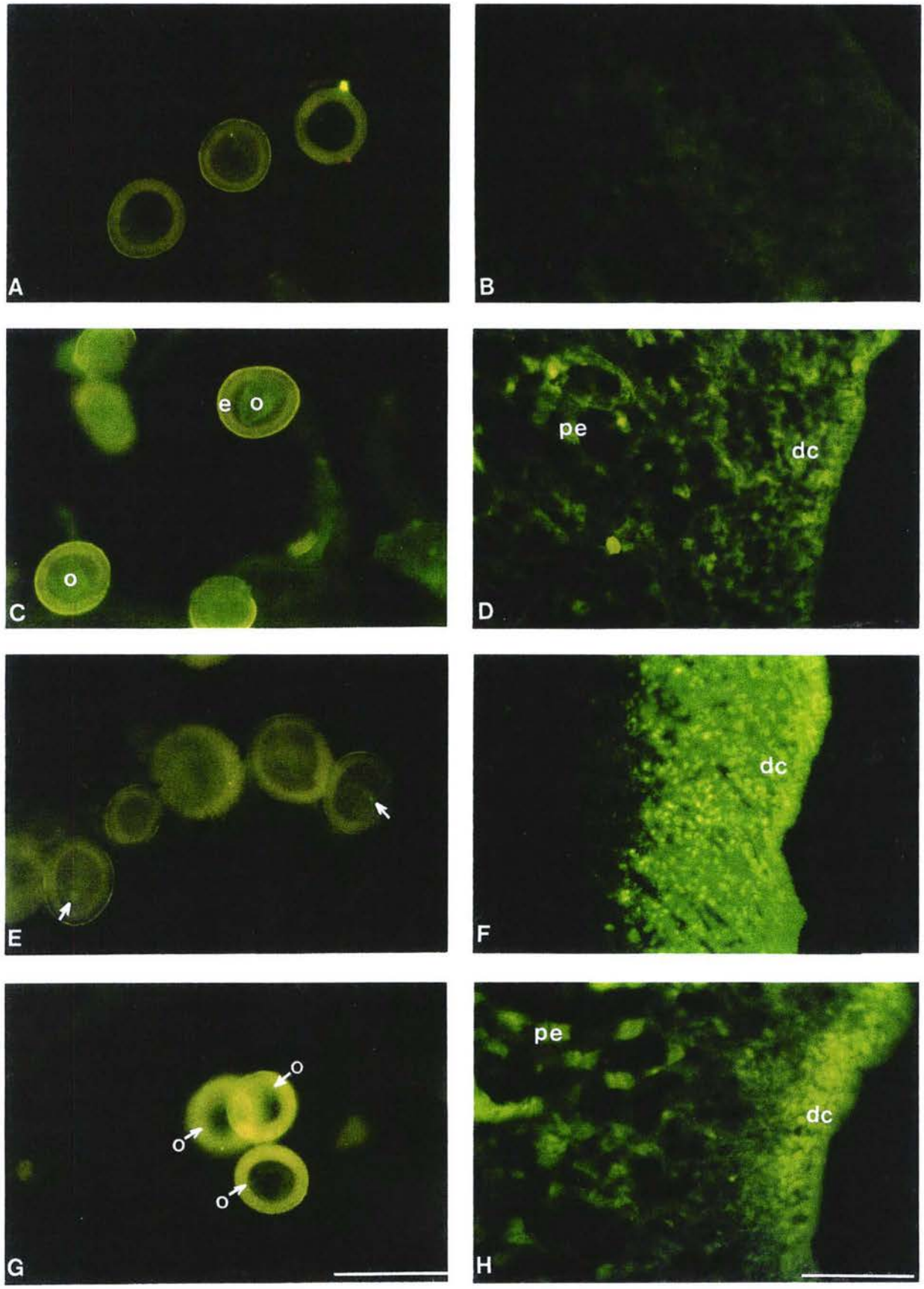
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TABLE 2. T-cell proliferative response of splenocytes from control and KETc1- and KETc12-immunized mice

Source of cells	Amt of [^{3}H]thymidine (cpm) incorporated after in vitro incubation with[a]:			
	Media	ConA	Peptide	*Tc*Ag
Controls	1,086 ± 370	183,643 ± 16,482	1,342 ± 248	754 ± 113
KETc1 mice	2,734 ± 1,364	119,030 ± 2,675	7,289 ± 2,675[b]	9,941 ± 1,802[b]
KETc12 mice	1,560 ± 262	155,125 ± 9,816	6,321 ± 2,021[b]	10,450 ± 2,238[b]

[a] Mean ± standard deviation of the [^{3}H]thymidine incorporated after 3 days of culture of splenocytes from three individual mice assayed separately. The data are representative of three repeat experiments, each performed in triplicate.

[b] Significantly increased proliferative responses were achieved when cells from immunized mice were stimulated with both peptides and whole *Tc*Ag. Comparisons between immunized and control values were considered statistically significant at ($P < 0.05$).

Assessment of T-cell epitopes on the KETc1 and KETc12 peptides. The proliferative response of spleen cells from mice immunized with KETc1 or KETc12 or saponin alone is reported in Table 2. Spleen cells from mice injected in vivo with free peptides or saponin were stimulated in vitro with the corresponding peptide, *Tc*Ag, or ConA in previously determined optimal concentrations. Table 2 shows that in vitro stimulation with KETc1 or KETc12, as well as with cysticercal antigens, induced a significantly greater proliferative response in cells from immunized mice than in those from control mice. Cells from mice injected with saponin (controls) showed no proliferative response above background levels.

Figure 4 shows that stimulated cells increased from 3.5 or 4.5% to 8–16.3% when the cells were primed with *Tc*Ag or the appropriate peptide, respectively. Stimulated cells were enriched in both $CD4^+$ and $CD8^+$ cells by factors of 1.2 to 2.0 for $CD4^+$ and 3.9 to 4.9 for $CD8^+$.

The proportion of cells capable of producing IL-2, IL-4, IL-10, and INF-γ was determined by FACscan analysis after intracellular staining for cytokines. The results are shown in Table 3. The proportion of cells producing IL-2 and INF-γ were significantly higher in KETc1-, KETc12-, or *Tc*Ag-stimulated cells than with media alone and more so in immunized than in control mice. The levels of IL-4- and IL-10-expressing cells were also increased but to a lesser extent.

DISCUSSION

Our results show that KETc1 and KETc12 induce protection against experimental murine *T. crassiceps* cysticercosis and that both are B- and T-cell epitopes. The protective capacity and the immunity induced by these two peptides closely resemble those induced by GK1, a previously reported protective epitope also shared by *T. crassiceps* and *T. solium* (25). It should be noted that there are two forms of expressing protection; i.e., reduction in parasite intensity and proportion of totally parasite-ridden mice. Because *T. crassiceps* cysticerci multiply asexually in the peritoneal cavity of infected mice, the reduction in parasite intensity is highly dependent on the time of assessment after infection; the effects of vaccination tending to disappear in late infections. Sterile immunity is attained only if the initial inoculum is totally destroyed by the immune system response. These and previous results are in accordance with the notion that if a single *T. crassiceps* cysticercus evades or survives the initial immune attack of the murine host, it will multiply and eventually reach very high parasite intensities indeed (3). This initial immune attack would appear must successful if a strong T1 response is induced, as we have shown here and others have shown previously (23). In older infections, when massive parasite intensities ($>10^3$) are achieved, T2 responses predominate and perhaps downregulate T1 (23). These two peptides would appear to touch off the protective T1 response more efficiently than the T2 response.

The immunologic assays performed in our experiments indicate the immune mechanisms involved in infection control. It has been repeatedly stated that protection induced by vaccination against *T. crassiceps* murine cysticercosis is T1 related whereas antibodies and other T2 molecules are less effective (1). In this study, results point in the same direction: while antibodies are erratically and weakly induced by both KETc1 and KETc12, IL-2 induction is noticeably increased 5.7- to 10.1-fold in immunized mice relative to control mice. The same is true for IFN-γ, the characteristic inflammatory cytokine, which activates macrophages in the vicinity of the parasite and triggers their well-known damaging effects (25). In addition to the preponderance of the inflammatory interleukins IFN-γ and IL-2, the low profiles of IL-4 and IL-10, which inhibit the proliferation of the T2 responses, could well explain the low levels of antibodies elicited by both peptides. Protective immunity in the context of a T1 response has also been related to innate resistance conditions (23, 27). Despite the low levels of specific antibodies induced by both peptides, their possible protective role cannot be excluded. This is of particular relevance considering the recent finding of the capacity of anti-GK1 antibodies to block *T. solium* cysticercus conversion to tapeworms (5). The fact that more than 50% of human neurocysticercosis patients make antibodies against KETc1 and KETc12 (8) strengthens our interest in these two epitopes.

Based on the different anatomic distribution of KETc1 and

FIG. 3. Immunofluorescence staining of the *T. solium* oncosphere (A, C, E, and G) and proglottid tegument (B, D, F, and H). Sections of 6 μm were processed and incubated with pooled sera from noninfected mice (A and B), *T. crassiceps*-infected mice (C and D), and KETc1-immunized (E and F) and KETc12-immunized (G and H) mice. It is evident that the oncosphere (o) and the distal cytoplasm region (dc) (C and D, respectively) stain positively. Some structures of the perinuclear cytoplasm region (pe), like the protoplasmic extensions of the tegumental cells, are also apparently positive. KETc1 antigen is almost negative in the oncosphere and appears as little positive spots (arrows); in contrast, in adult tissue (F) it is quite evident in the distal cytoplasm region of the tegument. The KETc12 antigen is only slightly present in the oncosphere (o) but is quite conspicuous in the distal cytoplasm region and in the perinuclear cytoplasm region of the adult tissue. Bar, 40 μm.

