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UNIVERSIDAD NACIONAL AUTONOMA DE MÉXICO

INSTITUTO DE INVESTIGACIONES BIOMÉDICAS

CISTICERCOSIS: EVALUACION DE LAS POSIBILIDADES DE LA INMUNIZACIÓN GÉNICA.

T E S I S

QUE PARA OBTENER EL GRADO ACADÉMICO
DE DOCTORA EN CIENCIAS BIOMÉDICAS
P R E S E N T A

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CIUDAD UNIVERSITARIA, 1998

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ABSTRACT: The purpose of this study was to evaluate DNA vaccination in cysticercosis prevention by using a *Taenia crassiceps* cDNA of a recombinant antigen (KETc7) which has been reported as protective against murine cysticercosis. The KETc7 cDNA was cloned into the pcDNA3 plasmid alone or with the betaglycan signal peptide sequence (pTc-7 and pTc-sp7, respectively). Positive expression of the pTc-sp7 product was confirmed by transfection of C33 cells and immunofluorescence using sera of mice infected with *T. crassiceps*. Immunization of mice with 3 injection of pTc-sp7 DNA at the higher dose (200 µg) was the most effective to induce antibody with or without bupivacaine. Immunization with pTc-sp7 induced protection against challenge with *T. crassiceps* cysticerci as successfully as previously observed with the KETc7 recombinant protein. Antibodies elicited by DNA immunization with pTc-sp7 specifically reacted with the native protein of 56 kDa previously reported, which is immunolocalized in the cuticle of *T. crassiceps* cysticerci. The 56 kDa antigen is also present in *Taenia solium* oncosphere, cysticerci, and adult tissue. The protection induced in DNA-immunized mice and the observation that the injected plasmid remains as an extracromosomal form within muscle cells, encouraged us to continue testing this procedure to prevent *Taenia solium* cysticercosis. To explore the possibilities of this form of immunization, mice were inyected intramuscularly with one or three doses of pTc-sp7 or with three intradermal doses (200 µg per mice). Intramuscular pTc-sp7 immunized mice developed similar levels of resistance, and higher levels of protection were observed with intradermal immunization. The immunization with either intramuscular or intradermal pcDNA3, significantly reduced the expected parasite load while no effect was observed with one immunization. The evaluation of induced immune response, indicates the relevance of the cellular response in the observed protection. Spleen or lymph node cells from DNA immunized mice induced a specific T-cell response to *T. crassiceps* antigens and to a synthetic peptide from the immunogen itself. Proliferated cells were enriched in CD8+CD4- T-lymphocytes. These results provide encouraging information on the possible use of KETc7 in the immunoprofilaxis of cysticercosis as a first insight into the characterization of the immune response induced by pTc-sp7.

El presente trabajo se realizó en el
Instituto de Investigaciones Biomédicas de la UNAM,
bajo la dirección de la doctora Edda Sciutto Conde

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RESUMEN

La cisticercosis causada por el estado larvario de la *Taenia solium* es una enfermedad parasitaria que afecta al hombre y al cerdo, orig
inando problemas sociales y económico en países en vías de desarrollo en donde predominan las condiciones que la favorecen. Considerando la alta frecuencia de esta enfermedad en nuestro país y su impacto en la salud humana, se han considerado diferentes estrategias para su prevención. Entre ellas se encuentra la interrupción de la transmición de esta parasitosis a través del aumnto de la resistencia del cerdo por medio de la vacunación. Esta estrategia parece una alternativa realista considerando que el cerdo es el hospedero intermediario obligatorio de este parásito. Para evaluar las diferentes estrategias de vacunación en contra de la cisticercosis porcina se ha utilizado el modelo experimental murino de cisticercosis causada por el metacéstodo de *Taenia crassiceps*.

A partir de una biblioteca de cDNA del cisticerco de *T. crassiceps* se identificaron 4 secuencias que codifican para las proteínas KETc1, KETc4, KETc7 y KETc12, que indujeron protección en contra de la cisticercosis murina. Utilizando la secuencia que codifica para la proteína KETc7 se evaluaron las posibilidades de la vacunación génica, lo que constituyó el objetivo principal de esta tesis. El cDNA de KETc7 fue insertado en el plásmido pcDNA3 con y sin la secuencia del péptido señal del receptor del betaglicano (receptor tipo III del TGFβ). Las construcciones fueron denominadas pTc-7 (sin péptido líder) y pTc-sp7 (con péptido lider). La capacidad de expresión de ambas construcciones se verificó por inmunofluorescencia indirecta de células C33 transfectadas con ambas construcciones. Se evaluó la capacidad inmunogénica de ambas construcciones utilizando dos diferentes dosis (120 y 200 μg de DNA/ratón), por vía intramuscular.

El mayor índice de seroconversión (85%) y de protección (58.63% en hembras y 100% en machos) se obtuvo al inmunizar tres veces con la construcción pTc-sp7 con 200 μg de DNA/ratón. Los niveles de protección alcanzados resultaron similares a los obtenidos por la inmunización con la proteína recombinante KETc-7. Los anticuerpos inducidos por la construcción pTc-sp7 reconocieron específicamente la fracción proteica de 56 kDa. Estos anticuerpos reconocieron estructuras en el cisticerco de *T. crassiceps*, así como en la oncosfera, cisticerco y adulto de *T. solium*. El plásmido se mantuvo extracromosomal al menos hasta 15 días después de la última inmunización. Con el fin de optimizar la vacunación con pTc-sp7 se evaluó su capacidad protectora bajo diferentes condiciones de inmunización. Los resultados indicaron que por vía intramuscular una sola inmunización induce niveles similares a los obtenidos con tres inmunizaciones. El porcentaje más alto de protección se obtuvo inmunizando por la vía intradérmica. La respuesta inmune asociada a la protección sugiere ser mediada por células T CD8+CD4- aunque aún queda por identificar el mecanismo involucrado.

En este trabajo de tesis se reportan las primeras evidencias en la literatura de la capacidad de controlar una cestodiasis a través de inmunización génica.

ABREVIATURAS USADAS EN EL TEXTO

CAT-. Cloranfenicol Acetil Transferasa

cDNA.- DNA complementario

CMV.- Citomegalovirus

CTL.-Linfocitos T Citotóxicos

DNA.- Acido Desoxiribonucleico

H_2O_2.- Peróxido de Hidrógeno

INF.- Interferón

MAP.- Multiples Antigenic Peptides

MHC I.- Complejo Principal de Histocompatibilidad de clase I

MHC II.- Complejo Principal de Histocompatibilidad de clase II

NO.- Oxido Nitroso

OMS.-Organización Mundial de la Salud

PBS.- Solución Amortiguadora de Fosfatos

RNA.- Acido Ribonucleico

RNAm.- Acido Ribonucleico mensajero

SSI.- Solución Salina Isotónica

SV40.- Simian Virus 40

TE.- Tris-EDTA

TNF.- Factor de Necrosis Tumoral

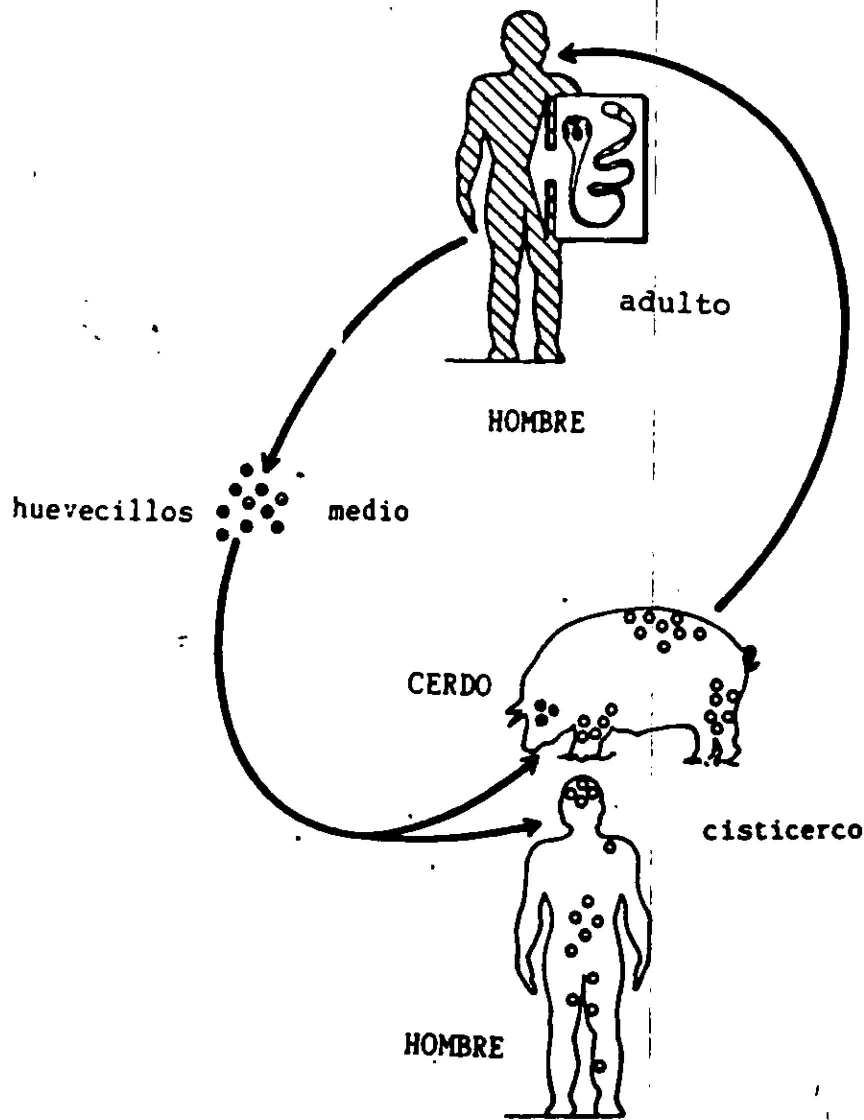

FIGURA 1- Ciclo de vida de *Taenia solium.*
Cuando el hombre come carne de cerdo infectada con cisticercos, éstos llegan al intestino delgado y se desarrollan a la forma adulta (tenia). Por reproducción sexual, la tenia produce millones de huevecillos que son expulsados al medio junto con las heces del hombre. La disposición inadecuada de las heces causa la diseminación de lo huevecillos en el medio, los cuales pueden ser ingeridos por el hombre ó por el cerdo. La ingestión de los huevecillos en ambos, causa el desarrollo de cisticercos en diferentes tejidos. En humanos se establece principalmente en el cerebro, ojo y músculo esquelético. En cerdos, el parásito de instala tanto en músculo como en cerebro. El ciclo de vida del parásito se cierra cuando el hombre come carne de cerdo infectada con cisticercos, los cuales se transforman en la tenia adulta en el tracto intestinal.

INTRODUCCION

La cisticercosis causada por *Taenia solium* es una enfermedad parasitaria que afecta al hombre y al cerdo y constituye un serio problema de salud y económico en países que tienen con malas condiciones sanitarias como son **los de** Asia, Africa y Latinoamérica. Es causada por ingerir los huevecillos del parásito y el posterior establecimiento y desarrollo de la larva de la *Taenia solium* en ambos hospederos (Fig. 1). En el hombre, la presencia de la larva en el sistema nervioso central ocasiona la neurocisticercosis enfermedad grave y frecuente en México (Flisser *et al.*, 1982) con importantes implicaciones sociales y económicas. Además, es la principal causa de hipertensión intracraneana y de consultas en instituciones neurológicas, al igual que es responsable del 50% de los cuadros convulsivos reportados (Flisser, 1980; Larralde *et al.*, 1992, Medina *et al.*, 1990). México se ha considerado como un país de alta endemicidad, así desde 1979 la OMS ha reportado un 1.9% de defunciones por cisticercosis cerebral y 3.5% de cisticercosis en necropsias (OMS, 1979). Estudios más recientes que incluyeron una muestra de 70,000 sueros colectados en una encuesta seroepidemiológica realizada en 1989 indicaron que existía una seroprevalencia nacional promedio del 1.2% lo cual indicó que hay un alto contacto de la población con este parásito (Larralde *et al.*, 1992).

La cisticercosis también afecta a la porcicultura rústica, que representa el 40% del total de la carne de cerdo que se producen en el país (Aluja, 1982). Esto ocasiona no sólo importantes pérdidas económicas por decomiso de carne infectada cuando ésta llega a inspección sanitaria, sino además, constituye una zoonosis que afecta a una proporción sustancial de la población humana que consume la carne parasitada, lo que permite la persistencia de esta cestodiasis (Aluja, 1982; Aluja, 1987; Mazón, 1991).

La elevada frecuencia con la que se presenta esta parasitosis así como los daños que ocasiona, justifican los intentos para prevenirla. Con la finalidad de interrumpir el ciclo de vida del parásito, se ha propuesto modificar la incidencia de la cisticercosis porcina a través de:

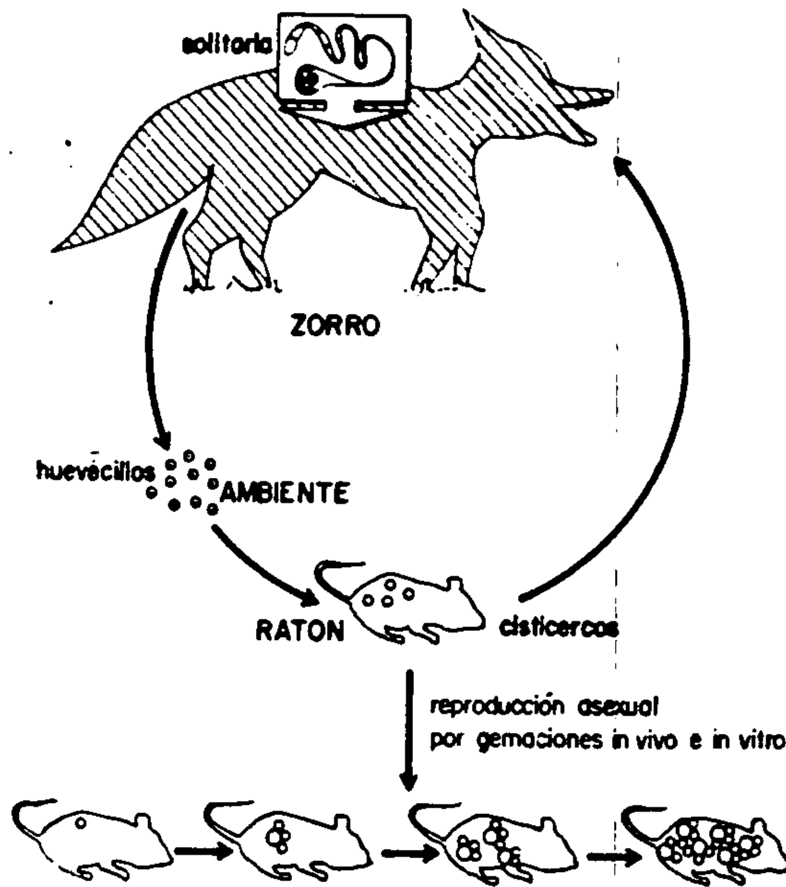

FIGURA 2- Ciclo de vida de *Taenia crassiceps*.

El cisticerco se desarrolla en la cavidad abdominal de roedores, los cuales adquieren la infección por ingestión de huevecillos dispersos en el medio ambiente provenientes de heces de cánidos infectados con la tenia adulta. En el roedor las larvas eclocionan de los huevecillos originando los cisticercos. Cuando un roedor infectado es devorado por algún cánido, los cisticercos se instalan en el intestino de éste y se desarrollan a la forma adulta. Al alcanzar la madurez sexual, la tenia produce huevecillos infectivos, cerrando de este modo el ciclo de vida del parásito. En la cisticercosis experimental murina, los cisticercos son inyectados en la cavidad abdominal del ratón donde se reproducen por gemación teniendo a lo largo de varios meses una cantidad considerable de parásitos

1) Campañas de educación y mejoramiento sanitario, considerando que la cisticercosis prevalece como consecuencia de condiciones inadecuadas de vivienda e higiene que favorecen su transmisión (Gemmel *et al.*, 1982).
2) Aumentar la resistencia del hospedero intermediario obligatorio (el cerdo) con el propósito de interrumpir la transmisión de la parasitosis, que puede ser a través de:
   a) Desarrollo de vacunas en contra de la cisticercosis porcina (Molinari *et al.*, 1993, 1997; Sciutto *et al.*, 1995, Manoutcharian *et al.*, 1996, Rosas *et al.*, 1998).
   b) Modificación genética de hospederos susceptibles para aumentar su resistencia innata mediante transferencia de genes de resistencia (Fragoso, 1998).
3) A través de la identificación y tratamiento del teniásico (Sarti *et al.*, 1994)

Considerando las dificultades económicas y prácticas involucradas en la experimentación con cerdos, se ha utilizado como modelo la cisticercosis experimental murina causada por el cisticerco de *Taenia crassiceps*, que ha permitido evaluar diferentes estrategias de vacunación. La cisticercosis murina presenta características atractivas para ser empleada como modelo, aunque con las limitaciones propias de un modelo experimental. Entre las ventajas podemos mencionar:

- Los cisticercos se reproducen por gemación en la cavidad abdominal del ratón, propiedad que permite mantenerlo y reproducirlo en condiciones controladas por pases intraperitoneales sucesivos (Freeman, 1962), lo que permite obtener proteínas y ácidos nucleicos del parásito fácilmente. (Larralde *et al.*, 1990).
- Su capacidad de crecer en ratones (especie muy estudiada tanto genética como fisiológicamente) permite realizar experimentos en condiciones controladas y sistemáticas.
- El ciclo de vida natural de ambos parásitos es semejante (Fig 2). Ambos involucran un hospedero intermediario y uno definitivo.

- Los cisticercos de *Taenia crassiceps* y de *T. solium* presentan alta antigenicidad e inmunogenicidad cruzada, lo que ha sido importante tanto para el diagnóstico como para la vacunación (Larralde *et al.*, 1989, 1992).

La búsqueda de antígenos de interés para vacunación, comenzó con la evaluación de la capacidad protectora de un extracto total del cisticerco de *T. crassiceps* en contra de la cisticercosis murina y porcina. Los resultados obtenidos mostraron que con este extracto y con la dosis apropiada se podía iinducir protección en contra de ambas parasitosis (90% de protección en contra de la cisticercosis murina y 50% en contra de la cisticercosis porcina) (Valdez *et al.*, 1994: Manoutcharian *et al.*, 1995). Sin embargo, la capacidad protectora resultó ser dependiente de la dosis empleada, ya que en el cerdo indujo facilitación cuando se utilizaron dosis altas (4 mg/kg de peso) e incrementó la carga parasitaria en más del 50% de lo esperado (Sciutto et al., 1995). Estas observaciones sugirieron la presencia de proteínas que pudieran inducir facilitación en lugar de protección en el estracto total. Con la finalidad de identificar sólo aquellas proteínas responsables de la protección, el extracto total fue separado electroforéticamente y se identificaron 3 fracciones antigénicas (56, 66 y 74 kDa) que indujeron mayor nivel de protección en contra la cisticercosis murina y porcina (Valdez *et al.*, 1994: Manoutcharian *et al.*, 1995). Con el objetivo de obtener solamente estas proteínas en mayor cantidad se construyó una biblioteca de cDNA en el vector Uni-ZapXR partiendo del RNAm del cisticerco de *T. crassiceps*, y por procesos de inmunodetección con sueros hiperinmunes de conejo, que fueron inmunizados con las fracciones antigénicas de 56, 66 y 74 kDa, y con sueros de cerdos infectados, se seleccionaron cuatro colonias que produjeron las proteínas recombinantes (Apéndice II) de interés para vacunación las cuales se denominaron: KETc1, KET4, KETc7 y KETc12. Estas proteínas fueron evaluadas en su capacidad protectora tanto en contra de la cisticercosis murina como porcina disminuyendo en ambos casos la carga parasitaria (Manoutcharian *et al.*,1995, 1996). A partir del conocimiento de la secuencia de la proteína KETc7 (Apéndice I) y con el fin de lograr mayor eficiencia en la vacunación, se exploraron dos procedimientos de experimentación: 1) La

identificación de 3 epítopes sintéticos (Apéndice III) (GK1, GK2, GK3), los cuales fueron posteriormente sintetizados químicamente y evaluados en su capacidad protectora en contra de la cisticercosis experimental murina (Gervorkian *et al.*, 1996; Toledo *et al.*, 1998) y 2) La inmunización génica con la secuencia de la proteína KETc7 utilizando un vector con capacidad de expresión en células eucariotes (Rosas *et al.*, 1998;. Cruz-Revilla *et al.*, 1998), la cual también se evaluó en el modelo experimental murino.

INMUNIZACIÓN GÉNICA.

Los primeros estudios realizados para conocer la capacidad funcional y de expresión de moléculas de DNA desnudo *in vivo*, se hicieron al inocular el DNA del virus de papiloma Shope en la piel de conejos sanos, induciéndoles la formación de tumores (Ito, 1960). Posteriormente, con este mismo procedimiento, se indujo en hamsters recién nacidos la producción de anticuerpos en contra de papiloma (Anastasiu en 1962) . En 1990 Wolff demostró que gracias a la transfección *in vivo* de células musculares de ratón con genes reporteros como el de CAT, luciferasa o beta-galactosidasa, las células producían la proteína con la actividad enzimática correcta. Estos antecedentes indicaron que las células animales transfectadas *in vivo* tenían la capacidad de capturar al DNA exógeno, introducirlo al núcleo, transcribirlo y finalmente traducirlo, lo que abrió la posibilidad de considerar esta forma de producción de proteínas *in vivo*, para inducir una respuesta inmune dirigida hacia agentes infecciosos con la esperanza de que pudiera inducirse una protección específica. (Tang *et al.*, 1992).

Este método de inmunización se apoya completamente en la tecnología del DNA recombinante ya que requiere de la selección de los genes que codifican para las proteínas idóneas para la vacunación. Depende de un plásmido como vector de expresión en el que se hacen las construcciones y si es necesario se puede agregar la secuencia de un péptido señal que ayude a la exportación de la proteína (Barry *et al.*, 1995; Lu *et al.*, 1996; Robinson y Torres, 1997).

Características de los plásmidos usados en inmunización génica.

Un plásmido ó replicón, es una molécula de origen bacteriano, circular, pequeña, de doble cadena e independiente del DNA bacteriano. Usualmente contiene un solo origen de replicación junto con sus elementos de control y secuencias de terminación, cualquier secuencia que se encuentre entre estas dos regiones será replicada como parte del plásmido. Esta molécula se replica al mismo tiempo que el cromosoma bacteriano, utilizando la maquinaria molecular de la célula que lo alberga (Sambrook *et al.*, 1989;

Lewin, 1996). Los plásmidos que se utilizan regularmente para inmunización génica están formados por los siguientes elementos:

- Secuencias de procariontes que facilitan la propagación del vector en bacterias:
  a) Origen de duplicación bacteriano funcional en *E. coli.*
  b) Genes de resistencia a antibióticos.
  c) Región de "polilinker", donde se introduce la secuencia exógena a través de cortes con enzimas de restricción

- Elementos requeridos para la expresión de la secuencia de DNA exógeno en células eucariontes:
  a) Los promotores, "enhancers" e intrones, son secuencias de DNA que median la transcripción de los genes.

  Los promotores son secuencias que controlan el inicio de la transcripción del gene, no codifican para proteínas funcionales, más bien son sitios a los que se les une primero los factores de transcripción y luego la RNA polimerasa. La eficacia del promotor depende de la afinidad que tengan los factores de transcripción celulares por él, por lo que no todos los promotores son eficientes en todos los tipos celulares, (Sambrook *et al.*, 1989). Algunos de los promotores más utilizados en los vectores para inmunización génica son:

  1) El de CMV.

  Es altamente eficiente y muy activo en muchos tipos celulares, ya que tiene muchos sitios de unión para factores de transcripción conocidos de diversos celulares. Se ha reportado que no induce la inserción del plásmido al genoma de la célula transfectada. Para aumentar su eficiencia puede estar acompañado de un enhancer e intrón A, como elementos reguladores de la transcripción (Montgomery *et al.,* 1993, Whalen, 1993).

  2) El de SV40.

  También es un promotor muy activo en muchos tipos celulares, Tiene múltiples enhancers a los que se le pueden unir muchos de los

factores de transcripción de las células en donde se encuentra (Kendrew, 1994).

3) Promotores funcionales en determinados tipos celulares.
Entre éstos se pueden mencionar: El promotor de la cadena β pesada de la miocina cardiaca de conejo, que permite una expresión muy eficiente de la proteína, cuando el plásmido se inyecta en músculo (Hansen *et al.*, 1991). Otro promotor específico es el de las inmunoglobulinas, que junto con su enhancer, induce la expresión de la proteína en linfocitos B. Aunque este ultimo promotor es muy eficiente, se ha reportado que permite que el plásmido se integre al genoma del linfocito (Xiong *et al.,* 1997).

Los "enhancers" son secuencias a los que se les unen los factores de transcripción de las células y pueden estimular la capacidad de transcripción de los promotores a los que estén unidos unas 1000 veces. Esta actividad no depende de la posición de enhancer con respecto al promotor ya que puede estar antes o después, además pueden funcionar en cualquier dirección. El enhancer del SV40 es de los más estudiados y muy utilizado junto con el de citomegalovirus para incrementar la eficiencia en la inmunización génica.

Los intrones son secuencias de DNA que no codifican para proteínas y son removidos del RNAm por el proceso de splicing, sin embargo, influyen mucho en controlar la expresión del gene, por ejemplo en el splicing alternativo, se pueden crear dos o mas formas funcionales del RNAm, lo que puede originar dos o más proteínas diferentes. Dependiendo del promotor, los intrones pueden influir en la estabilidad del RNAm en el citoplasma ya que pueden afectar la poliadenilación y transporte del RNA (Sambrook *et al.,* 1989.

b) Región de poliadenilación. Ayuda a la formación de un RNAm maduro, estable y le permite transladarse del núcleo al citoplasma. Está dotado de 20 a 30 nucleótidos del lado 5´ del gene. La longitud de esta región

incrementa la estabilidad del RNAm, entre más corto más se degrada. (Sambrook *et al.*, 1989.

c) Secuencias de DNA exógeno que codifican para proteínas no propias de las células hospederas. Estas secuencias codifican para la proteína que se quiere expresar. Cuando provienen de una biblioteca de cDNA carecen de intrones. Lo ideal es que las secuencias tengan el codón de inicio (AUG, que codifica para metionina) en contexto de la secuencia Kozak para unirse a ribosomas (Sambrook *et al.*, 1989).

Otra característica importante del DNA plasmídico es que posee secuencias CpG no metiladas que tienen actividad inmunoestimuladora (Pisetsky, 1996). Estas secuencias son muy abundantes en bacterias pero en eucariontes son raras, ya que en ellos se encuentran metiladas. Cada una de estas secuencias está formada por el hexámero PuPuCpGPyPy y se localizan en el promotor de CMV, en el replicón, y en el gen de resistencia a ampicilina. Estas secuencias pueden servir como adyuvantes en los procesos de inmunización génica, ya que incrementan la respuesta inmune contra la proteína codificada por el DNA del inserto. En sus formas no metiladas los motivos CpG estimulan monocitos y macrófagos para producir citocinas como: IL-12, TNF-α e IFN γ y β. Estas citocinas actúan sobre la células NK para inducir actividad lítica y secreción de IFN γ. Las CpG también estimulan la producción de IL-6, que promueve la activación de células B y la secreción de IgM (Krieg *et al.*, 1995; Yi A.K. 1996, Klinman *et al.*, 1996).

