UNIVERSIDAD NACIONAL AUTONOMA DE MEXICO

INSTITUTO DE INVESTIGACIONES BIOMEDICAS
UACPyP/CCH

FACTORES GENETICOS E INMUNOLOGICOS QUE PARTICIPAN EN LA SUSCEPTIBILIDAD A LA CISTICERCOSIS EXPERIMENTAL MURINA.

T E S I S

QUE PARA OBTENER EL GRADO ACADEMICO DE
DOCTORADO EN INVESTIGACION BIOMEDICA BASICA
P R E S E N T A:

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MEXICO D. F., 1995

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ABSTRACT

Cysticercosis is a parasitic disease caused by the larval stage of *Taenia solium*. Its high frequency and the severe consequences it bears on human heatlh and porciculture justify the attempts to control the disease. Two strategies are described in the present work: vaccination and genetic manipulation of the host to increase resistance to cysticercosis. The experimental model adopted to analyze these estrategies was murine cysticercosis caused by *Taenia crassiceps*.

Host genetic manipulation was intended to increase the innate resistance to cysticercosis by transferring genes associated to resistance. The genes of the Major Histocompatibility Complex (MHC) were considered likely candidates for resistance. The study of the genetic control of this parasitosis was therefore performed on diverse syngenic strains, syngenic BALB/c sublines, congenic and recombinant congenic mice strains for MHC genes. Protein Qa-2 encoded by the Q9 gene (a non-classic Class I Histocompatibility gene), was selected as probably associated to resistance against murine cysticercosis. To evaluate the effectiveness of this protein to transfer resistance, we generated transgenic mice by microinjection of Q9 gene. Two different transgenic mouse colonies were developed and both displayed greater resistance to parasitic growth than control mice, which implies that protein Qa-2 is in fact capable of effectively increasing resistance to murine cysticercosis by *T. crassiceps*.

Regarding the development of an efficient vaccine for host protection, we identified three antigenic fractions (56, 66 and 74 KD) in the *T. crassiceps* cysticercus, which seemed promising as protective agents both for pigs and for mice. These antigens were produced by recombination based on a genomic library of the *T. crassiceps* cysticercus. Five clones were selected from this library which were recognized by sera from cysticercotic pigs. Recombinant antigens of four of these named KETc1, KETc4, KETc7 and KETc12, were capable of significantly reducing the expected parasitic load in mice. This finding identifies these antigens as probable protective agents against pig cysticercosis.

The two studied strategies (vaccination and genetic manipulation) are considered complementary methods to increase host protection to cysticercosis.

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El presente trabajo se realizó en el
Instituto de investigaciones Biomédicas
de la UNAM, bajo la dirección de la Dra. Edda Sciutto Conde
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A aquellos a quienes mis ojos pudieron ver,
mis oidos escuchar y mi alma sentir.....
porque sin ellos esta no sería mi historia

A mis padres, hermanos y amigos

CONTENIDO

RESUMEN

La cisticercosis es una enfermedad parasitaria causada por el estadio larvario de *Taenia solium.* Su elevada frecuencia, así como las severas consecuencias que implica en la salud humana y en la porcicultura justifican los intentos para controlarla. En este trabajo se utilizaron dos estrategias: la vacunación y la modificación genética del hospedero con el fin de incrementar su protección contra esta parasitosis. Para acceder a estas estrategias se utilizó como modelo experimental al cisticerco murino de *Taenia crassiceps*. Con la modificación del hospedero se pretende incrementar su resistencia innata a la cisticercosis mediante la transferecia de gene(s) asociados a resistencia. Consideramos como genes candidatos de resistencia, a los del Complejo Principal de Histocompatibilidad (MHC) por lo cual el estudio del control genético de esta parasitosis se realizó en diversas cepas singénicas, sublíneas singénicas, congénicas y congénicas recombinantes en el MHC seleccionándose así a la proteína Qa-2 codificada por el gene Q9 (un gene no clásico de clase I de histocompatibilidad) como un candidato asociado a resistencia contra la cisticercosis murina. Con el fin de evaluar la efectividad de esta proteína para transferir resistencia, se generaron ratones transgénicos por microinyección del gene Q9. Dos colonias diferentes de ratones transgénicos se desarrollaron y en ambas se observó mayor resistencia al crecimiento parasitario que sus respectivos controles lo cual señala a la proteína Qa-2 con capacidad para incrementar efectivamente la resistencia a la cisticercosis murina causada por *T. crassiceps*. Respecto al desarrollo de una vacuna eficiente para la protección del hospedero, se identificaron en el cisticerco de *T. crassiceps* tres fracciones antigénicas, promisorias para proteger tanto al cerdo como al ratón (56, 66 y 74Kd). Estos antígenos se produjeron en forma recombinante a partir de una genoteca del cisticerco de *T. crassiceps*. De esta genoteca se seleccionaron cinco clonas que fueron reconocidas por sueros de cerdos cisticercosos de las cuales los antígenos recombinantes de cuatro, denominadas como KETc 1, KETc4, KETc7 y KETc12 fueron capaces de disminuir significativamente la carga parasitaria esperada en el ratón, hallazgo que las coloca como candidatos promisorios para proteger en contra de la cisticercosis porcina. Las dos estrategias estudiadas (vacunación y modificación genética) se contemplan como métodos complementarios para incrementar la protección del hospedero a la cisticercosis.

INTRODUCCION

El parasitismo es el resultado de una interacción biológica en la cual el parásito establece una relación de convivencia íntima y compleja con su hospedero (Behnke y Barnard, 1990). Evolutivamente se ha considerado que como consecuencia de esta interacción se produjeron una serie de adaptaciones mutuas que para el hospedero representaron modos de evitar o resistir la infección por el parásito, y para el parásito el utilizar al hospedero como medio idóneo en el cual vivir para reproducirse y asegurar su permanencia como especie (Sprent, 1962). Desde el punto de vista de frecuencia de adaptación del hospedero y del parásito, las modificaciones de cada uno no han sido proporcionales, es decir mientras que el hospedero ha recibido un mayor número de estímulos (diferentes parásitos, diferentes circunstancias de infección) a los que ha tenido que adaptarse, el parásito probablemente sólo ha tenido que modificarse para optimizar su relación con el hospedero (Dawkins y Krebs, 1979). Se ha establecido que las asociaciones hospedero-parásito se desarrollaron de un contacto inicial accidental entre ambas partes y que el establecimiento de una interacción bien definida se debió a que: 1) el parásito encontró en el hospedero un ambiente lo suficientemente propicio para vivir y/o 2) el parásito fue capaz de desarrollar una serie de mecanismos adaptativos que le permitieron establecerse y sobrevivir dentro de los parámetros fisiológicos y estructurales del hospedero (Wakelin, 1988). Cualquiera que sea el caso, la presencia del parásito generó una fuerte presión de selección sobre la población hospedera teniendo como consecuencia la evolución de mecanismos sofisticados capaces de controlar la extensión de la infección (Mitchison, 1990). Así, muchos organismos (en especial los vertebrados) desarrollaron mecanismos efectores de defensa controlados por sistemas regulatorios complejos y polimórficos, como el sistema inmune, lo que sugiere que su evolución estuvo influenciada para asegurar la sobrevivencia del hospedero (Klein, 1986).

CUADRO 1

Propiedades del parásito que favorecen su establecimiento en un hospedero inmunológicamente competente.

Modificación del parásito

Reducción de la inmunogenicidad de los antígenos parasitarios.

- Carencia de antigenicidad de las moléculas parasitarias: modificación estructural
- Expresión de moléculas parasitarias no inmunogénicas.
- Mimetismo molecular: producción de antígenos similares a los del hospedero por el parásito

Evasión de los mecanismos efectores del hospedero.

- Variación antigénica
- Liberación de antígenos

Resistencia a los mecanismos efectores del hospedero:

- Producción de enzimas anti-oxidantes
- Degradación enzimática de anticuerpos unidos a antígenos de superficie del parásito
- Liberación de factores anticomplementarios.

Modificación del hospedero

Modificación de las moléculas del hospedero por el parásito:

- Enmascaramiento con moléculas del hospedero y presentación como estructuras propias.

Modificación del sistema inmune del hospedero:

- Inducción para la producción de anticuerpos de baja afinidad
- Inducción para la producción de anticuerpos bloqueantes
- Inmunosupresión

Es importante señalar que esta respuesta inmune ocurre en un contexto en el que participan otros sistemas con los cuales interacciona como el sistema endócrino y el neuroendócrino (Besedovsky *et al.*, 1985; Grossman, 1984) y además puede verse influenciada por factores circunstanciales (micro-ambiente en el que ocurre la exposición del parásito al sistema inmune, estado fisiológico y edad del hospedero) que pueden modificarla y por lo tanto afectar el destino de la relación hospedero-parásito. Por otro lado, las variaciones del hospedero tendientes a modificar el ambiente al que el parásito se ha adaptado, han inducido a su vez cambios en el parásito, proceso conocido como coevolución, que le han proporcionado vías que le permitan continuar viviendo en su hospedero (Anderson, 1982). Respecto a los mecanismos desarrollados por los parásitos para establecerse en hospederos inmunológicamente competentes se han distinguido estrategias que pueden clasificarse en dos categorías: la modificación estructural del parásito y la modificación del hospedero (Cuadro 1).

La heterogeneidad genética del hospedero, en especial la generada como consecuencia del polimorfismo de los genes que regulan la respuesta inmune, permite una relación heterogénea en sus asociaciones con el mismo parásito. Así, algunos individuos desarrollaron estrategias más efectivas (resistentes) o menos efectivas (susceptibles) para el control de la parasitosis. Estos mecanismos de resistencia natural también se desarrollaron diferencialmente a nivel de especies lo cual condujo a una mayor especificidad del parasitismo. El concepto de resistencia natural fue definido por Frenkel y Caldweel (1975) como una combinación de componentes protectores del hospedero (barreras anatómicas, fagocitosis, digestión por neutrófilos y por células polimorfonucleares, respuesta inmune por células T y B) modulados por factores hormonales, nutricionales y genéticos. Esta resistencia depende no sólo de los factores intrínsecos del parásito y del hospedero, sino también de factores circunstanciales como localización geográfica del parásito y del hospedero, modo de vida del hospedero, etc.