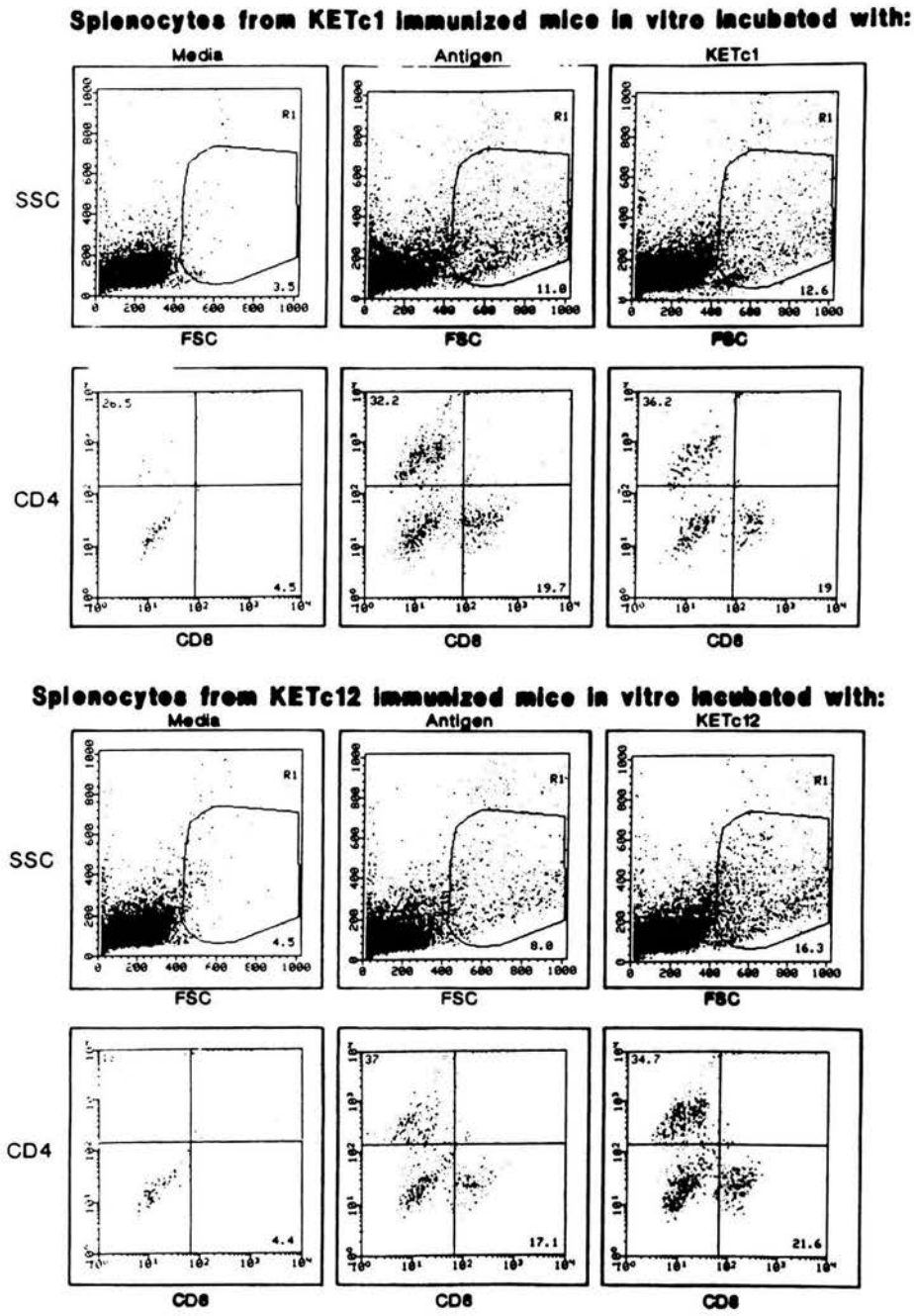

FIG. 4. Flow cytometer analysis of spleen cells from KETc1- and KETc12-immunized mice with or without in vitro stimulation with the respective peptide or antigen (*Tc*Ag) R1 denotes the region of proliferating cells in the SSC/FSC plot (side-scatter/forward-scatter plot), and the number below indicates the percentage of cells in this region. $CD4^+$ and $CD8^+$ cell percentage expression was determined in the defined R1 gate.

KETc12 in *T. solium* cysticerci and in oncospheres, which also differ from that of GK1, it would appear that all three peptides should be included in a vaccine against pig cysticercosis to maximize the number of targets. In spite of the risk associated with extrapolating from *T. crassiceps* and mice to *T. solium* and pigs, optimism about vaccine development prevails because of the many examples of effective immunity induced against different cestodes in diverse hosts. Also, the extensive similarities among cestode infections in terms of their natural history, pathology, and antigenic composition all point to possibly similar effects of vaccination (6, 7, 10). In fact, different sources of protective antigens have been successfully used as vaccines against porcine cysticercosis, one using recombinant antigen from *Taenia ovis* (12) and the other using antigen from *T. crassiceps* itself (13).

In the hope of increasing the efficiency of vaccination, it is advisable that KETc1 and KETc12 plus GK1, all of which induce high levels of protection in the murine model of cysticercosis and are present at all stages of *T. solium* development, be considered as candidates for inclusion in a mixed poly-

TABLE 3. Percentage of $CD3^+$ splenocytes expressing different intracellular cytokines with or without in vitro stimulation[a]

Stimulation	% of cytokine-expressing cells from mice immunized with[b]:							
	KETc1				KETc12			
	IL-2	IL-4	IL-10	INF-γ	IL-2	IL-4	IL-10	INF-γ
None (media)	1.4	0.7	0.6	0.6	1.6	0.9	1.2	0.7
Peptide	8.1	4.4	3.0	11.2	9.4	3.2	4.8	14.5
Cysticercal antigens	13.0	4.0	3.6	6.7	16.2	2.6	4.2	17.0

[a] Pooled splenocytes from three KETc1 or KETc12 immunized mice were analyzed for intracellular cytokines 60 h after in vitro stimulation.
[b] The percentages of $CD3^+$ cells expressing the four different interleukins (not included) were below 1% in splenocytes from control mice with and without in vitro stimulation. Data are representative of two different experiments using different mice.

epitopic synthetic vaccine to be used against *T. solium* cysticercosis in pigs.

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INHIBITORY ROLE OF ANTIBODIES IN THE DEVELOPMENT OF *TAENIA SOLIUM* AND *TAENIA CRASSICEPS* TOWARD REPRODUCTIVE AND PATHOGENIC STAGES

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ABSTRACT: Untreated *Taenia solium* cysticerci obtained from different naturally infected pigs vary notably in their capacity to develop into intestinal tapeworms in prednisolone-treated hamsters, whereas cells derived from *Taenia crassiceps* cysticerci after 2 mo of infection almost always develop to cysticerci in the peritoneal cavity of susceptible BALB/cAnN mice. Preincubation of whole cysticerci or parasite cells with mice immunoglobulins raised against an 18-mer peptide epitope (GK-1) common to both parasites significantly interferes with both transformations. These crippling effects of antiparasite antibodies suggest new forms of immunological interference with parasite biology other than simple killing. Antibodies that cripple biological functions of the parasite, e.g., their development to reproductive or pathogenic stages, make them important protagonists in taeniasis/cysticercosis disease as classic parasitocidal antibodies. Different serum levels of crippling antibodies in the infected pigs could be responsible for the varied ability of cysticerci to convert to tapeworms. Antigens capable of inducing crippling antibodies, e.g., GK-1, could be useful as a therapeutic vaccine for pigs in order to reduce parasite transmission.

Taeniasis–cysticercosis caused by *Taenia solium* is a very important public health problem in several parts of the world (Gemmell et al., 1985), leading to great suffering in the form of human neurocysticercosis (Sotelo et al., 1996) and important economic losses in pig breeding (Acevedo-Hernandez, 1982). In some geographic areas in Mexico, this endemic disease may affect more than 20% of the rurally bred pigs, and human seroprevalence levels vary from 0.06 to 2.97% (Larralde et al., 1992). Other developing countries may be affected to the extent that Mexico is, or worse (García et al., 1996).

Considering the essential role of pigs as intermediate hosts in the life cycle of *T. solium,* several research groups are trying to develop effective preventive vaccines against porcine cysticercosis as ways to reduce transmission to humans and pigs (Nascimento et al., 1995; Sciutto et al., 1995). We have recently reported a B- and T-cell response to an 18-mer peptidic epitope (GK-1: Gly Tyr Tyr Tyr Pro Ser Asp Pro Asn Thr Phe Tyr Ala Pro Pro Tyr Ser Ala), carried by the protective recombinant protein antigen KETc7 (Manoutcharian et al., 1996), that induces a high level of protection against murine cysticercosis concomitant with specific antibody and immune cellular responses (Toledo et al., 1999). This and other immunogens of potential interest in the design of an effective vaccine against porcine cysticercosis have been identified and tested with some degree of success (Molinari et al., 1993; Nascimento et al., 1995), but little is known about the molecular targets and mechanisms involved in their effectiveness. An important limitation of molecular research in taeniasis/cysticercosis is the need for expedient in vitro and in vivo laboratory models in which to explore vulnerability to immune attack for different developmental stages of cestodes. The use of *T. solium* eggs and oncospheres as targets to evaluate the effect of antibodies, or immune cells (Rickard, 1982), or both, is limited by the difficulty of obtaining eggs at an appropriate time, manner, and number, for experimentation. Evaluation of the effectiveness of immune attacks upon cysticerci is further complicated by their varying times of residence (years) in target organs (brain, skeletal muscles) that may go unnoticed (Aluja et al., 1996). Additional difficulties in exploring the parasite transformation from oncosphere to metacestode to tapeworm arise because of changes occurring when parasites are sequestered inside solid tissues or in the bowel's lumen. In other infectious diseases (Furuya, 1991), particularly in schistosomiasis, the use of parasite cell lines has allowed for considerable scientific advance (Bayne et al., 1994) and has been used in various immunodeficient or unnatural hosts in studying viral disease (Griep et al., 1993; Hanna et al., 1998). We have undertaken similar experimental approaches to study more closely the role of immunological molecules and cells in taeniasis/cysticercosis.

We have developed procedures to obtain cells from *Taenia crassiceps* cysticerci capable of infecting naive mice and developing into cysticerci (Toledo et al., 1997). Also, we adapted the immunodeficient hamster model of *T. solium* taeniasis (Verster, 1974) to study the rate of transformation of cysticerci to tapeworms. The relevance of immune attacks by anti-GK-1 antibodies upon both transformations are reported herein.

MATERIALS AND METHODS

Animals

We used female and male 4–6-wk-old BALB/cAnN mice bred in our animal facilities by brother–sister mating. Female golden hamsters, 4–6 wk old, bred in our animal facilities, were also used.

Synthetic peptide (GK-1)

The linear peptide GK-1 (Gly Tyr Tyr Tyr Pro Ser Asp Pro Asn Thr Phe Tyr Ala Pro Pro Tyr Ser [Ala]) corresponds to the 17-mer amino acids 69–85 of the KETc7 recombinant antigen of *T. crassiceps* (Manoutcharian et al., 1996) plus a terminal Ala from the resin. The peptide was synthesized by the multiple peptide solid-phase technique as described elsewhere (Gevorkian et al., 1996). The synthetic peptide was at least 95% pure when characterized by reverse-phase high-performance liquid chromatography. The same procedure also rendered the correct amino acid composition and sequence.

Immunization of mice and collection of sera

Groups of 10 male BALB/cAnN mice, were immunized subcutaneously (s.c.) with 100 μl of 1% v/v of GK-1 (10 μg/mouse) in 100 μg of saponin (Sigma Chemical Co., St. Louis, Missouri) as described elsewhere (Rosas et al., 1998). Two weeks later, the mice were given an s.c. booster with the same immunizing dose of the peptide and with the same adjuvant as used previously. Sera were obtained from each indi-

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vidual mouse 15 days after the last immunization and stored at −70 C until tested for the presence of specific antibodies.

Enzyme-linked immunosorbent assay (ELISA) for antibodies

Whole *T. crassiceps* soluble antigens obtained as previously described (Larralde et al., 1989) were used as antigen in an ELISA to measure serum antibody levels induced by immunization following the procedure of Rosas et al. (1998). Briefly, sample sera were used at 1:100 dilution in phosphate-buffered saline (PBS); bound immunoglobulins (Igs) were detected by the addition of anti-mouse IgG coupled to alkaline phosphatase (Sigma) diluted 1:1,000 in PBS containing 1% w/v bovine serum albumin and 0.1% v/v Tween 20 for 1 hr at 37 C. The enzyme activity was detected with *p*-nitrophenyl phosphate (Sigma). Optical density readings at 405 nm were carried out in a Humareader ELISA processor (Human Gessellschaft für Biochemica und Diagnostica, Taunnusstein, Germany).