Hasta la fecha muchos plásmidos han portado la secuencia de un sólo inmunógeno, sin embargo, es posible incorporarles una secuencia más, ubicada entre otro juego de promotor y cola de poliA, que puedan portar secuencias inmunoestimuladoras como las CpG, genes de linfocinas como GM-CSF ó IL12, al igual que moléculas coestimuladoras como B7.1 y B7.2 (Iwasaki *et al.*, 1997a; Wild *et al.*, 1998)

Administración del plásmido

Las vías de inmunización más frecuentes en la inmunización génica son la intramuscular y la intradérmica, esta última se ha probado tanto con

jeringa como con el sistema de pistola de genes o "gene gun". Este sistema ha mostrado ser altamente eficiente, ya que con cantidades muy pequeñas de DNA asociado a bolitas de oro, que funcionan como acarreadores, puede llegar a los núcleos de las células intradérmicas como las células de Langerhans y los queratinocitos (Raz *et al.,* 1994; Condon *et al.,* 1996; Hengge, 1996), incrementando la eficiencia de la transfección *in vivo* (Eisenbraun *et al.,* 1993; Wolff *et al.,* 1991). Sin embargo, su uso se ha limitado por la complejidad del equipo requerido. Cuando la inmunización se hace con jeringa (intramuscular o intradérmica) se requiere de 100 a 1000 veces más DNA que por bombardeo, ya que el DNA se inyecta fuera de las células, y solo un pequeño porcentaje del DNA podrá entrar a la célula y aún menos al núcleo y ser transcrito, lo cual disminuye la eficiencia de la vacunación (Warren *et al.,* 1995). La inmunización por la vía intramuscular tiene la desventaja de que se transfectan pocos miocitos, por lo que el nivel de expresión de la proteína en el organismo vacunado es a menudo tan bajo (del orden de nanogramos) que es muy difícil detectarlo (Wolff *et al.*, 1991), sin embargo, a pesar de su baja producción, el antígeno es capaz de inducir una respuesta inmune a largo plazo (Yankaukas *et al,* 1993). Con el fin de favorecer la entrada del DNA a las células musculares se ha reportado el uso de fármacos como la bupivacaina (anestésico local) y miotoxinas como la cardiotoxina, los cuales al dañar a los miocitos inducen a los mioblastos a transformarse en miocitos, proceso durante el cual se ha propuesto que el DNA entra a las células (Wolff *et al.,* 1991).

Otra vías de inmunización que se han probado han sido por mucosas, subcutánea e intravenosa con la intención de que el antígeno se produzca en la zona en que normalmente se da la infección, sin embargo por la sencillez y eficiencia para inducir una respuesta inmune adecuada se han empleado más frecuentemente la intradérmica e intramuscular (Wolff *et al.,* 1991; Davis *et al.,*. 1993; Fynan *et al.,* 1993; Kuklin *et al.,* 1997).

Ventajas de la inmunización génica

La vacunación génica posee características que la distinguen como más eficiente que otras formas de vacunación, como son:

- Las células del organismo inmunizado expresan solo fragmentos selectos del patógeno, de manera que no hay riesgo de infección como podría suceder con las atenuadas vivas (Griffiths, 1995), ni de efectos secundarios por la presencia de contaminantes presentes en las subunidades vacunales (Donelly *et al.*, 1997; Montgomery *et al.*, 1993; Ulmer,1993; Yankauckas *et al.*, 1993; Xiang, 1994).
- La respuesta inmune que induce puede llegar a ser muy prolongada (18 meses o más) (Temin *et al.*, 1990; Wolf *et al.*, 1992, Danko y Wolff, 1994).
- La proteína producida por las células transfectadas adquiere características estructurales (glicosilación y configuración terciaria) muy semejantes a la del agente infeccioso, por lo que se induce una respuesta inmune celular y humoral altamente específica que permita reconocer a la proteína nativa del agente infeccioso (Schödel *et al.*, 1994).
- El DNA es más estable que las proteínas y otros polímeros biológicos (Donnelly *et al.*, 1997).
- Podría ser de bajo costo si su aplicación se realizara con el sistema de "pistola génica" (gene gun) ya que solo requiere de nanogramos de DNA. para inducir una respuesta inmune efectiva,
- A diferencia de la vacunación con proteínas, con la inmunización con DNA no se quiere de la relación dosis/peso, ya que con dosis similares de DNA se puede inducir respuesta inmune en diferentes especies animales (ratones, cabras, vacas, cerdos). Una posible explicación de esto es que la cantidad de DNA necesario para que las células del organismo transfectado produzcan la proteína e induzcan una respuesta inmune eficiente es muy baja, incluso quizá menor que la inyectada (Cox *et al.*, 1993).

Respuesta inmune e inmunización génica

La vacunación con DNA ha contribuido para identificar el tipo de respuesta celular involucrada en el control de diferentes patógenos. Así, se ha observado que una misma construcción puede inducir una respuesta Th1 o Th2 dependiendo de la vía por la que sea administrada (Pertmer, 1997; Feltquate, 1997). Estas diferencias pueden ser consecuencia de los tipos celulares más

probablemente involucrados en cada forma de inmunización. Así, con "pistola génica" los inmunógenos que se producen pueden ser fagocitados y posteriormente presentados por las células de Langerhans, Th1, mientras que el antígeno producido por el DNA inyectado en músculo podría ser reconocido primero por células B, Th2 (Constant *et al.*, 1995; Condon *et al.*, 1996).

La inmunización génica es particularmente eficiente para inducir una respuesta de células T citotóxicas, lo que implica que probablemente a través de esta forma de inmunización se presente el antígeno en contexto del MHC de clase I. Esto puede ser consecuencia de que el plásmido le genere a la célula transfectada el mismo efecto que si ésta estuviera infectada por un virus.

Una de las interrogantes de la inmunización génica y su capacidad de inducir respuesta inmune, era conocer qué células se encargaban de la formación y presentación del antígeno. Para averiguarlo, se realizaron experimentos que señalaron que las células presentadoras de antígeno son las que provienen de la médula osea y no las células musculares como en un principio se sugerió, ya que los miocitos pueden expresar MHC de clase I en bajo nivel pero no expresan moléculas de clase II de manera constitutiva ni moléculas coestimuladoras, por lo que no parecen ser los candidatos para actuar como células presentadoras de antígeno (Hohlfeld y Engel 1994). Estos experimentos se realizaron usando ratones híbridos F1 provenientes de padres con 2 haplotipos de MHC diferentes, a los cuales se les irradió para eliminar sus células del sistema inmune y se les transplantó la médula osea de uno de los padres. Posteriormente se les inyectó el DNA, y se demostró que la presentación del antígeno codificado por las secuencias en los plásmidos fuea través de células presentadoras de antígeno que provenían de la médula osea que poseían el MHC I del haplotipo del padre donador y no el de las células musculares del ratón híbrido (Doe *et al.*, 1996; Iwasaki, 1997b).

También se ha estudiado el papel de las células transfectadas *in vivo* en la expresión del antígeno, para lo cual se retiró por cirugía la región del cuerpo del ratón que fue transfectada, ya sea piel ó músculo, lo que reveló que la piel es básica para iniciar la respuesta inmune, lo que no sucede con el músculo. Así, se observó que retirar el músculo inyectado un minuto después de haber inyectado el DNA, no afectó la magnitud ni el tiempo en que se produjeron los anticuerpos, lo cual sugirió que probablemente el DNA difundió a otras células o

que las células sanguíneas fueron transfectadas en ese momento y se alejaron rápidamente del sitio de inyección. Este proceso se observó utilizando las construcciones que codifican para proteínas que se asocian a la membrana (HA de la influenza), de secreción (Hormona de crecimiento humano) y proteínas intracelulares (proteína del virus de la influenza). Todas estas construcciones originaron una respuesta de LTC (Torres et al., 1997). En contraste, la piel transfectada con DNA demostró ser esencial para inducir una respuesta inmune tanto celular como humoral, ya que cuando se retira antes de tres días no se induce ningún tipo de respuesta inmune (Torres *et al.,* 1997). Esto puede deberse a que en la inmunización por piel se puedan transfectar directamente células pertenecientes al sistema inmune como las células de Langerhans (Salmon *et al.,* 1994).

Se ha propuesto que dependiendo de la vía de inmunización utilizada, la presentación del antígeno se realiza en diferentes órganos linfoides. Así, cuando se hace por bombardeo con "pistola génica" se ha identificado al ganglio inguinal como el lugar donde se presenta el antígeno, ya que este drena la linfa de la piel abdominal (Condon *et al.,* 1996; Boyle *et al.,* 1996), mientras que con la inoculación intramuscular la presentación del antígeno se localizó solo en bazo (Robinson *et al.,* 1997), probablemente sea consecuencia de los diferentes tipos celulares transfectados durante las diferentes formas de inmunización (Winegar *et al.,* 1996).

Relativamente poco se sabe de los mecanismos inmunológicos inducidos por la inmunización génica, aunque se han observado respuestas celulares y humorales por periodos prolongados de tiempo después de la inoculación, se han localizado células productoras de anticuerpos en médula ósea, así como células de memoria que probablemente se mantienen a consecuencia de la presencia continua del antígeno o de complejos antígeno-anticuerpo. Estas células de memoria pueden proveer nuevas células secretoras de antígeno al contactar con células que han mantenido por largo tiempo la expresión del plásmido (Ulmer *et al.,*1993; Robinson *et al.,*.1997; Yang *et al.,* 1997; Michel *et al.,* 1995; Yankauckas *et al.,* 1993). Originalmente, se había planteado que las células musculares podían ser las responsables de la producción del antígeno por tiempos prolongados. Sin embargo, actualmente se especula que las células musculares que sintetizan el antígeno y lo presentan en el contexto del MHC I

son eliminadas por el propio sistema inmune mediado por células (Davis *et al.*, 1997). Al parecer el tiempo de duración de la respuesta difiere entre la celular y la humoral. Así, en casos como el de la proteína Env del virus de la inmunodeficiencia la respuesta humoral es momentánea, mientras que la celular es bastante duradera (Lu *et al.*, 1996).

A la fecha, se han producido un gran número de reportes que han descrito la inducción de respuesta inmune efectiva tanto celular como humoral después de la vacunación con DNA. La Tabla 1 muestra algunos ejemplos de enfermedades que se han tratado de controlar por medio de la vacunación génica. Como se observa, este procedimiento ha sido más ampliamente utilizado para el control de enfermedades virales, sin embargo recientemente se han logrado avances en contra de parásitos pluricelulares como *Schistosoma japonicum* (Yang *et al.*, 1995) y el metacéstodo de *Taenia ovis* (Rothel *et al.*, 1997).

Este nuevo método de inmunización provee de protección efectiva en contra de virus, bacterias y parásitos, así como de nuevas **opciones** para diseñar estrategias alternativas para el control de infecciones. También esta metodología ofrece nuevas posibilidades para la manipulación terapéutica de la respuesta inmune en alergias y en enfermedades autoinmunes, el manejo del cáncer, además de permitirnos ahondar en el conocimiento del propio sistema inmune. Sin embargo, sólo experimentos cuidadosos y más investigación en el área pueden llegar a determinar el potencial de esta nueva forma de inmunización al igual que sus beneficios tanto en la salud humana, como en el diseño de estrategias para el control de enfermedades en especies animales.

JUSTIFICACION Y OBJETIVO DE ESTE TRABAJO

La inmunización génica es una de las formas más modernas y efectivas de vacunación, que ha demostrado tener la capacidad de inducir respuesta inmune celular, humoral y protección en contra de varios agentes infecciosos, sin embargo, en contra de parásitos complejos como el cisticerco de *Taenia ovis* o *Schistosoma japónicum* solo se ha logrado inducir respuesta inmune pero no protección. Desde hace varios años nuestro grupo de trabajo ha enfocado sus intereses en el desarrollo de una vacuna en contra de la cisticercosis porcina experimentando previamente en el modelo murino. El extracto crudo de la proteína recombinante KETc7, procedente del cisticerco de *T. crassiceps*, demostró tener la capacidad de proteger en contra de la cisticercosis murina, sin embargo, hasta la fecha no se ha logrado obtener en forma pura. La inmunización génica ofrece la posibilidad de evaluar la capacidad protectora de esta proteína ya que las células del organismo vacunado la sintetizan de forma pura y con una conformación muy semejante a la proteína nativa. Con base en estos antecedentes, se planteron los siguientes objetivos:

1) Evaluar las posibilidades de la inmunización génica para inducir respuesta inmune y protección en contra de la cisticercosis experimental murina
2) Evaluar diferentes protocolos de inmunización con el fin de identificar los más sencillos y más eficientes en inducir una respuesta inmune protectora.
3) Detectar a la proteína nativa (KETc7) en los diferentes estadios de la *Taenia solium* y en cisticercos de *Taenia crassiceps*.
4) Iniciar los estudios para identificar la respuesta inmune asociada a la protección.
5) Comparar la eficiencia de los diferentes métodos de inmunización usados en el laboratorio: proteínas recombinantes, péptidos sintéticos y vacunación génica.

RESUMEN DE LOS RESULTADOS PRESENTADOS EN LOS ARTÍCULOS

La secuencia de KETc7, se utilizó para evaluar las posibilidades de la vacunación génica, que constituye el objetivo principal de este trabajo. El cDNA del KETc7 fue insertado en el plásmido pcDNA3 con y sin la secuencia del péptido señal del receptor del betaglicano también llamado el receptor tipo III del TGFβ, (López-Casillas *et al.*, 199) dando lugar a las construcciones pTc-7 o pTc-sp7 respectivamente. La capacidad de expresión de ambas construcciones se comprobó por inmunofluorescencia indirecta en la línea de células C33 transfectadas con ambos plásmidos. En un primer estudio se evaluaron ambas construcciones en dos diferentes dosis de vacunación (120 y 200 μg de DNA/ratón) inmunizando por vía intramuscular, así como con y sin bupivacaina.

Se obtuvo el mayor índice de seroconversión (85%) y de protección (58.63 % en hembras y 100% en machos) utilizando tres inmunizaciones de la construcción pTc-sp7 con 200 μg de DNA cada uno sin bupivacaina. Los anticuerpos inducidos por la construcción pTc-sp7 reaccionaron especificamene con una proteína de 56 kDa, previamente reportada que por medio de inmunofluorescencia indirecta fue localizada en el tegumento del cisticerco de *T. crassiceps*, así como en la oncosfera, cisticerco y tejido adulto de *T. solium*. Para confirmar que la construcción se mantenía extracromosomal en las células musculares se utilizó el buffer de Hirt para separar el DNA extracromosomal del cromosomal, se amplificó la secuencia correspondiente al KETc7 y se identificó por autorradiografía comprobando que 15 días después de la última imunización, el plásmido pTc-sp7 se mantenía extracromosomal (Rosas *et al.*, 1998).

Con el fin de optimizar la vacunación con 200μg de pTc-sp7por dosis, se evaluó su capacidad protectora con tres inmunizaciones intradérmicas con una y tres inmunizaciónes intramusculares. Los resultados indican que una ó tres inmunizaciónes intramusculares inducen niveles de protección similares.

El porcentaje más alto de protección se obtuvo inmunizando por la vía intradérmica (79% en hembras que son los individuos más siceptibles a esta parasitosis). La respuesta inmune asociada a la protección sugiere ser

mediada por células T CD8+CD4- aunque el mecanismo aún no se ha estudiado (Cruz-Revilla *et al.*, 1998).

En estos artículos se reportan las primeras evidencias de la capacidad de controlar una cestodiasis a través de inmunización génica.

A continuación se presenta el artículo publicado y el manuscrito enviado para revisión que abarcan los resultados de esta tesis

J. Parasitol., 84(3), 1998 p. 516–523
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TAENIA CRASSICEPS CYSTICERCOSIS: HUMORAL IMMUNE RESPONSE AND PROTECTION ELICITED BY DNA IMMUNIZATION

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ABSTRACT: The purpose of this study was to evaluate DNA vaccination in cysticercosis prevention by using a *Taenia crassiceps* cDNA of a recombinant antigen (KETc7) that has been reported as protective against murine cysticercosis. The KETc7 cDNA was cloned into the pcDNA3 plasmid alone or with the betaglycan signal peptide sequence (pTc-7 and pTc-sp7, respectively). Positive expression of the pTc-sp7 product was confirmed by transfection of C33 cells and immunofluorescence using sera of mice infected with *T. crassiceps*. Immunization of mice with 3 injections of pTc-sp7 DNA at the higher dose (200 μg) was the most effective to induce antibody with or without bupivacaine. Immunization with pTc-sp7 induced protection against challenge with *T. crassiceps* cysticerci as successfully as previously observed with the KETc7 recombinant protein. Antibodies elicited by DNA immunization with pTc-sp7 specifically reacted with the native protein of 56 kDa previously reported, which is immunolocalized in the tegument of *T. crassiceps* cysticerci. The 56-kDa antigen is also present in *Taenia solium* oncospheres, cysticerci, and adult tissue. The protection induced in DNA-immunized mice and the observation that the injected plasmid remains as an episomic form within muscle cells, encouraged us to continue testing this procedure to prevent *T. solium* cysticercosis.

Cysticercosis caused by *Taenia solium* is a parasitic disease that seriously affects human health. It is responsible for important economic losses in the Third World (Gemmell et al., 1985; Aluja and Vargas, 1988; Larralde et al., 1992). The essential role of pigs as obligatory intermediate hosts in the life cycle of *T. solium* offers the possibility of applying control measures by interfering with transmission in order to decrease the prevalence of pig cysticercosis. During recent years, experimental murine cysticercosis induced by *Taenia crassiceps* has been successfully used as a model to test promising antigens in the prevention of *T. solium* cysticercosis (Sciutto et al., 1990). Recently, a recombinant proline-rich protective peptide (KETc7) from *T. crassiceps* cysticerci shared by *T. solium* was identified to be of interest in the design of a vaccine against pig cysticercosis (Manoutcharian et al., 1996).

Nucleic acid vaccination has been actively developed in the past few years against a variety of infectious agents including viruses, bacteria (Ulmer et al., 1996; Lai et al., 1997), and, more recently, against unicellular parasites (Xu and Liew, 1994; Yang et al., 1995; Gardner et al., 1996). Much less is known about the potential of DNA vaccination to induce a protective immune response against multicellular parasites (Rothel et al., 1997), although the procedure seems to be promising. It needs, however, to be assessed in each case, considering that its applicability will depend on the nature of the host to be immunized and on the type of immune response it generates. Because DNA immunization represents a new and potentially powerful approach for the development of vaccines (Fynan et al., 1993; Ulmer et al., 1993; Robinson, 1995; Wang et al., 1995) we decided to explore this possibility to control cysticercosis. In the present work, we identified the optimal conditions to elicit a specific antibody response and protection against murine cysticercosis by DNA immunization using the KETc7 cDNA as immunogen. The immunolocalization of the KETc7 native protein was also determined in *T. solium* and *T. crassiceps*.

Received 5 December 1997; revised 4 March 1998; 4 March 1998.
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MATERIALS AND METHODS

Mice

BALB/cAnN mice, bred in our animal facilities by brother-sister mating, were used, 4–6-wk old.

Parasites

The ORF strain of *T. crassiceps* (Zeder, 1800) (Rudolphi 1810), isolated by Freeman (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 8 yr at our Institute. Parasites for infection were harvested from the peritoneal cavity of mice 1–3 mo after inoculation of 10 cysticerci per animal.

Plasmid

The pTc-7 was constructed by cloning the cDNA of the KETc7 (305 pb) recombinant antigen of *T. crassiceps* cysticerci previously reported (Manoutcharian et al., 1996) into the Bam H1 and XhoI restriction sites of the pcDNA3 plasmid (Fig. 1A) (Promega, Madison, Wisconsin) under the control of the CMV early promoter (Fig. 1B). The pTc-sp7 was constructed by adding in frame the sequence of the signal peptide of the betaglycan receptor, also known as the type III transforming growth factor (TGF)-β receptor (López-Casillas et al., 1991). The signal peptide was added at the 5′ end of the KETc7 sequence (Fig. 1C), generating the sequence sp7 of 422 bp. The pTc-sp7 construct was verified by DNA sequencing. Plasmids used in this study were isolated in Promega DNA columns (Promega) according to the manufacturer's instructions. DNA was quantified by spectrophotometry at 260 nm and the final concentration of the solution was adjusted to 0.9% NaCl and 1 μg/μl of DNA.

Cell transfection

pTc-7 and pTc-sp7 plasmids were transfected into C33 cells. For this, C33 cells grown in Dulbecco's modified Eagle medium (DMEM) with 10% fetal calf serum were passaged the day before transfection at a density of about 1.5×10^4 cells/cm^2. The cells were added on poly-L-lysine-treated coverslips. Calcium phosphate precipitates of the pcDNA3, pTc-7, and pTc-sp7 constructions were made by standard procedure (Chandler et al., 1983). After 16 hr, the culture medium was changed, and 48 hr later, cells were washed with phosphate-buffered saline (PBS) and fixed with acetone for indirect immunofluorescence.

Immunocytochemistry

Sera from noninfected mice and from mice infected with *T. crassiceps* for 3 mo were used as the source for the initial antibody; sera were diluted 1:500 in PBS, plus 0.01% bovine serum albumin (BSA), and added to C33 cells transfected with the plasmids. Bound antibody was developed by a fluorescein isothiocyanate-labeled conjugated goat anti-mouse IgG (FITC-F[ab]′2) (Zymed, Lab., San Francisco, Califor-

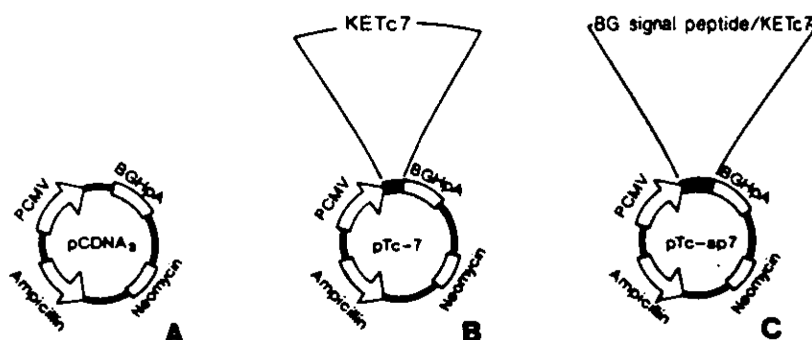

FIGURE 1. DNA constructs. Plasmids pTc-7 (B) and pTc-sp7 (C) derived from pcDNA3 (A) contain a eukaryotic cytomegalovirus immediate early gene (enhancer) and promoter (PCMV) driving the expression of the KETc-7 gene. Transcription is terminated by bovine growth hormone polyadenylation and termination signal (BGHp[A]). Ampicillin resistance gene a is also indicated. pTc-sp7 also contains the signal peptide sequence of betaglycan upstream to the KETc-7 sequence.

nia.) (1:1,000). Preparations were observed in an epifluorescent microscope Olympus BH2-RFCA.

Immunization protocol

Groups of 10 BALB/cAnN mice were injected with the pcDNA3, pTc-7, and pTc-sp7 plasmids into the quadriceps through the skin at 2 different doses (120 or 200 μg in 100 μl of saline solution) biweekly for a total of 3 inoculations. To enhance muscle cell uptake of plasmid DNA (Thomason and Booth, 1990), other groups of mice were injected into the same muscle with 100 μl of 0.5% bupivacaine hydrochloride (BPH) (Sigma Chemical Co., St. Louis, Missouri) in isotonic NaCl 24 hr before DNA injection.

Antibody response

Fifteen days after the last immunization before the infection, sera from immunized mice were collected and the antibody level was determined by enzyme-linked immunosorbent assay (ELISA) following the previously described procedure (Larralde et al., 1986). As a source of antigens, soluble *T. crassiceps* cysticerci antigens were used (Larralde et al., 1989). Briefly, Nunc-Immuno plates (NUNC Brand Products, Copenhagen, Denmark) were treated with 10 μg of *T. crassiceps* antigen per well diluted in carbonate buffer, pH 9.6, and incubated 1 hr at 37 C. Plates were washed 3 times for 5 min with 200 μl/well of 0.15 M saline solution containing 0.05% v/v Tween 20 and blocked with 200 μl PBS containing 1% w/v BSA and 0.1% v/v Tween 20 for 60 min at room temperature before washing again. Serum samples diluted 1:50 in PBS and 0.1% Tween-20 were added and incubated for 1 hr at 37 C, followed by the addition of anti-mouse IgG coupled to alkaline phosphatase (Sigma), which was developed with *p*-nitrophenyl phosphate (Sigma). Plates were washed 3 times after each reaction step. Optical density readings at 405 nm were carried out in a Humareader ELISA processor (Human Gessellschaft für Biochemica und Diagnostica, Taunusstein, Germany).

Mice infection

Fifteen days after the last immunization, control and mice immunized either with pcDNA3 or pTc-sp7 were injected i.p. with 10 small (2 mm in diameter), nonbudding *T. crassiceps* larvae suspended in PBS. Thirty days after infection, mice were killed and *T. crassiceps* cysts within the peritoneal cavity were counted.

Immunoelectrotransfer blot (EITB)

Electrophoresis and immunoblotting using *T. crassiceps* and *T. solium* antigens were performed as described elsewhere (Larralde et al., 1989). Sera from nonimmunized and immunized mice were diluted 1:20 in 0.05% PBS-Tween plus 1% v/v BSA and 5% v/v light milk. As a positive control, sera from *T. crassiceps*-infected mice were used. Bound antibodies were detected by incubating each strip in streptavidin-biotinylated peroxidase conjugate (Amersham Laboratories, Buckinghamshire, U.K.) diluted 1:2,000 for 30 min at room temperature. As a substrate, 0.5 mg/ml *o*-chloronaphthol with 3% v/v H_2O_2 in PBS, pH 7.3/CH_3OH (5:1) was used.

Immunolocalization of KETc7 protein

Host proteins joined to *T. crassiceps* and *T. solium* cysticerci were removed. For this process, cysticerci were placed on ice-cold PBS and the vesicular fluid was removed. Then, cysticerci were incubated with glycine buffer (50 mM glycine-HCl, pH 2.5; 0.1% Triton X-100; 0.15 mM NaCl) for 30 second, and pH was restored using Tris-HCl, pH 9. Cysticerci were extensively washed with cold PBS, embedded in Optimun-Cutting-Temperature Compound (O.C.T. Compound, Miles, Inc., California), and frozen at −70 C. Similar procedures were used to prepare eggs and adult *T. solium* tissues. Six-micrometer sections were cut with a cryostat, placed on poly-L-lysine-treated microslides, air-dried for 30 min, fixed in acetone for 10 min, and air-dried for 15 min at room temperature. Slides were rehydrated and blocked using 1% BSA in PBS plus 0.1% Triton X-100, pH 7.2 for 1 hr. For cysticerci slides, a second blocking was performed with sheep anti-mouse IgG whole antibody (Amersham) diluted 1:100 in PBS plus 0.1% BSA and incubated 1 hr at 4 C. Slides of adults and eggs were incubated 1 hr at 4 C with horse serum diluted 1:100 in PBS plus 0.1% BSA as a second blocking. Solutions were removed and the slides were incubated with the appropriate sera diluted at 1:10,000 in PBS and 0.1% BSA, from noninfected (negative control), infected (positive control), or pTc-sp7 immunized mice, overnight at 4 C. Slides were rinsed with PBS and then overlaid with FITC-F(ab)'2 goat anti-mouse IgG (Zymed, San Francisco, California) diluted 1:50 for 1 hr in darkness at room temperature. Slides were washed and mounted with Aquatek, polyvinyl alcohol (Merck, Darmstadt, Germany). Preparations were analyzed in an epifluorescence microscope Olympus BH2-RFCA. Slides were prepared in triplicate in a blind protocol and the fluorescence patterns were reproducible.