CUADRO 2

Tipos de ratones (hospederos) genéticamente controlados de interés para el estudio de enfermedades infecciosas

Clases de ratones desarrollados	Características
Cepas Singénicas **BALB/c C57BL/10J** **C57BL/6J** **C3H** **DBA**	**Se definen como cepas que han sido mantenidas por más de 20 generaciones, por apareamiento entre hermanos carnales. Es esencialmente homocigota en todos los loci genéticos (excepto en aquellos que den mutaciones surgidas espontáneamente.**
Cepas Congénicas Resistentes **BALB.B** **BALB.K** **B10.D2**	**Son aquellas en las que fueron transferidos segmentos cortos de un cromosoma entre cepas singénicas utilizando procedimientos de retrocruza y selección**
Cepas Congénicas Recombinantes **B10.D2(R103)** **B10.D2(R107)** **B10.A(2R)** **A/J**	**Se caracterizan por ser cepas que han sido derivadas del cruzamiento de dos cepas singénicas, altamente puras (progenitoras), tras una posterior cruza al azar de los pares F1 para la obtención de la generación F2 y así generar series recombinantes.**
Ratones hipotímicos • ***timectomizados*** • • ***Atímicos*** • ***Desnudos***	**Los ratones timectomizados son aquellos a los cuales desde neonatos se les extirpo el timo.** **Los ratones atímicos se definen como aquellos que nacen con carencia del timo por herencia congénita.** **Los ratones desnudos (nu/nu) se caracterizan por haber sufrido una mutación (nu) que tuvo como consecuencia la ausencia del timo causandoles numerosas deficiencias en su sistema inmune.**
Ratones SCID	**Son ratones que han tenido una mutación homocigota que los caracteriza por ser muy deficientes en linfocitos T y B funcionales. Su nombre se deriva de las siglas del inglés Severe Combined Immune Deficiency (SCID)**
Ratones transgénicos	**Ratones modificados genéticamente por microinyección de genes en el pronúcleo masculino de embriones de 18 horas de fecundación**
Ratones Knockout	**Son una variante de los ratones transgénicos, en los cuales la variación genética consiste en la deleción de una gene a través de la metodología del "gene targeting" en células estaminales embriónicas.**

La interacción entre parásitos y hospederos es un tema que se ha estudiado extensamente en los últimos años y muchos grupos de investigación han profundizado el estudio de la respuesta inmune del hospedero ante una parasitosis, en el intento de conocer la patogénesis de las infecciones y facilitar el desarrollo de nuevas estrategias para su diagnóstico y control (Behnke y Barnad 1990). Para acceder a estos estudios ha sido de gran utilidad el uso de modelos experimentales cuyas características genéticas estén bien conocidas. Debido a que los ratones fueron de los primeros animales experimentales en los cuales se comenzaron a desarrollar sistemas de crianza accesibles que permitieran contar con cepas puras, estos fueron los primeros hospederos utilizados en los modelos de estudio para diversos fines de la investigación biomédica. Su uso permitió que se conociera más acerca de su genética y de su fisiología, con lo cual se extendió su empleo al campo de la inmunoparasitología, con el fin de comprender mejor las relaciones hospedero-parásito.

Las necesidades en la investigación han conducido al desarrollo de una gran variedad de cepas de ratones (Cuadro 2), muchas de las cuales han permitido identificar genes específicos asociados a resistencia y/o susceptibilidad a infecciones parasitarias. Las primeras cepas de ratones producidas, genéticamente controladas, fueron las cepas conocidas como singénicas (Festing y Blackwell 1988; Altaman y Dittmer 1979), en las cuales se considera que genéticamente todos los individuos son practicamente idénticos. Con el uso de estas cepas se ha podido establecer si el control genético de una cierta parasitosis es una característica compleja gobernada por muchos genes o si depende de unos pocos genes. Así, cuando se infectan distintas cepas singénicas con el mismo parásito y se encuentran que algunas son muy susceptibles, otras muy resistentes y otras, de susceptibilidad intermedia, sugiere un fenotipo en el que participan muchos genes. Sin embargo, si se observa que la población se separa claramente en grupos de resistentes y susceptibles, entonces es muy probable que en el control de la parasitosis

CUADRO 3

Susceptibilidad y resistencia a diversas parasitosis en el modelo experimental murino

Parásito	Modelo experimental	Cepas estudiadas	
		Susceptibles	Resistentes
Plasmodium berghei	Singénico		C57BL/6J
	Congénico Recombinante	A/J	
Leishmania donovani	Singénico	BALB/c, B6	DBA/2, C3H/He
	Congénico Recombinante	B10.D2	A/J
Leishmania tropica	Singénico	BALB/c, B6, DBA	AKR, C3H/He
	Congénico Recombinante	B10.D2	A/J
Leishmania mexicana	Singénico	CBA, BALB/c	AKR, C3H/He, B6
Leishmania braziliensis	Singénico		B6, BALB/c, C3H, SWR
	Congénico Recombinante	A/J	
Toxoplasma gondii	Singénico	BALB/c	B6, C3H, DBA/1
	Congénico Recombinante		B10.D2
Giardia muris	Singénico	C3H/He	BALB/c
Trypanosoma cruzi	Singénico	DBA/2, AKR	B6, DBA/1, B10
		C3H	BALB/c
	Congénico Recombinante	A.BY, A/J	CBA/B10.BR
		C3H.SW	
Trypanosoma brucei	Singénico	C3H/He	B6
Trypanosoma congolense	Singénico		B6
	Congénico Recombinante	A/J	
Taenia crassiceps	Singénico	BALB/cAnN,	B6, B10, BALB/cJ
		DBA/2	BALB/cByJ
	Congénico		BALB.B, BALB.K
			B10.D2
	Congénico Recombinante	A/J	B10.D2 (R103)
			B10.D2 (R107)
			B10.A(2R)
Taenia taeniaeformis	Singénico	BALB/c, DBA/2, B6	C3H, AKR, CBA/H
Trichuris muris	Singénico	B10, CBA/2	BALB/c, CBA
	Congénico	B10.D2	BALB.B, BALB.K
	Congénico Recombinante	B10.BR. B10.6	
Trichinella spiralis	Singénico	B6,	BALB/c, C3H, DBA
	Congénico Recombinate	B10.M (17R)	B10.Q
		B10.A (IR)	B10.S
		B10.AM	A/J
		B10.A(4R)	
Ascaris sum	Singénico	C3H, CBA	B6
	Congénico	B10.Br	
Nematospiroides dubius	Singénico	C3H	BALB/c, B6
	Congénico Recombinante		A/J
Echinococcus multilocularis	Singénico	AKR	B10, BALB/c
			DBA/2, DBA/1
	Congénico Recombinante		A/J
Schistosoma japonicum	Singénica	B6	
Schistoma mansoni	Singénico		DBA/2, C3H, B6, B10
	Congénico	BALB/B, BALB/K	B10.D2
	Congénico Recombinante	C3H.B10	B10.A

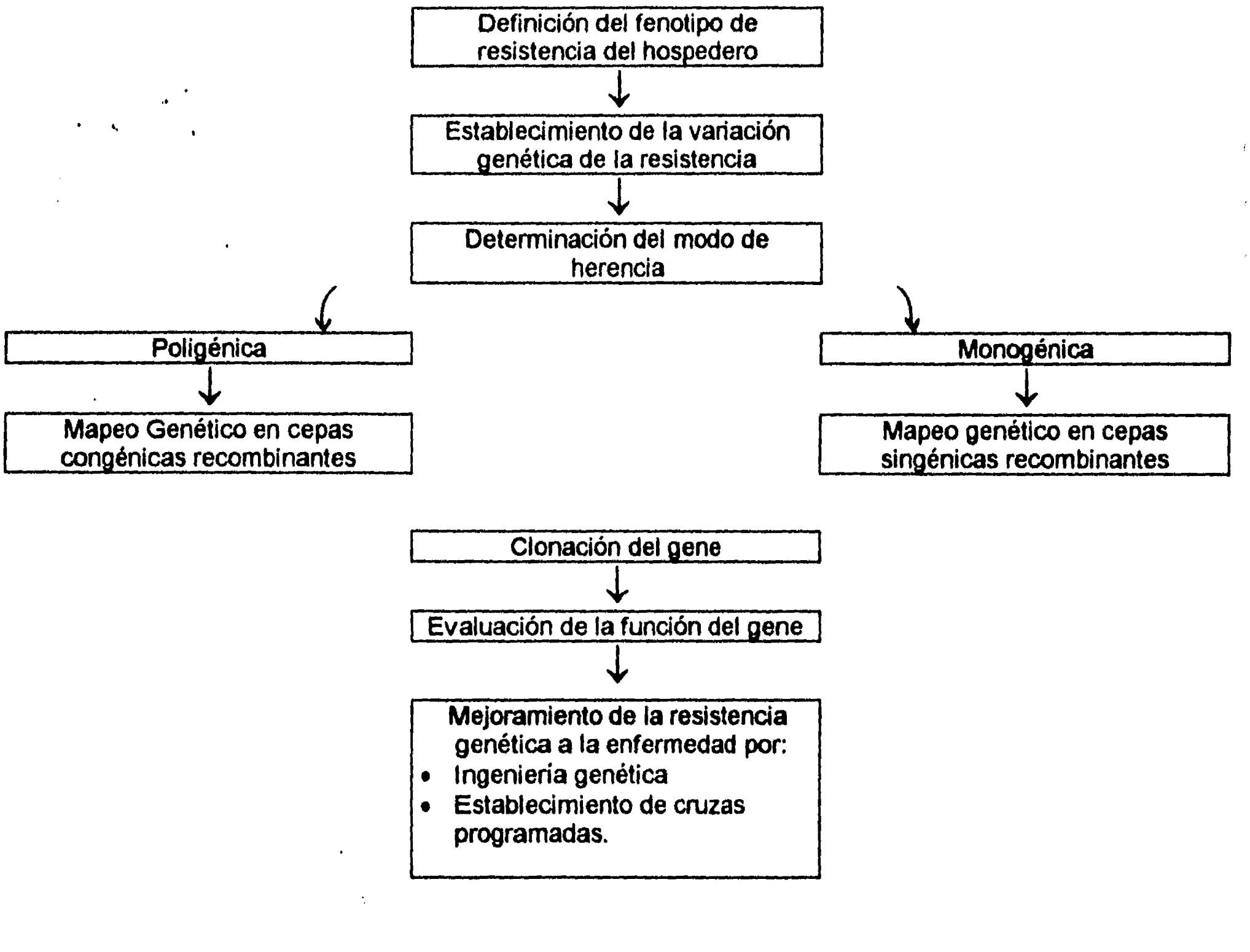
participen pocos genes o incluso un sólo locus con formas alélicas alternativas. A raíz del descubrimiento de que la respuesta inmune se encuentra controlada por un grupo de genes altamente polimórficos comprendidos en el Complejo Principal de Histocompatibilidad (MHC) conocido como H-2 en el ratón, se hicieron cruzas selectivas entre cepas singénicas particulares de ratones para la producción de cepas que solamente difieren entre sí en la región del H-2, pero que tienen el mismo fondo genómico (cepas congénicas), así como cruzas entre las diferentes líneas congénicas para la producción de cepas que solamente difieren en alguno(s) de los genes del H-2 (cepas congénicas recombinantes en H-2) (Klein, 1986).

Diversas parasitosis se han estudiado principalmente en las cepas singénicas, congénicas y congénicas recombinantes del ratón (Cuadro 3) encontrándose en todas ellas cepas resistentes y susceptibles al mismo parásito (Wakelin 1988: Fragoso *et al.*, 1991). Además en muchas de estas parasitosis puede observarse que no existe un patrón sencillo respecto a la influencia de los genes H-2, ya que cepas del mismo haplotipo pueden ser susceptibles y resistentes. Estas observaciones sugieren que el control genético de cada una de las parasitosis es complejo y sin duda se tendrá que recurrir al empleo de otros modelos experimentales específicos para cada parásito con el fin de identificar los genes que participan en la susceptibilidad o resistencia.