Taenia solium cysticerci

Whole *T. solium* cysticerci were dissected from skeletal muscles of (n = 19) highly infected pork carcasses 2–4 hr after slaughter in an abattoir at Zacatepec, Morelos, Mexico. Cysticerci used for infection were extensively washed with sterile PBS and placed in RPMI-1640 at 37 C for 1 hr before oral administration to hamsters.

Cyst transformation to tapeworms in hamsters

To estimate the rate of transformation of untreated cysticerci into tapeworms, cysticerci from 19 naturally infected pigs were separately collected as described above; 5 cysticerci from each pig were orally administered to each of 4 or 5 prednisolone-treated golden hamsters following the procedures described elsewhere (Verster, 1974; Allan et al., 1991). Hamsters were treated with 200 μl of Depo-Medrol (methyl prednisolone acetate, 80 μg/hamster, Upjohn, Mexico City, D.F.) as described (Aluja et al., 1993). Three weeks after infection, the hamsters were killed and the number of tapeworms found in their intestines was counted.

Effect of anti-GK1 antibodies on cysticercus–tapeworm transformation

Taenia solium cysticerci from each of 5 infected pigs (pigs 15–19) were incubated under sterile conditions with 200 μg/ml of Igs from immunized or control mice in a total volume of 5 ml of RPMI-1640 for 30 min at room temperature. After incubation, 5 cysts were fed to groups of 4 or 5 prednisolone-treated golden hamsters. Hamsters were killed 3 wk later and the number of live tapeworms was counted.

Taenia crassiceps cysticerci

The ORF strain of *T. crassiceps* isolated by Freeman (1962) and supplied by B. Enders (Behringwerke, Marburg, Germany) has been maintained by serial intraperitoneal (i.p.) passage in BALB/cAnN female mice for 12 yr at our Institute. Parasites for infection were harvested from the peritoneal cavity of experimentally infected mice following procedures previously described (Fragoso et al., 1998).

Cell isolation

Isolated *T. crassiceps* cells were obtained as previously described (Toledo et al., 1997). Briefly, cysticerci collected from peritoneal cavities of donor mice (2 mo of infection) were extensively washed with sterile 1% PBS solution, pH 7.2, and maintained in distilled water containing antibiotics (100 U/ml penicillin G and 100 μg/ml streptomycin) for 1 hr, to lyse adherent mouse cells. About 500 of these cysts were transferred to 50 ml RPMI-1640, supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, penicillin/streptomycin (as above), 0.01 mM nonessential amino acids, 0.2 mM L-glutamine, 1.6 μM β-mercaptoethanol, 25 mM HEPES, and 2 g/L $NaHCO_3$. Cysts were then cut into small, ~2-mm pieces with a scalpel and placed in 50-ml culture flasks (Falcon Labware, New Jersey) with 5 ml of culture medium. After 7 days of in vitro culture at 37 C, the fragments were transferred to fresh medium to which was added 1% trypsin-EDTA (Gibco BRL, Grand Island, New York) and maintained at room temperature with strong shaking for 15 min. The trypsin digest was washed 3 times after 10 min centrifugation of the lysate at 800 *g*, and the pellet was resuspended in culture medium. Viability of cells was tested using the trypan blue stain (Sigma) and counted using an optical microscope and a hemocytometer.

Mice inoculation with cells

Cells from parasites were collected after 7 days of in vitro incubation of the cysticercus in culture medium as previously reported (Toledo et al., 1997). Susceptible BALB/cAnN female mice (Fragoso et al., 1998) were injected i.p. with 0.5 ml of the cell suspension, containing 10^5 cells, and fed ad libitum. Mice were killed 30 days after infection, and the numbers of cysts in their peritoneal cavity were counted as described by Fragoso et al. (1998). To evaluate the capacity of cyst regeneration from cells of *T. crassiceps* cysticerci, mice were injected with cells from cysticerci obtained at different times after infection of donor mice (1–5 mo).

Effect of anti-GK-1 antibodies on cyst regeneration

Sera from mice immunized with GK-1 plus saponin or saponin alone (controls) (Toledo et al., 1999) were used to isolate purified total Igs following standard procedures that include thorough dialysis against PBS to rid the preparation from remaining and potentially toxic ammonium sulfate as tested by precipitation with $BaCl_2$ and conductivity assays (Sciutto et al., 1998). Purified Igs were adjusted to a concentration of 75 μg/ml and incubated with 10^6 *T. crassiceps*-isolated cells in 1 ml of RPMI-1640 during 30 min at room temperature under sterile conditions. After incubation of cells with RPMI-1640 or with purified Igs from GK-1-immunized or control mice, cells were injected into the peritoneal cavity of BALB/cAnN mice. After 30 days, mice were killed and the number of cysticerci recovered from each peritoneal cavity was counted.

Statistical analysis

The rate of transformation of untreated *T. solium* cysticerci into tapeworms (t/c) is expressed as the quotient of the number of cysticerci administered to each group of hamsters (c) and the number of tapeworms that developed in the same group (t). Statistical analysis required a nonparametric 1-way ANOVA, with pigs (n = 19) being the single variable and the response variable t/c estimated by 4–5 independent replicates (hamsters) in each pig. Thus, the between-pig variation would test the null hypothesis of all infected pigs bearing cysticerci with equal probability of transforming to tapeworms, whereas the variation within pigs confounds that from sampling cysticerci of the same pig and that from hamster differences in susceptibility. The effect of antibodies upon transformation of both *T. crassiceps* and *T. solium* was tested for significant differences by 2-way nonparametric ANOVA, 1-way being the 3 different treatments (3 columns: preincubation with culture medium, control Igs, and anti-GK-1 Igs) and the other way being the collection of cysticerci from 5 different pigs (5 rows); the response variable t/c was estimated in 5 different replicates (hamsters). As usual, the 95% confidence level was taken as statistically significant but, because of the intrinsic variation in these experimental procedures, statistical significance at 99% confidence level or greater was felt to strengthen the inferences.

RESULTS

Taenia solium

Transformation of untreated cysticerci to tapeworms in hamsters: Table I shows the ratio of tapeworm transformation of untreated cysticerci (t/c; t = number of tapeworms in bowel of each group of hamsters, c = total number of cysticerci administered per group) derived from 19 different collections of cysticerci, each collection from a different infected pig, and each collection tested in 4–5 different hamsters with 5 cysticerci per hamster. There is a very large and statistically significant variation between pigs ($F = 8.6$, $P < 0.001$), with some pigs bearing cysticerci having a transformation t/c index of 90% and

TABLE I. Ratio of tapeworm transformation in hamsters of untreated cysticerci derived from 19 different naturally infected cysticercotic pigs.

Pig number	Individual number of tapeworms found in each hamster*	Ratio (t/c), %†	
1	1, 1, 3, 3	(8/20)	40
2	2, 2, 4, 4	(12/20)	60
3	0, 4, 0, 3	(7/20)	35
4	0, 0, 0, 0	(0/20)	0
5	2, 3, 0, 0	(5/20)	25
6	3, 2, 2, 4	(11/20)	55
7	2, 1, 2, 0	(5/20)	25
8	2, 0, 2, 1	(5/20)	25
9	5, 3, 1, 4	(13/20)	65
10	4, 5, 4, 5	(18/20)	90
11	0, 3, 1, 0	(4/20)	20
12	3, 2, 3, 0	(8/20)	40
13	0, 0, 0, 0	(0/20)	0
14	4, 0, 4, 3, 4	(15/25)	60
15	0, 1, 1, 1, 0	(3/25)	15
16	4, 5, 4, 4, 4	(21/25)	85
17	1, 0, 0, 0, 0	(1/25)	4
18	4, 5, 5, 4, 4	(22/25)	88
19	4, 4, 4, 3, 4	(19/25)	76

* Four to 5 hamsters were orally infected each with 5 cysticerci.
† Percentage of transformed cysticerci. One-way ANOVA: Between pigs (df = 17, ssq = 175, Msq = 10.3), within pigs (df = 60, ssq = 71, Msq = 1.1); total (df = 77, ssq = 246), (F = 8.6, $P < 0.001$).

several with none (0%) or low (15%) t/c; the median was about 40%. The within-pig variation is also considerable, but, because it confounds the sources of variation due to differences in hamsters, cysticerci, and sampling errors, no reliable inference can be drawn from it other than its magnitude being suggestive of several factors being involved.

Effect of anti-GK1 antibodies on cysticercus–tapeworm transformation: Table II shows the effect of preincubation of the cysticerci with anti-GK-1 antibodies upon the development of *T. solium* cysticerci into tapeworms inside the intestine of immunodepressed hamsters. Statistical analysis revealed statistically significant sources of variation based both on the preincubation procedure (F = 73.3, df = 2.6, $P < 0.05$) and on the different pigs from which the cysticerci were collected (F = 77.7, df = 4.6, $P < 0.05$). The variance due to interaction was also significant (F = 11.1, df = 8.6, $P < 0.05$) and thus points to the effect of preincubation not being the same for all pigs.

Taenia crassiceps

Effect of anti-GK-1 antibodies on cyst regeneration: To optimize cyst regeneration from the cell lines, we first estimated the time of infection at which the collected cysticerci best developed in recipient mice. Cyst cells were recovered from cysticerci 1, 2, 3, 4, or 5 mo after infection and were injected i.p. into BALB/cAnN mice (10^5 cells per mouse). Cells from cysticerci harvested from 1- and 2-mo-old infections best reproduced in the majority of the recipients, with parasite intensities of 83.8 ± 5.2 and 84.2 ± 6.0 cysts/mouse, respectively. As for the effects of preincubation with antiparasite Igs, Table III shows the number of cysticerci recovered from mice injected

TABLE II. Effect of anti-GK-1 antibodies on *Taenia solium* cysticerci.

	Cysticerci were incubated with		
		*Purified Igs from	
Exp.	RPMI-1640	Control mice	GK-1-immunized mice
1	†0, 1, 1, 1, 0	2, 0, 1, 0, 1	0, 0, 0, 0, 0
2	4, 5, 4, 4, 4	4, 4, 5, 3, 4	4, 1, 2, 3, 1,†
3	1, 0, 0, 0, 0	0, 0, 0, 0, 0	0, 0, 0. 0, 0
4	4, 5, 5, 4, 4	3, 2, 2, 1, 1	0, 0, 0, 1, 0‡
5	4, 4, 4, 3, 4	3, 3, 2, 2, 2	1, 1, 0, 0, 0‡

* Igs were purified from immunized mice separated by 15 days with saponin (control) or saponin (100 μg/mice) plus GK-1 peptide (10 μg/mice).
† Number of tapeworm(s) found in each hamster orally infected with 5 *T. solium* cysticerci, each experiment from a different pig. Cysticerci were previously incubated with medium or purified immunoglobulins (200 μg in 5 ml).
‡ Significantly different from controls (Kruskal–Wallis nonparametric ANOVA test, $P < 0.05$).

with cysticercus cells harvested from a 2-mo-old infection previously incubated with either culture medium, Igs from saponin-injected mice (mean ELISA OD = 0.2), or Igs from mice immunized with GK-1 in saponin (mean ELISA OD = 0.6). Although a statistically significant protective effect upon parasite intensity is caused by nonspecific Igs from adjuvant-treated mice (66.8 vs. 24.4, $P < 0.01$), specific anti-GK-1 antibodies purified from GK-1-immunized mice more effectively reduced the parasite intensity expected from control values (66.8 vs. 8.6, $P < 0.01$).