Detection of plasmid DNA in the muscle

Quadriceps were separated from mice and carefully minced with scissors. Muscle pieces were incubated in DMEM medium in the presence of DNAse I (100 μg/ml) for 1 hr at 37 C. After the enzymatic treatment, the suspension was washed once with PBS and incubated with trypsin (0.25%) for 30 min at 37 C. Recovered cells were washed with PBS resuspended in Hirt buffer (Hirt, 1967) and incubated for 20 min at room temperature. NaCl to a final concentration of 1 M was added to the suspension and incubated for 8 hr at 4 C. After centrifugation at 14,000 rpm (1 hr, 4 C), the supernatant (containing episomal DNA) and the pellet (genomic DNA) were phenol/$CHCl_3$ extracted and precipitated at −20 C for DNA detection by polymerase chain reaction (PCR) analysis. DNA was amplified by PCR using as sense primer: 5'GAATTCGGCACGAGCATTTATGCAGCCGCAT3' and 3'CATAAGATTCTTCTTATCTTCTGGTTCCAT5' as the antisense. These oligonucleotides correspond to bases 1–30 of the 5'end and 276–305 of the 3' end of the KETc7 sequence, and therefore, they must amplify a fragment of 305 bp in the intact pTc-sp7 plasmid. To label the PCR reac-

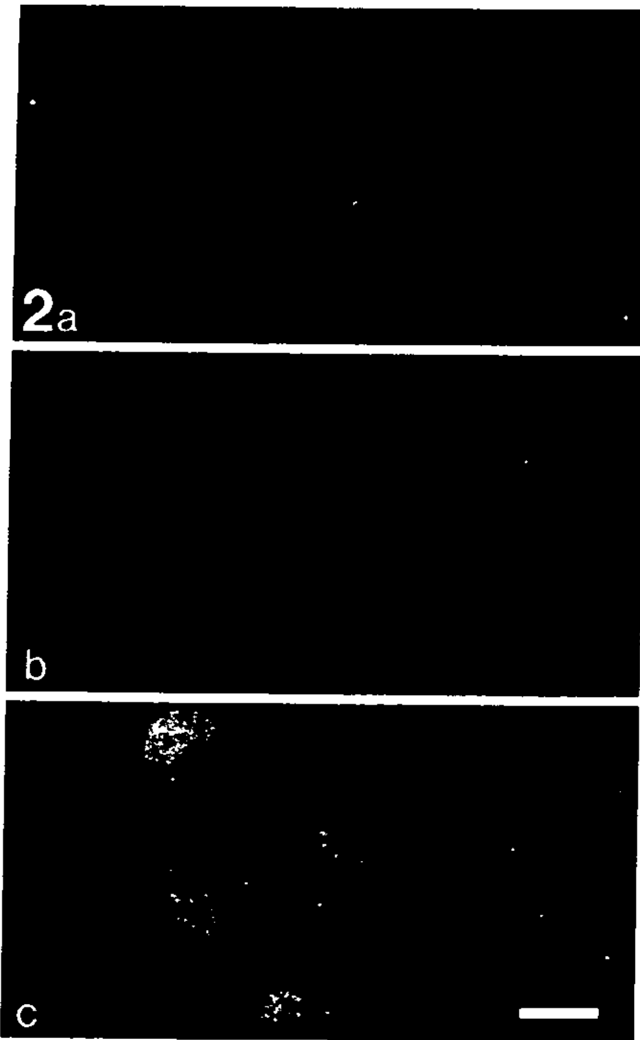

FIGURE 2. Analysis of KETc7 expression by immunofluorescent staining. C33 cells transfected with 10 μg of pCDNA3 (a), pTc-7 (b), and pTc-sp7 (c) were stained 48 hr after transfection by indirect immunofluorescence. Sera from *Taenia crassiceps*-infected mice were used as a primary antibody. Bar = 40 μm.

tions, 30 cycles of 94 C for 1 min, 57 C for 2 min, and 72 C for 30 sec were performed in the presence of [^{32}P]dCTP (Amersham). The radioactive PCR product was resolved on a 5% polyacrylamide gel and exposed to autoradiography for 48 hr at −70 C using Kodak film.

Statistical analysis

Statistical comparisons between groups were carried out by the Kruskal–Wallis and Dunn's multiple comparisons test.

RESULTS

Expression of the KETc7 protein in C33 cells

Cells were transfected with pcDNA3, pTc-7, and pTc-sp7 plasmids, and expression of KETc7 was determined by indirect immunofluorescence using sera of mice infected with *T. crassiceps*. Clear differences were observed between pTc-7- and pTc-sp7-transfected cells. The former showed small granules, randomly distributed, throughout the cytoplasm (Fig. 2b) and the latter showed a higher level of immunofluorescence, granular and diffuse, probably including the nuclear envelope and the plasma membrane (Fig. 2c). Fluorescence was not observed in cells transfected with the plasmid pcDNA3 (Fig. 2a) or in cells transfected with constructions and developed with sera from noninfected mice (data not shown).

Antibody response induced by DNA immunization

To identify the optimal conditions for the induction of immunity, levels of antibodies produced by DNA inoculation were determined by ELISA. Sera from control mice inoculated with 100 μl of saline or different concentrations of pcDNA3 in presence or absence of BPH did not show reactivity against cysticercal antigens. Mice injected with either pTc-7 or pTc-sp7 plasmids developed specific antibodies, although the response differed. In 5 of 7 mice, increased levels of antibodies were generated when injected with the plasmid pTc-sp7 at the higher concentration (200 μg), as well as in 4 out of 7 with the lower concentration (120 μg) in the presence or absence of BPH. In contrast, a weak antibody response was observed in mice inoculated with pTc-7 when BPH was included (Fig. 3).

The specificity of the antibody response was determined by western blot. Sera from mice immunized with the pTc-sp7 plasmid strongly recognized a protein of 56 kDa of *T. crassiceps* cysticerci (Manoutcharian et al., 1996), which corresponded to the expected molecular weight of the KETc7 protein band from the *T. solium* fraction that was also detected (Fig. 4).

Immunolocalization of KETc7 protein

Specific antibodies induced by DNA immunization with pTc-sp7 were employed to locate the native protein in *T. crassiceps* and *T. solium* cysticerci and in *T. solium* oncospheres and tissue. Sera from DNA immunized mice were obtained 15 days after the last of 3 injections of 200 μg of DNA from the orbital sinus. Control sera were obtained from the same mice before DNA immunization. In *T. crassiceps* cysticerci, immunofluorescence was restricted to the tegument (T) (Fig. 5e) and in *T. solium* cysticerci to the vestibular wall and the tegument of the fold of the spiral canal (Fig. 5f). Interestingly, the KETc7 antigen was intensively detected in the oncosphere of the egg (Fig. 6e) and in the tegument of the *T. solium* adult (Fig. 6f). When sera from infected mice were used, all structures were fluorescent (Figs. 5c–d, 6c–d); in contrast, only low fluorescence was observed with sera from non-treated mice (Figs. 5a, b, 6a, b).

Mice protection after DNA immunization

The protective effect induced by DNA immunization was determined by counting the number of parasites after challenge. As Table 1 shows, the female mice immunized with pTc-sp7 carried a significantly reduced number of cysticerci (32.6 ± 17.1) compared to control mice immunized only with saline (78.8 ± 19.7). Male mice immunized with pTc-sp7 were completely protected, whereas in only 2 out of 5 pcDNA3 immunized mice no cysticerci were found.

Plasmid localization after immunization

Due to the health regulations, we examined the localization of plasmid DNA in the muscle cells 2 wk after the last immunization. For this purpose, we prepared genomic and episomal DNA from the injected muscles of the immunized mice

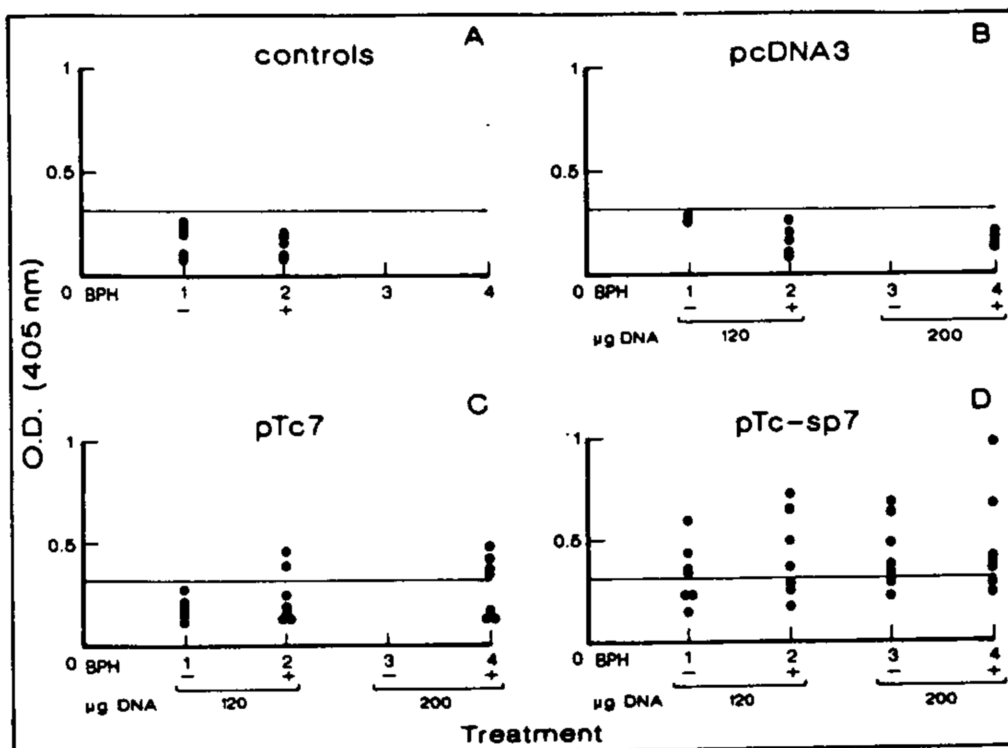

FIGURE 3. Individual antibody response induced after DNA inoculation. IgG anti-cysticercal antibodies were measured by the ELISA method using *Taenia crassiceps* total antigens. Each point represents the optical density (OD) detected in each mouse. A serum sample was considered to be positive when its ELISA OD reading exceeded the mean negative control value + 2 SDs. Control mice were intramuscularly injected with saline solution. BPH^- (1A) or BPH^+ (2A). pcDNA3 (B), pTc-7 (C), and pTc-sp7 (D) show the antibody response of individual mice immunized with 120 µg of DNA, BPH^- (1), or BPH^+ (2) and with 200 µg of DNA, BPH^- (3), or BPH^+ (4).

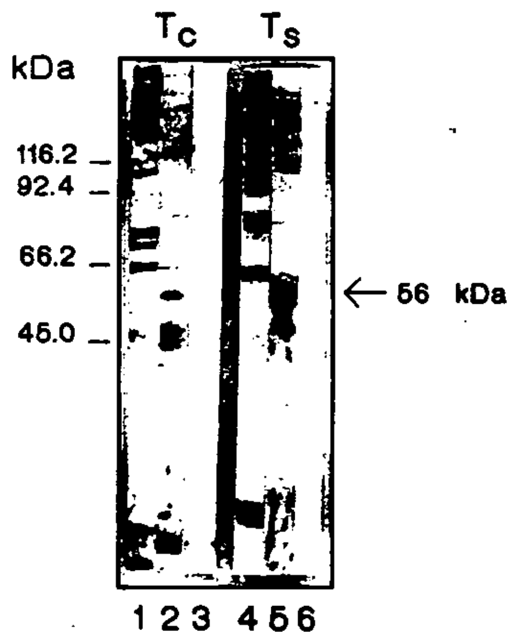

FIGURE 4. Identification of a native protein of 56 kDa in the total extract from *Taenia crassiceps* (Tc) and *Taenia solium* (Ts) cysticerci by western blot. Strips were developed with pool sera from infected mice (1, 4), pool sera from the highest OD pTc-sp7 immunized mice (2, 5), and pool sera from nontreated mice (3, 6). The arrow shows the band of interest.

and used it to detect the plasmid sequence by PCR procedures. The plasmid DNA was found mainly as an episomic form in all animals injected; no plasmid was detected in the genomic fraction after PCR analysis (Fig. 7). These results show that the procedure used with this plasmid is able to stimulate the immune system.

DISCUSSION

In the present study, we demonstrate the induction of a specific antibody response and protection induced by DNA immunization with a plasmid carrying the cDNA coding for the previously reported KETc-7 protective antigen against *T. crassiceps* murine cysticercosis as previously reported (Manoutcharian et al., 1996). Comparative studies reported herein show that, although it is possible to obtain a degree of detectable antibody response by administration of pTc-7, a successful response was obtained when the signal peptide of a cell surface betaglycan receptor (López-Casillas et al., 1991) was added to the construction (pTc-sp7) (Fig. 3). The increased antibody response, as well as the higher number of seroconverted mice, could be in accordance with the increased expression observed when cells were transfected in vitro with pTc-sp7 versus the expression observed when they were transfected with pTc-7 (Fig. 2). In fact, cells transfected with the construction pTc-7 were lightly fluorescent with small granules distributed randomly throughout the cytoplasm (Fig. 2b), whereas the signal peptide sequence not only modified the pattern of distribution of the peptide but also effectively increased its level of expression

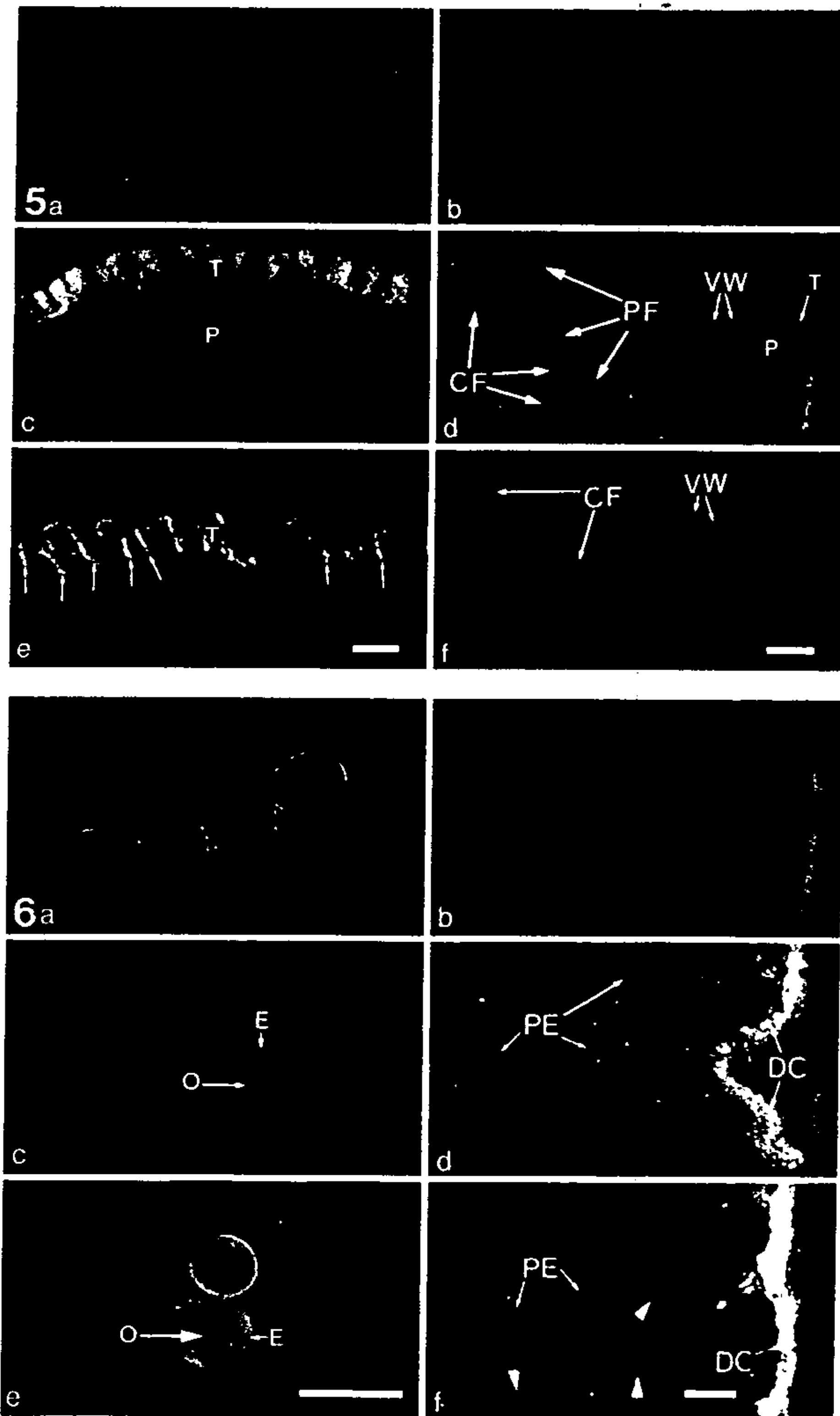

FIGURE 5. Immunofluorescence staining of *Taenia crassiceps* (a, c, e) and *Taenia solium* (b, d, f) cysticerci. The sections were incubated with sera from nontreated mice (a, b), *Taenia crassiceps*-infected mice (c, d), and pTc-sp7-immunized mice (e, f). *Taenia crassiceps* tegument (T) shows a protruding and intensively positive wall surface and the parenchyma (P) is evident (c). In *T. solium* (d) the T, P, vestibular wall (VW), parenchymal folds (PF), and cuticular folds of the spiral canal (CF) are evident. In 5e, only T was positive, particularly the grooves located between the protuberances (arrows). In 5f, only VW and CF were stained positively. Bar = 40 μm.

FIGURE 6. Immunofluorescence of eggs (a, c, e) and adult tegument (b, d, f) of *Taenia solium*. The sections were stained with sera from nontreated (a, b), from *T. crassiceps*-infected (c, d), and pTc-sp7-immunized mice (e, f). In 6c, the oncosphere (O) is intensively stained and in 6d the distal cytoplasm (DC) and some structures of the perinuclear cytoplasm region, like the protoplasmic extensions (PE) of the tegumental cells are positive. In 6e and 6f, O, PE and DC are more fluorescent than the positive control (c, d). In 6f, PE contain structures that are probably the rhabdiform organelles (arrowhead). Bar = 40 μm.

TABLE I. Protective response induced against *Taenia crassiceps* cysticercosis by DNA immunization.

	Females		Males	
Treatment*	Number of parasites (mean ± SD)	% Protection	Number of parasites (mean ± SD)	% Protection
Saline solution	78.8 ± 19.7	—	6.14 ± 1.55	—
pcDNA3	52.5 ± 14.3	33.37	4.0 ± 4.85	34.4
pTc-sp7	32.6 ± 17.1	58.63	0	100

* Eight male and female mice per group were intramuscularly injected 3 times with 200 μg of DNA per mouse or with isotonic saline solution. Fifteen days after the last immunization, mice were infected with 10 cysticerci each. Cysticerci were counted after 30 days of infection. The number of cysticerci found in female mice vaccinated with pTc-sp7 was statistically lower ($P < 0.001$) than in those who received isotonic saline solution (ISS). No significant differences were observed between ISS and pcDNA3 and between pcDNA3 and pTc-sp7. Although no significant differences were observed between control and pcDNA3-immunized male mice, in 2 out of 5 pcDNA3-immunized mice no cysticerci were found. All pTc-sp7-immunized male mice were completely protected.

(Fig. 2c). The pTc-sp7 construct should direct the expression of a secretory protein; thus, the immunofluorescence pattern may be due to molecules in transit. Interestingly, the immunization with pTc-sp7 not only increased the number of seroconverted mice, but its effect was also independent of a previous inoculation with BPH (Fig. 3). Bupivacaine has been reported to improve DNA uptake by cells (Thomason and Booth. 1990) during the degeneration and regeneration of muscle fibers (Davis et al., 1995). In addition, it produces rapid destruction of striated muscle fibers followed by phagocytosis (Benoit and Belt, 1970). It is probable that the effective expression of the KETc-7 protein by the use of the pTc-sp7 construction bypassed the requirement of BPH in the treated mice. In contrast, in the case of mice immunized with pTc-7, seroconversions were obtained only when mice were pretreated with the BPH. This result points to the relevance of the increased inflammatory response induced by BPH that allows the antigen be detected by the immune system.

Figure 3 shows that the level of antibodies generated in this study was highly variable among animals and was relatively low in most mice. This finding is in accordance with another report in which only 1 of 15 sheep injected with pcDNA3-45W produced serum antibody to a level that would be considered

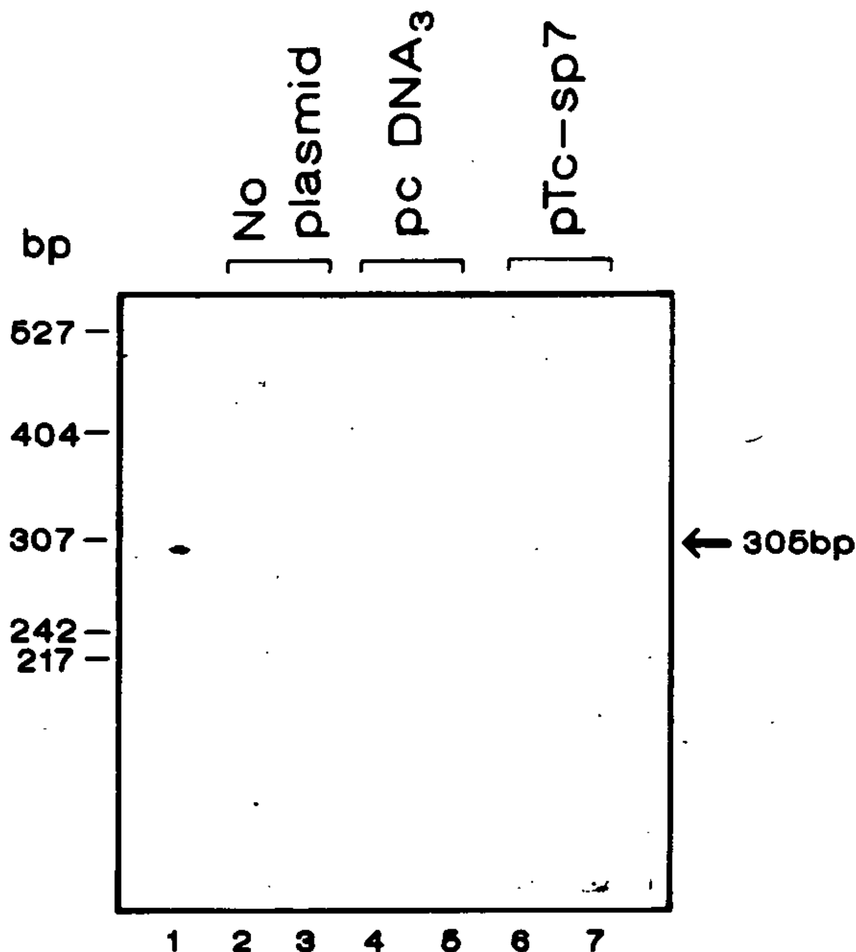

FIGURE 7. Plasmid detection within muscle cells by PCR: plasmid pTc-sp7(lane 1), chromosomal DNA from untreated, pcDNA3 and pTc-sp7 immunized mice (lane 2, 4 and 6, respectively), and extrachromosomal DNA from untreated, pcDNA3, and pTc-sp7-immunized mice (lanes 3, 5, and 7, respectively). Chromosomal and extrachromosomal fractions were isolated by using the Hirt method.

protective (Rothel et al., 1997). However, whereas in *Taenia ovis* cysticercosis, protection correlates with serum antibody levels, the antibody response in *T. crassiceps* cysticercosis induced by vaccination is not closely related to protection (Sciutto et al., 1995). In fact, no correlation was found between the antibody response and the individual protection observed (data not shown). Herein, we show a significant level of protection induced by the immunization with pTc-sp7 (58.6% in females and 94.7% in males). Although the mechanism underlying this protection is not yet known, we are now studying the cellular immune response related to this protection. Interestingly, the immunization with pcDNA3 also reduced by almost 34% the number of expected parasites. Similar effects have been previously observed by Lin et al. (1998), probably due to a nonspecific adjuvant effect of the plasmid DNA (Lathe et al., 1987; Roman et al., 1997). In fact, it has been observed that pcDNA3 has short immunostimulatory DNA sequences that contain CpG dinucleotides flanked by 5′ purines and 2, 3′ pyrimidines found in the ampicillin resistance gene, which induce B, T, and natural killer cells to secrete cytokines that favor a polyclonal immune response (Klinman et al., 1996; Sato et al 1996).

In a previous study, we reported that the immunization with KETc7 recombinant antigen induced a similar level of protection (51.75%) in the murine model of cysticercosis to that found here (58.6%) by DNA immunization. Thus, the results reported in the present study indicate that DNA immunization is at least as efficient as the conventional procedures using KETc7 protein to immunize (Manoutcharian et al., 1996).

The specificity of antibodies induced by pTc-sp7 immunization was tested by immunoelectrotransference. As Figure 4 shows, antibodies recognized a 56-kDa *T. crassiceps* native antigen as previously reported (Manoutcharian et al., 1996) and an antigen of similar molecular weight in *T. solium*. Considering that antibodies specifically detect the expected antigen, they were used to immunolocalize the native antigen in both parasites. Several points merit comment regarding the localization of KETc7 antigen in the parasite: First, antibodies recognized a major antigen component present in *T. crassiceps* cysticerci. Second, and most important, is the strong presence of the antigen in the oncosphere because these are thought to be most susceptible to immune attack (Rajasekariah et al., 1982). Although it has been reported that several antigens are stage specific (Harrison and Parkhouse, 1986), KETc7 antigen is not the case. Interestingly, we found that this protein is highly conserved in the different stages of *T. solium*. Its presence in the cysticerci also speaks in favor of its availability to the immune system, especially in the early metacestode stage.

Finally, the protection induced by DNA immunization using pTc-sp7 and the presence of the plasmid in the nucleoplasm, rather than integrated into the host-cell genome (Fig. 6), gives us some optimism as to the possible use of this construction for vaccine purposes, although improvements are needed before progressing to test the system in swine.

ACKNOWLEDGMENTS

The authors thank, Miriam Guido, Martha Ustarroz, Diana Millan, Ma. Luisa Rodriguez, Gerardo Arrellin, Georgina Díaz Herrera, and Nelly Villalobos for technical support, and Karen Manoutcharian and Marisela Hernández for the pTc-7 construction. Isabel Pérez Montfort corrected the English version of the manuscript. This study was supported by grant IN 208395 from the Dirección General de Asuntos de Personal Académico, Universidad Nacional Autónoma de México, CONACyT, México, The European Union INCO-DC STD3 Programme, and Programa Universitario de Investigación en Salud (PUIS).