Con base en lo observado en diversas parasitosis se ha considerado que la susceptibilidad o resistencia a una cierta parasitosis puede ser el resultado de una deficiencia en algún componente que participe en la respuesta inmune del huésped o bien estar determinada por la presencia de genes que afecten los componentes regulatorios involucrados en el control homeostático del sistema inmune (Wakelin 1988). Respecto a esta última opción, se han hecho una serie de proposiciones acerca de las estrategias generales que se

FIGURA 1

Diagrama General de Flujo propuesto para el estudio y modificación de la susceptibilidad genética a infecciones parasitarias



pueden seguir con el fin de identificar los genes asociados a resistencia y/o susceptibilidad a una infección (Figura 1) (Gavora 1990; Festing y Blackwell 1988). Un aspecto importante a considerar para el estudio del control genético de enfermedades parasitarias es la definición del parámetro de resistencia y el modo como se heredan los genes ya que de las observaciones obtenidas dependerán las condiciones que se establezcan para intentar modificar el destino de una parasitosis por modificación genética. Respecto a la definición del parámetro de resistencia, se han utilizado diversos parámetros de medición, todos ellos sencillos y cuantificables en un gran número de animales. Entre los más utilizados se encuentran a) los signos y el tiempo que dura la enfermedad, b) la determinación del número de parásitos presentes después de un tiempo determinado del desafío c) la medición de componentes básicos de la respuesta inmune como anticuerpos, factores del complemento, citocinas, linfocitos, etc. d) muerte del hospedero. Respecto al modo de herencia de estos genes, en general se utiliza el sistema de cruzas dialélicas entre la cepa más resistente y la más susceptible estudiando las poblaciones derivadas de la F1, y la F2 así como las retrocruzas hacia los parentales resistentes y susceptibles (Festing y Blackwell, 1988). Así, es posible no sólo identificar si los genes son dominantes y recesivos, sino también estimar si la herencia de la resistencia está dada por un sólo gene o bien si el control es poligénico. Utilizando este tipo de estrategia se ha podido identificar la forma como se hereda la resistencia y/o susceptibilidad en varias infecciones parasitarias del ratón como en la leishmaniasis, tripanosomiasis, toxoplasmosis, triquinosis (Wakelin, 1988).

Con el conocimiento de la influencia crítica de los productos del MHC, (H-2 en el ratón), sobre las funciones básicas del sistema inmunológico del hospedero (Klein, 1986) (Apéndice I), se enfatizó la búsqueda de genes asociados a resistencia y/o susceptibiidad en este conjunto génico (Else y Wakelin, 1988; Sciutto *et al.* 1991; Wasson *et al.* 1979; Wünderlich *et al.* 1988; Wakelin y Donachie 1983; Blackwell *et al.* 1993)). Asi, mediante el uso de las

CUADRO 4

Genes de Respuesta Inmune del ratón que se han asociado a diversas parasitosis	
Genes del MHC	*Trichinella spiralis* y *Nematospiroides dubius*. En ambas, las cepas resistentes se caracterizan por ser I-E- (la presentación de antígenos en el contexto de I-E parece favorecer la instalación del parásito. *Plasmodium chabaudi*. Las cepas I-A^b se caracterizan por ser resistentes..
Genes que codifican para los receptores de células T	*Trypanosoma cruzi*. Las cepas susceptibles tienen un defecto en el receptor de la IL-2
Genes que codifican para las Inmunoglobulinas	*Taenia taeniaeformis*. Las cepas resistentes presentan mayores títulos de anticuerpos protectores.
Genes que codifican para linfocinas	*Trypanosoma cruzi*. No hay una estimulación del sistema inmune debido a una disminución de la IL-2
Genes específicos que confieren resistencia	*Leishmania tropica* (Lsh) y *Trichinella spiralis* (Ts)

cepas congénicas y congénicas recombinantes y siguiendo algunas de las proposiciones de Gavora se han logrado identificar genes específicos del H-2 que se encuentran asociados a la resistencia y/o susceptibilidad a algunas parasitosis como en *Trichinella spiralis* (Wasson *et al.* 1979) y *Toxoplasma gondii* (Blackwell *et al.* 1993). Los resultados obtenidos han establecido las bases para acceder a una nueva alternativa para el control de enfermedades parasitarias a través del mejoramiento genético de las especies. Dentro de estas estrategias genéticas se pueden distinguir dos alternativas principales: la transferencia de genes asociados a resistencia y el mejoramiento genético de las especies mediante cruzas selectivas. Respecto a la primera alternativa, se han propuesto cinco categorías de genes (asociados todos ellos a la respuesta inmune) como los candidatos más inmediatos para transferir resistencia: 1) genes del MHC, 2) genes que codifican para el receptor de células T, 3) genes que codifican para las inmunoglobulinas, 4) genes que codifican para linfocinas y 5) categoría de genes de resistencia específicos de una determinada enfermedad parasitaria no asociados a la respuesta inmune (Cuadro 4). Otra estrategia que se ha propuesto para el control de enfermedades infecciosas y que también involucra manipulación genética del hospedero es la inmunización *in vivo* con genes que codifican para anticuerpos monoclonales protectores (Müller y Brem, 1991). Esta posibilidad surgió como una variante de la transferencia de genes de inmunoglobulinas anticuerpo-específicos en la línea germinal del ratón (Storb, 1987). Entre estas alternativas, la transferencia de genes que se integren en la línea germinal del animal (animales transgénicos) ha sido la metodología más empleada en diversas áreas de la investigación biológica, sin embargo, su aplicación como alternativa para modificar la susceptibilidad innata a enfermedades infecciosas aún se contempla como estrategia potencial de aplicación y el único modelo experimental que se desarrolló con estos fines fue el de la influenza murina, en el cual produjeron un ratón transgénico utilizando el gene Mx1, en el cual se logró transferir resistencia contra esta enfermedad (Arnheiter *et al.* 1990).

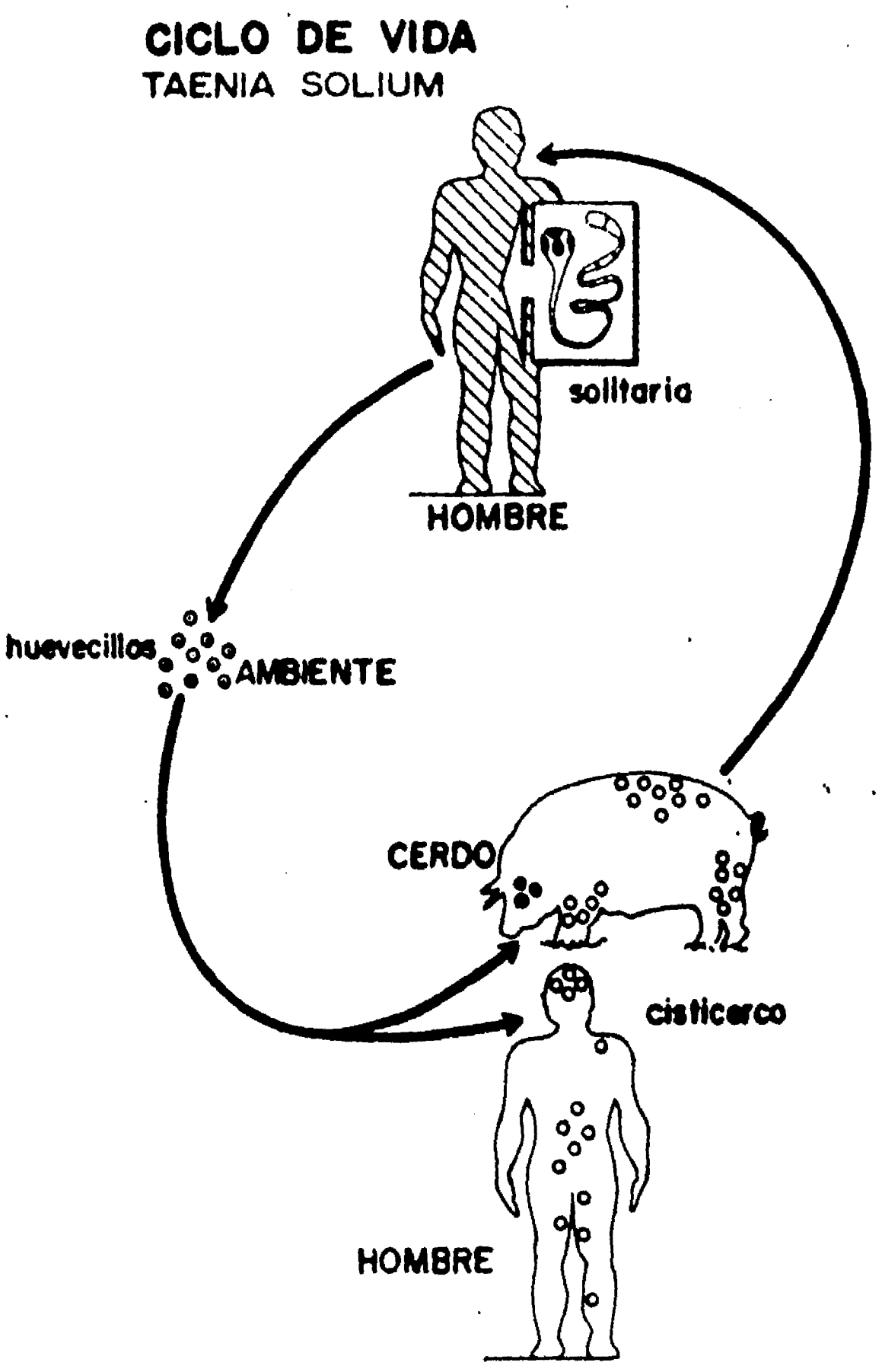


FIGURA 2. Ciclo Biológico de *Taenia solium*

En México, una parasitosis importante por su elevada frecuencia y por la grave enfermedad que causa en el humano es la cisticercosis causada por el establecimiento de la fase de metacéstodo de la *Taenia solium* . En el hombre la cisticercosis causa una enfermedad neurológica muy seria y en los cerdos es una de las principales causas de su decomiso en los rastros. Estudios seroepidemiológicos realizados utilizando los sueros de 70,000 individuos representativos de la población mexicana indican que la seropositividad en la población abierta es del orden del 1.2% en promedio (Larralde *et al.* 1992), lo cual implica un alto riesgo de contacto con el parásito. Si bien esta enfermedad ha sido erradicada en países desarrollados gracias al mejoramiento de las condiciones higiénicas e inspección sanitaria, en los países en vía de desarrollo las condiciones socioeconómicas han favorecido la persistencia de la transmisión (Aluja *et al.* 1987). Las posibilidades de transmisión se agravan si consideramos además, que la crianza rústica de este tipo de cerdos, representa aproximadamente el 40% del total de la producción porcina en México (Mazon, 1991).

El ciclo biológico de la *Taenia solium* (Figura 2) incluye una fase larvaria la cual puede ocurrir tanto en el cerdo (huésped intermediario) como en el hombre, y una fase adulta que solamente se presenta en el hombre (huésped definitivo). La ingesta de huevecillos de *T. solium* por el hombre o el cerdo conducen al desarrollo de cisticercos los cuales se alojan en diferentes compartimientos anatómicos (preferencialmente el músculo y el cerebro). El ciclo se completa cuando el hombre ingiere carne de cerdo infectada con el cisticerco, a partir del cual se desarrolla el gusano el cual se aloja en el intestino delgado del individuo infectado.