DISCUSSION

A most striking finding was the great variation between pigs of their untreated cysticerci's ability to transform into tapeworms, i.e., from 0 to 90%. Thus, in spite of the great variation within pigs, our work firmly establishes that not all infected pigs, and perhaps not all cysticerci within the same pig, equally participate in transmission. This fact is of great consequence for understanding the epidemiology of cysticercosis, as it points to concealment of the viable cysts in the multitude of seemingly normal, but irrelevant, crippled cysts. For example, a classic riddle in *T. solium* epidemiology is the difficulty in finding the tapeworm carrier in localities with high (10–20%) endemic levels of pig cysticercosis (Keilbach et al., 1989) and in regions or countries with high levels of seroprevalence in humans (Sarti-Gutierrez et al., 1988; Larralde et al., 1992). A number of explanations have been proposed for the tapeworms' comparatively low epidemiological profile. Some have suggested that the large number of eggs produced by a single tapeworm is responsible for the high prevalence of cysticercosis (Gemmell et al., 1985). The long life span and resistance of infective eggs (Laclette et al., 1982), coupled to the geographically expansive migratory habits of definitive hosts that contaminate extensive areas with viable eggs (Gemmell et al., 1985) are also invoked. The differential difficulties in diagnosis of pig cysticercosis and human taeniasis (Keilbach et al., 1989) may also contribute to the tapeworm's apparently low prevalence. None of these reasons are mutually exclusive. The new crippled cysticercus notion would explain such an unbalanced ratio of metacestode/adult numbers by proposing that only a few of the seemingly

TABLE III. Effect of anti-GK-1 antibodies on *Taenia crassiceps* cyst regeneration from isolated cysticercal cells.

Cysticercal cells incubated with	*Individual parasite load	Mean number ± SD
RPMI-1640	67, 45, 70, 65, 60, 73, 88, 59, 71, 70	66.8 ± 11.11[a]§
Immunoglobulins purified from†		
Control mice (0.2 ± 0.02)‡	45, 36, 30, 10, 28, 0, 23, 21, 15, 36	24.4 ± 13.50[b]
GK-1-immunized mice (0.6 ± 0.03)‡	0, 7, 12, 0, 18, 15, 9, 0, 13, 12	8.6 ± 6.60[c]

* Number of cysticerci found in each mouse after infection with 10^6 *T. crassiceps* cysticercal cells previously incubated with medium or purified immunoglobulins (75 μg/ml).
† Igs were purified from twice-immunized mice separated by 15 days with saponin (control) or saponin (100 μg/mouse) plus GK-1 peptide (10 μg/mouse).
‡ Optical density (ELISA) of the sera of mice immunized with saponin or saponin plus GK-1.
§ Data labeled with different letters are significantly different from each other (Kruskal–Wallis nonparametric ANOVA test, $P < 0.01$).

viable cysticerci are indeed capable of transforming into tapeworms, even if ingested by a susceptible host. The impact of the crippled cysticercus notion upon control programs based only on meat inspection or treatment is ominous: such programs would need to confiscate and/or destroy all infected cysticerci lest a few viable ones escape and reproduce by the millions.

A potentially very important and useful finding is the blocking effect of anti-GK-1 antibodies upon *T. solium*'s transformation from cysticerci to tapeworm in prednisolone-treated, presumably immunosuppressed, hamsters used as bioincubators (the only available way of assessing such capacity in the parasite) (Verster, 1974). The issue was studied herein using 5 different collections of cysticerci, each from a different infected pig. Experiments proved that anti-GK-1 Igs significantly reduce the ability of cysticerci to transform to tapeworms and, to a lesser extent, so did Igs from adjuvant (saponin)-treated control mice. The specific antibodies must act very fast in vitro, as they performed their crippling function within an hour of incubation before being exposed to the acidic conditions of the stomach of the hamster. Or else, the antibodies may penetrate or localize in sites less accessible to degradation but quite vulnerable sites of the cysticercus, e.g., the spiral channel (Rosas et al., 1998; Toledo et al., 1999). The comparatively smaller but significant effects of Igs from saponin-treated control mice most probably derive from polyclonal expansion. The ELISA values of preimmunization bleedings of these same saponin-injected mice being somewhat lower than after 1 or 2 saponin injections (0.15 vs. 0.18 and 0.23, respectively) (Toledo et al., 1999) support this possibility. The fairly frequent mimotype cross-reactivity of this GK-1 peptide (Gevorkian et al., 1996) could also be involved in the adjuvant effect. We assume that under natural conditions, specific antibodies would perform their potent crippling function during the prolonged (months or years) residence of the metacestodes within the skeletal muscle and other tissues of the intermediate host. This could in part explain the variability of untreated cysticerci in transforming to tapeworms.

Thus, one may speculate that the antibody-mediated crippling effect could have significant biological and epidemiological consequences, as not all infected pigs, and perhaps not all cysticerci within the same pig, would equally participate in transmission. Also, antibody crippling effects may have important applications in endemic areas as an additional way to reduce the transmission potential of *T. solium* cysticerci. Thus, immunotherapy with GK-1 of the infected pigs, directed at raising the serum levels of crippling antibodies, might lower the transmission potential of the infected pigs.

Results also show that anti-GK-1 Igs can block the regeneration of cystic structures from *T. crassiceps* cells. This finding adds to the significance of the GK-1 epitope in the biology of this parasite. GK-1 is an 18-mer peptide corresponding to a 17amino acid stretch of KETc7, with an additional alanine. KETc7 is a protein shared by both *T. crassiceps* and *T. solium* (Rosas et al., 1998). Also, GK-1 has considerable sequence homology with the family of extensins and has been proposed as an effective candidate antigen for a preventive vaccine against pig cysticercosis (Hernández, 1998). GK-1 is expressed in all life-cycle stages of *T. solium* including eggs, cysticerci, and adults, and is quite accessible for binding anti-GK-1 antibodies in solution (Toledo et al., 1999). Thus, the interest in GK-1 epitope is furthered by our finding that it is also related to the development of isolated *T. crassiceps* cells into cysticerci.

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Synthetic peptide vaccine against *Taenia solium* pig cysticercosis: successful vaccination in a controlled field trial in rural Mexico

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Abstract

Taenia solium cysticercosis seriously affects human health when localised in the central nervous system (CNS) and causes great economic loss in pig husbandry in rural areas of endemic countries. Increasing the resistance to the parasite in the obligatory host pig may help in curbing transmission. Three synthetic peptides based on protein sequences of the murine parasite *Taenia crassiceps*, which had previously been shown to induce protection in mice against homologous challenge, were tested as a vaccine against *T. solium* cysticercosis in pigs. Vaccinated and unvaccinated piglets (240 in all) were distributed in pairs among the peasants' households of two rural villages in Mexico in which 14% of the native pigs were cysticercotic. Ten to twelve months later, the effect of vaccination was evaluated at necropsy. Vaccination decreased the total number of *T. solium* cysticerci (98.7%) and reduced the prevalence (52.6%). The natural challenge conditions used in this field trial strengthen the likelihood of successful transmission control to both pig and human through a large-scale pig vaccination program. We believe this is a major contribution in anticysticercosis vaccine development as these rather simple yet protective peptides are potentially more cost-effective to produce and less variable in results than antigens that are more complex.

Keywords: Cysticercosis; Synthetic peptides; Vaccines

1. Introduction

Taenia solium cysticercosis is a common parasitic disease of the central nervous system (CNS) of humans and one of the most frequent neurological disorders in several countries of Latin America, Africa and Asia, where it causes enormous human suffering and great economic loss [1–3]. It is also considered a re-emerging disease in the USA because of immigration of tapeworm carriers from endemic areas [4,5]. Autochthonous cases are still recognised in some countries of eastern Europe, Spain and Portugal [6].

The life cycle of *T. solium* includes a larval phase (metacestode or cysticercus) that develops in both pigs and humans from ingested eggs contaminating the environment. When humans ingest improperly cooked pork meat infected with live cysticerci, the cysticerci develop to the stage of an adult intestinal tapeworm, which produces millions of eggs that are then shed to the environment in human faeces.

The hope of controlling transmission by massively improving socio-economic standards of the very poor in the developing countries is not foreseeable in the near future. Nevertheless, since the pig is an indispensable intermediate host, it is conceivable to curb transmission by reducing pig cysticercosis through their effective vaccination. Vaccination against metacestode infections with what seems a wide and capricious variety of vaccine preparations has been surprisingly effective (relative to the difficulties found in other antiparasite vaccines) in a number of different cestodes and hosts [7–13].

Developing an effective vaccine against *T. solium* pig cysticercosis is also being pursued by a number of scientists with promising results. However, most reports are based on experimental trials with very few pigs, that do not represent realistic transmission conditions [9,10,12,13] (i.e. in dose and frequency of challenge, age of the host in initial and

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subsequent challenges, genetic and nutritional status of pigs). On the other hand, when performed in the field, results are expressed only as yearly changes of in vivo prevalence of pig cysticercosis in the region by tongue inspection [14,15], but do not measure the impact of vaccination upon infection intensity. In addition, changes in prevalence in the next couple of years after vaccination would imply an unlikely immediate and potent effect of vaccination upon the number of tapeworm-carriers in the rural communities. These experimental and preliminary field measurements of the promising effects of pig vaccination, demand a controlled field trial followed by thorough post-mortem counts of viable and damaged cysticerci to critically evaluate the vaccine's effects upon the prevalence and the intensity of pig cysticercosis in highly endemic rural areas.

The composition of the antigens to be included in a *T. solium* vaccine is a major issue. The use of crude antigen extracts from wild cysticerci collected haphazardly from naturally infected cysticercotic pigs can hardly be reproducible, as the mixtures usually include at least 20–50 or so different protein antigens recognisable in Western Blots [16], each carrying unknown numbers of peptidic and possibly glycosidic epitopes. Antigenic diversity among *T. solium*, although largely unexplored, has been shown to be significant [17] and could influence results. Further, some of the natural antigens have protective or infection-enhancing properties in murine *Taenia crassiceps* cysticercosis, whilst others are irrelevant [18,19]. With this in view, simplification of the vaccine composition was considered desirable. Purified natural antigens [9,18] and recombinant proteins of other cestodes [13,20] that have proved to be effective in homologous or heterologous systems are also being tried in *T. solium* pig cysticercosis. We have chosen to test synthetic peptides with a view of eventually expressing them in a more cost-effective biotech system (i.e. phage vectors, transgenic plant expression).