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Taenia crassiceps cysticercosis: protective effect and immune response elicited by DNA immunization

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ABSTRACT: The sequence of a protective recombinant antigen of *Taenia crassiceps* cysticerci present at all stages of *Taenia solium*, namely KETc7 cloned into pcDNA3 plasmid with the signal peptide sequence of the betaglycan receptor (pTc-sp7) has been reported as useful in DNA immunization. To explore the possibilities of this form of immunization, mice were injected intramuscularly with one or three doses of pTc-sp7 or with three intradermal doses (200 μg per mice). Intramuscular pTc-sp7 immunized mice developed similar levels of resistance, and higher levels of protection were observed with intradermal immunization. Three immunizations with either intramuscular or intradermal pcDNA3, significantly reduced the expected parasite load (355) while no effect was observed with one immunization. The evaluation of the induced immune response, indicates the relevance of the cellular response in the observed protection. Spleen or lymph node cells from DNA immunized mice induced a specific T-cell response to *T. crassiceps* antigens and to a synthetic peptide from the immunogen itself. Proliferated cells were enriched in $CD8^+$ $CD4^-$ T-lymphocytes. These results provide encouraging information on the possible use of KETc7 in the immunoprophylaxis of cysticercosis as well as a first insight into the characterization of the immune response induced by pTc-sp7.

Indexing terms: *Taenia solium*, *Taenia crassiceps*, cysticercosis, DNA immunization, pcDNA3, protective immunity, vaccination.

Taenia solium cysticercosis is still an important socio-economic problem in non-developing countries in Latin America, Asia and Africa, where poverty and lack of hygiene prevail and favor transmission. In humans, the more severe form of the disease is neurocysticercosis (Sotelo et al., 1996). In developed countries, its frequency is extremely low, but due to immigrants from endemic areas in Mexico and Central American countries an increase has been reported in the United States (Loo and Braude, 1982; McCormick et al., 1985; Grisolia and Wiederholt, 1982; Richards and Schantz,1991).

In order to interrupt the life cycle of the parasite by preventing pigs from acquiring the larval stage, vaccination studies have been initiated (Molinari et al., 1983, 1993; Nascimento et al., 1995; Sciutto et al., 1995; Manoutcharian, 1996). Our research group identified a set of recombinant antigens in a *Taenia crassiceps* cDNA library shared by *T. solium* (Manoutcharian et al., 1996). Four of them induced high levels of protection in an experimental murine model of cysticercosis caused by *T. crassiceps,* which has been successfully used to identify promising antigens in the prevention of *T. solium* cysticercosis (Sciutto et al., 1990; 1995). Recent reports have demonstrated the effectiveness of nucleic acid immunization that results in the production of the protein or peptide in a target tissue of the host. Encouraging results have been obtained using this vaccine approach, specially against unicellular parasites (Connell et al., 1993; Mor et al., 1995; Doolan et al., 1998). However, DNA vaccines may also be effective against multicellular parasites, although this possibility has been less explored (Yang et al., 1995; Rothel et al., 1997). In cysticercosis, we recently reported encouraging data in DNA vaccination using the KETc7 sequence, one of the protective recombinant antigens previously identified (Manoutcharian et al., 1996). Using the sequence which codes for this antigen, we prepared an immunogenic KETc7

cDNA construction (pTc-sp7). This construction induced a similar level of protection by DNA immunization as that obtained with the respective recombinant protein (Rosas et al., 1998). In addition, pTc-sp7 immunization elicited a specific antibody response that let us detect the native protein in the external tegument of *T. crassiceps* and *T. solium*. Interestingly, the antibodies also recognized the KETc7 antigen in the tapeworm and oncosphere stages (Rosas et al., 1998). The present work was carried out to continue the study of DNA immunization in cysticercosis prevention using the murine model of cysticercosis and to identify the type of immune response elicited by this form of immunization.

MATERIALS AND METHODS

Mice

A syngenic BALB/cAnN strain of mice, previously characterized as susceptible to murine cysticercosis (Fragoso et al., 1998), was used. Original stocks were purchased from Jackson Laboratory, Bar Harbor, Maine, and then bred and kept in our animal facilities by the "single-line systems" for twenty generations. All mice were 4-6 wk-old at the start of the experiments. All experiments were carried out according to the principles set forth in the Guide for Care and Use of Laboratory Animals, Institute of Laboratory Animals Resources, National Research Council, Washington, D.C.

Parasites

The ORF strain of *T. crassiceps* cysticerci (Zeder, 1800) (Rudolphi 1810), isolated by Freeman (Freeman, 1962) and supplied by Dr. B. Enders, 1984 (Behringwerke, Marburg, Germany), has been maintained by serial intraperitoneal (i.p.) passage in BALB/cAnN female mice for 10 yr at

our Institute. Parasites for infection were harvested from the peritoneal cavity of mice 1 to 3 mo after inoculation of 10 small (2-3 mm in diameter), non-budding cysticerci per mouse.

Plasmid

The expression vectors used in this study have been previously described (Rosas et al., 1988). Briefly, the pTc-sp7 was constructed by cloning the cDNA of the KETc7 (305 pb) recombinant antigen of *T. crassiceps* cysticerci previously reported (Manoutcharian et al., 1996) into the Bam H1 and Xho1 restriction sites of the pcDNA3 plasmid (Promega, Madison, Wisconsin) under the control of the CMV early promoter. The pTc-sp7 was constructed by adding in frame the sequence of the signal peptide of the betaglycan receptor, also known as the type III TGF-β receptor (López-Casillas et al., 1991). The signal peptide was added at the 5' end of the KETc7 sequence, generating the sequence sp7 of 422 pb. The pTc-sp7 construct was verified by DNA sequencing. Plasmids were isolated in Qiagen plasmid purification columns (Qiagen Inc. Santa Clarita, California), the solutions used were prepared in our laboratory, according to the manufacturer's instructions. DNA was quantified by spectrophotometry at 260 nm and the final concentration of the solution was adjusted to 0.9% NaCl (SS) and 1 μg/μl of DNA. DNA quality was determined by electrophoresis on 1.5 % agarose gels. The expression of KETc7 was confirmed by indirect immunofluorescence in C33 pTc-sp7 transfected cells (Rosas et al., 1998).

Immunization protocol

To determine the protective capacity induced by immunization with the pTc-sp7 construction, groups of 10 BALB/cAnN mice were injected with the pcDNA3 or pTc-sp7 into the quadriceps muscles through the skin or intradermically using a 27-gauge needle. Each construct was injected intramuscularly (100 μg) into an individual quadriceps muscle or intradermically into the ventral

area (200 μg) of each mouse three times at 2-wk intervals. To increase the possibilities of the participation of the ganglionic chain, intradermal immunization was performed by the inoculation of DNA at six different sites in the ventral region of mice. As control, 10 mice were injected intramuscularly into each quadriceps with 100 μl of SS or intradermically injected at six sites in the ventral area with 200 μl of SS, three times at 2-wk intervals. To determine the requirement of a booster in the intramuscular immunization, groups of 9 to 10 mice were immunized only with one dose.

Antibody response

Sample serum from immunized mice were collected individually before the infection and stored at -70 C until individually tested for the presence of specific antibodies. Antibody levels were determined by ELISA following the previously described procedure (Larralde et al., 1986). As a source of antigens, soluble *T. crassiceps* cysticerci antigens were used (Larralde et al., 1989). Briefly, 96-well flat-bottom microtitration plates (NUNC Brand Products, Copenhagen, Denmark) were coated with 1 μg of *T. crassiceps* antigen per well diluted in carbonate buffer, pH 9.6, and incubated overnight at 4 C. Plates were washed 3 times for 5 min with phosphate-buffered saline (PBS) containing 0.1% v/v Tween 20 (PBS-T) and blocked with 200 μl of PBS containing 1% w/v BSA (blocking buffer) at 37 C for 1 hr before washing again with PBS-T. Serum samples were used at 1:100 dilution in blocking buffer as noted above, and 100 μl per well were incubated for 1 hr at 37 C and added in duplicate to the well. Plates were washed three times for 5 min with PBS-T. Bound mouse Igs were detected using the alkaline phosphatase-conjugated anti-mouse IgG (whole molecule, Sigma Chemical Co., St. Louis, Missouri) diluted 1:1000 in blocking buffer for 1 hr at 37 C. The substrate used was detected using *p*-nitrophenyl phosphate (Sigma) in

diethanolamine buffer for 10 min at room temperature. The reaction was stopped with 2N NaOH. Optical density readings at 405 nm (A_{405}) were carried out in a Humareader ELISA processor (Human Gesellschaft für Biochemica und Diagnostica, Taunusstein, Germany).

Proliferation assay

Spleen or lymph node cells from control and intramuscularly or intradermally immunized mice either with pcDNA3 or pTc-sp7 were cultured in RPMI 1640 medium (GIBCO, Gaithersburg, Md) supplemented with L-glutamine (0.2 mM), nonessential amino acids (0.01 mM), penicillin (100U/ml), streptomycin (100μg/ml) and FBS 10%. Cells were cultured at a concentration of $2x10^5$ cells per 200 μl of final volume and incubated with Con A (5 μg/ml) or *T. crassiceps* total antigens (10 μg/ml), obtained as previously described (Sciutto et al., 1990). An immunogenic synthetic peptide from the KETc7 sequence namely GK-1 (Gevorkian et al., 1996), was also used at 10 μg/ml and incubated at 37 C in a 5% CO_2 humidified atmosphere in flat-bottom microtiter plates. Ten thousand peritoneal cells recovered from the same mice were added to each well at a volume of 50 μl. Peritoneal cells were obtained by ex vivo lavage with 5 ml of RPMI-1640. The cells were sedimented by centrifugation at 800 g for 10 min. The pellets were resuspended in an additional 3 ml of supplemented RPMI medium thus adjusting the volume to contain $2x10^5$ cells/ml. After 72 hr, the cultured cells were pulsed (1 μCi per well) for a further 18 hr with (Methyl ^{3}H thymidine) (Amersham, Life Science, U.K.). Then, all cells were harvested and the amount of incorporated label was measured by counting in a 1205 β-plate spectrometer (Wallac). All assays were performed in triplicate in three individual mice per group.

Flow cytometry

After 72 hr of in vitro culture with mitogen, whole antigens or peptide, splenocytes were harvested and CD8, CD4 expression was determined by two color stainings with FITC-conjugated anti-CD8 (Pharmingen, San Diego, California), phycoerytherin (PE)-labeled anti-CD4 (Pharmingen). CD3 cells were determined by a single color using a phycoerytherin (PE)-labeled anti-CD3 (Pharmingen), conducted according to the protocol previously described (Fragoso et al., 1998). Briefly, cells were washed with PBS plus 10 % of FBS γ-globulin-depleted and 0.02% NaN_3 and incubated with the indicated antibodies at 4 C, 30 min. After washing, splenocytes were resuspended in 3% v/v formaldehyde in isotonic solution and analyzed by FACScan (Becton Dickinson, San Jose, California). Results were expressed as percent of positive cells.

Mouse infection

Eight weeks after the first immunization, control and mice immunized either with pcDNA3 or pTc-sp7 were infected with 10 small non-budding cysticerci (2 mm in diameter) suspended in PBS as previously reported (Fragoso et al., 1998). Thirty days after infection, mice were killed and the cysts found in the peritoneal cavity were counted.

Statistical analysis

The effect of DNA immunization on the parasite load in challenged mice was analyzed statistically using the non-parametric Mann-Whitney U Test, considering that in the immunized groups many mice bore zero parasites, and parasite density is a discontinuous variable (i.e. 0,1,2,.....n parasites). The statistical significance of the difference between mean values of binding activity in ELISA , proliferative response and flow cytometry was carried out by the unpaired T Welch's test (Alternate T test). Both statistical analyses were performed by the Instat Software Program (GraphPad, San Diego, California). Differences were considered significant when the two-tailed P

values of <0.05 were obtained in both statistical evaluations, confidence limits at 95 % significance for the difference between the means.

RESULTS

Antibody responses induced by DNA immunization

Figure 1 shows the level of antibodies induced by intradermal immunization with 200 μg of pTc-sp7. Antibodies that recognized total *T. crassiceps* antigens by ELISA were detected in 5 of the 9 pTc-sp7 immunized mice (62%) while no antibodies were detected in control or pcDNA3 immunized mice. Surprisingly, no seroconvertion was observed in mice immunized intramuscularly one or three times with 200 μg of all tested plasmid purified with the Qiagen columns (data not shown). The same plasmid DNA was used both for intramuscular and intradermal immunization.

Proliferative response

Figure 2 shows the level of proliferation obtained when spleen or lymph node cells from non-immunized and immunized mice injected intramuscularly or intradermically, were stimulated in vitro. A significant level of proliferation was induced by whole antigens from *T. crassiceps* or GK-1, in mice immunized either intradermically or intramuscularly, while no proliferation was observed in those injected with SS or pcDNA3.

Cytometry Analysis

Figure 3 shows the percent composition of proliferated cells determined by FACS analysis. A clear increase in $CD3^+$ cells was observed in pTc-sp7 intramuscularly immunized mice when either whole antigen or GK-1 were used for in vitro stimulation. A clear and significant increase in $CD8^+$

cells was detected ($P<0.01$), and to lesser level, a significant increase in the $CD4^+$ population was also observed.

Protective effect against *T. crassiceps* cysticercosis induced by different number of DNA immunizations

The effect of one or three DNA immunizations upon the number of cysticerci recovered from mice thirty days after infection is shown in Table I. One or three pTc-sp7 intramuscular immunizations significantly reduced the expected parasite load to a similar extent (52 % and 63 % respectively). However, with three intradermal or intramuscular immunizations, the pcDNA3 by itself reduced the parasite load about 35 % while no protective effect was observed when only one intramuscular immunization was administered. pTc-sp7 intradermically immunized mice induced a higher level of protection than that obtained under similar conditions by intramuscular immunization (80% vs 63%, respectively).

DISCUSSION

The KETc7 peptide has been considered a possible candidate antigen for a vaccine against *T. solium* cysticercosis (Manoutcharian et al., 1996). It is detected in the external tegument of the *T. solium* cysticercus and of the tapeworm and it is also expressed in the onchosphere stage (Rosas et al., 1998). In previous work, we evaluated DNA immunization using this KETc7 sequence. Best results were obtained when KETc7 was added to the type III TGF-β receptor signal peptide sequence (pTc-sp7). Thereafter, this construction was tested under different conditions to maximize the effectiveness of this form of immunization and to analyze the immune response induced.

In the present study, we confirmed the protective capacity of pTc-sp7 DNA immunization. In addition, almost identical levels of protection were displayed in mice immunized with a single (52%) or three (63%) intramuscular injections. These results are agree with those observed by Davis et al., (1995) who reported that a single injection of DNA induced a rapid, strong and sustained humoral and cell-mediated immune response against the hepatitis B virus. It is of interest that between 34 and 36 % of protection was induced by intramuscular or intradermal immunization with the empty cDNA3. This result confirms the previously observed protective effect, probably mediated by a non-specific polyclonal immune response, induced by short immunostimulatory DNA sequences that contain CpG dinucleotide in a particular base context, flanked by two 5' purines and two 3' pyrimidines (Klinman et al., 1996, 1997; Roman et al., 1997; Kline et al., 1998; Krieg et al., 1998) included in the cytomegalovirus promoter as well as in the ampicillin resistance gene. Interestingly, only one dose of pcDNA3 did not modify the expected parasite load (1%), allowing a specific evaluation of the relatively protective capacity induced by the protein coded by pTc-sp7 (52%).

Results obtained here let us propose that a single individual dose of DNA is a much simpler alternative to test promising candidates for a vaccine showing the trouble of producing in vitro the recombinant proteins. This is of special interest because in our experience, serious limitations have been encountered to express *T. crassiceps* genes in prokaryotes expressions systems.

Table I also shows higher levels of protection induced by intradermal immunization (80%) than by the intramuscular (63%) route as reported by other authors (Raz et al., 1994; Davis et al., 1995). Although this is encouraging information, increased protection obtained by intradermal

immunization must be considered with reserve, taking into account the variations between experimental sessions which is a common finding in this form of cysticercosis (Sciutto et al., 1990).

In previous work, we obtained almost 85% of seroconversion (Rosas et al., 1998) while in the present study we did not detect antibodies induced by intramuscularly immunized mice with pTc-sp7 (Data not shown) In contrast, 62% of seroconversion were observed in mice immunized with the same batch of DNA intradermically (Fig. 1). It should be noted that, in this work, DNA was purified by Qiagen plasmid purification columns which differs from the previous study in which the Promega kit was used. This probably leads to differences in the conformational state of the purified DNA (relative amount of superhelicoidal DNA). However, since the presence (Rosas et al., 1998) or absence of antibodies reported in this study did not affect the level of protection (58.6% and 63%, respectively); the antibody response does not seem to be transcendental for parasite growth control, as suggested elsewhere (Bojalil et al., 1993). In addition, variations in effectivity to induce an antibody response using DNA of different quality obtained by diverse procedures could explain some conflicting results that have been reported with respect to the antibodies elicited by DNA immunization (Yang et al., 1995; Waine et al., 1997). Furthermore, our results show that intradermal immunization is a more effective alternative to produce antibodies, as has been also reported by other authors (Boyle et al., 1997; Gramzinski et al., 1998).

These results suggest an important participation of mostly cell-dependent mechanisms in conferred immunity. Thus, the cellular immune response induced by both intramuscular and intradermal injection was explored. Although the cells used for proliferation stemmed from

different lymphoid organs (spleen for intramuscular and lymph node for intradermal immunization), results were very similar (Fig. 2). Albeit the percentage of $CD4^+$ cells was increased in the proliferated T-cells from intramuscularly immunized mice, the percentage of $CD8^+$ cells was rather high (Fig. 3). It is probable that this $CD8^+$ response could be a consequence of the features of KETc7, more than the form of immunization, specifically considering that immunization with the GK-1 epitope stemming from this antigen induces a very similar immune response (Andrea Toledo, pers. comm). We are now attempting to determine which cytokines are involved in this cell-mediated response induced by DNA immunization.

One concern on the advantages of nucleic acid vaccines in terms of public health is the amount of high quality DNA required for immunization which implies high costs. However, the possible solution of DNA delivery by particle bombardment (Fynan et al., 1993), should be thoroughly examined, particularly considering recent reports that clearly show that a different quality of immune response is elicited by DNA injection or by particle bombardment with DNA (Feltquate et al., 1997). It is also possible that fused antigens used to increase the delivery to APCs cells can reduce the DNA required to induce an effective immune response (Boyle et al., 1998). In short, further research is needed to procure a more simple and less costly vaccine. Finally, results obtained in this study encourage us to use the KETc7 antigen in the design of a multivalent vaccine, and establish conditions for DNA immunization that offer a simple and very advantageous alternative to test additional candidates for the design of this vaccine.

ACKNOWLEDGMENTS

The authors thank, Gerardo Arrellin, Georgina Díaz Herrera and Marisela Hernández for technical assistance. Isabel Pérez Montfort corrected the English version of the manuscript. This work was supported by grant IN 208395 and IN 212798 from the Dirección General de Asuntos de Personal Académico, Universidad Nacional Autónoma de México, CONACyT (grant G25955m), México and a grant INCO-DC STD3 Programme from The European Union.

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FIGURE LEGENDS

Figure 1. Individual antibody response in control and pcDNA3 or pTc-sp7 intradermically immunized mice determined by ELISA. The cut-off values were obtained from two SD plus the mean optical density (OD) reading of the control serum sample of each mouse. The bars represent mean OD for each experimental group injected.

Figure 2. T-cell proliferative response of lymph node or spleen cells from mice injected intradermically (A) or intramuscularly (B) respectively, were determined by [^{3}H] thymidine incorporation on day 3 of culture and incubated with *Taenia crassiceps* antigens or GK-1 (10 μg/ml) or with Con A (5 μg/ml) as a positive control. The data show the mean ± SD of three individual mice. Differences were significant when $P<0.05$ (*).

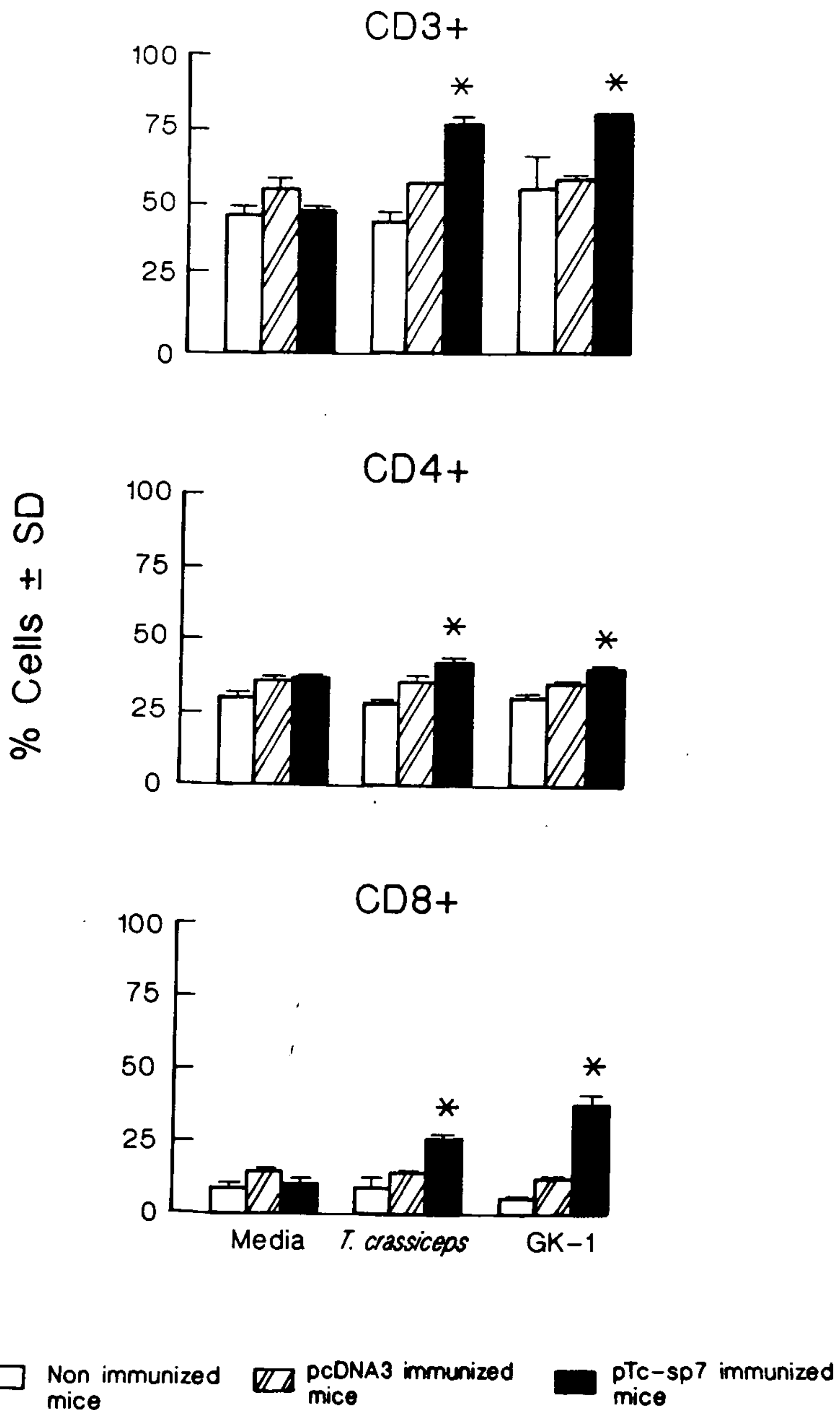
Figure 3. Flow cytometer analyses were performed on spleen cells from control and immunized mice injected intramuscularly either with pcDNA3 or pTc-sp7. After 3 days of in vitro culture with *Taenia crassiceps* antigens or GK-1 (10 μg/ml) or with Con A (5 μg/ml) as positive control, spleen cells were harvested and CD8, CD4 and CD3 expression was determined. Each bar represents the mean ± SD of positive cells obtained from three individual mice. Differences were significant when $P<0.05$ (*).

Table I. Protective effect induced against *Taenia crassiceps* murine cysticercosis by intramuscular and intradermal DNA immunization.