CUADRO 5

Nuevas estrategias propuestas para el control de la Cisticercosis Porcina

GENETICAS Aumentar la resistencia innata a la cisticercosis.	INMUNOLOGICAS Inducir protección aumentando la inmunidad por vacunación
Identificar genes asociados a resistencia →	Estudiar el control genético de los genes asociados a la resistencia: Herencia y Dominancia
↓	↓
Evaluación de la funcionalidad de los genes asociados a resisencia. →	Transferir genes asociados a resistencia a embriones de ratones susceptibles
	↓
	Evaluar la funcionalidad del transgene en el control de la cisticercosis murina
	↓
	Desarrollar metodologías de Transgénesis en cerdos
	↓
	Evaluar la capacidad de transferir resistencia en el cerdo incorporando en su genoma los genes asociados resistencia evaluados en el ratón
	↓

Evaluar los antígenos del cisticerco murino para prevenir la cisticercosis porcina	
↓	
Identificar antígenos protectores utilizando el modelo murino (fracciones de 56, 66 y 74. Kd)	
↓	
Confirmar el papel protector contra la cisticercosis porcina	
↓	
Producir los antígenos recombinantes (56, 66 y 74 KD) por clonación	
↓	
Evaluar la capacidad protectora de los antígenos recombinantes en ratones y cerdos.	
↓	

Evaluar la capacidad de incrementar la protección vacunando con los antígenos recombinantes a ratones y cerdos transgénicos

En los últimos años las investigaciones sobre cisticercosis se han incrementado de manera muy importante. Varios grupos de investigación han abordado este problema con diversos fines como el desarrollo de técnicas para el inmunodiagnóstico específicas, sensibles y reproducibles, capaces de permitir una detección temprana de pacientes cisticercosos (Larralde *et al.* 1986; Ramos-Kuri *et al.* 1992, Flisser y Larralde 1986). El conocimiento de los factores biológicos que intervienen en la relación de huésped-parásito, ha sido otro campo que se ha venido estudiando, si bien las limitaciones impuestas por los huéspedes afectados (hombre y cerdo) así como por el mismo parásito, han dificultado los avances alcanzados en esta área (Larralde *et al.* 1989). Otro campo de gran interés lo constituye el desarrollo de estrategias que permitan la prevención y control de la cisticercosis. Dentro de las estrategias propuestas, la mayor parte de los enfoques se han concentrado en establecer programas tendientes a mejorar las condiciones de saneamiento de las regiones altamente afectadas; el uso de drogas cisticidas y el desarrollo de vacunas eficaces aplicadas en el huésped intermediario, el cerdo, que logren interrumpir el ciclo biológico de esta parasitosis.

Considerando que el cerdo es indispensable para mantener el ciclo de vida de éste parásito, se abre la posibilidad de interferir con su ciclo de vida modificando la prevalencia de la cisticercosis porcina. En el grupo de investigación de la Dra. Edda Sciutto se ha considerado reducir la susceptibilidad innata de los cerdos tanto por vacunación como por manipulación genética de los hospederos susceptibles a la cisticercosis (Cuadro 5). Sin embargo considerando la dificultades económicas y experimentales involucradas en la experimentación con cerdos, se decidió comenzar con un modelo experimental de cisticercosis causada por el cisticerco de *Taenia crassiceps*. Este parásito infecta naturalmente a los roedores y puede mantenerse fácilmente en la cavidad peritoneal de ratones donde se reproduce rápidamente por gemación (Fremman, 1962). El ciclo biológico de este parásito (Figura 3) es muy

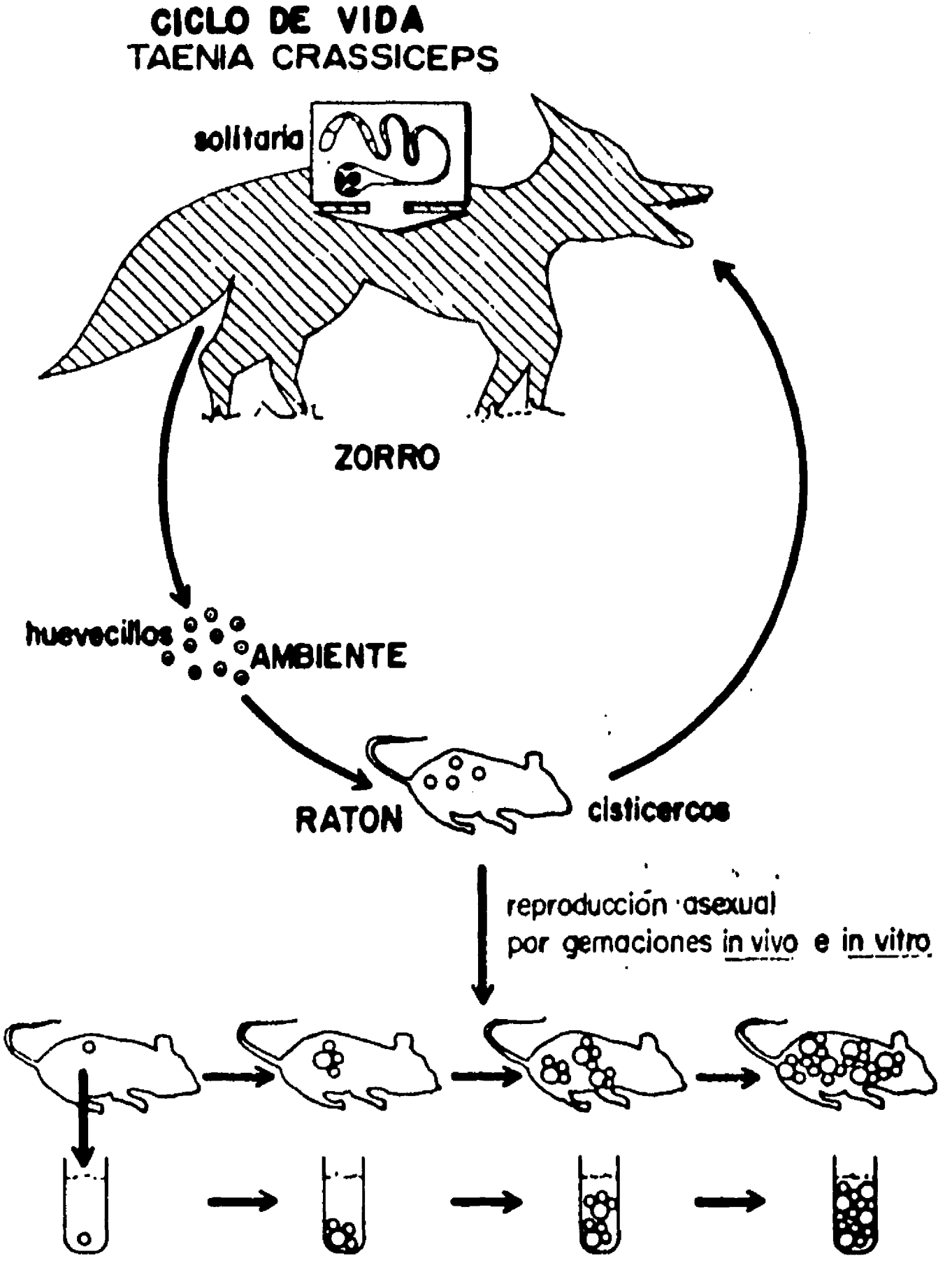


FIGURA 3. Ciclo Biológico de *Taenia crassiceps*

parecido al de la *Taenia solium*, e involucra como hospederos definitivos a los cánidos y como hospederos intermediarios a los roedores. En esta especie, se desarrolla un metacéstodo (*Taenia crassiceps*) que tiene la característica de ser muy similar antigénicamente al cisticerco de la *Taenia solium* (Larralde *et al.* 1989) y además se ha observado en experimentos de vacunación que induce inmunoprotección cruzada (Sciutto *et al.* 1990, Sciutto *et al.* 1995). Dado que infecta a los ratones, ofrece la posibilidad de explorar sistemáticamente factores biológicos involucrados en la susceptibilidad del hospedero así como antígenos de potencial interés para vacunación. En este trabajo de investigación, utilizamos este modelo experimental para intentar modificar la relación hospedero-parásito, tanto por vacunación como por manipulación genética del hospedero con el fin de establecer estrategias complementarias para el control de este parasitosis.

OBJETIVOS

OBJETIVOS

Este trabajo de tesis doctoral tuvo como objetivos:

1. Modificar la susceptibilidad a la cisticercosis experimental murina por modificación genética del hospedero susceptible.
    - Identificar genes asociados a resistencia a la cisticercosis murina y confirmar su funcionalidad.
    - Desarrollar la metodología de transgénesis en ratones.

2. Colaborar en la producción y evaluación de una vacuna contra la cisticercosis porcina.

3. Colaborar en el estudio de la respuesta inmune asociada a la parasitosis

ARTICULOS COMO PRIMER AUTOR

Journal of Clinical Laboratory Analysis 7:343–352 (1993)

Immunoenzymatic Assay That Measures the Expression of Murine Histocompatibility Antigens in Macrophages and Lymphocytes

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A convenient—simple, sensitive, rapid and reproducible—enzyme immunoassay to measure H-2 particulated and solubilized cellular antigens is described. Cellular antigens were measured by ELISA through the binding of specific biotinylated antibodies and streptoavidin-peroxidase conjugate to cells in suspension. Endogenous peroxidase activity of activated cells was inhibited by addition of sodium azide and H_2O_2 in acid conditions. The assay proved capable of distinguishing between two cell lines (EL-4/H-2^b and P-815/H-2^d) and even between the cells of three congenic mouse strains (BALB/B, H-2^b; BALB/c, H-2^d, and BALB/K, H-2^k) and was sensitive to as few as 2.5×10^4 cells/well. Results were comparable to those obtained with FACS. An inhibition version of this assay was found to be very useful for the detection of H-2 antigens present in whole antigen cells extracts.

Key words. MHC-ELISA, H-2 antigens, surface antigens

INTRODUCTION

Cell-surface antigens can be detected through a variety of methods including microscopic immunofluorescence (1,2), complement-mediated cytotoxicity (3,4), radioimmunoassay (RIA) (5,6), ELISA (7,8), and fluorescence-activated cell monitoring and sorting (FACS) (9–11). All these methods are adequate, but some are costly, whereas others require delicate standardization to yield reproducible and quantitative results or cannot be applied to activated cells because of their high enzyme content. We presently describe an immunoenzymatic assay (ELISA) to study H-2 surface antigen expression that is reproducible and capable of processing a large number of samples in a few hours, very economically, and useful for all cellular types.

The performance of this ELISA in evaluating the expression of MHC products in cell suspensions of different types of murine cells at different stages and in whole antigen cell extracts was tested. The basic idea behind this method is the detection of surface MHC antigens by reaction with biotinylated specific polyclonal or monoclonal antibodies. Ag-Ab binding is developed using the peroxidase-avidin system. One of the advantages of this method is that phagocytic cells can also be studied without modifying cellular antigens and retaining sensitivity to different levels of antigen expression, when inhibiting their endogenous peroxidase activity with sodium azide and H_2O_2. The method is also useful to detect MHC antigens in a complex mixture of antigens by inhibition of subsequent specific binding of anti-MHC antibodies to control cells.

MATERIALS AND METHODS

Antibodies: Purification and Biotinylation

Polyclonal anti-H-2^d and anti-H-2^b alloantisera were obtained by eight serial weekly intraperitoneal immunizations of H-2^d (BALB/c) and H-2^b (BALB/B) spleen cells of 10 BALB/B and 10 BALB/c mice, respectively. One week after the last immunization, the animals were bled (previously anaesthesized with Avertine) and their sera pooled within strains. Each alloantiserum was found to agglutinate erythrocytes of the strain used for immunization at dilutions $\geq$1:1,360, and not to react with other congenic haplotype red cells at dilutions $\geq$1:80 (data not shown).

Monoclonal antibodies in ascitic fluid, M1/42, rat-anti-mouse; M.8.114, rat-antimouse IE^{dk}-IA^{bds} (12); 34.2.12, mouse anti H-2D^d (13); 28.14.8S, mouse anti H-2D^b (14) were provided by Dr. Carol Reiss (Dana-Farber Institute); three IgG2a monoclonal antibodies: anti K^{k}D^k, anti K^{b}D^b, and anti K^{d}D^d from three cell lines (15-3-1S, 28-8-6S, and

Received April 3, 1992; accepted June 12, 1993.