Because of the high costs and slow data retrieval involved in testing pigs in order to identify vaccine candidates, we first used the experimental disease caused in mice by another tapeworm (*T. crassiceps*) [21]. This murine parasite exhibits extensive antigen cross-reactivity [16] and cross-immunity with *T. solium* [22], and its metacestodes easily and rapidly develop in the peritoneal cavity of mice [21]. Thus, we identified and isolated antigens from *T. crassiceps* that effectively protect against experimental *T. crassiceps* murine and experimental *T. solium* porcine cysticercosis [18]. Antibodies against these protective protein fractions were then employed to identify three recombinant antigens in a cDNA library [20]. One of them, namely, KETc7, has been proved to effectively protect mice against *T. crassiceps* cysticercosis by DNA vaccination [23,24], via at least one known peptide stretch of 18 amino acids (GK-1) [25]. From the other two, KETc1 and KETc12, two additional peptides (KETc1/p of 13 and KETc12/p of 9 amino acids) were synthetically produced and confirmed in their capacity to protect mice [26]. The three protective peptidic epitopes identified were expressed in the different stages of *T. solium*, and represent different immunological targets in the parasite [26].

Thus, we decided to evaluate a vaccine against porcine *T. solium* cysticercosis that is composed of these three chemically synthesised peptidic epitopes (GK-1, KETc1/p, KETc12/p), under realistic field conditions of exposure to infection and under thorough assessment of the infection's prevalence and intensity in vaccinated and control pigs.

2. Materials and methods

2.1. Experimental design

The experimental design is that of a vaccinated and control study with 120 and 120 pigs in each group, distributed in matching pairs (one vaccinated and one control) in 70 village households. The study was performed under the natural exposure conditions of two different rural villages of Mexico that are endemic for pig-cysticercosis (prevalence 14%). The response variables were prevalence (the number of pigs with at least one parasite/total number of pigs in the group) and intensity (the parasite load in each pig, classified as total cysticerci, viable or necrotic) collected from half the carcass of each pig in the study.

2.2. Selection of the communities

Two rural communities, Huatlatlauca and Tepetzetzintla, in the state of Puebla, Mexico, were selected. In both, the conditions which favour the transmission of *T. solium* were present, namely, the high prevalence of cysticercosis in live pigs (14%, determined by tongue inspection), open air faecalism, local pork meat consumption and extensive domestic pig slaughtering, low personal hygiene and low sanitary conditions in household and village.

2.3. Vaccine

This vaccine consists of the following synthetic peptides: GK-1 (amino acids [aa] from 69 to 85 of KETc7; (Gly Tyr Tyr Tyr Pro Ser Asp Pro Asn Thr Phe Thr Ala Pro Pro Tyr Ser **Ala**); KETc1 (Ala Pro Met Ser Thr Pro Ser Ala Thr Ser Val Arg **Gly**) and KETc12 (Gly Asn Leu Leu Leu Ser Cys Leu **Gly**). They were prepared by manual stepwise solid-phase synthesis with *N*-tertibutyloxycarbonyl derivatives of L-amino acids on phenylacetamidomethyl resin (Sigma Chemical Co., St. Louis, MO). Bold letters indicate the extra amino acids that were coupled to the commercial resin. All peptides were 95% pure as judged by high-pressure liquid chromatography on analytical C18 reversed phase columns (3.9 by 150 nm; Delta Park; Waters). The correct amino acid sequence of each peptide was confirmed by protein sequencing on a pulsed liquid-phase protein sequencer (Applied Biosystems). The vaccine was

transported refrigerated to the communities and immediately applied to the animals.

2.4. Pigs and vaccination

Previous research had shown that piglets in villages might be infected in the first few days after birth, as soon as they can follow their mothers in her search for food [27]. We were not certain that an incompletely developed immune system in newborn piglets would respond to vaccination, therefore, the sows of the piglets to be vaccinated were themselves vaccinated during gestation in the hope of reducing the risk of infection of the new-born. Thus, we purchased 49 pregnant sows in the selected communities. Twenty-five of these sows, those that were to breed the vaccinated piglets, were themselves vaccinated subcutaneously at the base of the ear during the last month of gestation with 500 μg of each peptide plus 300 μg of adjuvant (saponin). The remaining 24 pregnant sows, which were to breed the control piglets, were injected with 300 μg of adjuvant, at the same time and site of injection as the vaccinated sows. It was estimated that one hundred and twenty piglets per group (vaccinated and control) was adequate to evaluate the vaccine's effect with 95% confidence intervals at an expected prevalence of 14% of cysticercosis in the control group [28]. One hundred and thirty-eight piglets were vaccinated and 140 that received only adjuvant, served as controls. Of all these, 18 vaccinated and 20 control pigs died of causes not related to vaccination nor to cysticercosis and were excluded from the study. The piglets born to the vaccinated sows were vaccinated twice, at 60 and 90 days of age with 250 μg of each peptide plus 150 μg of saponin in saline isotonic solution at the base of the ear. Piglets born to the control sows received only 150 μg of saponin each at the same ages and under the same conditions as the vaccinated piglets and were kept as controls. After receiving the second dose of vaccine or adjuvant, the piglets were distributed in matched pairs (one vaccinated, one control) among 70 villagers' households, preference being given to those villagers that had been previously identified as having owned infected animals. Each household received one or two matched pairs, thus, possible variations in management and exposure levels among the different households were controlled for all pairs. Villagers were instructed to treat and feed the experimental pigs as they usually do with their own pigs.

2.5. Necropsies

All pigs were humanely killed between the ages of 10 and 12 months. Half the carcass of each animal, together with liver, tongue, masseter muscle and heart were carefully dissected and all parasites counted. Additionally, the cysticerci were macroscopically classified in either viable or necrotic according to their macroscopic aspect (viable cysticerci being those well-limited neatly conserved cystic structures containing clear vesicular fluid, whilst necrotic cysticerci were no longer cystic but rather semi-solid caseous masses without discernible parasite structures). In addition, samples of parasitised tissues were fixed in buffered 10% formalin (pH 7.2). The samples were then processed for histological observation and stained with haematoxylin-eosin to confirm the macroscopic diagnosis by microscopy [27].

2.6. Statistical analysis

The statistical analyses used were the marginal χ^2 Mac Nemar test to compare the relative risk of being infected in vaccinated versus control pigs according to the results of necropsies in the 120 matched pairs. The non-parametric Mann–Whitney *U*-test was used to compare the number of cysticerci recovered in both groups. The statistical programmes of EPI-Info and SSPS were used.

3. Results

Table 1 shows the effect of vaccination on the reduction of the number of cysticerci in infected pigs (15.8–7.5%). Table 2 summarises the distribution of infected pigs among vaccinated and controls groups. It also shows the number of total, viable and necrotic cysticerci found in each pig. Of the 120 control pigs, 19 (15.8%) were infected: 2 (10.5%) of these with one parasite, 13 (68.4%) with less than 10, and 6 (31.6%) with thousands of cysticerci. From these control pigs, 66,565 cysticerci were collected, (63,951 vesicular and 2614 necrotic). From the 120 vaccinated animals, 9 (7.5%) were infected with at least one parasite: 6 (66%) of these with less than 10, 2 (22%) with less than 100, and only one with 1286 cysticerci. Of these vaccinated pigs, 1369 cysticerci were collected (806 vesicular and 563 necrotic). At 95% level of confidence, results indicate 52.6% efficiency of the vaccine in reducing the number of infected pigs and 97.9% reduction of the total parasite load. The vaccine's reduction of parasite load relevant for transmission would be closer to 99%, if the necrotic cysticerci were of no consequence for transmission. The skewed distribution of the number of cysticerci found in each group should be noted: there being a number of low intensity cases (<10 cysticerci

Table 1
Effect of pig vaccination against *T. solium* cysticercosis on the number of non-infected and infected pigs following field exposure

	Pigs inoculated with	
	Adjuvant (controls)	Adjuvant and peptides (vaccinated)
Non-infected	101[a]	111
Infected	19	9
Percent	15.8%[b]	7.5%

[a] Number of pigs per group.

[b] Percent of infected pigs. The relative risk of being infected in vaccinated pigs was significantly lower than in controls ($P < 0.05$, χ^2 marginal test).

Table 2
Effect of pig vaccination against *T. solium* cysticercosis on the numbers of larvae found in the carcasses of infected pigs following field exposure[a]

Experimental group	Number of infected pigs	Intensity class (number of cysticerci/pig)					
		1–10	11–100	101–1000	1001–10000	>10000	Total (necrotic/vesicular)
Control							
Number of infected pigs in each intensity class	19	13	0	0	2	4	
Number of cysticerci in each pig		1, 1, 2, 2, 2, 2, 2, 3, 3, 3, 4, 9			2700, 3500	13000, 14950, 15000, 17378	66565 (2614/63951)
Vaccinated							
Number of infected pigs in each intensity class	9	6	2	0	1	0	
Number of cysticerci in each pig		1, 1, 2, 2, 4, 4	34, 35		1286		1364 (563/806)

[a] The number of cysticerci recovered in controls was significantly higher than in vaccinated pigs ($P < 0.05$, Mann–Whitney U-test).

per pig) in both groups (15 in control and only 6 in vaccinated pigs), whilst massive intensities (>1000 cysticerci per pig) were more commonly found in the control pigs (6 versus 1).

4. Discussion

The prevalence of cysticercosis in the control pigs introduced in these rural villages very closely reproduced the previously estimated in vivo prevalence of pig cysticercosis, (15.8% obtained herein versus 14% reported by Huerta et al. [31]). This validates our approach and lends credence and significance to results in the vaccinated group.

The most direct and simple demonstration of the vaccine's effects is that it reduced the prevalence of cysticercosis among the vaccinated pigs by 52.6% and, most significantly, reduced the intensity of infection with viable cysts by 97.9%. That these potent effects were obtained with a mixture of synthetic peptides tested in realistic exposure conditions argues in favour of the effectiveness of vaccination against pig cysticercosis. It is also a strong technical argument towards the development of an effective, less variable, more stable and cost-effective vaccine based on peptides to be used on a wider scale. Even if it were the vaccine's only effects, the reduction in pig cysticercosis prevalence and parasite intensity is greatly beneficial for the villagers' economy because pork meat without cysticercosis sells at much higher prices. In addition, protecting pigs against cysticercosis by vaccination has the economic benefit of allowing the villagers' rustic low-cost form of rearing pigs to the only one they can afford, to continue. On the other hand, whether pig vaccination actually reduces the number of tapeworms that would eventually develop from lightly parasitised pigs will have to be ascertained in a study designed differently, as there is no known clear-cut correlation between the number of viable cysts in a pig and their probability of becoming a tapeworm upon ingestion by a human host.

The vaccination protocol starting in the gestating sows of the vaccinated piglets leads to the question of which of the vaccinations is the one relevant for the protective effects: the sows' or the piglets' or both? While this point needs to be clarified, the success of the vaccination protocol suggests that vaccination of both pregnant sows and piglets should be considered. Experimentation is under way to determine the cellular and molecular immunological protagonists responsible for the vaccine's efficiency against pig cysticercosis. Should results from *T. crassiceps* murine cysticercosis hold for *T. solium* in pigs, one would expect the participation of TH1-mediated immune responses under the strong influence of genetic and endocrine factors of the host [29–31].