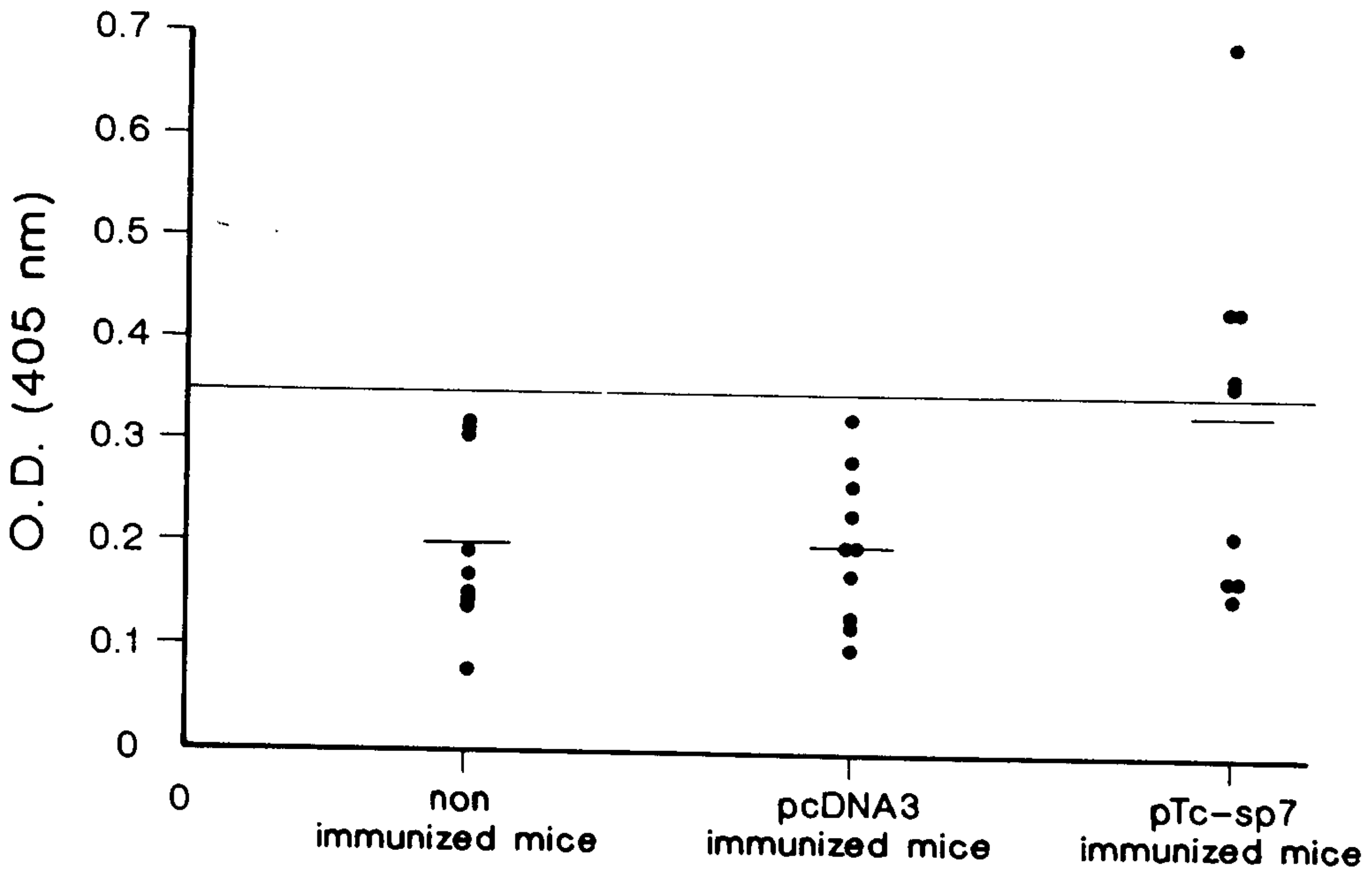
	Intramuscular	Intradermal
Controls	79.7 ± 21.1[a*]	44.3 ± 26.4[e*]
Immunized with one dose of:		
pcDNA3	78.9 ± 44.4[a b] (1%)†	ND‡
pTc-sp7	37.1 ± 20.2[c d] (52%)	ND
Immunized with three doses of:		
pcDNA3	52.3±15.4[b c] (34%)	28.2 ± 25.6[e f] (36%)
pTc-sp7	29.1 ± 15.1[d] (63%)	9.2 ± 5.1[f] (80%)

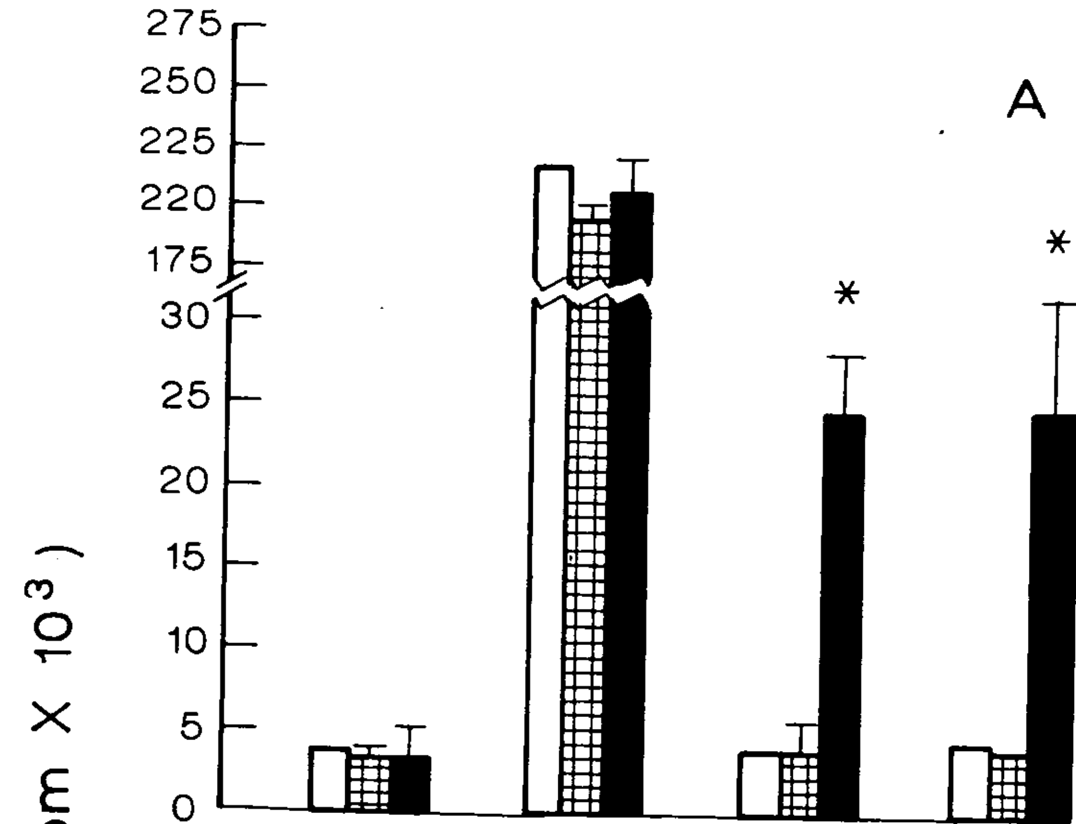
*Mean ± SD of the parasite load in each group. †Percent of protection in each group. Statistic comparisons were done between intramuscularly [(abcd)] or intradermally[(ef)] immunized mice. Data labeled with the same letter are not significantly (NS) different from each other, whereas those with different letters are significantly different using the non-parametric Mann-Whitney U Test ($P<0.05$).

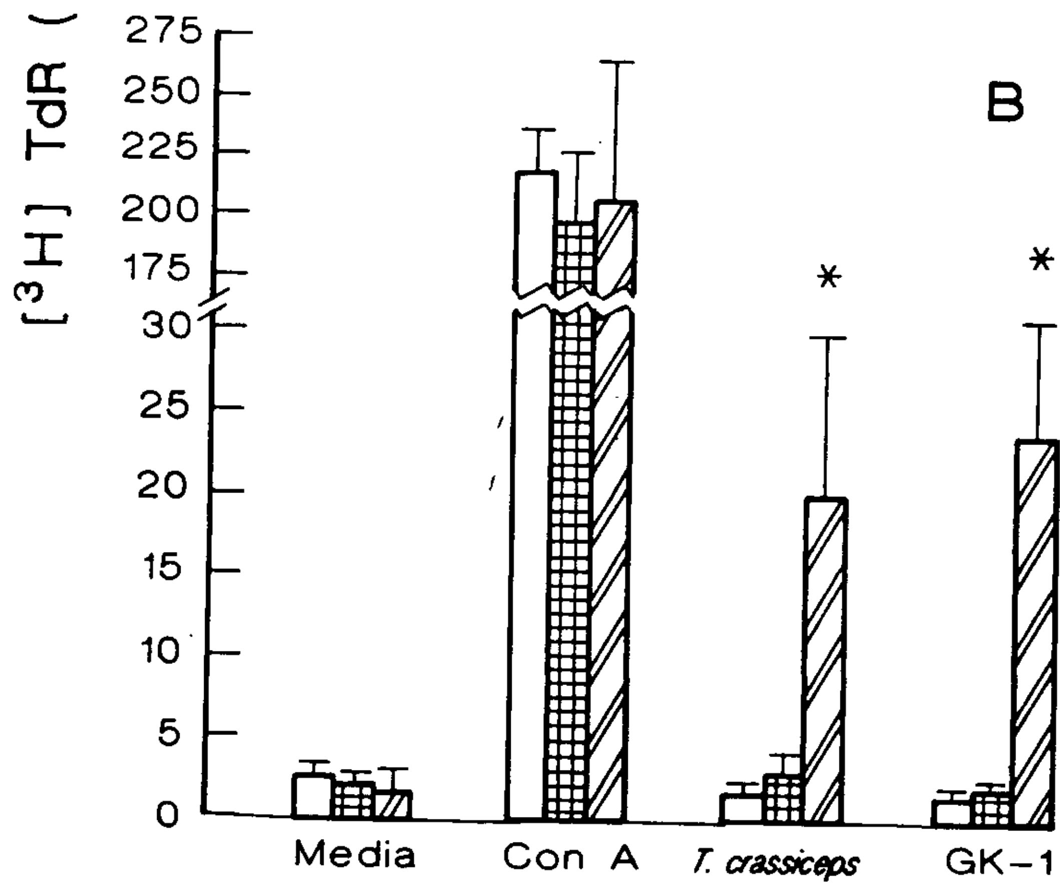
‡ND; Not determined.



CD3+
CD4+
CD8+
% Cells ± SD
100
75
50
25
0
*
Media
T. crassiceps
GK-1
Non immunized mice
pcDNA3 immunized mice
pTc-sp7 immunized mice


O.D. (405 nm)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
non
immunized mice
pcDNA3
immunized mice
pTc-sp7
immunized mice





non immunized mice pcDNA3 pTc-sp7 intramuscularly pTc-sp7 intradermically

DISCUSION

En este trabajo de tesis se reportan los resultados de protección en contra de la cisticercosis experimental murina a través de diferentes formas de vacunación, entre las que se incluyen: proteínas recombinantes (Manoutcharian *et al.*,1996), péptidos sintéticos (Toledo *et al.*, 1998) y dado que cada uno de los trabajos tiene su propia discusión, en la presente sección se analizarán bajo un punto de vista general y comparativo de los resultados de protección y respuesta inmune reportados en dichos artículos.

Se observó que dependiendo del protocolo de inmunización utilizado se pueden inducir diferentes niveles de protección, utilizando a la proteína KETc7 ó algunos de sus epítopes antigénicos.

En el apéndice IV se encuentra el artículo de Manoutharian *et al.*, 1996, en el que se identificaron 5 antígenos recombinantes, KETc1, KETc4, KETc7, KETc11 y KETc12. De los cuales el antígeno KETc11 resultó ser irrelevante en la protección, mientras que los 4 restantes tuviero la capacidad de inducir protección en contra de la cisticercosis murina al emplearse como lisados crudos. Como controles de este experimento se emplearon lisados crudos de bacterias que contenían solo el vector de expresión (fago λ). De las proteínas recombinantes, la primera secuencia que se determinó fue la de KETc7 (Apéndice 1).

La eficiencia de la vacunación utilizando la proteína recombinante KETc 7 se reflejó en su capacidad de desminuir la carga parasitaria de los ratones inmunizados a un 30.5% en hembras y a un 73% en machos, aunque no se logró eliminar completamente la carga parasitaria en los individuos inmunizados.

El conocer la secuencia que codifica para la proteína de 100 aminoácidos, permitió desarrollar dos diferentes estrategias de inmunización:

a) Por medio de péptidos sintéticos (GK1, GK2 y GK3), que fueron elaborados a partir de los probables epítopes inmunogénicos (Gevorkian *et al.*, 1996) y sintetizados quimicamente en forma lineal (Toledo *et al.*, 1998) (Apéndice IV).

b) Por inmunización génica (con las construcciónes pTc-sp7 y pTc-7), en la cual el organismo inmunizado sintetiza la proteína que inducirá la respuesta inmune (Rosas *et al.*,1998, Cruz-Revilla *et al.*, 1998).

A través de ambos procedimientos se ha podido avanzar en el conocimiento de los mecanismos inmunológicos asociados a la protección inducida por la proteína. Cabe aclarar que estos últimos procesos resultaron ser altamente efectivos en contra de la cisticercosis murina, especialmente en machos en los que en ambos casos se logró elevar el índice de protección a 91.3% en animales inmunizados con el péptido GK1, y del 100% por in unmunización génica utilizando la construcción pTc-sp7 (tabla 4). La inmunización con el péptido GK1 se realizó por vía subcutanea utilizando dos dosis, mientras que con el DNA la inmunización se realizó por vía intramuscular con tre dosis. Es importante mencionar que en experimentos posteriores confirmamos que niveles similares de protección pueden observarse con una sola inmunización utilizando la construcción pTc-sp7 por vía intramuscular, lo cual destaca el interés de este procedimiento de vacunación, en especial cuando la vacuna está destinada a animales a los que no es fácil ponerles varios refuerzos.

En general, la protección en contra de esta parasitosis fue más efectiva en machos que en hembras, lo cual sugiere que por medio de la vacunación se puede aumentar la resistencia de los individuos que son naturalmente más suceptibles (Sciutto *et al.*, 1991). Es importante destacar que la inmunización génica por vía intradérmica logró inducir el mayor porcentaje de protección en hembras, 79%. (condiciones de máxima susceptibilidad) comparado con todos los otros protocolos empleados (Ver tabla 4). Si bien la inmunización se realizó con tres refuerzos por vía intradérmica es probable que con una sola dosis se pueda alcanzar un nivel similar de protección considerando los resultados obtenidos por la vía intramuscular.

La eficiencia de la protección demostrada por la inmunización génica se puede deber a que en este sistema las proteínas se sintetizan con la conformación adecuada. En este sentido, se ha observado que utilizando genes que codifican para proteínas cama la luciferasa, cuya función es altamente dependiete de su conformación, e independientemente de que su origen sea de un insecto como la luciernaga, al ser sintetizada por las células

musculares del ratón, la enzima tiene la capacidad de actuar sobre su sustrato natural igual que la producida por la luciernaga misma (Hartikka *et al.*, 1996).

Resultados semejantes se han observado al utilizar plásmidos portadores de la secuencias que codifican para las enzimas cloranfenicol acetil transferasa y β galactosidasa (Wolff *et al.*, 1990, Jiao *et al.*, 1992). Cabe mencionar que algunos autores han sugerido que la expresión de antígenos parasitarios en células de mamíferos podrían conducir a una modificación estructural, como serían diferentes patrones de glicosilación, lo que podría influir adversamente en la inmunogenicidad de las proteínas y en su capacidad de inducir una respueta inmune protectora (Griffiths, 1995). Sin embargo, en la mayor parte de los estudios de vacunación génica reportados, no se ha observado que la expresión de las proteínas en células de eucariontes pierda la capacidad de inducir una respuesta inmune (Robinson and Torres, 1997, Gardner *et al.*, 1996, Rothel *et al.*, 1997, Waine *et al.*, 1997). Con base en estas observaciones se puede asumir que si la secuencia de la proteína vacunal es de origen eucarionte y es sintetizada por las células musculares del ratón, es probable que pueda presentar una estructura conformacional muy semejante a la proteína nativa, y por lo tanto, inducir una respuesta inmune tan eficaz como la inducida por la proteína nativa del patógeno (Ulmer *et al* 1993, Wang *et al.*, 1993).

El contar con los péptidos sintéticos, permitió avanzar en el conocimiento de la respuesta inmune asociada a la protección inducida por la proteína KETc7. De manera interesante se observó que tanto la inmunización con GK1 como con pTc.sp7 lograron protección esterilizante en un alto porcentaje de los ratones machos, y en ambos casos los linfocitos CD8+CD4- fueron los más abundantes en ensayos de proliferación con antígeno total ó con péptido GK1. Sin embargo, la inmunización con GK1 incrementó en un 15% las células CD8+CD4- con ambos antígenos, respecto a los controles, mientras que en los ratones inmunizados con pTc-sp7 el incremento fue 26.9% en las células estimuladas con antígeno total y 38.4% en las estimulados con GK1. Esto podría indicar que la proteína KETc7 producida por las células del ratón pudiesen presentar otros epítopes inmunógenicos, que justifiquen la mayor proliferación de las células CD8+CD4- con antígeno total procedentes

de ratones inmunizados con pTc-sp7 que con GK1 y además, que inmunizando con pTc-sp7 se activan más células CD8+CD4- que con GK1 que tienen la capacidad de reconocer a GK1. Estos resultados en conjunto indican que el péptido GK1 constituye uno de los epítopes mas inmunogénicos de la proteína, aunque debe considerarse que aún falta estudiar de manera más profunda otros posibles epítopes de KETc7.

Las similitudes en la respuesta inmune inducida por el peptido GK1 y por inmunización con DNA nos permiten suponer que esta última también **involucra** interleucinas inflamatorias (T1). Esta capacidad de que las células CD8+ produzcan IFN γ en estados de activación, también ha sido reportado por otros autores (Maraskovsky *et al.*, 1992; Di fabio *et al.*, 1994; Morvan *et al.*, 1995; Noble *et al.*, 1995; Sad *et al.*, 1995; Engwerda *et al.*, 1996; Mosman *et al.*, 1997). Se ha observado que el IFN γ está muy relacionado con el proceso de activación de macrófagos (Wynn *et al.*, 1994) así como el TNF α que al actuar sobre el macrófago lo inducen a producir la enzima NO sintetasa, la cual al realizar la deiminación oxidativa de 1-arginina a 1-citrulina produce el NO (Oswald y James 1996) con lo que es capaz de aniquilar, en cultivo, a parásitos como *Brugia malayi* y *Oncocerca linealis* (Taylor *et al.*, 1996), así como larvas de *Schistosoma mansoni* en ratones vacunados y con una respuesta inducida por la vacuna de tipo T1 (Fouad *et al.*, 1997). Se han reportado otras moléculas producidas por el macrófago inducidas por una respuesta T1, que también inducen daño y muerte de los parásitos mencionados como el H_2O_2 y el peroxinitrito (Capron, 1982; Taylor *et al.*, 1996; Thomas *et al.*, 1997; Ahmed *et al.*, 1997). Resulta entonces razonable proponer que la protección inducida por la vacunación es una posible consecuencia de una respuesta inmune inflamatoria producida por las interleucinas como el IFN γ secretadas por los linfocitos CD8+.

Al respecto del posible papel de la proteína KETc-7 en el parásito, estudios preliminares realizados en el laboratorio en los que se analizó la secuencia de aminoácidos de la proteína, sugieren que podría ser considerada como un miembro de la familia de las extensinas por la similitud que existe con ellas. Con base en las funciones reportadas para las extensinas en otros organismos se podría pensar que estas proteínas participan en el desarrollo y

mecanismos de defensa del parásito. Así, cuando se induce una respuesta anti-KETc7 por vacunación, es factible que se logre inhibir el crecimiento del parásito así como su capacidad de defenderse del sistema inmune del hospedero (Uribe, 1998).

La inmunización con GK1 y con el pTc-sp7 indujeron niveles bajos de anticuerpos en contra del antígeno total del cisticerco de *Taenia crassiceps*. Sin embargo, estos anticuerpos nos permitieron localizar a la proteína nativa en el cisticerco de *Taenia crassiceps* así como en huevo, cisticerco y adulto de *Taenia solium* por medio de inmunofluorescencia. Al ser GK1 parte de KETc7 ambos sueros reconocieron las mismas estructuras. Se encontró que esta proteína se presenta en todos los estadios del parásito así como en regiones muy expuestas, por lo que se puede pensar que se encuentran muy accesibles al sistema inmune. Es interesante notar que la inmunización con pTc-sp7 al igual que con la proteína KETc7 recombinante, indujeron anticuerpos capaces de reconocer proteínas en el rango de 56 kDa por Western Blot, lo que no fue observado con los anticuerpos procedentes de ratones inmunizados con GK1 (datos no mostrados) a pesar de que sí reconocieron la proteína nativa por inmunofluorescencia. Es probable que estos anticuerpos reconozcan un epítope conformacional que se pierde durante el proceso de preparación de la muestra del antígeno total de *T. crassiceps* para su corrimiento electroforético y/o transferencia.

Una de las características que ha sido reportadas para la inmunización génica es que tiene la capacidad de inducir tanto respuesta inmune humoral como celular (Donnelli *et al.*, 1995; Pertmer *et al.*, 1995; Boyle *et al.*, 1997), lo cual pudo ser observado en los dos trabajos de inmunización génica presentados en esta tesis. Resulta interesante que en el primer manuscrito (Rosas *et al.*, 1998) en que se informo la mejor construcción, la mejor dosis y la capacidad de protección entre otras cosas se utilizaron kits de promega para la purificación del DNA. En estas condiciones sí se pudieron detecar ratones seroconvertidos. Sin embargo, en el segundo trabajo (Cruz-Revilla *et al.*, 1998), utilizando las mismas condiciones de inmunización pero con DNA purificado por columnas de Qia-Gen y con reactivos producidos en el laboratorio, no se detectaron anticuerpos en los ratones inmunizados intramuscularmente. Estas diferencias probablemente se deben a que en el

segundo caso la forma y las condiciones físicas del DNA inyectado no fueron las mismas que en el primero, lo cual pudiera repercutir en la calidad y cantidad del DNA que puede expresarse en las células productoras de la proteína KETc-7.

Es interesante hacer notar el efecto que indujo la inmunización con el vector solo (pcDNA3) tanto en la protección como en la proliferación celular. Se observó que cuando se inmunizaba 3 veces con éste vector, se inducía alrededor del 35% de protección en todos los casos. Por otro lado, el porcentaje de linfocitos CD3+, CD4+CD8- y CD4-CD8+ proliferados es muy semejante sin importar las condiciones en que se pusieran a proliferar a las células. En el caso de las células CD3+, aproximadamente constituyen alrededor del 60%, las CD4+CD8- el 34% y las CD4-CD8+ el 15%. En estos resultados se refleja lo que ha sido reportado por otros autores, al respecto de las propiedades inmunogénicas de las secuencias CpG presentes en el plásmido pCDNA3, que tienen la capacidad de inducir una respuesta policlonal inespecífica (Klinman *et al.*, 1996). Sin embargo, en los ratones inmunizados con el pTc-sp7 hay una marcada diferencia entre el fenotipo de las células incubadas en medio controles y las células incubadas con antígeno total, o con GK1, (Tabla 5), (Fig 2 Cruz-Revilla *et al.*, 1998). Así, aún cuando el fenotipo de las poblaciones celulares proliferadas fue primordialmente del tipo CD8+CD4-, hubo un ligero incremento de células CD4+CD8- en los cultivos estimulados con antígeno (Tabla 5), (Fig2 Cruz-Revilla *et al.*, 1998). Las células CD4+CD8- pudiesen ser de tipo inflamatorio considerando la baja producción de anticuerpos obtenidos en la inmunización intramuscular , observaciones que han sido reportadas por otros autores (Tighe *et al.*, 1998)

Cuando se inmunizó sólo una vez con pcDNA3 su efecto fué irrelevante con respecto a la protección, a diferencia de la inmunización con el pTc-sp7 con la que se lograron niveles muy semejantes de protección a los inducidos con tres inmunizaciones intramusculares.

La inmunización por la vía intradérmica demostró ser más eficiente en protección que la intramuscular (63 vs 79%), y además fue capaz de inducir un 60% de ratones seroconvertidos y niveles similares de proliferación en cultivo comparado con la inmunización intramuscular y utilizando el DNA obtenido con las columnas de Qia-gen. Es probable que esto se deba a que la transfección

de las células *in vivo* por la vía intradérmica sea más efectiva, al igual que del sistema inmune relacionado con piel.

Un aspecto que preocupa con respecto a la seguridad de este tipo de inmunización, es la probabilidad de que el plásmido se pueda integrar al genoma de las células hospederas. Sin embargo, se ha demostrado que la probabilidad de daño al genoma de las células hospederas por la integración del plásmido es menor que la probabilidad normal de mutación de las células, lo cual en la naturaleza es muy baja (Warren, 1995). Al respecto de esta situación, y siguiendo metodologías muy semejantes a las reportadas por el mismo autor, en nuestro trabajo (Rosas *et al.*, 1998) detectamos que al tiempo en que se sacrificaron los ratones, el plásmido no se había integrado al genoma de las células musculares transfectadas.

Con base en lo que se ha discutido, es interesante considerar las posibilidades de la inmunización génica como un método viable de ser aplicado en trabajos prácticos, aunque actualmente es más costosa que otras formas de inmunización. Sin embargo, una de la principales ventajas que ofrece esta forma de inmunización es que se puede inducir respuesta inmune por más de 19 meses, con posibilidades de ser aún más prolongada (Wolff *et al.*, 1992). Creemos que este procedimiento será factible de ser aplicado en los próximos años, cuando se hayan optimizado los métodos de inmunización que permitan el uso de bajas cantidades de DNA reduciendo así los costos del mismo. Los conocimientos establecidos en este trabajo aunados a la identificación de otros antígenos resultarán críticos para el diseño de estrategias para el control de esta parasitosis.

PERSPECTIVAS

Al ser este el primer trabajo de inmunización génica en el que se induce respuesta inmune y protección en contra de un parásito tan complejo como el cisticerco *de T. crassiceps*, el campo de trabajo futuro es muy promisorio, ya que aún quedan por explorar múltiples aspectos relacionados con la protección y la respuesta inmune asociada a ella. Considerando que estos esfuerzos van encaminados a poder aplicar esta vacuna en cerdos, es necesario mejorar tanto los protocolos de inmunización como las construcciones, con la intención de que con menor cantidad de DNA se logren alcanzar, cuando menos, los mismos niveles de protección aquí reportados, lo que bajaría los costos de la vacuna. Con respecto a los protocolos se podría explorar más profundamente la vía que resultó ser más eficiente, que fue la intradérmica, para lo que se podría usar la "pistola de genes". Para las nuevas construcciones se podría pensar en aquellas que permitan la expresión simultánea de la proteína KETc7 con interleucina 2, 6 ó IFNγ, así como construcciones que permitan dirigir a la proteína hacia las células presentadoras de antígeno lo que se podría conseguir fusionando la proteína KETc7 con la proteína CTLA-4 por ejemplo.

Con respecto a la respuesta inmune generada por este tipo de vacunación, queda aún por conocer cuáles son las células que se transfectan por las diferentes vías de inmunización, así como cuáles son las células presentadoras y cuáles son la respondedoras.

El hecho de contar con la biblioteca de cDNA del cisticerco también nos permitirá explorar las posibilidades de otras secuencias que aún no han sido probadas para inducir protección. Al ser la tenia o su estado larvario, el cisticerco, un parásito tan complejo, entre más elementos se tengan para inducir una respuesta inmune protectora, será más fácil poder llegar al control de esta parasitosis.

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G AAT TCG GCA CGA GCA TTT **ATG** CAG CCG CAT CCT TCC TTC TCT CCA

N S A R A F M Q P H P S F S P

CCG CCA GTT GAT TAT CTG TAC CAG ACA AAC TCT CCA CCG CCC TAT

P P V D Y L Y Q T N S P P P Y

GGG GGC GCT GTC CCT CCA CCT TAC GCG CCG AAT CCA GGT CCA CCG

G G A V P P P Y A P N P G P P

CCG CCA TAC ACG GGT GCG GCA AGT TCG ATG CCG CCT TAT CCG ACC

P P Y T G A A S S M P P Y P T

GGT GGT CCG CCA CCC GTC AAC ACC GGT TAT TAC TAT CCA TCT GAT

G G P P P V N T G Y Y Y P S D

CCA AAT ACC TTC TAC GCT CCA CCC TAC AGC CAG GCT TCA GCA CCT

P N T F Y A P P Y S Q A S A P

CCT ATG GAA CCA GAA GAT AAG AAG AAT CTT TAG

P M E P E D K K N L

FIGURA 3. Características de la secuencia de nucleótidos que codifican para la proteina KETc7

Presenta:

- *G AAT TCG:* Secuencia reconocida por *Eco* RI
- **ATG (AUG):** codón de iniciación de la transcripción (metionina)
- No tiene secuencia de reconocimiento para las enzimas de restricción *Bam*HI (GGATCC) Está constituida por 304 nucleótidos

APENDICE I

PROTEÍNA KETc7

La proteína KETc7 surgió de una biblioteca de cDNA del cisticerco de *T. crassiceps*, cuya secuencia es de 305 pb y representa el segmento 3´del gene nativo (Manoutcharian *et al.*, 1996) (Fig. 3). La secuencia de esta proteína fue determinada con base al marco de lectura que codificó para la proteína más grande. Es una proteína formada por 100 aa y es parte de la proteína nativa de 56 Kda (Fig. 4).

Esta proteína posee un 30% de prolinas, y al hacer un alineamiento múltiple de KETc-7 con otras proteinas también ricas en prolinas se detectó que había de un 35-45% de similitud con otras proteínas ricas en glicina, prolina e hidroxiprolina. Comparte dos motivos con las extensinas: (Ser- Pro_{3-7} , XP_{2-7} donde X puede representar cualquier aminoácido). Estas extensinas se localizan en las paredes celulares de algunos vegetales, pero también ha sido localizada en el huevo de *Squistosoma japonicum* y *Aematobium* (Bobek *et al.*, 1989, 1991). Estas proteínas se han relacionado con mecanismos de resistencia a patógenos dada la estructura celular en los vegetales así como con la expansión de su pared celular (McNeil *et al.*, 1984) , sin embargo su función en parásitos aún no es clara.