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Abbreviations: BSA, bovine serum albumin; ELISA, enzyme linked immunosorbent assay; FACS, fluorescence activated cell monitoring and sorting; MHC, mayor histocompatibility complex; PBS, phosphate-buffered saline.

34-1-2S) from ATCC were purified from the respective culture medium (15). Sera from intact BALB/B and BALB/c mice were also used to determine levels of background reactions. Antibodies from immune sera and ascitic fluid were purified over an Affi-Gel Protein A MAPS (Monoclonal Antibody Purification Systems II, BIO-RAD).

For biotinylation, purified antibodies at concentrations of 100 μg/ml were dialyzed with 100 mM sodium bicarbonate, pH 8. Biotin N-hydroxysuccinimide ester (Sigma) was dissolved in dimethylsulphoxide (Merck) at 100 μg/ml, and 125 μl of this solution were added to each ml of antibody solution. The mixture was allowed to stand at room temperature for 2 hours and the reaction was stopped by addition of 1 M NH_4Cl, pH 7.2 in 1/10 the volume of the total reaction volume. Then, the mixture was dialyzed against phosphate buffered saline (PBS: 0.15 M NaCl, 10 mM sodium phosphate buffer, pH 7.2), sterilized by filtration (Milliphore No. 0.22) and stored at 4°C until used. Finally, antibody dilutions were freshly prepared for each assay.

Cells

Mice peritoneal cells and tumoral cell lines were used in these immunoenzymatic assays to study the expression of H-2 surface antigens. Peritoneal cells from BALB/c and BALB/B mice were isolated from intact mice as well as from mice infected intraperitoneally with *Taenia crassiceps* cysticerci (16,17). The EL-4 (H-2^b) cells, a gift from Dr. Guillermo Alfaro (Instituto de Cancerología, México, D. F.) were maintained in C57BL/6J mice (18), whereas P815 (H-2^d) cells were maintained in (BALB/c × DBA/2)F1 mice (19). Lymph node cells obtained from BALB/c, BALB/B, and BALB/K mice by standard procedures were also used.

Inhibition of Endogenous Cellular Peroxidase Activity

Endogenous peroxidase inhibition in activated peritoneal mouse cells and cell lines was carried out following the technique described by Malorney (20), as follows: one volume of the cell package was treated with 50 volumes of a solution containing 100 mM sodium azide (Sigma) in isotonic saline (150 mM NaCl), adding 0.04% of H_2O_2 (Merck) at room temperature for 20 min. Cells were washed twice with PBS and the pellet resuspended in a 100 mM citrate/phosphate buffer, pH 6.5 (one volume of cells per 50 volumes of solution) for 20 min at room temperature. After this, the cells in the pellet were washed three times with PBS and resuspended in 1% BSA in PBS to obtain the concentration used in the assay.

Cellular Immunoenzymatic Assay

To determine H-2 cell expression, the cells were added to 100 μl per well in V-shaped Immulon plates (Dynatech) at various cell concentrations (2.5×10^4, 5×10^4, 10^5, 2.5×10^5, 5×10^5), and spun for 7 min at 1,500 rpm. The supernatant was discarded and 100 μl per well of the diluted biotinylated antibodies (20 μg/ml) were added. One to 5 μg of antibody per well were used depending on the antibody employed. Plates were incubated for 90 min at 4°C and then washed three times. The bound antibodies were developed by adding 100 μl of 1:2,000 streptoavidine-peroxidase (Amersham) conjugate in 1% BSA in PBS during 60 min. After washing three times, the enzyme was detected on the plate by reaction with 100 μl of 0.4 mg/ml orthophenylendiamine (Sigma) and 0.03% H_2O_2 in 100 mM citrate phosphate buffer (pH 5) at room temperature for 20 min. The orthophenylendiamine reaction was stopped with 50 μl of 4 M sulphuric acid. Optical density readings at 492 nm were obtained with a Behring ELISA processor.

Flow Cytometer Assay

Lymph node cells from BALB/c and BALB/B mice were obtained by standard procedures. One million cells per well were reacted with anti-H-2K^dD^d and anti-H-2K^bD^b antibodies (diluted in saline with 5% fetal calf serum, FCS) at 1 μg/well, during 60 min at 4°C. Cells were then washed three times in saline (5% FCS) and incubated with anti-mouse IgG FITC conjugated (Sigma), for 1 hour at 4°C, washed three times and then fixed with 3% formaldehyde in saline. Fixed cells were then analysed by flow cytometry (FACSTAR plus, Becton-Dickson).

RESULTS

Measure of Murine Histocompatibility Antigen Expression

Figure 1 shows that the amount of anti-H-2 antibody (M1.42) specifically bound to H-2 molecules in resident peri

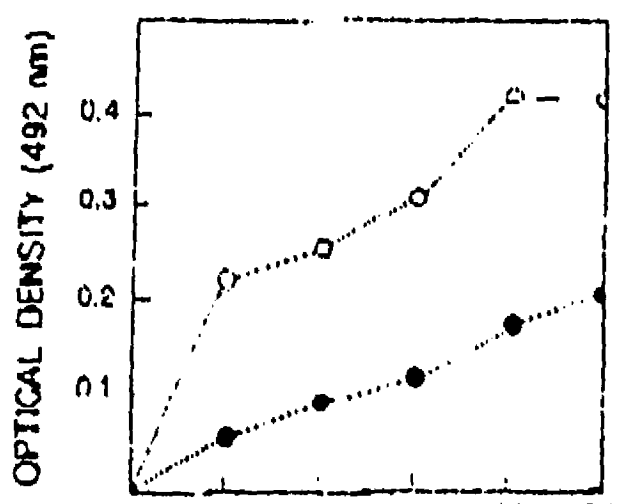

Fig. 1. Optical densities obtained for different numbers of cells using cells plus mouse IgG immunoglobulins (●), as control for nonspecific binding, and cells plus MoAb anti H-2 (○: M1.42. Each optical density value is the average of three determinations per sample.

TABLE 1. Identification of H-2 Antigens by Cell ELISA in Peritoneal Cells

Antibody		Optical Density (492 nm) ± S.D Peritoneal cells[b] (5×10^5 cells per well)	
Name	Specificity[a]	$H\text{-}2^d$	$H\text{-}2^b$
Polyclonal	anti-$H\text{-}2^d$	0.67 ± 0.21[c]	0.35 ± 0.03
34.2.12	anti-$I\text{-}2D^d$	0.59 ± 0.03	0.40 ± 0.01
M.5.114	anti-$IE^{dk}\text{-}1A^{dkq}$	0.51 ± 0.01	0.51 ± 0.01
Polyclonal	anti-$H\text{-}2^b$	0.35 ± 0.034	0.82 ± 0.01
28.14.8S	anti-$H\text{-}2D^b$	0.33 ± 0.033	0.66 ± 0.01
Pool of IgG mouse immunoglobulins from intact mice		0.17 ± 0.066	0.21 ± 0.01

[a]For full analysis of specificities of mAbs used, see references in Material and Methods.
[b]Peritoneal cells (50% macrophages, 30% lymphocytes) used in this study were recovered from peritoneum of BALB/c and BALB/B mice, 30 days after infection with 10 cysts of *Taenia crassiceps*. Cells were previously treated to inhibit intrinsic peroxidase activity.
[c]Each value is the average of three determinations per sample ± s.d.

toneal BALB/c macrophages increased with the amount of cells present per well. The assay produced reliable readings with as few as 2.5×10^4 cells per well. That this cell ELISA performed equally well with monoclonal as with polyclonal antibodies of different specificities in the determination of a variety of surface antigens is shown with peritoneal cells (Table 1), with different cell lines (Table 2), and with lymph node cells (Table 3). Table 3 also shows the reaction of the $H\text{-}2^d$ (BALB/c), $H\text{-}2^k$ (BALB/K), and $H\text{-}2^b$ (BALB/B) cell antigens specifically recognized by anti-$H\text{-}2K^dD^d$, anti-$H\text{-}2K^kD^k$, and anti $H\text{-}2K^bD^b$, by FACS analysis.

Identification of Cellular Haplotypes by Cell ELISA Inhibition

The identification of cellular haplotypes by inhibition of the specific ELISA reaction ($H\text{-}2^d$/anti $H\text{-}2^d$) is shown in Figure 2. A greater percentage of inhibition (70%) was observed when P815 ($H\text{-}2^d$) cells were used to inhibit the reaction than with EL-4 ($H\text{-}2^b$) cells (30%). Similar results were obtained when soluble $H\text{-}2^d$ and $H\text{-}2^b$ antigens were used as inhibitors of the $H\text{-}2^d$ anti $H\text{-}2^d$ reaction (data not shown).

TABLE 2. Identification of H-2 Antigens by Cell ELISA in Cell Lines

Antibody		Optical Density (492 nm) ± S.D Cell lines (2×10^5 cells per well)	
Name	Specificity[a]	EL-4 ($H\text{-}2^b$)	P815 ($H\text{-}2^d$)
Polyclonal	anti-$H\text{-}2^d$	0.19 ± 0.001[b]	0.96 ± 0.03
34.2.12	anti $H\text{-}2D^d$	0.18 ± 0.01	0.56 ± 0.03
M.5.114	anti-Ik^{dk},IA^{dkq}	0.39 ± 0.01	0.46 ± 0.28
Pool of IgG mouse immunoglobulins from intact mice		0.16 ± 0.04	0.21 ± 0.01

[a]For full analysis of specificities of mAbs used, see references in Material and Methods.
[b]Each value is the average of three determinations per sample ± s.d.

TABLE 3. Identification of H-2 Antigens by Cell ELISA and Flow Cytometer Analysis in Lymph Node Cells

	Cells		
Antibodies	BALB/c ($H\text{-}2^d$)	BALB/B ($H\text{-}2^b$)	BALB/K ($H\text{-}2^k$)
anti-$H\text{-}2K^dD^d$	0.7 ± 0.01[a]	0.28 ± 0.02	0.09 ± 0.01
	80%[b]	37%	
anti-$H\text{-}2K^bD^b$	0.14 ± 0.002	0.48 ± 0.01	0.117 ± 0.02
	6.7%	42%	
anti $H\text{-}2K^kD^k$	0.026 ± 0.001	0.12 ± 0.03	0.219 ± 0.015

[a]Average of the optical density (492 nm) ± s.d. obtained by cell ELISA per triplicate.
[b]Percentage of fluorescence obtained by FACS analysis.

Inhibition of Endogenous Cellular Peroxidase Activity

Two of the cellular types used, macrophages and lymphocytes, have considerable intrinsic peroxidase activity. This activity was completely inhibited in macrophages by treatment with sodium azide in isotonic saline solution containing H_2O_2, and only partially inhibited in lymphocytes (Table 4). This acid substrate treatment did not modify the surface antigenic properties as shown in Table 1.

DISCUSSION

The ELISA method described herein was developed to measure MHC product expression in different cell types. The assay uses cells in suspension, thereby decreasing possible modifications of cell surface antigens (21) upon their fixation to solid surfaces, and lessening the complication of nonspecific reagent binding commonly seen when poly-L-lysine is used to facilitate adhesion of cells to plastic (22).

Most other ELISA methods require at least $1-10 \times 10^5$ cells per well (8,22), whereas this method can detect different classes of histocompatibility antigens with quantities as low as 10^4 cells per well. The assay detected differences in the amount of histocompatibility antigens of different numbers of cells, and it also distinguished between H-2d, H-2b and H-2k antigens, despite the presence of major sequence homologies (23). Optical densities varied (Tables 1–3; Fig. 1) because of differences in the number and type of cells and in the antibodies employed. Since the assay was carried out at 4°C, the possibility of internalization and exchange of membrane proteins was reduced to a minimum. Nonspecific binding of immunoglobulins was evaluated using normal immunoglobulins (purified from sera of intact mice) and the values obtained were very low, as shown in Tables 1 and 2. No interference by Fc receptors was detected, as described in other assays (7,24), possibly due to the effect of biotinylation of the Fc receptor, although this suggestion must be tested.