Finally, it is also worth noticing the over-dispersed distribution of parasite intensities in both vaccinated and control groups. There are at least two modes: one of very light intensity infections (<10 parasites per pig) that is sensitive to vaccination (approximately 50% reduction in the vaccinated group), and another of massive intensity infections (with thousands or tens of thousands of parasites per pig), the most vulnerable to vaccination. An over-dispersed distribution of parasite intensities in naturally acquired pig cysticercosis has been documented before [31] and has led to suspect the involvement of host genetics in susceptibility and resistance to *T. solium*, as shown for *T. crassiceps* in mice [29]. Advisability of an extensive pig vaccination program for public health reasons would be strengthened by evaluation of the differential transmission roles of the pigs afflicted with few cysticerci versus the massively infected ones. There would be no question about the public health benefits of vaccination if the total number of cysticerci were the only thing that mattered.

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Th1 and Th2 indices of the immune response in pigs vaccinated against *Taenia solium* cysticercosis suggest various host immune strategies against the parasite

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Abstract

Kinetics of the production of serum antibody levels and Th1 (IL-2, IFN-γ) and Th2 (IL-4, IL-10) cytokines was studied in five pigs vaccinated with a synthetic tri-peptide vaccine (S3Pvac) against *Taenia solium*, a vaccine that has been shown protects pigs against naturally acquired infection. Healthy pigs of mixed genetic background, similar to those bred in rural villages of Mexico, were vaccinated with S3Pvac or with adjuvant alone, kept in sanitary conditions and bled at different times after vaccination to study the development of their specific immune response. Peripheral blood mononuclear cells (PBMCs) of vaccinated pigs showed a significant increment in the production of Th1 cytokines (IL-2 and IFN-γ) but not of Th2 cytokines (IL-4 and IL-10) after specific PBLs stimulation with all the individual peptides. A Th1-inclined cytokine profile leading to an exacerbated local inflammation at the early installation stage of the cysticercus may possibly interfere with their successful establishment in the serum antibodies against total cysticercus antigens and against each of the three different peptides comprising S3Pvac were detected 7–51 days after vaccination. Antibodies against GK-1 interfered with the cysticerci development into intestinal tapeworms in prednisolone-treated hamsters. The sub-lethal crippling effect of anti-GK-1 antibodies upon cysticerci indicates to a therapeutic application of S3Pvac in infected pigs having potential epidemiological consequences, as it could aid in decreasing the number of tapeworms expected to develop from the few cysticerci that survive in the vaccinated pigs.

Keywords: Vaccination; Immune response; Th1/Th2; Synthetic peptides; Pig cysticercosis; *Taenia solium*

1. Introduction

Taenia solium taeniasis/cysticercosis is a major parasitic disease of global proportions that poses

Abbreviations: S3Pvac, synthetic tri-peptide vaccine; *Ts*Ag, total *Taenia solium* antigens; SI, stimulation index

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serious threats to human health and economy in the third world (Del Brutto et al., 1998; Sciutto et al., 2000). The life cycle of this parasite includes a larval (cysticercus) phase affecting both pigs and humans after ingestion of eggs excreted in the faeces of human tapeworm carriers. The parasite's transmission strategy depends largely in rustic pig rearing, open-air defecation and low hygienic standards of the population (Schantz et al., 1993), and more profoundly in the very slow rate of social and educational development in rural areas of endemic countries. A number of control measures (i.e., meat inspection) have been tried in the third world. However, despite their past success in Europe and its reported effectiveness in local studies (i.e., treatment of tapeworm carriers, uplifting of sanitary conditions) (Hitchcock, 1987; Sotelo et al., 1996), the disease persists and extends worldwide, now threatening millions of poor people in Latin America, Asia and Africa and reemerging in the first world due to human migration (Schantz et al., 1998).

There have been several efforts to develop a vaccine against *T. solium* pig cysticercosis as a way to interfere with transmission. Different candidate immunogens have been tried with promising results in experimental disease (Molinari et al., 1993; Nascimento et al., 1995; Sciutto et al., 1995; Plancarte et al., 1999) or in rural conditions in naturally acquired disease (Molinari et al., 1997). Recently, we developed a synthetic tripeptide vaccine (S3Pvac) comprised of three synthetic peptides of 18, 12 and 8 amino acids present in a *Taenia crassiceps* cDNA library (Manoutcharian et al., 1996; Toledo et al., 1999, 2001) and also in *T. solium* (Huerta et al., 2001). S3Pvac was shown to be effective in a controlled field trial against naturally acquired pig cysticercosis, reducing prevalence (50%) and, more notably, intensity (98%) (Huerta et al., 2001).

The identification of the type and mechanisms of protection of the immune response induced in pigs by S3Pvac has been little explored. Gathering information from various taenia/host relationships, there is general agreement that antibodies can harm early cestode larvae but not developed metacestodes (cysticerci) because of the parasite not exposing vital molecules accessible to antibodies at the metacestode stage (Parkhouse and Harrison, 1989) or by inhibiting complement mediated damage (Laclette et al., 1992). Thus, the prevailing notion is that cysticerci may only be damaged by cellular immune mechanisms if at all (Bojalil et al., 1993; Terrazas et al., 1999; Mooney et al., 2000). However, in many of the taenia/host relationships studied, an inflammatory process ensues in the host–parasite interface that includes all types of leukocytes responsive to immune signals and considered capable of exerting parasitocidal effects (de Aluja and Vargas, 1988; Padilla et al., 2001; Londono et al., 2002), as they stand or after activation by an additional signal from parasite or host (i.e., antigen liberation, drug treatment). More recently, a novel form of antibody anti-parasite action, characterized not by killing the cysticerci but by interfering with its transformation into tapeworm, has possible epidemiological consequences and brings back antibodies as effective immunological actors in cysticercosis control (García et al., 2001).

Advances in the understanding of the immunological response leading to protection in pigs could help devise strategies by which to enhance its effectiveness. Thus, we moved to characterize the immune response of pigs vaccinated with S3Pvac, focusing on only the most prominent of the Th1 and Th2 markers (IL-4, IL-10, IL-2 and TNF-γ production by PBLs) and serum antibody levels at different times after vaccination as assessed in vitro with each separate peptide comprised in the S3Pvac.

2. Materials and methods

2.1. Anticysticercus synthetic-peptide vaccine (S3Pvac), peptides and total antigen extract from T. solium

The vaccine against cysticercosis (S3Pvac) consists of a 1:1:1 mixture of the three following synthetic peptides: GK-1 (amino acids [aa], (GYYYPSDPNTFFYAPPYSA), KETc1 (APMSTPSATSVR) and KETc12 (GNLLLSCL). The peptides were prepared in our laboratory by manual stepwise solid-phase synthesis with *N*-tertibutyloxycarbonyl derivatives of L-amino acids on phenylacetamidomethyl resin (Sigma Chemical, St. Louis, MO). All peptides were 95% pure as judged by high-pressure liquid chromatography on analytical C18 reversed phase columns. The correct amino acid sequence of each peptide was confirmed by protein sequencing on a pulsed

liquid-phase protein sequencer (Applied Biosystems). S3Pvac and the separate peptides were maintained refrigerated (2–4 °C) until they were used. For total antigen extract whole *T. solium* cysticerci were obtained from the skeletal muscle of infected pigs', washed with phosphate-buffered saline solution, homogenized, and centrifuged at 25 000 × *g* for 45 min at 4 °C. The soluble antigens in the supernatant were recovered; calcium was precipitated with ammonium oxalate 0.3 M and ammonium hydroxide 1:10 and centrifuged at 25 000 × *g* for further 40 min at 4 °C. The supernatant was recovered and filtered with 0.22 mm in sterile conditions, and protein content quantified by the method of Lowry, and frozen at −20 °C until used as a whole antigen fraction (*Ts*Ag).

2.2. Pigs and vaccination

To study the immune response induced by S3Pvac 10 piglets of both sexes of mixed genetic background, 2 months of age and free of cysticercosis, were purchased in a rural farm in the state of Puebla, transferred to Mexico City and kept in the farm of the Veterinary School of the Universidad Nacional Autónoma de México in controlled sanitary conditions to prevent infections. Five of the piglets were injected with S3Pvac twice, at 60 and 90 days of age, with 250 μg of each peptide plus 100 μg of saponin in distilled water, subcutaneously (s.c.) at the base of the ear. The other five piglets to be used as controls were also injected s.c. with only 100 μg of saponin at the same ages as were the vaccinated ones. Blood samples from all injected pigs were obtained by intravenous puncture before and at different days (7, 14, 30, 37 and 51) after vaccination to study the kinetics of the humoral and cellular immune responses induced by S3Pvac. To provide with antibodies for the bioassay of cysticerci–tapeworm transformation, four other additional groups of five piglets each were immunized three times at 0, 30 and 60 days with 250 μg of either GK-1, KETc1 or KETc12 mixed with 100 μg of saponin or with 100 μg saponin alone, and bled 30 days after last immunization.

2.3. Antibody detection by ELISA

Sera from pigs immunized with S3Pvac or with each peptide or saponin alone (controls), were processed to measure specific antibodies by ELISA. To reduce unspecific binding of sera pig components, an enriched Ig fraction of the sera was precipitated by ammonium sulphate, chromatographed and rediluted in 0.15 M PBS before assessment of serum antibody levels by ELISA (Harrison et al., 1989). *T. solium* cyst fluid or each of the peptides (1 μg per well) were used as antigen in ELISA. Briefly, policarbonate 96 well microtitration plates (Nunc, Roskilde, Denmark) were incubated with 100 μl per well of either *T. solium* cysticerci antigen (*Ts*Ag) or each of the peptides (1 μg per well of each one) in carbonate buffered saline, pH = 9.6, for 60 min at 37 °C. The wells were then washed and incubated for 60 min at 37 °C with 100 μl of the precipitated Ig fractions diluted in 0.03% PBS-Tween 1:200. Specific antibodies bound to the well were detected with 100 μl of rabbit anti-pig IgG (whole molecule) peroxidase conjugate (Sigma) diluted to optimum concentration (1:1000). The substrate used was 3,3′,5,5′-tetra-methyl-benzidine, 5 mg/ml (Sigma). The reaction was stopped using 1 N H_2SO_4, 50 μl per well, and the absorbance values were measured at 450 nm in an ELISA reader (LP400, Pasteur Diagnostics, Paris, France).

2.4. Bioassay of the effects of anti-peptide antibodies on T. solium cysticercus-tapeworm transformation

T. solium cysticerci used to infect hamsters were dissected from the skeletal muscle of three different naturally infected pig carcasses, 2–4 h after slaughter in an abattoir of Zacatepec, Morelos. The cysticerci were extensively washed with sterile PBS and placed in RPMI 1640 at 37 °C during 1 h before use. Five cysticerci from each pig were incubated under sterile conditions with 200 μg/ml of enriched Ig fractions from the peptide vaccinated or control pigs in a total volume of 5 ml of RPMI 1640 for 30 min at room temperature. Afterwards, to evaluate the cysticerci capacity to transform into tapeworms (Verster, 1974; García et al., 2001), the cysticerci were orally administrated to five groups of immunodepressed golden hamsters, each group comprised of 4–5 hamsters. Hamsters were immunodepressed with 200 μl of Depo-Medrol (methyl prednisolone acetate, Upjohn, Mexico, 80 μg per hamster), as described (Allan et al., 1991). Hamsters were sacrificed 3 weeks later, and

their intestines were completely dissected to count the number of live tapeworms.