El perfil antigénico de esta proteína se analizó por el método de Jemeson and Wolff (1988) el cual predijo regiones de alta antigenicidad para células B de potencial interés (Tabla 6). De ellas se seleccionaron las denominadas GK1, GK2, y GK3 (Gervorkian, 1996).

Estos epítopes fueron sintetizados químicamente, y se confirmó su capacidad antigénica por ser reconocidos por sueros de individuos cisticercosos. El GK3 resultó de interés para el diagnóstico de cisticercosis en humanos, detectando anticuerpos tanto en suero como en líquido cefalorraquídeo. Pero solo el GK1 tuvo la capacidad de inducir del 70% al 90% de protección en ratones, llegando en algunos casos a reducir la carga parasitaria a cero (Toledo *et al.*, 1998).

Al realizar pruebas de ELISA utilizando suero de conejo inmunizado con los péptidos GK1 y GK2 se pudo detectar que probablemente estos epítopes estén compartido por varios parásitos, como *Giardia lamblia, Entamoeba histolítica*, al

<u>GAAT TCG</u> GCA CGA GCA TTT **ATG** CAG CCG CAT CCT TCC TTC TCT CCA

N° S° A R^+ A F M Q° P H^+ P S° F **S°** **P**

CCG CCA GTT GAT TAT CTG TAC CAG ACA AAC TCT CCA CCG CCC TAT

P **P** V D^- Y° L Y° Q° T° N° **S°** **P** **P** **P** Y°

GGG GGC GCT GTC CCT CCA CCT TAC GCG CCG AAT CCA GGT CCA CCG

G° G° A **V** **P** **P** **P** Y° A P N° P **G°** **P** **P**

CCG CCA TAC ACG GGT GCG GCA AGT TCG ATG CCG CCT TAT CCG ACC

P **P** Y° T° G° A A S° S° **M** **P** **P** Y° P T°

GGT GGT CCG CCA CCC GTC AAC ACC GGT TAT TAC TAT CCA TCT GAT

G° **G°** **P** **P** **P** V N° T° G° Y° Y° Y° P S° D^-

CCA AAT ACC TTC TAC GCT CCA CCC TAC AGC CAG GCT TCA GCA CCT

P N° T° F Y° **A** **P** **P** Y° S° Q° A S° **A** **P**

CCT ATG GAA CCA GAA GAT AAG AAG AAT CTT TAG

P M E^- P E^- D^- K^+ K^+ N° L

FIGURA 4. Características de la proteína KETc7 obtenidas con base al programa PC/Gene Intelligent.

- Peso Molecular 10.235 kDa
- Punto isoeléctrico: 3.85
- Carece de péptido señal
- Carece de segmentos asociados a la membrana
- + Aminoácidos con carga positiva
- - Aminoácidos con carga negativa
- ° Aminoácidos polares sin carga
- Carece de secuencias de glicosilación. Ninguna Serina o treonina se encuentran agrupadas en conjuntos de serinas o de treoninas. Tampoco hay Asparaginas en el contexto Asn-X-Ser(Thr). Donde X es cualquier aminoácido menos prolina.
- Está constituida por 100 aminoácidos y es altamente insoluble.
- Tiene un alto contenido de prolina (30% de prolina). Su análisis de identidad indica 35-43 % de homología con secuencias protéicas ricas en prolina (extensinas). La proteína KETc7 comparte con las extensinas 2 motivos: Ser-Prolina $_{(3-7)}$ y $XP_{(2-7)}$ (donde X puede representar cualquier aminoácido), ambos interrumpidos por diferentes secuencias de aminoácidos.

igual que por *Taenia solium* (Uribe, 1998) . La presencia del epítope GK1 los diferentes estadios de *T. solium* también fue comprobada por medio de immunofluorescencia, en donde se detectó en las mismas estructuras que la proteina KETc7.

Posteriores análisis del perfil antigénico de KETc7 con el fin de identificar epítopes para células T con los métodos propuestos por Margalit et al., 1987 (Algoratismo de Fauchere and Pliska de 1983); Rothbard and Taylor (1988) y Sette *et al.*, (1989) se detectaron 4 posibles epítopes que se ilustran en la Tabla 7. Es interesante destacar que dos epítopos coinciden, el T1 y el T4 por ambos métodos, y que GK1 coincide con T2

PEPTIDO	SECUENCIA DE ANIMOACIDOS
GK1	GYYYPSDPNTFYAPPYS 69-85*
GK2	MPPYPTGGPPPV 55-66*
GK3	PPPYAPNPGPPPPYTGA 35-51*

FIGURA 5-Secuencia de aminoácidos de tres epítopes B determinados por el programa pCG intelligent (Jameson and Wolf, 1988) con base en el índice de antigenicidad de la proteína KETc7. *Posición de los aminoácidos que integran el epítope.

PEPTIDO	SECUENCIA DE ANIMOACIDOS	CITA
T1	DYLYQTNSPP (15-23)	Margalit et al., 1987
T2	DPNTFYAPP (71-79)	Margalit et al., 1987
T3	GAAS (46-49)	Rothbard and Taylor, 1988 Sette *et al.*, 1989
T4	YLYQT (15-19)	Rothbard and Taylor, 1988 Sette *et al.*, 1989

FIGURA 6-. Secuencia de aminoácidos de epítopes T determinados por el programa EPI PLOT, con base a la secuencia de la proteína KETc7: hélices anfipáticas. Programa EPI PLOT 1989 (Menendez, 1990)

.APENDICE II

VACUNACION CON PROTEÍNAS RECOMBINANTES

La aplicación de la tecnología del DNA recombinante ha abierto nuevas posibilidades en el área de las vacunas, éstas técnicas se aplican ampliamente en la identificación y aislamiento de antígenos. Permiten clonar y expresar de manera individual los antígenos de un organismo, y en la mayoría de los casos, proveer altas cantidades de antígeno puro para realizar con él las pruebas necesarias. Estas metodologías nos permiten utilizar componentes aislados del organismo, lo que en muchas ocasiones puede resultar benéfico, como sería una aplicación más controladas (Ada , 1993).

Las técnicas de DNA recombinante permiten la expresión en una célula de genes exógenos obtenidos de especies distintas, incluso el procedente de mamíferos superiores en bacterias como *Escherichia coli.* Esta tecnología se basa en que el código genético es casi universal, lo que quiere decir que la secuencia de DNA que codifica para una proteína en un organismo, puede expresarse en otro, produciendo una proteína con la misma secuencia de aminoácidos (Binns, 1993). Esto funcionó muy bien al transformar a las bacterias con el DNA de eucariotes inferiores como *Sacharomices cerevisiae* o *Neurospora crassa*. Desafortunadamente, cuando se realizaron experimentos semejantes con DNA que provenía de eucariotas superiores no siempre se ha logrado obtener la expresión de dichos genes con alta eficiencia, probablemente debido a las diferencias entre los mecanismos de expresión de los genes entre los procariotes y eucariotes superiores. Entre las diferencias más importantes que podemos mencionar se encuentran: la presencia de intrones en el DNA eucariote, la existencia de distintas secuencias promotoras en bacterias y células eucariotes, la ausencia de unión al ribosoma (secuencias Shine-Delgarno) en el RNAm eucariote, el uso preferencial de codones y, en muchos casos, la necesidad de una modificación postraduccional como la glicosilación, para que el péptido sea totalmente funcional (Estrada, 1992).

Figuran entre las limitaciones para la expresión de proteínas de eucariotes en sistemas procariotes que las proteínas recombinantes pueden ser letales para

TABLA 2.- Aplicación experimental de algunas proteínas recombinantes producidas para el control de enfermedades

ENFERMEDADES VIRALES	
Virus	**Referencia**
Herpes Simple 1	Rooney *et al.*, 1992
Virus de la hepatitis B	Iwarson, 1995
Virus del papiloma humano	Hines *et al.*, 1995
ENFERMEDADES BACTERIANAS	
Bacteria	**Referencia**
Vibrio Cholerae	Levine *et al.*, 1988
Shigella sonnei	Formal *et al.*, 1981
Brucella abortus	Stabel *et al.*, 1990
Borrelia burfdorferi	Stover *et al.*, 1991,1992a, 1993, 1994
ENFERMEDADES PARASITARIAS	
Parásito	**Referencia**
Taenia ovis ,	Johnson *et al.*, 1989 Lightowlers *et al.*, 1996
Taenia crassiceps	Manoutcharian *et al.*, 1996

la bacteria, ó son rapidamente degradadas por las enzimas proteolíticas bacterianas. A pesar de estas dificultades en algunos casos se han logrado resultados satisfactorios, en la Tabla 2 se mencionan algunos ejemplos de estos casos, en especial cuando se han elaborado proteínas híbridas, parte procariote y parte eucariote. Algunas de ellas tienen la característica de insolubilizarse después de la síntesis, propiedad que favorece su purificación (Bolivar-Zapata, 1986).

Las secuencias de las proteínas recombinantes procedentes de la biblioteca de cDNA del cisticerco de *T. crassiceps* no han podido ser expresadas en sistemas bacterianos, a diferencia de otros casos exitosos como la Paramiocina de *T. solium* (Landa *et al.,* 1993), Paramiocina de *S. mansoni* (Laclette *et al.,* 1991), proteína HP6 (Benitez *et al.*, 1996) y *Taenia ovis* de 48 kDa (Johnson *et al.,* 1989; Lightowlers *et al.*, 1996)

TABLA 3.- Aplicación experimental de algunos peptidos sintéticos producidos para el control de enfermedades

ENFERMEDADES VIRALES	
Virus	**Referencia**
Virus de la Influenza	Arnon y Levi, 1995
Virus de la encefalomielitis murina	Yahikozawa *et al.*, 1997
ENFERMEDADES BACTERIANAS	
Bacteria	**Referencia**
Salmonella sp.	Russmann et al., 1998
Pseudomona sp	Sheth et al 1995
Neisseria sp	van den Elsen et al., 1997
ENFERMEDADES PARASITARIAS	
Parásito	**Referencia**
Plasmodium sp.	Nardin *et al.*, 1995; Patarroyo *et al.*, 1988
Entamoeba histolytica	Lotter *et al.*, 1997
Taenia ovis	Lightowlers *et al.*, 1996
Taenia crassiceps	Toledo *et al.*, 1998
Toxoplasma gondii	Velge-Roussel *et al.*, 1997
Tripanosoma sp.	Reynolds *et al.*, 1994

APENDICE III

VACUNACIÓN CON PEPTIDOS SINTÉTICOS

La producción de antígenos sintéticos fue propuesta desde principios de 1980 cuando se logró inmunizar exitosamente con una vacuna totalmente sintética en contra de la difteria (Audibert *et al.*, 1982). Posteriormente, surgen otros dos trabajos que apoyan el uso de péptidos sintéticos como vacuna, en uno utilizaron segmentos de 4, 6 y 8 aminoácidos de la toxina diftérica con muramil-dipéptido como adyuvante, y como acarreador una cadena de poli-(DL)-alanina, con lo que lograron inducir anticuerpos contra la toxina completa en cobayos (Arnon y Sela 1982). En ese mismo año, se protegieron cobayos en contra de la fiebre aftosa. por medio de un péptido de 20 aa de una de las proteínas del virus (Bittle *et al.*, 1982). Sin embargo, **ésta** no funcionó para proteger en ganado por el tipo de anticuerpos que generaba. A partir de entonces se han continuado los estudios para evaluar la capacidad protectora de peptidos sintéticos en contra de diferentes agentes infecciosos. Actualmente, las vacunas sintéticas se producen previa identificación y aislamiento de los antígenos protectores, así como la caracterización completa de su secuencia de aminoácidos con base a la secuenciación de sus genes. Con esta información se pueden identificar diferentes secuencias peptídicas de la molécula protectora que tengan una antigenicidad elevada. Dado que el sitio activo de los anticuerpos y el receptor de los linfocitos T solamente reconocen pequeñas porciones de los antígenos proteicos o epítopes, (5 a 6 residuos de aminoácidos para los sitios activos de los anticuerpos, ó 9 a 12 para el sitio activo del receptor del linfocito T). Éstos epítopes pueden ser fácilmente obtenidos en el laboratorio mediante técnicas para la síntesis de péptidos, con las que se pueden formar moléculas de 2 a 50 aminoácidos, se purifican y posteriormente se evalúa su habilidad para inducir una respuesta inmune protectora (Arnon and Van Regenmortel, 1992).

En general los péptidos sintéticos no son muy inmunogénicos, pero esto puede resolverse usando moléculas acarreadoras o adyuvantes apropiados (Estrada, 1992)

En principio esta tecnología permitiría la producción de una vacuna completamente sintética, evitando las limitaciones presentadas por las vacunas vivas (Dyson and Wright, 1995) mencionadas previamente en otras secciones de este trabajo. Sin embargo, la síntesis de péptidos se complica conforme el tamaño del péptido aumenta.

Otro aspecto que hay que considerar en el diseño de epítopes sintéticos es que la secuencia peptídica debe contener secuencias que le permitan unirse a moléculas del MHC de clase I y/o II a fin de asegurar una respuesta inmune humoral y/o celular. Una manera segura para elegir una secuencia que tenga la capacidad de unirse a moléculas del MHC consiste en purificar a partir de las células infectadas ó presentadoras de antígeno, las moléculas del MHC y examinar los péptidos que tienen unidos. Así se podrían detectar solo los péptidos que son finalmente procesados y presentados por las células infectadas

Se han propuesto tres estrategias para el desarrollo de vacunas sintéticas:

1) La utilización de péptidos polimerizados o unidos a moléculas ó proteínas acarreadoras que contengan secuencias capaces de unirse a moléculas del MHC (Adorini *et al.,* 1979).

2) Se ha considerado importante, que la secuencia peptídica debe contener epítopes para linfocitos T y B, debido a que en ciertas situaciones la inducción de una respuesta de anticuerpos contra antígenos timo dependientes involucra que los linfocitos T y B reconozcan diferentes determinantes de la misma molécula. Se sabe que las secuencias de dichos epítopos no necesariamente se sobreponen en una misma molécula (Adorini *et al.,* 1979). Recientemente, se ha ideado la síntesis de MAPs los cuales utilizan una matríz peptídica base para construir una macromolécula sintética que puede ser inmunogénica aún en ausencia de una proteina acarreadora. Utilizando este sistema, se pueden definir parámetros como tamaño, número, radio y posición relativa de epítopes de células T y B. El potencial inmunoprofiláctico del MAP se ha evaluado en algunas enfermedades infecciosas al igual que en la inducción de anticuerpos protectores y respuesta de células T en contra de malaria y esquistosomiasis y la generación de anticuerpos neutralizantes en contra del HIV. Una posibilidad a considerar con

respecto a esta opción es que si se sabe cuales son las características de la respuesta inmune eficiente para la destrucción del patógeno se sintetize la vacuna solo con epítopes T ó B, según sea el caso.

3) La incorporación de un péptido promiscuo capaz de unirse a un amplio repertorio de moléculas de MHC II (Estrada, 1992).

En la Tabla 3 se presentan algunos ejemplos de vacunas producidas con péptidos sintéticos que han sido evaluadas para el control de algunas enfermedades virales, bacterianas y parasitarias.

APENDICE IV

ARTICULOS EN COLABORACIÓN

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J. Parasitol., 82(2), 1996, p. 250–254
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CYSTICERCOSIS: IDENTIFICATION AND CLONING OF PROTECTIVE RECOMBINANT ANTIGENS

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ABSTRACT: We describe the cloning and the evaluation of the protective capacity of 5 recombinant antigens expressed during the cysticercus stage of both *Taenia crassiceps* and *Taenia solium*. A cDNA library was constructed in bacteriophage λZAP using mRNA isolated from larvae of *T. crassiceps* of the ORF strain. The recombinant phage library was screened with polyclonal antibodies against 56- and 74-kDa protective antigen fractions. This screening identified 13 recombinant clones, 5 of which were also strongly recognized by pooled sera from pigs experimentally infected with *T. solium*. The native antigens are proteins of 56 (clones KETc1, 4, 7) and 74 and 78 kDa (clones KETc11, 12) of *T. crassiceps* cysticerci. Vaccination experiments using these 5 recombinant clones against murine cysticercosis point to the relevance of KETc1, 4, 7, and 12 in host protection, whereas immunization with the clone KETc11 does not modify the parasite load in females and facilitates the parasitosis in males. We report here the DNA and the deduced amino acid sequence (100 amino acids) of the first protective antigen (KETc7) of potential interest for *T. solium* pig cysticercosis prevention.

Cysticercosis, caused by *Taenia solium* seriously affects human health and is responsible for important economic losses in developing countries (Gemmell et al., 1985; Aluja and Vargas, 1988; Larralde et al., 1992). In developed countries its frequency is extremely low, but recently, due to immigrants from endemic areas in Mexico and Central America, its prevalence has risen in the United States (Loo and Braude, 1982; Richards and Schantz, 1991).

The essential role of pigs as obligatory intermediate hosts offers the possibility of a realistic control measure by interfering with transmission by vaccinating pigs, thus modifying the prevalence of cysticercosis.

Several antigenic preparations from oncospheral and larval stages of different cestodes have been useful in the development of vaccines against cysticercosis (Molinari et al., 1983, 1993; Nascimiento et al., 1987) and 1 recombinant vaccine effective against *Taenia ovis* has been developed (Johnson et al., 1989). Among these antigenic preparations, *Taenia crassiceps* cysticerci antigens are capable of protecting mice against *T. crassiceps* in experimental infections (Sciutto et al., 1990), as well as rats and pigs, respectively exposed to eggs of *Taenia taeniaeformis* (Ito, Takami, and Itoh, 1991) or *T. solium* (Sciutto et al., 1995). In addition, *T. crassiceps* experimental murine cysticercosis has proved to be a useful and suitable model in which to test promising antigens in the prevention of pig cysticercosis caused by *T. solium* (Sciutto et al., 1995). From several *T. crassiceps* antigen fractions previously evaluated, 3 (56, 66, and 74 kDa) were selected for further experiments considering their protective capacity, availability, and cross-reactivity with *T. solium* antigens (Valdez et al., 1994). Here, we report the protective capacity of these *T. crassiceps* antigen fractions against pig cysticercosis, the production of 5 of these as recombinant antigens, and the evaluation of their protective effect against *T. crassiceps* murine cysticercosis.

Received 1 May 1995; revised 25 October 1995; accepted 25 October 1995.

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MATERIALS AND METHODS

Animals

Female and male BALB/cAnN mice, bred in our animal facilities by brother–sister mating, were used. All mice used were 5–7 wk old at the start 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 Animal Resources, National Research Council, Washington, DC.

Female Yorkshire and Landrace hybrid hogs were used. Pigs were immunized with anticholera vaccine 20–45 days after birth and housed in the Facultad de Medicina Veterinaria y Zootecnia, UNAM, where no transmission of cysticercosis occurs until used for experimentation. Pigs were humanely killed with a captive bolt stunner (Schermer, Germany).

Parasite antigens and anti-parasite antibodies

Soluble antigens fractions of *T. crassiceps* cysticerci were obtained from cysticerci recovered from the peritoneal cavity of BALB/cAnN female mice after 30–60 days of infection by a procedure described by Valdez et al. (1994). Mice and pigs were experimentally infected with *T. crassiceps* cysticerci and *T. solium* eggs, respectively, and 30 days later sera from these animals were obtained and pooled. The presence of specific antibodies were tested by enzyme-linked immunosorbent assay (ELISA; data not shown) following the technique previously described (Larralde et al., 1992).

Affinity purification of anti-parasite antibodies reactive with recombinant clones and immunoblot analysis

New Zealand white rabbits were immunized by subcutaneous (s.c.) injection with acrylamide gel including 56- and 74-kDa *T. crassiceps* cysticerci antigen fractions in Freund's complete adjuvant (FCA). After 3 biweekly boosts with antigens in Freund's incomplete adjuvant, blood samples were taken, and the presence and specificity of antibodies produced were tested by western blot analysis according to a previously reported protocol (Larralde et al., 1989). Antibodies to antigens expressed by recombinant clones were affinity-purified from these immune sera according to the method described by Ozaki et al. (1986). Affinity-purified antibodies from the KETc1, 4, 7, 11, and 12 were used to probe immunoblots of *T. crassiceps* vesicular fluid antigens to identify the native antigens that correspond to recombinant proteins.

Construction of cDNA library

Messenger RNA from 15 ml of *T. crassiceps* cysticerci was isolated using a commercial kit (Stratagene, La Jolla, California) according to the manufacturer's instructions. The mRNA (5 μg) was transcribed into cDNA using the ZAP-cDNA synthesis kit (Stratagene). The cDNA was

TABLE I. Protective effect of vaccination with *T. crassiceps* antigens on *T. solium* pig cysticercosis.*

Group	Number of cysticerci per individual	Mean	% Protection
Control pigs	6, 6, 7, 3, 3, 5	5	–
Pigs immunized with:			
Vesicular fluid antigens	1, 3, 3, 2, 0, 0, 9	2.5†	48.5
Gel cut-out antigen fractions (56 + 66 + 74 kDa)	0, 0, 0, 0, 0, 1	0.16†	96.8

* Pigs were immunized with antigen preparations in FCA, 60 days before challenge. Pigs were killed 60 days after the challenge and the individual parasite load was determined.
† Significantly different from nonimmunized pigs at $P < 0.05$.

then ligated in Uni-ZAP XR vector (Stratagene). The ligated cDNA was packaged into lambda heads using Gigapack Packaging extract (Stratagene) and amplified once on XL1-Blue MRF' cells (Stratagene). The direct titer of pools of 4 packagings was 1.8×10^5 plaque-forming units (pfu).

Screening of the *T. crassiceps* cDNA library

To detect clones that code for antigens of 56 and 74 kDa, the specific rabbit immune sera, produced as described above, were used following conventional techniques (Snyder et al., 1987). The cDNA library was plated at a concentration of 10^4 pfu/plate on XL1-Blue cells and 2×10^4 clones were screened using specific polyclonal rabbit antisera, diluted 1:100 in phosphate-buffered saline (PBS) containing 0.05% Tween (PBS-T), after removal of anti-*Escherichia coli* antibodies (Sambrook et al., 1989). To detect positive clones ^{125}I-Protein A (Amersham, Buckinghamshire, U.K.) was used in accordance with the manufacturer's instructions. To detect those of interest for *T. solium* vaccination, a screening with pooled sera from *T. solium*-infected pigs diluted 1:50 in PBS-T was performed.

Immunization

A group of 7 York-Landrace pigs, 40 days old, was immunized (s.c.) with *T. crassiceps* vesicular fluid antigens at the base of the ear with a single dose of 0.4 mg per kg (2.5 ml), homogenized in the same volume of FCA. Another group of 6 pigs was immunized (s.c.) under equivalent conditions with gel cut-out bands of 56, 66, and 74 kDa together, in a single dose of 60 μg per kg. Antigens were prepared as previously reported (Valdez et al., 1994). Control pigs were inoculated with FCA with saline at a dose of adjuvant similar to that received by the immunized animals, considering previous observations that acrylamide does not modify the number of parasites recovered after infection.

Crude lysates of recombinant clones were prepared following the procedure described by Snyder et al. (1987). Soluble antigens from crude lysates were used for immunization. Groups of 5 male and 8 female BALB/cAnN mice were immunized by injecting (s.c.) 400 μl of a crude lysate from each recombinant clone homogenized in an equal volume of FCA. Control mice were immunized with a crude lysate from λ phage without insert emulsified in FCA.

Parasites, challenge, and measurement of the parasite load

The ORF strain of *T. crassiceps* (Zeder, 1800) Rudolphi 1810, 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 8 yr in our Institute. Parasites for infection were harvested from the peritoneal cavities of mice, 1–3 mo after inoculation of 10 cysticerci per mouse.

Immunized mice were i.p. infected with 10 small cysticerci 15 days after immunization as described elsewhere (Valdez et al., 1994). Control mice were infected at the same time. Parasite load was measured in each mouse by counting the number of larvae recovered from the peritoneal cavity 30 days after infection.

Taenia solium eggs used for challenging pigs were isolated from a tapeworm recovered from 1 man in the state of Chiapas, México, after treatment with a single oral dose (2 g) of Niclosamide (Yomesan, Bayer, México City, México). The tapeworm was identified and eggs were recovered as previously described (Sciutto et al., 1995). For infection, pigs were administered 10,000 eggs each, orally, in a single meal of Purina Chow, 60 days after immunization. Sixty days after infection, pigs were killed and the parasite load of each pig was measured by counting the number of parasites in complete masseters, tongue, and diaphragm, in 200 g of right and left abdominal muscles, in intercostal muscles, and in 400 g of right and left shoulders and hind legs.

DNA sequencing

After immunological screening, positive plaques were converted into phagemid (pSK) clones using the excision procedure. λDNA and rescued phagemid DNA were obtained using the Lambda DNA Purification Kit (Stratagene) and commonly used plasmid DNA preparation procedures (Sambrook et al., 1989). Sequencing was performed by the dideoxynucleotide chain termination method (Sanger et al., 1977) using "−40" and Reverse primers to sequence the 2 chains and [α-^{35}S]dATP (Amersham). Double-stranded plasmid DNA was employed for sequencing using Sequenase (U.S. Biochemicals, Cleveland, Ohio) as recommended by the manufacturer. The resultant DNA sequences and deduced amino acid sequences were compared with the GenBank DNA Databases (Altschul et al., 1990) and other available databases.

Statistics

Results were statistically analyzed by multifactorial analysis of variance ANOVA (Anonymous, 1985).

RESULTS

Effect of immunization with *T. crassiceps* antigens against *T. solium* pig cysticercosis

Table I shows the parasite load in control pigs after 60 days of oral infection with *T. solium* eggs and those obtained in pigs immunized either with total antigens from vesicular fluid or a mixture of gel cut-out *T. crassiceps* antigens (74 + 66 + 56 kDa). Immunization with vesicular fluid antigens resulted in a significant decrease of the parasite load. Higher protection was observed in pigs immunized with gel cut-out antigens of 56, 66, and 74 kDa, where 5 out of 6 showed no parasites.