Similar levels of discrimination among cells types by their reactions with specific antibodies were found by FACS in the $H\text{-}2^d$ and $H\text{-}2^b$ haplotypes ($H\text{-}2^k$ was not tested), thus confirming the usefulness and reliability of the method proposed here.

Our results also show that the high intrinsic peroxidase ac-

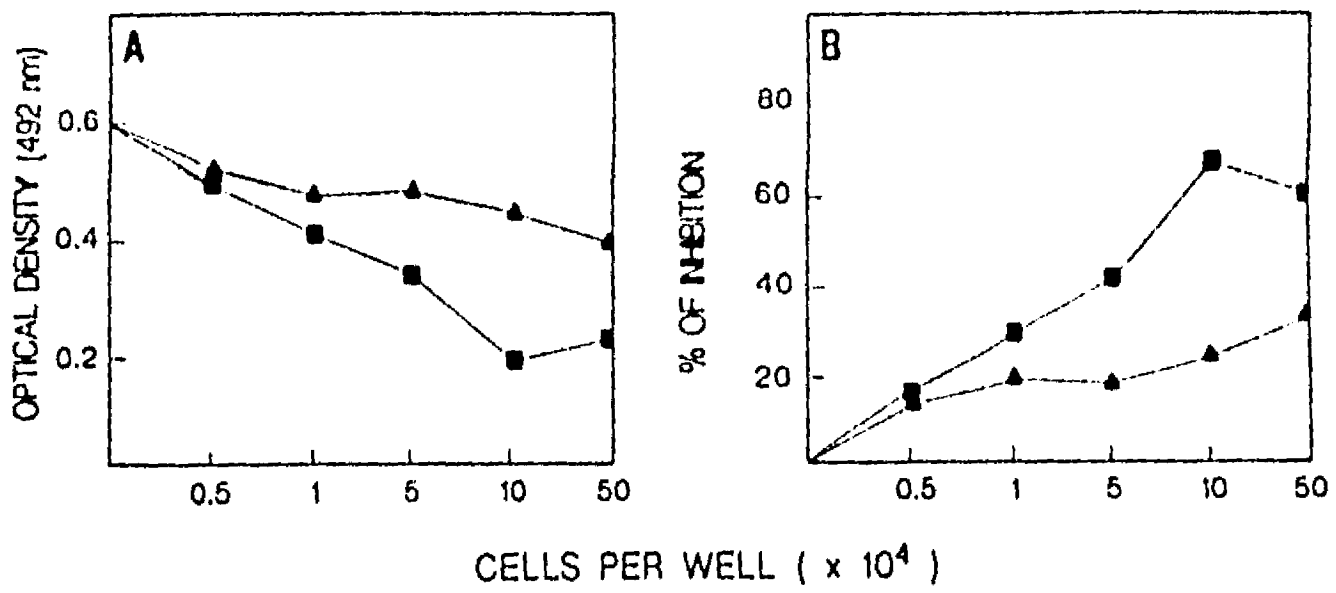

Fig. 2. Optical densities (A) and Percentage of Inhibition (B) obtained with P815 (H-2^d) (■), and EL-4 (H-2^b) (▲) as inhibitor cells of the specific reaction of H-2^d node lymphatic cells from BALB/c mice with H-2^d polyclonal biotinylated antibody.

TABLE 4. Inhibition of Intrinsic Cellular Peroxidase by Acid Substrate Treatment[a]

	Optical Density (492 nm)			
	Macrophages (Resident peritoneal BALB/c cells)		Lymphocytes EL-4 (H-2b)	
Number of cells per well	Noninhibited	Inhibited	Noninhibited	Inhibited
10^4	0.10[b]	0.01	0.10	0.07
10^5	0.48	0.01	0.16	0.10
10^6	0.74	0.01	0.41	0.23

[a]Peroxidase activity quantification in intact and treated cells for the inhibition of the intrinsic peroxidase activity.
[b]Average of three determinations.

tivity of macrophages was significantly inhibited by treatment of cells with sodium azide in isotonic saline solution containing H_2O_2, which did not affect the expression of MHC antigens. This was not observed with the low intrinsic peroxidase activity of lymphocytes.

Thus this ELISA method of quantifying MHC antigens on cells is a reliable and accessible tool for measuring the expression of MHC antigens and other surface antigens in different types of cells and microenvironments, alternative to others more cumbersome or costly.

ACKNOWLEDGMENTS

This project was supported by CONACyT and by the Universidad Nacional Autónoma de México (DGAPA, IN-207489). The authors thank Mrs. Violeta Aguilar for her secretarial work and Isabel Pérez Montfort for her edition of the original manuscript in English.

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Dear Dr. Sciutto:

This is to acknowledge receipt of your manuscript entitled *Genetic control of susceptibility to Taenia crassiceps cysticercosos*. The manuscript will be sent to the Board of Editors, and you will be informed as quickly as possible concerning their decision.

Yours sincerely,

Donna Devine

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GENETIC CONTROL OF SUSCEPTIBILITY TO *Taenia crassiceps* CYSTICERCOSIS

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SUMMARY

Our laboratory previously reported that genes within the major histocompatibility complex influence the intensity of *Taenia crassiceps* murine cysticercosis. This genetic control which is readily apparent in mice of BALB background was further studied in *H-2* congenic and recombinant B10 mice as well as in BALB/c substrains differing in expression of Class I Qa-2 antigens. After intraperitoneal infection, similarly low parasite numbers were found in all B10-derived strains tested, regardless of *H-2* haplotype, indicating that, contrary to the observations in BALB mice, the effect of *H-2* genes in controlling susceptibility is overridden in mice of B10 background. On the other hand, BALB/c substrains differed significantly in susceptibility: BALB/cAnN was highly susceptible, whereas BALB/cJ, in contrast, was highly resistant and BALB/cByJ showed intermediate susceptibility. We suggest that susceptibility or resistance in BALB/c substrains may be associated to differences known to distinguish them, such as serum testosterone levels and Qa-2 protein expression. In bidirectional F1 hybrids of C57BL/6J and BALB/cAnN resistance to cysticercosis was inherited as a dominant autosomal trait. In F1 hybrids of BALB/cJ with BALB/cAnN, BALB/cByJ and BALB.K resistance was also inherited as a dominant trait. However, in (BALB/cAnN x BALB/cByJ)F1 and (BALB/cAnN x BALB.K)F1 hybrids, dominant susceptibility to cysticercosis was observed.

INTRODUCTION

It has been extensively reported that the immune response to parasites is influenced by the genotype of the host (Wakelin, 1985). Considerable attention has been directed to the involvement of genes linked to the Major Histocompatibility Complex (MHC) in controlling the response to infection. Much of this work derives from the well established role of MHC gene products in the regulation of T–cell functions and the T–dependent nature of anti-helminth immunity. Thus, it has been demonstrated that *H-2* linked genes play a role in susceptibility or resistance of mice to several parasites (Blackwell *et al.* 1980; Vadas, 1980; Lloyd, 1987; Wassom *et al.* 1987; Backwell & Roberts, 1987; Else & Wakelin, 1988). However, the effects of *H-2* genes is known to be modified by the mouse genetic background (Carter *et al.* 1993; Behnke & Wahid, 1991).

Cysticercosis by *Taenia solium* is a frequent cause of serious illness of humans and pigs in Mexico and other countries of Latin America, Asia and Africa. The risk of contracting cysticercosis is thought to be related to the magnitude and frequency of exposure to eggs of *T. solium*. Evidence implicating biological factors in susceptibility

antigens has been found (Del Bruto *et al.* 1991). Further, the very heterogeneous clinical pictures and forms of evolution of the disease, argue for a complicated network of factors and events belonging to parasite and host concurring in the pathogenesis of cysticercosis. Systematic exploration of the role of genetic factors in susceptibility to cysticercosis is hardly possible studying man, and most laborious and costly in pigs. However, other cestodes -*Taenia crassiceps* and *Taenia taeniaeformis*- that affect mice are suitable for experimentation. *Taenia crassiceps* experimental infection is simply attained by injection of metacestodes in the peritoneal cavity of mice where they reproduce by budding. Initial observations in *Taenia crassiceps* murine cysticercosis, showed that genes linked to *H-2* influence parasite growth. Thus, significant differences in the extent of parasitosis were found between mice carrying the $H\text{-}2^d$ haplotype (BALB/cAnN, DBA/2J), which were the most susceptible, and mice with $H\text{-}2^b$ (BALB.B, C57BL/6J) or $H\text{-}2^k$ (BALB.K, C3H/HeJ, C3H/FeJ) haplotypes, which were comparatively resistant. A preliminary analysis in the naturally recombinant A/J strain, which was also susceptible, suggested that $H\text{-}2D^d$ and/or $H\text{-}2S^d$ genes, could be involved in susceptibility to this parasitosis (Sciutto *et al.* 1991).

The identification of genes involved in host resistance may be of relevance for the development of new strategies for the control of infectious disease in commercially important livestock and humans (Malo & Skamene, 1994). *T. crassiceps* cysticercosis seems to be a convenient model in which to identify genes involved in host innate resistance to cestodes and to study the immunological mechanisms underlying it. Therefore, we have undertaken a systematic study of the genetic control of resistance to *T. crassiceps* as a first step in the identification of the genes involved.

MATERIALS AND METHODS

Mice

BALB/cByJ, C57BL/6J, A/J, C3H/HeJ, BALB/cAnN, BALB.B and BALB.K strains were obtained from our animal facility. Original stocks were from The Jackson Laboratories (Bar Harbor, Maine), and Dr. Michael Bevan (Seattle University). C57BL/10J (B10), B10.D2nSn, B10.D2 (R103), B10.D2(R107), B10.A(2R)/ SgSn were obtained from the Jackson Laboratory (Bar Harbor, Maine). BALB/cJ mice were obtained from the National Institute for Medical Research, Animal Centre, London UK. Female (BALB/cAnN x BALB/cJ)F1, (BALB/cJ x BALB/cByJ)F1, (BALB/cJ x BALB.K)F1, (BALB/cAnN x BALB.K)F1, (BALB/cAnN x BALB/cByJ), (C57BL/6J x BALB/cAnN)F1 and (BALB/cAnN x C57BL/6J)F1 hybrids were produced in our animal facility. The experiments reported herein were conducted according to the principles set forth in the Guide for the Care and Use of Laboratory Animals, (NIH Publication No. 86-23, revised 1985)

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Parasites and Infections

Taenia crassiceps ORF strain (Freeman, 1962) has been maintained by serial intraperitoneal passage in BALB/cAnN female mice for eight years in our Institute. Parasites for infection were harvested from the peritoneal cavity of mice, one to three months after inoculation of ten cysticerci per mouse as described (Sciutto *et al.* 1990). For the evaluation of susceptibility to cysticercosis, male and female mice of the strains above were injected intraperitoneally with ten small (2mm in diameter), non-budding *Taenia crassiceps* larvae, suspended in phosphate-buffered saline (PBS). Thirty days after infection, mice were sacrificed and *T. crassiceps* cysts inside the peritoneal cavity were counted.

Statistical Analysis

Statistical comparisons between groups were carried out by the Wilcoxon Ranked Sum test. On the basis of the parasite load, the mouse strains tested were grouped into three statistically different ($P<0.01$) clusters: highly susceptible, intermediate susceptible and resistant.