2.5. Proliferation assay and cytokine quantitation

PBMC were harvested from the blood of vaccinated and control pigs the day of immunization (day 0) and at 7, 14, 30, 37 and 51 days after immunization, and immediately passed through a Ficoll-Hypaque gradient 1.077 (Sigma, St. Louis, MO). PBMC were cultured in RPMI 1640 medium supplemented with L-glutamine (0.2 mM), non-essential amino acids (0.01 mM), penicillin (100 U/ml), streptomycin (100 μg/ml) and FBS (10%). ConA (5 μg/ml) or each of the three peptides (10 μg/ml) or *Ts*Ag at 10 μg/ml were added to the culture wells containing 2×10^5 PBMC in 200 μl of culture medium and incubated at 37 °C in a 5% CO_2 humidified atmosphere, in flat-bottomed microtiter plates (Costar, Cambridge, MA). After 5 days of incubation, the cellular cultures were pulsed with 1 μCi per well of tritriated methyl thymidine (Amersham Life Science, UK) and incubated for 20 h more. Then, all cells were harvested and the amount of incorporated thymidine was measured in a 1205 β-plate liquid scintillation counter (Wallac Oy, 20101 Turku 10, Finland). Assays were done in triplicate and data are presented as stimulation indexes (SIs were calculated as ratios of cpm from Ag-stimulated cells to matched unstimulated cells from the same pig at the same time-point). Additionally, supernatants from replicate cultures were collected after 72 h of incubation with GK-1, KETc1, KETc12 or *Ts*Ag for cytokine determination. IL-2, IFN-γ, IL-4 and IL-10 were measured using the ELISA Kit following the recommendations suggested by the manufacturer (Biosource International, California). OD values are reported in pg/ml in a logarithmic scale.

2.6. Statistical analysis

All statistical analyses were performed using Spss 10.0. Although the design is multifactorial, the number of pigs employed is insufficient for a multifactorial analysis. Thus, we focused the analysis on whether there are immune response differences between pigs injected with S3Pvac plus saponin (vaccinated) and those injected with saponin alone (control) in the six different immunological parameters at five different days after injection. The data for each immunological parameter was analyzed by two-way ANOVA, with treatments (vaccinated, control) and days after vaccination (0, 7, 15, 30, 37 and 51) as independent variables, and the average value of duplicate or triplicate measurements of each response parameter in each of the five pigs as the dependent variable.

3. Results

There is considerable variation in response between pigs in all graphs, more pronounced in the vaccinated group than in controls.

3.1. Antibody response

The levels of total antibodies elicited by vaccination against total *Ts*Ag, or GK-1, KETc1 and KETc12 peptide is shown in Fig. 1. Levels of antibodies against GK-1, KETc1 and KETc12 are significantly greater than those of controls after the first immunization ($P < 0.001$) and remain so throughout the experiment (51 days later). Antibodies against *Ts*Ag show a tendency to increase in the vaccinated pigs after day 14th but it is not statistically significant.

3.2. Cellular immune response

The proliferative responses of PBMC from vaccinated and control pigs throughout the experiment are shown in Fig. 2, and are expressed as the SI calculated as the ratio of cpm from stimulated to matched unstimulated cells in each pig at each day after vaccination and as elicited by each antigen or peptide. The PBMC response to in vitro stimulation with the peptides and with whole *Ts*Ag varied considerably among pigs and days after vaccination but there is a statistically significant trend to increased values in the vaccinated pigs for KETc12 and KETc1.

The levels of IL-2, IL-4, IL-10 and INF-γ were determined in the supernatant of PBLs from control and vaccinated pigs at 7, 30 and 37 days postimmunization and after inducing their specific proliferation with GK-1, KETc1 and KETc12 (Fig. 3). Here again there was considerable variation among pigs. IL-2 and INF-γ levels were significantly higher in vaccinated pigs than in controls ($P < 0.05$) when they were

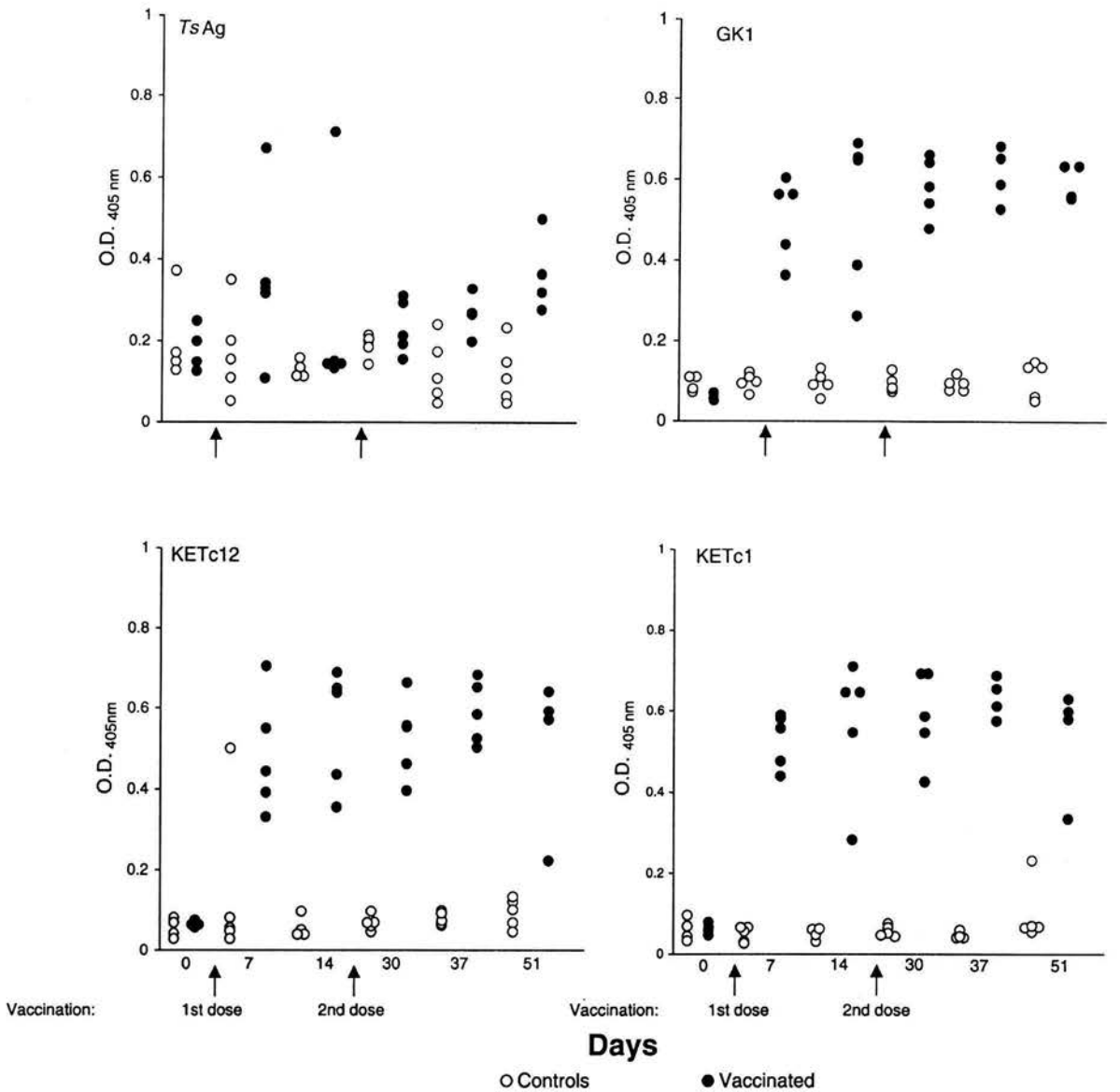

Fig. 1. Antibody response of pigs immunized with saponine (control) or S3Pvac against *Ts*Ag or GK-1, KETc1 and KETc12 peptides. Control and vaccinated pigs were bled before immunization and after the first and second dose of S3Pvac. The levels of antibodies were determined by ELISA in total Igs purified from sera. Each point represents the antibody level per pig.

stimulated in vitro with GK-1, KETc1 and KETc12. The increased levels of these cytokines did not change at the different days after vaccination. Thus, values for each cytokine from all days in each pig were pooled and then contrasted by two-way ANOVA (with *vaccinated* and *control* pigs and type of *interleukin* as independent variables and amount of cytokine produced by each sample of each pig as dependent variable). IL-2 and INF-γ levels were significantly higher in vaccinated pigs than in controls ($P < 0.0001$) when they were stimulated in vitro with GK-1, KETc1 and KETc12 (Fig. 3) but no differences were detected neither in IL-4 ($P > 0.17$) nor in IL-10 ($P > 0.56$) cytokines.

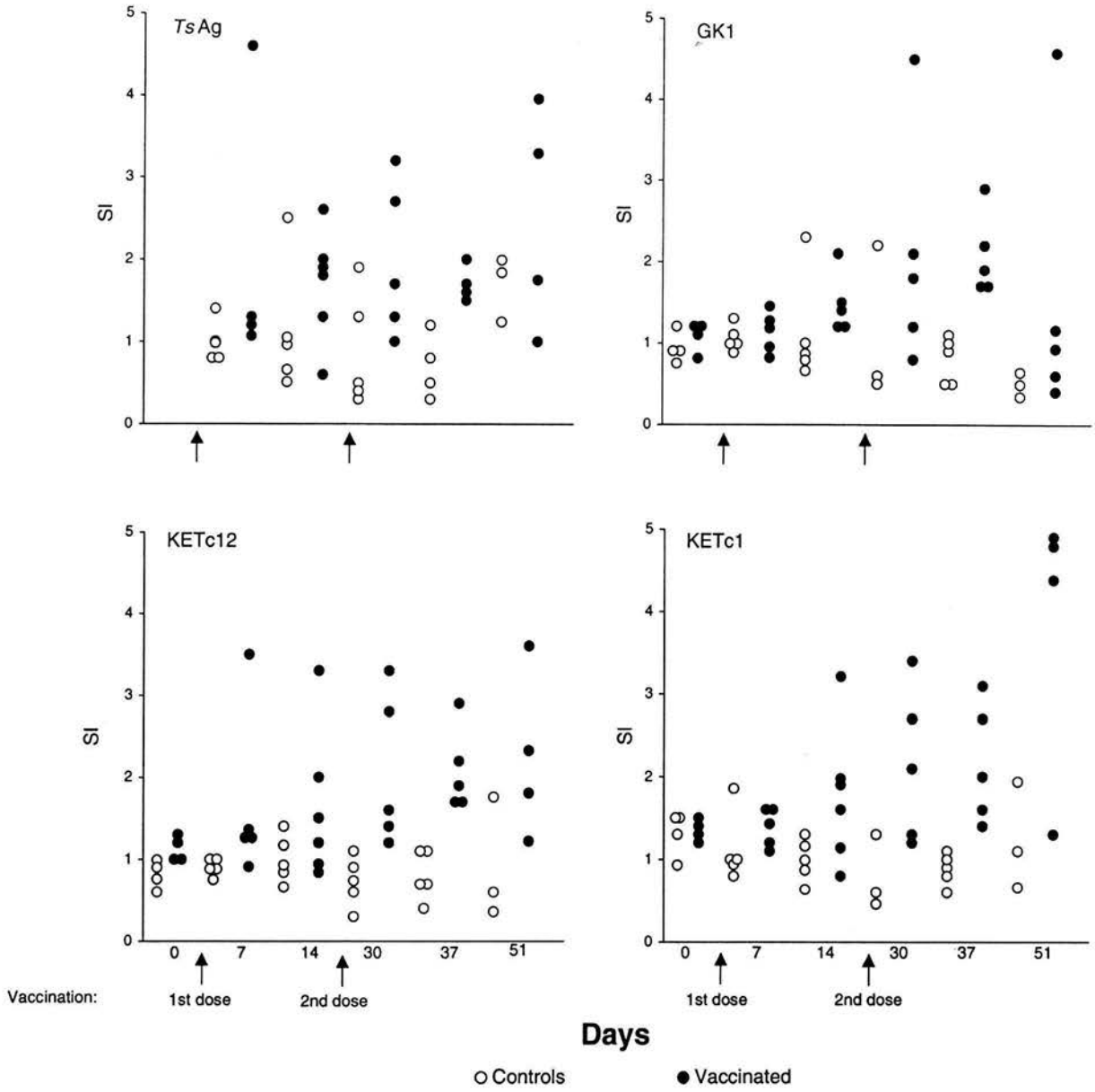

Fig. 2. Proliferative response of PBL cells from pigs immunized with saponine (control) or S3Pvac. Proliferation was measured by [methyl-^{3}H] thymidine incorporation on day 5 of culture either with *Ts*Ag (10 μg per well) or with the peptide GK-1, KETc1 or KETc12 peptides (10 μg per well). Data is shown as SI (SI = cpm after vs. before stimulation from the same pig). Each point represents the SI of each individual pig.