Immunoscreening of a *T. crassiceps* cDNA library

A cDNA library was constructed using mRNA isolated from *T. crassiceps* cysticerci. In order to perform an antibody screening of the cDNA library, specific polyclonal antibodies were prepared in rabbits against 56-, 66-, and 74-kDa *T. crassiceps* cysticerci antigens. Screening of approximately 2×10^4 recombinant phage yielded 13 clones, recognized by antibodies against

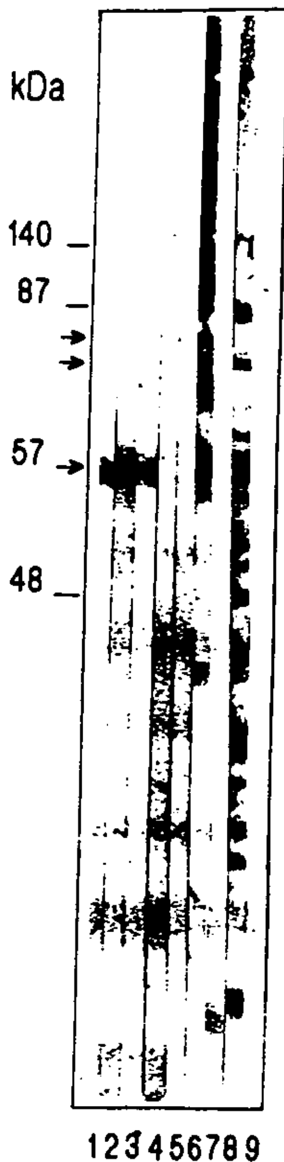

FIGURE 1. Identification of native proteins of *T. crassiceps* cysticerci encoded by the recombinant clones by western blot of vesicular fluid antigens. Lanes probed with: rabbit anti-56-kDa antibodies affinity-purified from clone KETc 1 (1), from clone KETc 4 (2), and from clone KETc 7 (3), and rabbit anti-74-kDa antibodies affinity-purified from clones KETc11 (4) and from clone KETc12 (5). Lanes 6 and 7 were probed with pooled serum from pigs infected or noninfected with *T. solium*, respectively. Lanes 8 and 9 were probed with pooled serum of mice infected or noninfected with *T. crassiceps*, respectively.