RESULTS

Susceptibility of H-2 congenic and recombinant congenic B10 mouse strains to T. crassiceps

Susceptibility to cysticercosis was studied in B10 and its *H-2*^{d} congenic and recombinant strains; BALB/cAnN and C57BL/6J were included for comparison. Table 1 shows the mean number of larvae found in each strain 30 days after infection. As expected, BALB/cAnN was highly susceptible (132.96 cysticerci in females and 27.49 in males) and C57BL/6J was resistant (18.6 cysticerci in females and 0.34 in males). Differences in susceptibility between sexes, noted before (Sciutto *et al.* 1990) were also mantained; i.e., males were consistently less susceptible than females. C57BL/10J, (*H-2*^{b}) and its congenic B10.D2, (*H-2*^{d}) were as resistant as C57BL/6J; again males bore a smaller parasite burden than females. The three recombinant congenic strains B10.D2(R103), B10.D2(R107) and B10.A(2R) were also resistant. Thus, the apparent effect of *H-2* in controlling parasite load, observed previousiy in *H-2* congenic BALB mice (Sciutto *et al* 1991), is not seen in C57BL-derived strains. This indicates that gene(s) in C57BL background confer resistance to the parasitosis such that override the effect of *H-2* if any.

Susceptibility and resistance to murine cysticercosis in BALB mouse strains

To further explore the effect of MHC on susceptibility to *T. crassiceps*, a second series of experiments was carried out in mice of BALB genetic background, including the substrains BALB/cByJ and BALB/cJ in addition to BALB/cAnN. Interestingly,

different degrees of susceptibility among these three *H-2*^{d} BALB/c substrains were found, both in males and females (Table 2). BALB/cAnN, was highly susceptible (122.1 cysticerci in females and 30.29 in males), whereas BALB/cByJ showed intermediate susceptibility (31.1 cysticerci in females and 4.46 in males) and BALB/cJ was the most resistant (7.2 cysticerci in females and 0.39 in males). In fact, BALB/cJ was the most resistant of all strains we tested, including mice of the B10 series. Parasite growth in females of the BALB.B (*H-2*^{b}) and BALB.K (*H-2*^{k}) strains was similar to that observed in BALB/cByJ females ($P<0.01$), whereas males of these two congenic strains were as resistant as BALB/cJ male mice.

Susceptibility to murine cysticercosis in F1 hybrids

To study the inheritance of resistance and susceptibility to murine cysticercosis, (susceptible x resistant)F1 hybrids of two strains of disparate *H-2* and background, namely, (BALB/cAnN x C57BL/6J)F1 and (C57BL/6J x BALB/cAnN)F1 were infected. As shown in Figure 1 resistance was inherited as a dominant autosomic trait; there were no significant differences in disease susceptibility between the F1 hybrids and the resistant C57BL/6J parent ($P<0.01$). Susceptibility was also examined in F1 hybrids of the three BALB/c substrains and F1 mice crosses of BALB/cAnN and BALB/cJ with BALB.K. Figure 2 shows that female F1 hybrids having BALB/cJ as one of the parents, namely, (BALB/cAnN x BALB/cJ)F1, (BALB/cJ x BALB/cByJ)F1 and (BALB/cJ x BALB.K)F1 resistance was also inherited as a dominant trait. In contrast, (BALB/cAnN x BALB.K)F1 and (BALB/cAnN x BALB/cByJ) F1 female mice were found to be as susceptible to cysticercosis as the highly susceptible parent, BALB/cAnN, indicating that in these two crosses susceptibility was inherited as a dominant trait (Figure 2). Same patterns of inheritance were observed in studies of susceptibility done in F1 male mice (data not shown).

DISCUSSION

In the present study we examined the influence of *H-2* and background genes on the susceptibility and resistance to *T. crassiceps* infection. Collectively, our results indicate that background genes play a major role in the control of cysticercosis. The contention, based on the different parasite loads of *T. crassiceps*-infected BALB/cAnN (*H-2*^{d}), and its congenics BALB.B (*H-2*^{b}) and BALB.K (*H-2*^{k}), that genes linked to *H-2*^{d} were a factor confering high susceptibility (Sciutto *et al* 1991) did not hold on the C57BL/10J background where an intermediate degree of susceptibility was observed regardless of *H-2* haplotype. Thus, the participation of *H-2*, evidenced in congenic BALB mice and in a set of inbred strains (Sciutto *et al* 1991), in determining the outcome of the infection was not confirmed in mice of the B10 series. One possible explanation for these findings is that

MHC does not participate in an important way in the control of *T. crassiceps* infection, however, it may be that the effect of C57BL background genes overrides any function played by *H-2*. Variations in the influence of MHC genes in different host genetic backgrounds on the response to several parasites have been previously observed; the BALB background seems to provide a more favourable genetic environment in which to evaluate the effect of MHC genes on the susceptibility or resistance to toxoplasmosis (Blackwell *et al.* 1993), leishmaniasis (Carter *et al.* 1993), trypanosomiasis (Trishmann & Bloom, 1982) and filariasis (Petit *et al.* 1992).

The effect of genes outside *H-2* on the response to *T. crassiceps* was also revealed by the differential susceptibility of three $H\text{-}2^d$ BALB/c substrains: BALB/cAnN was highly susceptible, confirming our previous report (Sciutto *et al.* 1991); whereas BALB/cJ was the most resistant and BALB/cByJ displayed an intermediate degree of susceptibility, similar to that observed in all B10-derived strains. The differences in susceptibility to cysticercosis found between $H\text{-}2^d$ BALB/c substrains is not totally unexpected in view of previous reports describing variable susceptibility to *Listeria monocytogenes*, (Skamene, 1985), Orthopoxviruses (Buller, 1985), and *Coxiella burnetti* (Williams *et al.* 1985) among them. These findings raise the possibility that the differential susceptibility of BALB/cAnN, BALB.B and BALB.K to *T. crassiceps*, previously attributed to the effect of *H-2* genes (Sciutto *et al* 1991) may in fact be due to unidentified non MHC related subline differences. At present we cannot distinguish between these alternatives.

The BALB/cJ substrain is unique within the BALB/c family since it differs from the Andervont's BALB/c substrains, BALB/cAnN and BALB/cByJ, in several phenotypes (reviewed by Potter, 1985) such as aggressive behavior, higher brain weight, low expression of androgen-sensitive major urinary protein, increased levels of three adrenal enzymes involved in catecholamine biosynthesis, high levels of testosterone in male mice (Roderick *et al.* 1985), elevated levels of serum alpha-fetoprotein in adult mice as a consequence of the mutation in the *Afr-1* gene (Blankkenhorn *et al.* 1985); and expression of the non-classic class I Qa-2 protein due to the presence of two intact genes (*Q6* and *Q7*) (Mellor *et al.* 1985) Although linkage analysis of susceptibility to cysticercosis and these traits has not yet been carried out it appears that resistance of BALB/cJ to cysticercosis does not correlate with the mutant $Afr\text{-}1^b$ locus since it is a recessive gene (Olson *et al.* 1977) while in BALB/cJ crosses, resistance is inherited as a dominant trait (Figure 2). On the other hand, BALB/cJ is resistant and expresses Qa-2 whereas BALB/cAnN and BALB/cByJ are both more susceptible to *T. crassiceps* and Qa-2 negative. Although the function of Qa-2 is unclear, it serves as a peptide receptor (Rotzschke *et al.* 1993) and is involved in skin graft rejection (Mellor *et al.* 1991). One might speculate that Qa-2 is somehow

related to differences in susceptibility to *T. crassiceps*. We are currently using transgenic mice to explore this intriguing possibility. Hormonal factors such as testosterone could also play a role in the resistance of BALB/cJ to cysticercosis, specially considering that female mice are consistently more susceptible than males (Larralde *et al.* 1989; Sciutto *et al.* 1991) and that susceptibility is under strong regulation by gonadal factors (Huerta *et al.* 1992; Terrazas *et al.* 1994). In this regard, are of interest the observations that levels of testosterone seem to be under control of genes linked to *H-2* (Ivanyi *et al.* 1976) and that testosterone and *H-2* genes apparently interact in the control of intestinal nematodes (Wunderlich *et al.* 1988; Harder *et al.* 1994). It is also possible that other as yet unidentified loci are responsible for the natural resistance of BALB/cJ to cysticercosis

The high resistance displayed by BALB/cJ mice is of particular interest since BALB/c substrains are known to be genetically quite similar (Roderick *et al.* 1985) and therefore, the identification of the gene(s) that confer resistance to *T. crassiceps* is plausible.

ACKNOWLEDGMENTS

This project was supported by Grant M9105-0008 from Consejo Nacional de Ciencia y Tecnologia, México and by Grant IN209393 from Dirección General de Asuntos de Personal Académico, Universidad Nacional Autónoma de México. The authors wish to thank Isabel Pérez Montfort for English corrections, and Mercedes Baca and Marisela Hernández for excellent technical assistance

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Table 1. Resistance of C57BL mice strains to *Taenia crassiceps* cysticercosis

Strain	*H-2*								Parasite Load*	
	I									
	K	*Aα*	*Aβ*	*Eα*	*Eβ*	*S*	*DL*	Qa-2	Female	Male
BALB/cAnN	*d*	*d*	*d*	*d*	*d*	*d*	*d*	-	132.96± 6.88[a]	7.49 ± 1.95[b]
C57BL/6J	*b*	*b*	*b*	*b*	-	*b*	*b*	+	18.60± 5.38[c]	0.34 ± 0.14[d]
C57BL/10J	*b*	*b*	*b*	*b*	-	*b*	*b*	+	23.00± 5.52[c,b]	0[d]
B10.D2	*d*	*d*	*d*	*d*	*d*	*d*	*d*	+	16.04± 3.47[c]	0[d]
B10.D2(R103)	*d*	*d*	*d*	*d*	*d*	*d*	*b*	+	18.38± 2.61[c]	0.70 ± 0.46[d]
B10.D2(R107)	*b*	*b*	*b*	*b*	-	*b*	*d*	+	21.68± 4.42[c,b]	0.36 ± 0.36[d]
B10.A(2R)	*k*	*k*	*k*	*k*	*k*	*d*	*b*	+	18.05± 4.70[c]	0[d]

*Mean ± Standar error of the number of cysticerci recovered 30 days after challenge with 10 parasites per mice.

[a,b,c,d] Data labeled with the same letter are not significantly different from each other, whereas those with different letters are significantly different ($P < 0.01$).

Table 2. Susceptibility of BALB/c substrains and BALB *H-2* congenic mice to infection with *Taenia crassiceps* cysticerci

Strain	*H-2* haplotype	Qa-2	Parasite Load*	
			Female	Male
BALB /cAnN	*d*	-	122.1± 8.07[a]	30.29± 2.15[b]
BALB/cByJ	*d*	-	31.1± 4.80[b]	4.46± 1.84[c]
BALB/cJ	*d*	+	7.2± 1.98[d,c]	0.39± 0.16[e]
BALB.B	*b*	+	23.7± 3.76[b]	0.53± 0.27[e]
BALB.K	*k*	-	27.7± 5.49[b]	0[e]

*Mean ± Standar error of the number of cysticerci recovered 30 days after challenge with10 parasites per mice

[a,b,c,d,e] Data labeled with the same letter are not significantly different from each other, whereas those with different letters are significantly different ($P<0.01$).