3.3. Effect of anti-peptide antibodies on T. solium cyst transformation

Table 1 shows the number of cysticerci that transformed into tapeworms after preincubation with medium, non-immune pig enriched-Igs and anti-peptide pig enriched-Igs. Transformation was tested in cysticerci collected from three different infected pigs, and each collection was tested in 4–5 different hamsters, 5 cysticerci per hamster per preincubation treatment.

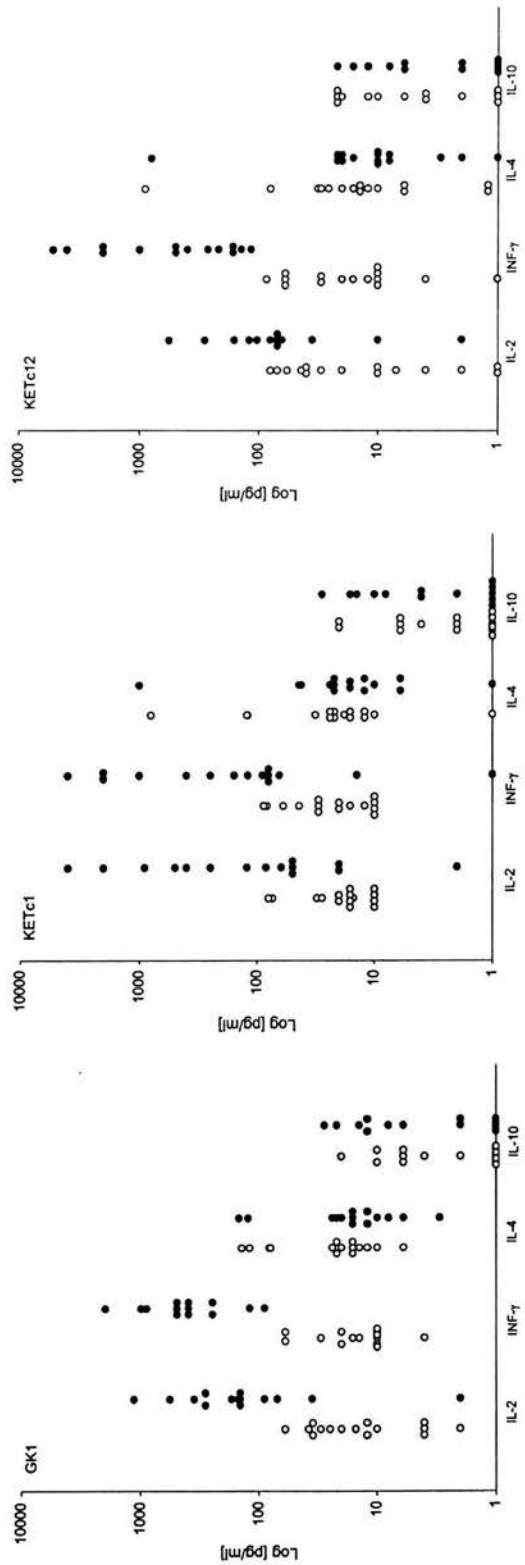

Fig. 3. Levels of IL-2, IL-4, IL-10 and INF-γ measured in the supernatant of cells from pigs immunized with saponine (control) or S3Pvac after 5 days of in vitro stimulation with the corresponding peptide GK-1, KETc1 or KETc12. All values from vaccinated pigs obtained at 7, 30 and 37 days postimmunization in each pig were pooled to illustrate the results. Statistically significant differences between vaccinated and control pigs were found in IL-2 and IFN-γ responses ($P < 0.0001$), using the t-Student's test.

Table 1
Effect of antibodies induced by the immunization with each synthetic peptide that constitutes the S3Pvac vaccine on *T. solium* cysticerci

	Donor pig		
	1	2	3
Cysticerci were incubated with			
Medium	1, 2, 1, 1[a]	3, 0, 1, 1	2, 1, 2, 0
Pigs immunized with[b] adjuvant (saponin)	1, 2, 0, 1	0, 1, 0, 1	1, 2, 0, 1
GK-1[c]	0, 0, 0, 0	0, 0, 0, 0	0, 0, 0, 0
KETc1	2, 0, 0, 2	0, 2, 1, 2	2, 1, 4, 1
KETc12	0, 1, 4, 1	2, 5, 0, 1	1, 1, 4, 2

[a] Number of tapeworm(s) found in each hamster 21 days after oral infection with five *T. solium* cysticerci per hamster. Cysticerci used in each experiment were from three different pigs. Cysticerci were previously incubated with medium or Ig-rich fraction (200 μg in 5 ml).

[b] Igs were purified from pigs immunized with saponin (control) or saponin plus GK-1, KETc1 or KETc12, respectively.

[c] The only preincubation treatment that ANOVA significantly contributed to variance ($F = 4.956, P < 0.002$).

There is no statistically significant transformation effect of preincubation of the cysticerci with medium alone, nor with anti-KETc1 and anti-KETc12 pig Igs. Only those cysticerci that were cultured with anti-GK-1 enriched-Igs were totally inhibited in their transformation to tapeworms in all the hamsters tested and in all three different collections of cysticerci. Statistical significance of these results was studied by two-way ANOVA, with preincubation treatment (five levels, rows) and identity of infected donor pigs from which cysticerci were collected (three levels, columns) as independent variables, and hamsters as replications (four replications). ANOVA revealed that only preincubation of cysticerci with GK-1 enriched-Igs had significantly contributed to the total variance in the number of tapeworms found in the intestines of the hamsters ($F = 4.956$, $P < 0.002$).

4. Discussion

A synthetic tripeptide vaccine (S3Pvac) against *T. solium* pig cysticercosis was recently shown to be effective in rural pigs exposed to naturally acquired infection in a field trial (Huerta et al., 2001). Herein, we report of the involvement of both Th1 and Th2 effectors in the vaccinated pigs reaction to infection and discuss the possible mechanisms involved in protection.

Firstly, we found considerable variation within pigs in the magnitude of their different responses. This is an important observation possibly related to the highly diverse genetic background of rural pigs in Mexico. This may explain the SP3vac's incomplete efficiency in the reduction of prevalence (50%), as well as the variable reductions in the individual pigs parasite intensities and in the proportions of damaged cysticerci that establish in each individual vaccinated pig (Huerta et al., 2001).

Despite between pigs variation, statistically significant higher levels of antibodies were found in the group of vaccinated pigs compared with controls. The higher levels of anti-GK-1, KETc1 and KETc12 antibodies, SI and Th1 cytokines were observed as early as 7 days after S3Pvac immunization in all pigs and remained unchanged until the end of the experiment (51 days), in spite of the booster given in the 30th day. Differences in antibody levels between vaccinated and control pigs are more evident when using as ligand the isolated peptides than when using the complete antigen preparation, a fact we suspect results from the peptides low relative concentration in the complete antigen preparation and from the high background of non-specific binding that is common when complex antigen mixtures are used in ELISA (Toledo et al., 1999).

Thus, our results of increased levels of specific anti-peptide antibodies and higher PBMC stimulation indexes in vaccinated pigs, with and emphasis in Th1 cytokine production resembles that induced in mice by S3Pvac (Toledo et al., 1999, 2001). A Th1 response is also implicated in early experimental infection of mice as the immune mechanism that limits parasite successful establishment and eventual multiplication (Villa and Kuhn, 1996; Terrazas et al., 1998). In our study of pigs vaccinated with S3Pvac, results also indicate to a strong Th1 response, in at least IFN-γ, IL-2, and to no response of Th2, as measured by IL-4 and IL-10, other than antibody production. Enhanced lymphocyte proliferation to the three peptides, GK-1, KETc1 and KETc12, and *Ts*Ag occurred in pigs as early as 14 days after vaccination and remained at similar high levels for the rest of the study (51 days), and so did the Th1 cytokines

(IL-2 and IFN-γ) but not the Th2 cytokines that did never exceed the control values.

With respect to the biological activity of the anti-peptide antibodies elicited by S3Pvac, our results show that pig anti-GK-1 antibodies but not the other anti-peptide antibodies block *T. solium's* transformation from cysticerci to tapeworm in prednisolone-treated hamsters (a model of *T. solium* taeniasis developed in immunodeficient hamsters (Verster, 1974)). It would seem that the pigs' anti-GK-1 antibodies do just as the mice anti-GK-1 antibodies did when this crippling effect was first described: a subtle yet effective antiparasite activity blocking its reproduction, as opposed to the more ostensible killing effects (García et al., 2001). The crippling function of pigs anti-GK-1 antibodies has possible application as a way to lower the transmission potential of *T. solium* cysticerci of S3Pvac vaccinated pigs. Anti-parasite activity differences among the anti-peptide Igs could relate with the localization of the native antigen bearing the peptide epitopes in the live cysticerci and with the varying consequences for the parasite of the binding of antibody to each epitope (Toledo et al., 1999, 2001).

The between pigs variability in the immune response induced by the different peptides comprised in the S3Pvac follows from so complicated a parasite as *T. solium* and from so diverse a genetic background as that of the rural pigs in Mexico, and is possibly enhanced by varying degrees of exposure to infection. Nonetheless, naturally occurring infection is the real scenario where vaccines have to show their real worthiness. The description of the immune response of vaccinated pigs contained in this paper is far from complete, we dealt here with some of the more conspicuous indices. But even at this level of simplification, the complexity of this host–parasite relationship is already showing and would advise to consider the use of multiepitope vaccines as a way to complement a more effective defense strategy against the parasites various stages and multifaceted strategies (Toledo et al., 1999, 2001; Shi et al., 1999).

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