antigen fractions of 56 and 74 kDa, with estimated sizes of inserts ranging from 0.4 to 2 kb. To detect those of interest for *T. solium* vaccination, positive clones were secondarily screened with pooled anti-*T. solium* sera from infected pigs using the spot-lysis approach. By this procedure, 5 positive clones were detected. These clones were designated as KETc1, 4, 7, 11, and 12. Inserts from these 5 clones were analyzed. Restriction mapping showed that the 5 clones had inserts from 0.4 to 1.4 kb (data not shown).

```
1G AAT TCG GCA CGA GCA TTT ATG CAG CCG CAT CCT TCC TTC TCT CCA CCG
1   N   S   A   R   A   F   M   Q   P   H   P   S   F   S   P   P
50  CCA GTT GAT TAT CTG TAC CAG ACA AAC TCT CCA CCG CCC TAT GGG GGC
17  P   V   D   Y   L   Y   Q   T   N   S   P   P   P   Y   G   G
98  GCT GTC CCT CCA CCT TAC GCG CCG AAT CCA GGT CCA CCG CCG CCA TAC
33  A   V   P   P   P   Y   A   P   N   P   G   P   P   P   P   Y
146 ACG GGT GCG GCA AGT TCG ATG CCG CCT TAT CCG ACC GGT GGT CCG CCA
49  T   G   A   A   S   S   M   P   P   Y   P   T   G   G   P   P
194 CCC GTC AAC ACC GGT TAT TAC TAT CCA TCT GAT CCA AAT ACC TTC TAC
65  P   V   N   T   G   Y   Y   Y   P   S   D   P   N   T   F   Y
242 GCT CCA CCC TAC AGC CAG GCT TCA GCA CCT CCT ATG GAA CCA GAA GAT
81  A   P   P   Y   S   Q   A   S   A   P   P   M   E   P   E   D
290 AAG AAG AAT CTT TAG TTTCTGTCGGGTCTCACTTACATCTCTGCTCTCACCATC
97  K   K   N   L
344 GGAATTAATATCACTTGAGAATCCTTTTTGGTTTGGCTAATTTTTCTTCTTAATAAA
401 ATTTCGTCTCCATACGCAAAAAAAAAAAAAAAAAAAAAAACTCGAG
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FIGURE 2. Nucleotide sequence and predicted amino acid sequence of the KETc7 clone. The DNA sequence of 445 nucleotides and 100 amino acids are numbered from the left starting at the *Eco*RI adaptor sequence. TAG termination codon, *Xho*I restriction enzyme recognition site from oligo, and putative polyadenylation signal, AATAAA, are indicated. The amino acid sequence is given below the DNA sequence (GenBank accession number U31524).

Identification of the native parasite proteins corresponding to the recombinant antigens

Taenia crassiceps vesicular fluid antigens were separated by sodium dodecyl sulfate polyacrylamide gel electrophorosis (SDS-PAGE) and electrophoretically transferred to nitrocellulose filters. The blots were reacted with anti-KETc1, 4, 7, 11, and 12 (Fig. 1). A single band representing approximately 56 kDa was detected with antibodies purified with KETc1, 4, and 7, and 2 bands of 74 and 78 kDa were observed with antibodies purified with KETc11 and 12.

Nucleotide sequencing

Sequence analysis of inserts from the 5 clones demonstrated that all clones were unique in view of the fact that no homology was found in the approximately 400 bp of the 3′ portion of them except for the insert of KETc11 from which 361 bp were previously reported in *T. crassiceps* HYG as full-length sequence (Zarlenga et al., 1994). Figure 2 shows the complete sequence of KETc7, which was the only one entirely sequenced. As shown, this sequence is 445 bp in length. The coding strand was identified by the presence of a polyA tail. The presence of *Eco*RI and *Xho*I sites in flanking regions of the gene shows that the cloning procedure was carried out correctly. From 3 possible reading frames of these sequences, only 1 appeared to be appropriate (300 bp); it was also in frame with the lacZ gene of λZAP and coded for a 100 amino acid polypeptide. The sequencing of the remaining 4 clones is now in process.

Effect of immunization with λ lysates from recombinant clones against murine cysticercosis

To test the potential of recombinant antigens on anti-cysticerci immunity, crude lysates of the 5 clones in FCA were used to immunize BALB/cAnN female and male mice (Table II). As controls, mice injected with lysates from a clone without an insert in FCA were included. As shown in Table II, significant protection was obtained with 4 (KETc1, 4, 7, 12) of the 5 clones tested. For these 4 clones, higher levels of protection were observed in males than in females. The level of protection ranged from 73 to 84.4% in males and from 53.5 to 64.1% in females. In contrast, immunization with KETc11 clone lysate significantly increased the expected parasite load in males and did not significantly modify that obtained in females.

DISCUSSION

In this report we confirm, in pigs, the host-protective nature of 56-, 66-, and 74-kDa *T. crassiceps* antigens previously observed in mice against murine cysticercosis. Pigs immunized with 56-, 66-, and 74-kDa *T. crassiceps* cysticerci antigen fractions were significantly (96.8%) protected against infection with *T. solium*, although few parasites were recovered from orally challenged animals, which is not unusual (Sciutto et al., 1995). This indicates the protective effect of isolated antigen fractions of *T. crassiceps* against pig cysticercosis and attempts to modify cysticercosis transmission can therefore be considered. To produce these antigens in plentiful supply, we prepared a cDNA library from *T. crassiceps* cysticerci. Specific antibodies against the 56- and 74-kDa antigen fractions, produced in rabbits, enabled us to isolate clones that represent parts of the serologically related molecules of these 2 fractions. With this screening, we identified 13 recombinant clones. Only 5 of these proved to be of interest in the prevention of *T. solium* pig cysticercosis because they reacted with pooled serum from pigs infected with *T. solium*. The western blots using affinity-purified antibodies against each recombinant clone confirmed that KETc1, 4, and 7 correspond to a native antigen of 56 kDa, whereas KETc11 and 12 correspond to 74–78 kDa (Fig. 1).

We determined the efficacy of these 5 recombinant antigens in their protective capacity in inbred mice. Considering previous findings that BALB/cAnN is the strain with higher susceptibility to *T. crassiceps* cysticercosis and the strong differences in susceptibility observed between sexes (Sciutto et al., 1991), males and females of this strain were employed in the study. As Table II shows, 4 of these 5 recombinant antigens induce high levels of protection in mice (but not complete). This result points toward the existence of recombinant antigens of potential interest for *T. solium* pig cysticercosis prevention. On the other hand, mice immunized with lysates from KETc11 showed a higher parasite load than control male mice, whereas no significant effect was observed in susceptible females in which the parasitosis facilitating effect could be masked by the naturally high susceptibility of females. A systematic study of the immune response underlying this phenomenon is in process, with the aim of understanding the immunological mechanisms involved in this host–parasite relationship. Comparisons of the nucleic acid sequences and predicted amino acid sequences of our clones with databases revealed significant homology only with KETc11. A 100% homologous sequence has been previously reported by 2 different authors (Fischer et al., 1994; Zarlenga et al., 1994). Interestingly, in these reports evidence of a native protein of 10 kDa was presented, which we did not detect. As we found, anti-KETc11 monospecific antibodies react against native antigens of 74–78 kDa. At present, we have no explanation for this discrepancy, and more experiments are needed to gain further insight.

Although we only have the 3′ portion of the KETc7 gene, this short segment encodes at least 1 protective epitope of the native protein as demonstrated in the vaccination experiments shown on Table II. This information opens the possibility of identifying epitope(s) more precisely by the use of synthetic peptides. Interestingly, in the KETc7 gene we found an open reading frame, encoding a polypeptide of 100 amino acids which is highly hydrophobic and proline rich (29%) as shown in Figure 2.

Varying degrees of protective immunity against some cestodes have been shown to be effectively induced in their mammalian hosts (Ito, Bogh, et al., 1991; Ito, Takami, and Itoh, 1991; Sciutto et al., 1991, 1995) with a variety of antigens; nevertheless, the epidemiological impact of a widespread vaccination program upon transmission dynamics should not be trivial (Gemmell et al., 1985). Oncosphere antigens, natural or recombinant, are held to be the most effective in protecting rats and sheep against challenge with eggs of *T. taeniaeformis* (Ito, Bogh et al., 1991) and *T. ovis* (Johnson et al., 1989), respectively, allegedly because of the onchosphere's greater sensitivity to antibodies compared with the metacestode stage (Johnson et al., 1989). It is interesting in this context that we were able to identify larval stage protective antigens. This may be due to the fact that they were from *T. crassiceps* cysticerci, and this species can divide by asexual budding, apart from going through a sexual stage and, therefore, probably displays a much more heterogeneous group of antigens, some of which could share oncosphere determinants. So far, it is not known if the protective antigens

TABLE II. Effect of immunization against *T. crassiceps* cysticercosis in BALB/cAnN mice.*

	Mean number of the parasite load ± standard deviation (% protection)	
Group	Female	Male
Control	61.3 ± 29.7	28.2 ± 18.4
Immunized with soluble antigens from crude lysates of:		
KETc1	28.5 ± 17.5† (53.5%)	6.6 ± 4.7† (76%)
KETc4	22.0 ± 15.9† (64.1%)	N.D.
KETc7	42.5 ± 6.4 (30.5%)	7.6 ± 7.6† (73%)
KETc11	46.0 ± 17.4 (24.9%)	40.6 ± 22.2† (−44%)
KETc12	26.3 ± 7.2† (57.1%)	4.4 ± 5.3† (84.4%)

* Fifty days after immunization with the recombinant antigen in FCA, mice were challenged with 10 cysticerci each and killed 30 days later. Control mice were immunized with crude lysate from λ phage without insert emulsified in FCA. Groups of 5 males and 8 females were used. N.D.—not determined.

† Significantly different from control mice at $P < 0.05$.

we identified are present in the oncospheral stage. However, the early cysticerci antigens used here would protect against oncospheres that managed to reach the metacestode stage. Although 100% protection has not been achieved yet, higher levels of protection could be obtained through an optimal combination of the purified protective antigens. The use of more appropriate adjuvants to increase levels of protection remains to be studied. Furthermore, considering the heterogeneity of the natural challenge, which includes variations in the host (genetic composition, health status, nutrition), in the parasite (antigenic variability), and in the circumstances of the natural infection, we consider highly unlikely that only 1 antigen could serve as an efficient vaccine; therefore, in this study we used a mixture of recombinant antigens. At the present time, we are evaluating our recombinant antigens against the challenge of pigs with *T. solium* eggs and with encouraging results.

ACKNOWLEDGMENTS

We thank Roberto Coria for help with database comparison, Mercedes Baca for her assistance, and Carlos Larralde and Edmundo Lamoyi for their comments. Original stocks of BALB/cAnN were obtained from M. Bevan (Seattle University). Isabel Pérez Montfort corrected the English version of the manuscript. This work was supported by grants from CONACyT and DGAPA, UNAM, México.

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Towards a *Taenia solium* cysticercosis vaccine: An epitope shared by *Taenia crassiceps* and *Taenia solium* protects mice against experimental cysticercosis.

Running tittle: T and B cell-epitope protects mice against cysticercosis.

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ABSTRACT

The *Taenia crassiceps* recombinant antigen KETc7 has been shown to be effective as a vaccine against experimental murine cysticercosis, a laboratory model used to test potentially promising molecules against porcine *Taenia solium* cysticercosis. Based on the deduced amino acid sequence of this proline-rich polypeptide, three fragments, GK-1, GK-2 and GK-3, were chemically synthesized in linear form. Of the three peptides, only GK-1 induced sterile protection in 40-70% of BALB/cAnN male mice against *T. crassiceps* cysticercosis. GK-1 is an 18 amino acid peptide which contains at least one B-cell epitope as demonstrated by its ability to induce an antibody response to the peptide and *T.crassiceps* antigen without need of a carrier protein. Immunofluorescence studies revealed that anti-GK1 antibodies strongly react with the native protein in the tegument of *T. crassiceps* and also with anatomical structures of *T. solium* eggs, oncospheres, cysticercus and tapeworm. GK-1 also contains at least one T-cell epitope, capable of stimulating the proliferation of CD8 T cells primed either with the free peptide or *T.crassiceps* total antigen. The supernatant of the stimulated cells contained high levels of IFNγ and low levels of IL-4, an indication of the peptide's capacity to induce an inflammatory response. The remarkable protection induced by GK-1 immunization, its physicochemical properties and its presence in all the developmental stages of *T. solium*, point to this synthetic peptide as a strong candidate in the construction of a synthetic vaccine against *T. solium* pig cysticercosis.

INTRODUCTION

Taenia solium cysticercosis is highly prevalent in humans and pigs in Latin America, Asia and Africa (23) and has serious health and economic consequences (9). Although cysticercosis has been practically eradicated in developed countries, it is a major concern in the developing world and of consideration as a reemerging disease in the U. S. because of immigration from endemic areas (19). Moreover, a recent publication indicates that European countries may not be totally rid of human neurocysticercosis caused by *T. solium* (24). The life cycle of this parasite includes a larval (cysticercus) phase affecting both pigs and humans after ingestion of eggs present in human faeces. The eggs are produced by the adult tapeworm localized in the gut of humans who ingested live cysticerci present in improperly cooked pork meat. The tapeworm produces millions of eggs that are passed to the environment. Transmission is thus clearly related to prevailing low sanitary standards in personal hygiene and enviromental control and also with rustic rearing of pigs in impoverished sectors of the rural population. Control of transmission by general improvement of the social, economical and educational status of developing countries is not within reach in the near future. But, since the pig is an indispensable intermediate host, transmission could be hindered by lowering the prevalence of pig cysticercosis through their effective vaccination. Development, of an effective vaccine to be applied in pigs is being pursued by a number of scientists (13,15, 22).

Because experimentation leading to a vaccine against porcine cysticercosis is hampered by the high cost and slow data retrieval involved in testing pigs, another cestode, *Taenià crassiceps*, which exhibits extensive antigen similarities with *T. solium* and whose metacestodes easily and rapidly develop

in the peritoneal cavity of mice (3, 6, 9) has been used as an experimental model to test and preselect promising antigens before testing them in pigs (10, 11, 21, 27). Thus, we have shown that total *T. crassiceps* antigens can partially protect pigs against *T. solium* cysticercosis; however, effects of vaccination with antigen extracts strongly depended on the dose used, some being protective whilst others lead to facilitation of the infection (22), a finding that oriented our research to the identification of individual protective antigens and their peptidic epitopes (10, 11 27). We identifed and cloned 4 recombinant *T. crassiceps* antigens (KETc 1, 4, 7, and 12) which conferred mice with different levels of resistance to murine cysticercosis (11). The antigenicity profile of the deduced 100 amino acid sequence of the KETc7 clone was structurally assessed to detect potentially immunologically active epitopes (7). Three of the peptide candidates of KETc 7 (GK-1, GK-2, GK-3) were chemically synthesized and their antigenicity was tested with sera from *T. solium* and *T. crassiceps* infected hosts (humans, pigs and mice). Since the three peptides were extensively reactive with these sera (7), we assessed their protective capacity and studied the immune response they elicit in immunized mice. We also searched for the peptide's presence in *T. solium* specimens to provide indications as to its potential inclusion in a vaccine against porcine cysticercosis, especially if found in oncospheres and early larvae, the parasite's developmental stages most vulnerable to immunological attack by antibodies (16). Also, studies on the peptide's physicochemical properties and structural characteristics were performed in order to explain its immunological functions.

MATERIALS AND METHODS

Peptides. The peptides GK-1 (aa69-85) GYYYPSDPNTFYAPPYS(A), GK-2 (aa 55-66) (KK)MPPYPTGGPPPV(K) and GK-3 (aa 35-51) PPYAPNPGPPPPYTGA were manually prepared 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.). All peptides were 95% pure as judged by HPLC on analytical C_{18} reversed-phase column (3.9 x 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. GK-1 was coupled to bovine serum albumin (BSA) by standard procedures (25) using glutaraldehyde. Also, GK-1 was prepared as MAP (multiple-antigen peptide), containing eight copies of the GK-1 sequence coupled to a core matrix comprising oligomeric lysine (25).

Mice. A syngenic BALB/cAnN strain of mice, previously characterized as susceptible to cysticercosis (21) was used for vaccine trials. Original stocks were purchased from M . Bevan (Seattle University) and then bred and kept in our animal facilities by the "single-line breeding" system for twenty generations . All mice used were males of 5-7 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 collection of sera. Groups of five to ten BALB/c AnN mice each, were immunized s.c., with different doses (0.5, 10 and 50 μg/mouse) of each peptide (GK-1, GK-2, GK-3) emulsified either in Freund's Complete Adjuvant (FCA) prepared as previously reported (27). Different forms of GK-1 were used at 10 μg per mice in saponin (Sigma Chemical Co., St. Louis, MO) at a concentration of 100 μg/mouse as reported elsewhere (12).This concentration of peptide was determined as optimal when saponin was used as adjuvant in colateral 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 each immunization and stored at -70°C until individually tested for the presence of specific antibodies.

Parasites and cysticercal antigens. The ORF strain of *Taenia crassiceps* (Zeder 1800) Rudolphi 1810, isolated by Freeman (1962), and supplied by Dr. B. Enders in 1984 (Behringwerke, Marburg, GE) has been maintained by serial passage in BALB/cAnN female mice for 14 years in our animal facilities. Cysticerci for infection were harvested from the peritoneal cavity of mice 1 to 3 months after inoculation of 10 non-budding small cysticerci (2-3 mm in diameter) per animal. The soluble antigens were recovered from similar cysticerci by the procedure previously described (9).
Whole *T. solium* cysticerci were dissected from skeletal muscle of highly infected pork carcasses between 2 and 4 hours after slaughter in an abattoir in Zacatepec, Morelos, Mexico. Segments from *T. solium* tapeworm and eggs were obtained from the faeces of an infected child in the state of Puebla, Mexico. The tapeworm was recovered after the child's treatment with a single oral dose (2 g) of niclosamide (Yomesan; kindly supplied by Bayer, Mexico).

After washing in saline plus antibiotics (100 U per ml penicillin + 100μg/ml streptomycin), several gravid proglottids were separated for immunofluorescence assays and eggs were obtained by cutting the proglottids with fine sharp scissors and then teasing the fragments. The eggs were then washed in saline before inclusion to immunolocalization studies.

ELISA for antibody measurements. *T. crassiceps* antigens obtained as previously described (9), were used as antigen in ELISA to measure the antibody response induced by peptide immunization following the procedure described elsewhere (18). Briefly, 96 well flat-bottomed microtitration plates (Nunc, Denmark) were coated with the respective antigen preparation (1μg/per well) and incubated overnight at 4 °C. Sera were used at 1:100 dilution in PBS containing 1% of BSA. Bound mouse Igs were detected using the alkaline phosphatase-conjugated anti-mouse IgG (whole molecule, Sigma Chemical Co., St. Louis, MO) diluted 1:1000 for 1 hr at 37°C. The substrate used was detected using p-nitrophenyl phosphate (Sigma) in diethanolamine buffer for 10 min at room temperature. The reaction was stopped with 2N NaOH. Optical density readings at 405 nm were carried out in a Humareader ELISA processor (Human Gessellschaft Für Biochemica und Diagnostica, Taunusstein, Germany).

Proliferation assay. Spleen cells from non-immunized and GK-1 immunized mice were harvested 15 days after the second immunization with GK-1 plus saponin or saponin respectively and cultured in RPMI 1640 medium supplemented with L-glutamine (0.2mM), nonessential amino acids (0.01m M), penicillin (100U/ml), streptomycin (100μg/ml) and FBS(10%). Cells were cultured with the appropriate concentration of 5 μg/ml of ConA, 10 μg/ml of GK-

1 or *Taenia crassiceps* antigens 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 200µl of final volume. Ten thousand peritoneal cells recovered from the same mice were added to each well at a volume of 50µl. Peritoneal cells were obtained by *ex vivo* lavage with 5 ml of RPMI-1640. The cells were sedimented by centrifugation at 800 g for 10 min. The pellets was resuspended in an additional 3 ml of supplemented RPMI media and were adjusted in volume to contain 2×10^5 cells/ ml. After 72 h, the cultured cells were pulsed (1µCi per well) for a further 18 h with [Methyl ^{3}H thymidine] (Amersham Life Science U.K.) Then, all cells were harvested and the amount of incorporated label was measured by counting in a 1205 β-plate spectrometer (Wallac). All assays were performed in triplicate in at least four individual mice.

Cytokine measurements. Supernatant from non-stimulated and stimulated cells described above were harvested after 48 and 72 h. The solid phase ELISAs for measurement of IL-4 and INF-γ were used as previously described (26), and according to the manufacturer's instructions (Pharmingen). The pairs of cytokine-specific monoclonal antibodies and recombinant cytokines were all obtained from Pharmingen.

Flow cytometry. After 3 days of *in vitro* culture with different doses of mitogen, antigen or peptide, splenocytes were harvested and CD8, CD4 and CD3 expression was determined by staining with FITC-conjugated anti-CD8 (Pharmingen), phycoerytherin (PE)-labeled anti-CD4 (Pharmingen) and phycoerytherin-labeled anti-CD3 (Pharmingen), following the procedure previously reported (5). Briefly, cells were washed with PBS plus 10% of FBS γ-globulin-depleted and 0.02% NaN_3 and incubated with the indicated antibodies

at 4°C, 30 min. After washing, splenocytes were resuspended in cold PBS 3% formaldehyde in isotonic solution and analyzed by FACScan (Becton Dickinson, San Jose, CA). Results were expressed as percent of positive cells.

Experimental challenge. Metacestodes used in challenge infections were harvested from BALB/cAnN female mice carrying the ORF strain of *T. crassiceps* cysticerci. Ten small (diameter aprox. 2mm), non-budding larvae were suspended in 0.5 M NaCl, 0.01 M sodium phosphate buffer, pH 7.2 and injected i.p., into each mouse using a 27-gauge needle. 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. Variation in individual parasite intensities within groups we attribute to differences in the infectivity of each parasite inoculum, which also varies between the different parasite harvests used in challenge infection. In consequence, experimental designs measuring levels of immunity by parasite intensity must include in each and all vaccination sessions a session–specific measurement of each inoculum infectivity in unvaccinated control mice.

Immunolocalization of GK-1 protein. *T. crassiceps* cysticerci and *T. solium*'s specimens (cysticerci, eggs and tapeworm segments) were placed on ice into a 50 ml conical plastic bottom centrifuge tube with ice-cold PBS. The vesicular fluid was removed from cysticerci. After this, the tissues were incubated for 30 seconds with glycine chloride buffer (50 mM glycine-HCl, pH 2.5; 0.1% Triton X-100; 0.15 mM NaCl), to reduce contamination of host protein and the pH was restored using Tris-Cl pH= 9. After further washing, tissues were included in Tissue-Tek O.C.T. compound (Miles, Inc), frozen at -70°C and sectioned 6 μm thick. Sections were placed on poly-L-lysine treated microslides, airdried for 30

min, fixed in acetone for 10 min and dried for 15 min at room temperature. The slides were rehydratated and blocked using 1% BSA in PBS plus 0.1% Triton X-100; pH 7.2 (PAT) during 1 h. In cysticerci tissue sections, a second blocking was performed with anti-mouse sheep IgG (whole antibody , Amersham, U.K.) diluted 1:100 in PBS plus 0.1% BSA and incubated 1 h at 4°C. Slides of *T. solium* tapeworm and egg slides were incubated 1 h at 4°C with horse serum diluted 1:100 in PBS plus 0.1% BSA as a second blocking agent. Solutions were removed and the slides were overlaid with the appropriate sera from non-infected (negative control), infected (positive control) or anti-GK1 immunized mice diluted 1:10000 in PBS 0.1% BSA, overnight at 4°C, and then washed twice in PBS (pH 7.2). Finally, sections were incubated with FITC-labeled goat antimouse IgG (Zymed) diluted 1:50 for 1 h at room temperature. 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 were performed by the Wilcoxon Ranked Sum test, because many mice bore zero parasites in the immunized groups and because parasite intensity is really a discontinuous variable (i.e. 0,1,2,..n parasites). Data were considered as statistically significant at $P < 0.05$.

Computational methods in peptide structural analysis. Theoretical chemistry calculations of GK-1 started with its geometry optimization by methods based in molecular mechanics (1). Subsequently, the peptide was submitted to a single point calculation with the Austin Model 1(AM 1) semiempirical quantum chemistry method (2). In this way the electrostatic

charges, the electron density, the electrostatic potentials and the dipole moment of the molecule were obtained. Additionally, the log octanol/water partition coefficient, and distributed hydrophobicity of GK-1 were calculated. The software utilized was SPARTAN 4.0. (Wave function Inc, Irvine Ca U.S.A.), Insight II (Biosym/MSI, San Diego Ca U.S.A.) and Chem Plus (Hypercube, Inc. Ontario Canada).

RESULTS

Protective effect of peptide immunization against *T. crassiceps* cysticercosis. The effect of peptide immunization upon the number of cysticerci recovered from mice immunized with GK-1, GK-2 and GK-3 at different doses (0.5, 10, 50 μg per mice) in FCA is shown in Table I. Immunization with the GK-2 and GK-3 peptides did not confer protection, whereas three out of five mice immunized with GK-1 were completely protected at a dose of 50 μg per mice. To further evaluate this protective capacity, free GK-1 as well as BSA conjugated GK-1 and MAP-GK-1 emulsified in saponin were used for mice immunization in several repeated experiments. Table II confirms, in several instances, the high level of protection induced by GK-1 when used as an immunogen either free of carrier or conjugated to BSA.. Mice immunized with MAP-GK-1 did not lower mean parasite intensity although some mice were totally protected.

Determination of B cell epitope(s) on GK-1. To test for the presence of B-cell epitope(s) within the GK-1 peptide, we studied whether GK-1 immunization induced antibodies against the peptide and against whole *T. crassiceps* antigen by ELISA. Mice immunized with the monomeric non-conjugated form of GK-1 produced low but detectable levels of serum antibodies that reacted with GK-1 as well as with *T. crassiceps* in ELISA (Table III). The examination of anti-GK-1 antisera reactivity against histological sections of *T. crassiceps* revealed that these antibodies specifically react with *T. crassiceps* cysticerci at the tegument of the parasite. Furthermore, the anti-GK-1 antisera also reacted with all developmental stages of *T. solium* (Figure 1). A clear reaction was detected in the oncosphere contained inside the eggs and also in the egg wall. In

T. solium cysticerci, the reacting protein is concentrated in the spiral canal whilst in the tapeworm it is located in all the distal tegument. The specificity of all these antibody reactions in ELISA and immunofluorescence was demonstrated by specific preabsorption of antisera with free GK-1 and lack of reactivity of normal mouse serum.

Assessment of T-cell epitopes on GK-1 peptide. To identify the presence of T-cell epitope(s) on the GK-1 peptide we studied the proliferative response of spleen cells from mice immunized both with GK-1 or saponin alone. Spleen cells from mice primed *in vivo* with free peptide or saponin were stimulated *in vitro* with the same peptide (10 μg/ml), with *T. crassiceps* whole antigen (10 μ g/ml), or with ConA (5 μg/ml) as positive control. Results show that *in vitro* stimulation with GK-1 as well as with cysticercal antigens induced a strong proliferative response in cells from GK-1 immunized mice (Figure 2). Cells from mice injected with saponin (non-immunized mice) showed no proliferative response above background levels. These results confirm the presence in GK-1 of T-cell epitope(s).

In vitro stimulation of spleen cells with GK-1 increases $CD8^+$ T cells. To determine the responding lymphocyte subset by two-color fluorometry, the relative proportion of $CD4^+$ and $CD8^+$ T cells was examined in splenocytes stimulated with GK-1 peptide. The proportion of $CD8^+$ cells increased in 15 % whilst the $CD4^+$ cells remained constant (Figure 3). These findings indicate that immunization with the GK-1 peptide favored $CD8^+$ cytotoxic cell expansion.

T cell proliferation induced by GK-1 up-regulates IFN-γ production and down-regulates IL-4 production. Next, we examined the cytokine response expressed by GK-1 stimulated spleen cells in which an increased level of $CD8^+$ T-cell phenotype was detected. Splenocytes from non-immunized control mice produced a small amount of IL-4 and INF-γ that increased only after stimulation with ConA. In contrast, a clear increased amount of INF-γ and a low amount of IL-4 was found after stimulation of the splenocytes of GK-1 immunized mice both with GK-1 and with whole cysticercal antigens (Figure 2). These results together indicate that GK-1 induces growth and differentiation of $CD8^+$ T cells of the Tc1 phenotype.

GK-1 physicochemical properties. The dipole moment was directed towards the tyrosine end of GK-1. The octanol/water partition coefficient of the peptide was 7.92. However, its hydrophobicity was distributed in zones in accordance with the amino acid composition. Thus, the phenylalanine region was the most hydrophobic while alanine was the most hydrophilic and the glycine end was more hydrophobic than the tyrosine end. Planar rings from the tyrosine and phenylalanine showed high electron density. However, the higher electron density was observed for the oxygen atoms belonging to the carbonyl and hydroxy functional groups. Interestingly, the electrostatic potentials were displayed emerging mostly from these functional groups. Figure 4 shows the physicochemical properties of GK-1.

DISCUSSION

High levels of sterile immunity to experimental *T. crassiceps* cysticercosis were conferred to male mice vaccinated with a synthetic 18 amino acid peptide (GK-1) from the recombinant protein KETc7 of the parasite (11). The proportion of totally protected mice varied in experiments performed on different occasions from 40-70 %, whilst the average decrement in the immunized group's parasite intensity was 85-95 % of that expected from challenged control male mice. Variation in parasite intensity within experimental groups and between experimental sessions is a common finding in this form of cysticercosis due to factors not quite well identified, but that we attribute to variation in infectivity of each parasite harvest and inoculum. The statistical validity of the inferences drawn from these experiments is, however, not weakened if each experimental session includes its own internal control. Coupling of GK-1 to BSA or rearranging the peptide in an eight-pointed MAP construct did not result in an increased immunogenicity of the peptide, if not in fact reduced it somewhat. Sterile immunity is seldom induced in this form of cysticercosis by purified, natural or recombinant antigens (9, 21, 27); however, GK-1 induced higher levels of protection than those observed with the whole KETc7 recombinant protein published elsewhere (11). Research into the reasons why this peptide is so effective relative to other forms of antigen preparation, including the complete recombinant antigen KETc7 from which the peptide is derived (11), could perhaps reveal general principles of immunogenicity applicable to this and other vaccine preparations. Assuming that the binding properties of GK-1 relate to its immunogenicity and since these depend on its stereoelectronic properties, it is of interest to note the high dipole moment and the asymmetry in the electronic distribution of GK-1. Moreover, GK-1 showed high hydrophobic areas alternating with hydrophilic ones (Fig 4b), this dual hydrophilic-

hydrophobic property gives interesting possibilities of water and lipid interaction that may perhaps help in the peptide's reaction with B and T membrane-bound receptors. The external distribution of the hydroxyl groups favor water or hydrogen bonding judging by the rich and complex electrostatic potential of these hydrophilic groups, whilst the abundance of aromatic amino acids in GK-1 defines steric regions with high non-covalent electrostatic interactions capable of enhancing binding affinity that could also favor the peptide presentation by antigen-presenting cells (17). Peptide immunogenicity being altered by the structural changes inflicted by chemical coupling to BSA and the MAP construction, also points to a strong structure dependence of its biological functions. GK-1's electronic polarity, adequately positioned anchor motifs and similarities to motifs reactive with class I MHC molecules may explain the peptide's ability to induce a $CD8^+$ proliferative response (17). The involvement of a B cell response after immunization is documented by the presence of serum specific anti-GK-1 antibodies in vaccinated mice. Immune reactivity against the whole parasite antigens was greater than that against the GK-1 peptide itself, probably because of loss of reactivity of GK-1 once bound to the plate. T cell involvement is shown by the *in vitro* proliferative assays with spleen cells from GK-1 immunized mice which strongly responded to both GK-1 and cysticercal antigens. The composition of the resultant lymphocyte population was enriched in $CD8^+$ cells, which suggests the presence of T-cell class I epitope(s) in the GK-1 peptide. Although the direct participation of a cytotoxic response in the control of this parasite's reproduction remains to be thoroughly elucidated, the immune response elicited by this peptide features a prominent $CD8^+$ T cells response. If these cells aid in controlling cysticercosis, as they seem to do in intracellular parasitic diseases (14), cysticerci would have to present the epitope in a class I MHC context, a condition requiring that

the cysticercus bear a protein analogous to class I MHC molecules in it's cell contact surface, a possibility that has been raised before (28). Other factors contributing to parasite damage may be related to the IFN-γ, a cytokine that plays a central role in cell-mediated effector mechanisms in the protection observed in mice vaccinated against other parasites (4). The large amount of IFN-γ detected which could have increased the inflammatory response, and to the ensuing of macrophage activation in the parasite's vicinity. All this is also consistent with the low levels of antibodies induced by GK-1 immunization, which can be the consequence of the low levels of IL-4 preventing the activation of helper T cells. Low antibody levels and high T1 responses are well in keeping with recent trends in opinion about immune resistance to metacestodes diseases which place T1 cells in the forefront of protection (26), and add to well-established views that stress the role of antibody only in the destruction of early larvae developing from egg infection (20). Both mechanisms are, of course, non-disjunctive, and GK-1's high protective efficiency may well result from the synergic action of its capacity to trigger both the B and the T cell immune responses.

Additional features of GK-1 that deserve mention is that it is represented in an antigen fraction of 56 kDa in *Taenia crassiceps* cysticerci which induces high levels of protection against *T. solium* pig cysticercosis (27). This GK-1 peptide is also recognized by sera from *T. solium* infected humans (7). Furthermore, the identification of GK-1 by immunofluorescence at all stages of *T. solium* -the infecting egg, the hexacanth embryo, the metacestode and the tapeworm- make GK-1 a likely effective target for immune attack and an interesting candidate for vaccine design against the much dreaded *T. solium* cysticercosis.

ACKNOWLEDGMENTS.

This investigation was supported by Dirección General de Asuntos de Personal Académico IN 208395 and IN212798, Universidad Nacional Autónoma de México, and CONACYT G25955m, México.

The authors thank Dr. Felipe Massó for performing the peptides' sequence analysis and Nelly Villalobos for support in the obtention of the *T. solium* taperworm. Isabel Pérez Montfort aided in the translation of the manuscript.

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Figure Legends

FIG 1. Immunofluorescence staining of *T. crassiceps* (1) and *T. solium* (2,3) cysticerci; eggs (4) and adult tegument (5) of *T.solium*, incubated with sera from normal mice (a), *T. crassiceps* infected mice (b) and GK-1 immunized mice (c). The labeled epitope is clearly evident in structures accesible to the immune system. It is intensively expressed in the tegument (T) of *T. crassiceps* cysticerci (1c) and weakly in the *T. solium* cysticerci (2c). It is strong expressed in the cuticular folds of the spiral canal (SC) (3c), in the oncosphere (O) (4c) and in the distal tegument (T) of the tapeworm (5c). The arrowheads (3c) indicates the protonehridia.

FIG 2. T-cell proliferative response of spleen cells from non-immunized (control) and immunized mice determined by [^{3}H] thymidine incorporation on day 3 of culture. Data presented are mean ± SD of four individual mice separately assayed. Cytokine production (INF-γ and IL-4) was determined in collected cultured supernatant obtained 72 h poststimulation. Data are the means of four mice and are representative of two repeat experiments. Significantly increased proliferative response and INF-γ levels was achieved when cells from immunized mice were stimulated both with *T. crassiceps* antigens or GK-1 peptide.

FIG 3. Immunofluorescence and flow cytometer analysis were performed on spleen cells of non-immunized and GK-1 immunized mice after 3 days of culture without or with the presence of GK-1. Each bar represents the mean ± SD of positive cells obtained from 4 individual mice and are representative of three repeats experiments. The figure shows a significant increased in the percentage of $CD8^+$ and $CD3^+$ in immune mice compared with those non-

immunized ($P<0.05$) whilst the percentage of $CD4^+$ remained constant in both groups.

FIG 4. a) Amino acid arrangement of GK-1 and its optimized geometry. The red arrow shows the dipole moment pointing the vector's negative end towards tyrosine, b) Depicts a spacefilling model of GK-1, showing different degrees of hydrophobicity (red) or hidrophilicity (blue); c) Shows the encoded electronic density elicited by the molecule and its calibration bar at the right where oxygen atoms display red whilst negative zones corresponding to lone pairs of electrons in atoms displayed as yellow; d) Shows the bulk of electrostatic potential emerging mostly from the negative zones of the electron density surface.

Table I. ***Effect of immunization with three immunogenic peptides from KETc7 T. crassiceps recombinant protective antigen upon individual parasite intensities***

	Dose of peptide (μg /mouse)		
	0.5	10	50
Immunized with:			
GK-1	9.4 ± 10.9[a]	12.4 ±14.3	3.8 ± 5.8
	(0)[b]	(1)	(3)
GK-2	13.0 ± 7.9	9.2 ± 4.9	7.4 ± 2.7
	(0)	(0)	(0)
GK-3	9.9 ± 6.6	7.2 ± 3.8	7.4 ± 2.0
	(0)	(0)	(0)
Controls	14 ± 8.4		

[a] Mean ± SD of individual parasite intensity (i.e. number of cysticerci in each mouse) in groups of five mice. [b] Number of mice totally protected (i.e. bearing zero cysts). Groups of 5 male mice each were immunized with FCA (controls) or the respective peptides in FCA and challenged 15 days after the second immunization. Thirty days after challenge, mice were sacrificed and the parasite intensity determined.

Table II. *Protective immunity against murine T. crassiceps cysticercosis by immunization with cysticercal antigens and different forms of GK-1*

		Number of experiment:		
		1	2	3
Linear GK-1	Controls	6.9 ± 2.4[a]	14.0 ± 3.3	25.1 ± 4.6
		0/8[b]	0/5	0/9
	Immunized	*0.7± 0.8[c]	*2 ± 2	*1.3 ± 2.3
		4/7	2/5	7/10
MAP-GK-1	Controls	27.4 ± 27.1	24.6 ± 24.1	
		0/5	0/5	
	Immunized	48.1 ± 0.2	21.3 ± 28.8	N.D.
		2/10	4/10	
BSA-GK-1	Controls	53.20 ± 7.5	27.6 ± 21.9	12.3 ± 9.07
		0/5	0/5	0/3
	Immunized	*17.2 ± 10.7	*2.7 ± 7.2	*3.7 ± 6.01
		0/5	8/10	6/10
All antigens	Controls	17 ± 4.7	14.7 ± 3.7	
		0/5	0/5	
	Immunized	*1.47 ± 3.7	*1.33 ± 1.97	N.D.
		4/6	5/7	

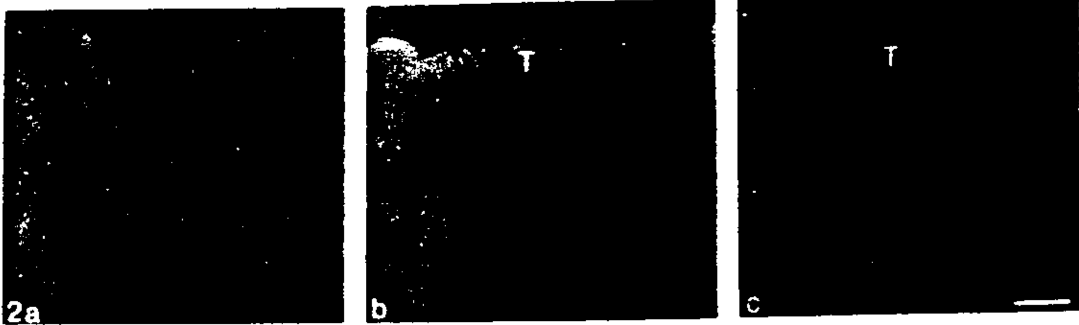
[a] Mean ± SD of individual parasite intensities in control mice injected with adjuvant alone or together with an immunizing antigen[c]. [b] Proportion of mice without a single parasite in the group. * Statistically significant differences between control and immunized mice at the 95 % confidence interval. Mice were immunized twice with soluble *T. crassiceps* cysticercal antigens (100μg per mice), GK-1 free of carrier, BSA conjugated or GK-1-MAP (10 μg per mice) in saponin. Control mice were injected twice with saponin in saline. Fifteen days after the booster, mice were challenged and sacrificed 30 days later. The parasite intensity affecting by each mouse was determined.

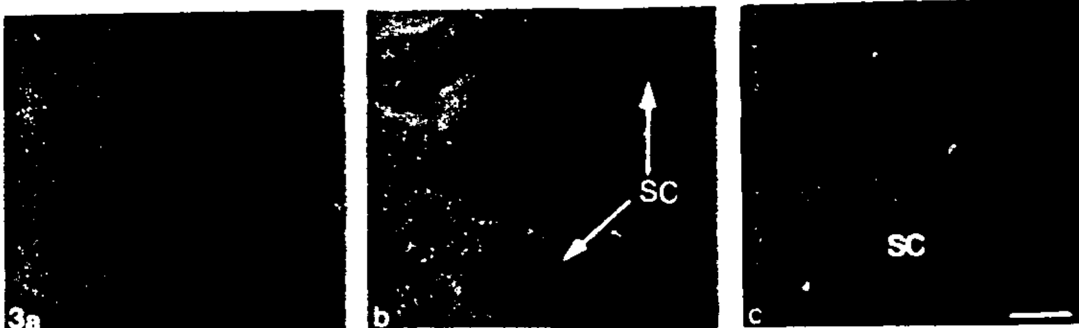
Table III. ***Level of antibodies measured by ELISA (O.D. $_{405}$ nm) during the immunization protocol.***

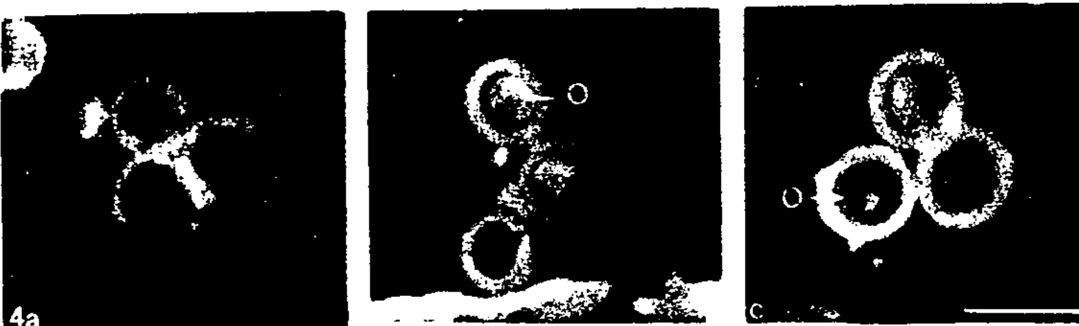
Non immunized	Immunized with: Saponin		Saponin + GK-1	
	1st Dose	2nd Dose	1st Dose	2nd Dose
0.129 ± 0.007^{+}	0.146 ± 0.005^{+}	0.164 ± 0.010^{+}	0.164 ± 0.020^{+*}	0.190 ± 0.020^{+*}
0.150 ± 0.01^{++}	0.189 ± 0.006^{++}	0.230 ± 0.027^{++}	0.247 ± 0.105^{++*}	0.431 ± 0.131^{+*}

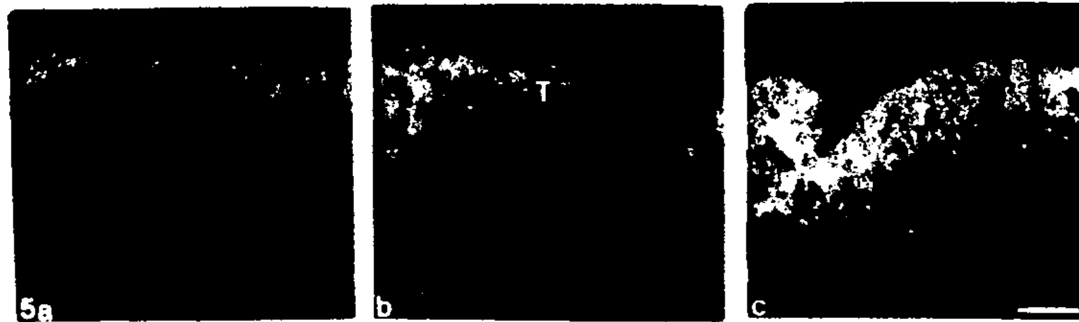
$^{+}$ Level of antibodies detected against GK-1 peptide or $^{++}$ *Taenia crassiceps* antigens in groups of ten mice each ± SD. *Statistically significant form the respective control values at 95 % confidence interval. Levels of murine antibodies raised during the course of immunization with GK-1 peptide. Mice were immunized and tested two weeks after each immunization. The serum Ab levels against GK-1 and *T. crassiceps* cysticercal antigens were determined by ELISA in immunized and non-immunized mice.

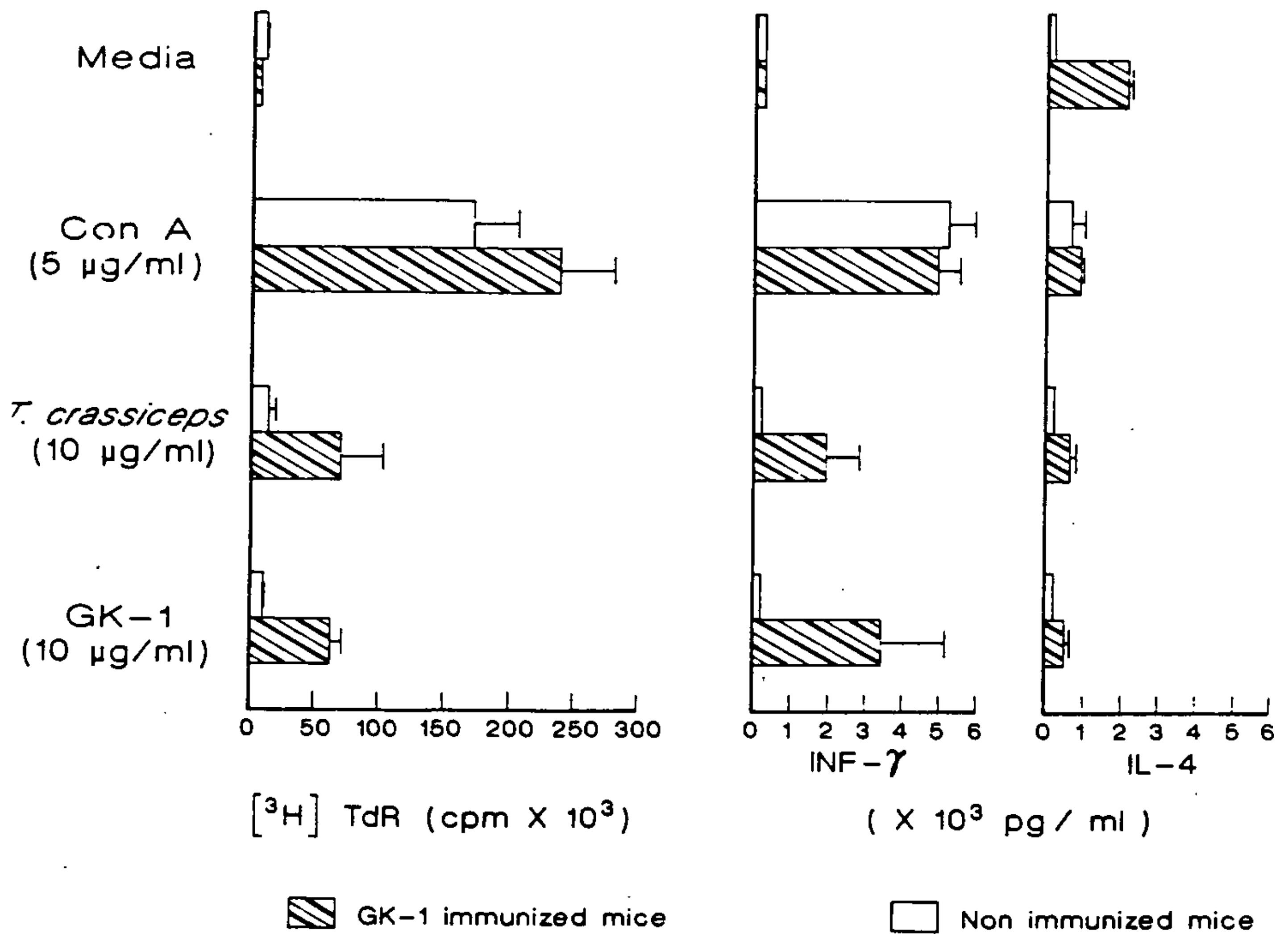
1a
b
c

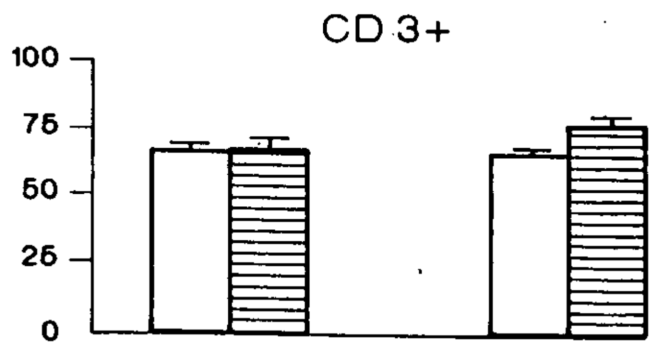

2a
b
T
T
c

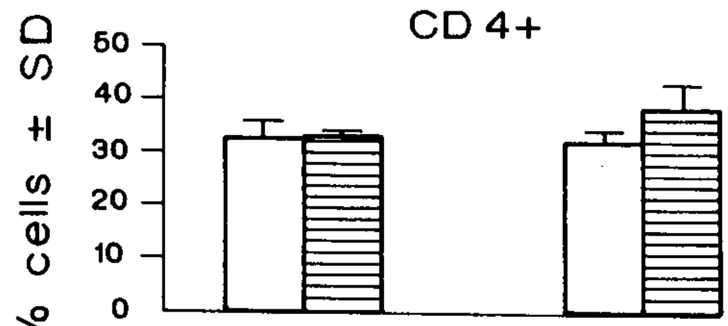

3a
b
SC
c
SC

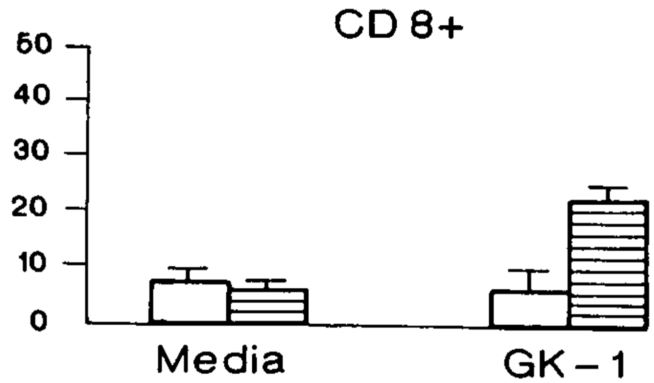

4a
O
O
c

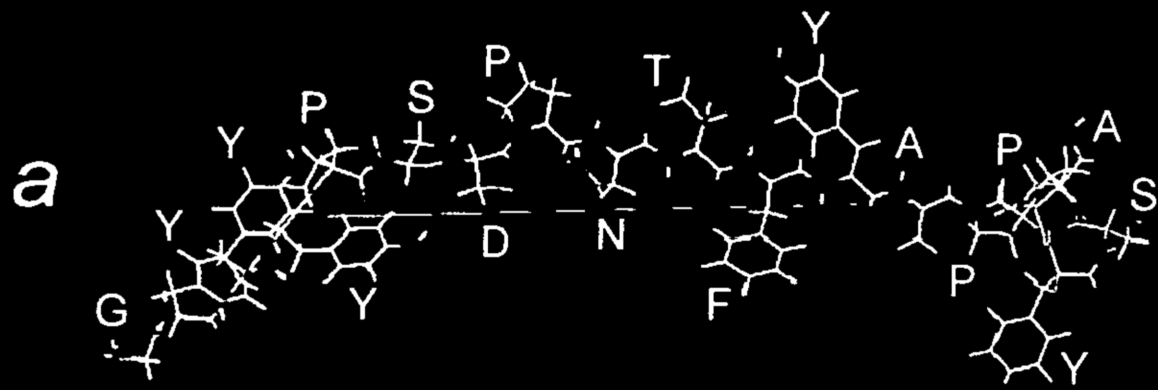

5a
b
T
c


Media
Con A
(5 µg/ml)
T. crassiceps
(10 µg/ml)
GK-1
(10 µg/ml)
0 50 100 150 200 250 300
[3H] TdR (cpm X 10³)
0 1 2 3 4 5 6
INF-γ
0 1 2 3 4 5 6
IL-4
(X 10³ pg / ml)
GK-1 immunized mice
Non immunized mice


CD 3+
100
75
50
25
0


% cells ± SD
CD 4+
50
40
30
20
10
0

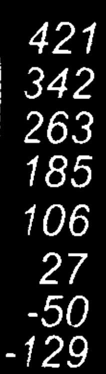

CD 8+
50
40
30
20
10
0
Media
GK-1
Non immunized mice
GK-1 immunized mice


a
Y
T
P
S
P
Y
A
P
A
S
Y
D
N
G
Y
F
P
Y

b

13.0
11.3
9.6
7.9
4.5
2.8
1.1
-0.6
-2.3
-4.0

c


421
342
263
185
106
27
-50
-129

d