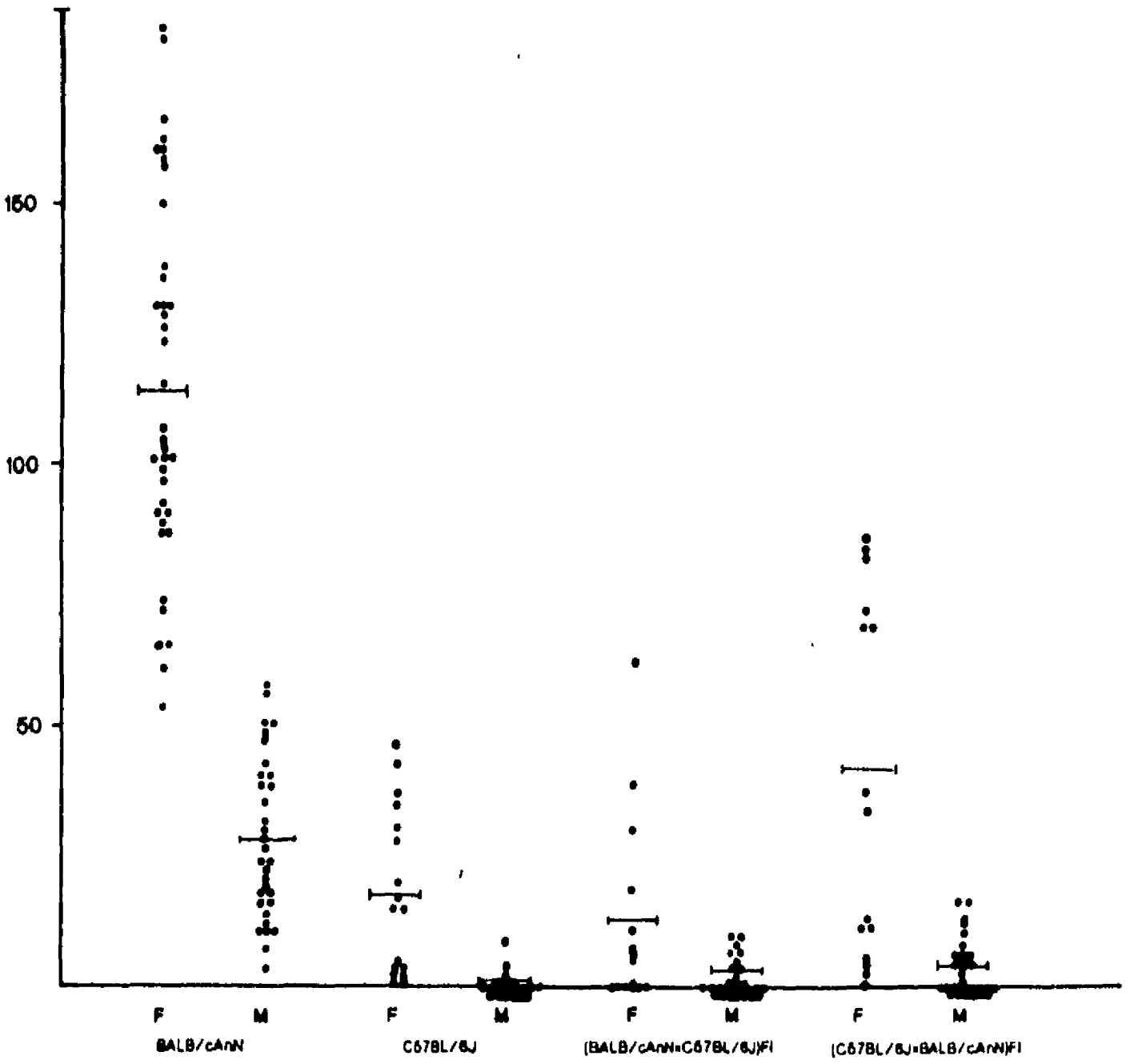

Fig. 1. Individual number of parasites recovered from each parental and F1 progeny mouse 30 days after intraperitoneal infection with 10 cysticerci each. Mean number of parasite load is indicated in each group.

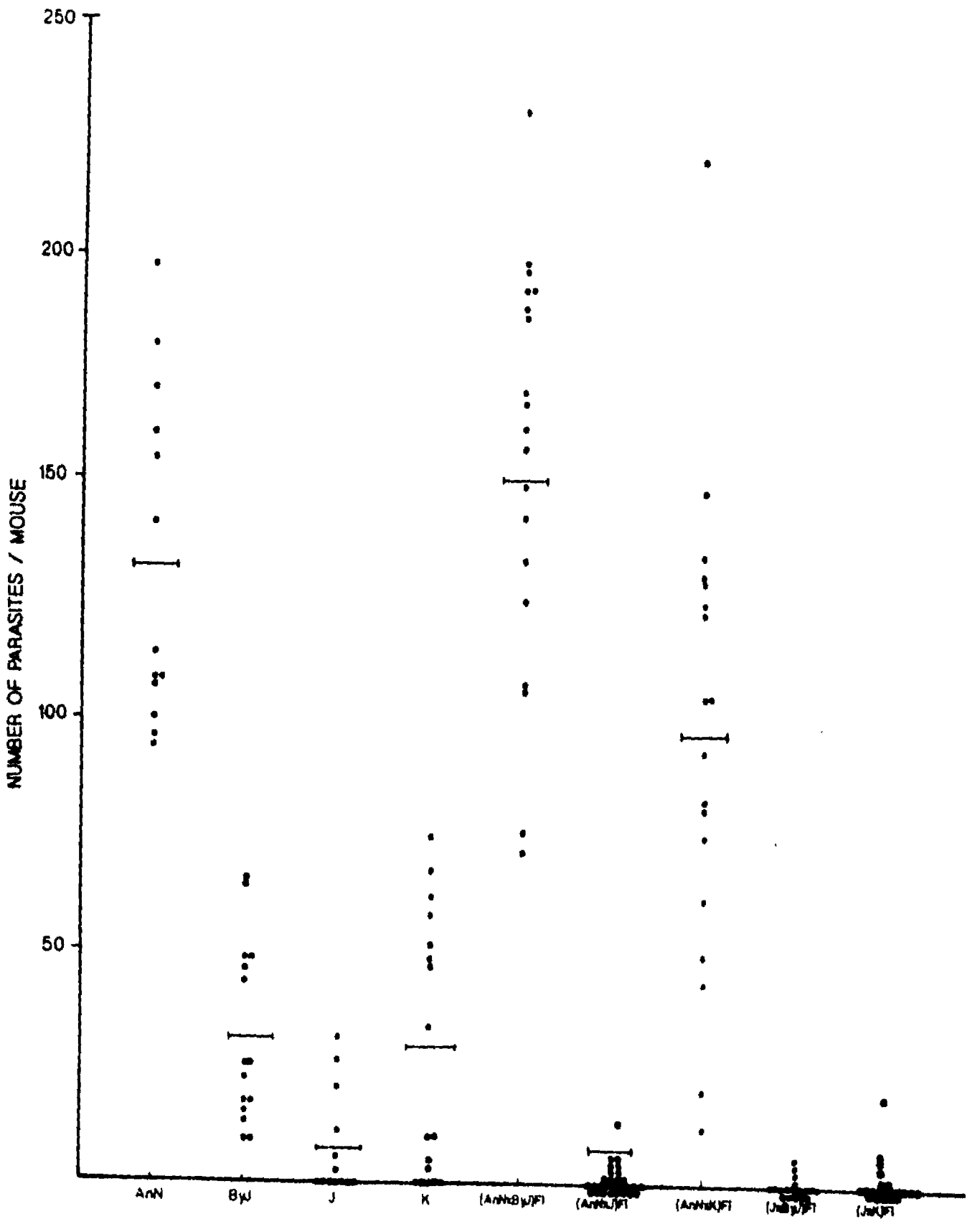

Fig. 2. Number of parasites recovered from each parental female and F1 progeny mouse 30 days after intraperitoneal infection with 10 cysticerci each. AnN: BALB/cAnN, J: BALB/cJ, ByJ: BALB/cByJ, K: BALB.K. Mean number of parasite load is indicated in each group.

INCREASED INNATE RESISTANCE AGAINST *T. crassiceps* MURINE CYSTICERCOSIS IN Qa-2 TRANSGENIC MICE

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Murine cysticercosis caused by *Taenia crassiceps* cysticerci is a parasitic disease controlled by a network of factors , i.e. *H-2* locus, gonads and the immune system[1]. We have used it as an experimental model of the disease caused by *Taenia solium* cysticerci, that exclusively afect pigs and humans, to study the genetic factors involved in the host susceptibility . We had found significant differences in the extent of the *T. crassiceps* cysticercosis between BALB/c sublines[2] in which very few phenotypic differences have been reported[3]. Two of such phenotypes may be associated to resistance cysticercosis: the high levels of testosterone and the expression of the non-classical (class I-b) Qa-2 histocompatibility protein. The possibility that Qa-2 protein could be involved in the high resistance was tested with transgenic mice expressing high levels of this protein. Both female and male transgenic mice were significantly more resistant than controls. Thus the high expression of Qa-2 protein can increase in at least 50% the innate resistance to *T. crassiceps* cysticercosis.

Taenia solium cysticercosis is a parasitic disease that seriously affect human health and porciculture of developing countries. In humans this parasite cause neurocysticercosis which is a serious and very frequent neurological illness in Latin America, Africa and Asia. In pigs this parasitosis is a cause of economically losses by the confiscation of infected meat in abbatoirs. Meanwhile it has been erradicated in developed countries by the general improvement in living conditions, in developing countries conditions that favour the transmission persist. Considering that porcine cysticercosis is indispensable in maintaining the life cycle this offer the possibility of interfering with it by modifying the prevalence of pig cysticercosis through the modification of the natural susceptibility of pigs by vaccination or by the improvement of cysticercosis resistance in pigs.

Systematic exploration of the role of genetic factors in susceptibility is hardly possible, laborious and costly in pigs. Considering this, we decided to use an experimental model of cysticercosis caused by the *T. crassiceps* cysticerci to systematically explore the role of biological factors involved in host susceptibility. This is a suitable model caused by a parasite that

cysticerci in its peritoneal cavity where they rapidly reproduce by budding[4]. Most interesting, *T. crassiceps* cysticercal antigens cross-react with those of *T. solium*[5] and provide some degree of cross-immunity between the pig and the mouse parasite[6,7].

Significant differences in the extent of the parasitosis were found between mice carrying the *H-2*^{d} haplotype (BALB/cAnN and DBA/2J), which were the most susceptible, and mice with *H-2*^{b} haplotype (BALB.B, C57BL/6J) or *H-2*^{k} (BALB.K, C3H/HeJ, C3H/FeJ) which were comparatively resistant[8]. A preliminary analysis in the naturally recombinant A/J strain, which was also susceptible suggested that *H-2D*^{d} and /or *H-2S*^{d} genes could be involved in susceptibility to this parasitosis.

This effect of *H-2* was not observed in B10 mice because gene(s) in this background confer resistance to this parasitosis such that override the effect of *H-2*. In other series of experiments carried out in mice of BALB genetic background different degrees of susceptibility were observed: BALB/c AnN was highly susceptible, whereas BALB/cByJ showed intermediate susceptibility and BALB/cJ was the most resistant of all the strains tested[2]. The high resistace in BALB/cJ is of special interest because BALB/cJ susbstrain is unique within the BALB/c family and differs from the other substrains by only a few phenotypes[3]. Among such phenotypes, the two candidates which may be associated with resistance to cysticercosis are: the elevated levels of testosterone[9] and the expression of the Qa-2 non-classical (class I-b) histocompatibility antigen[10].

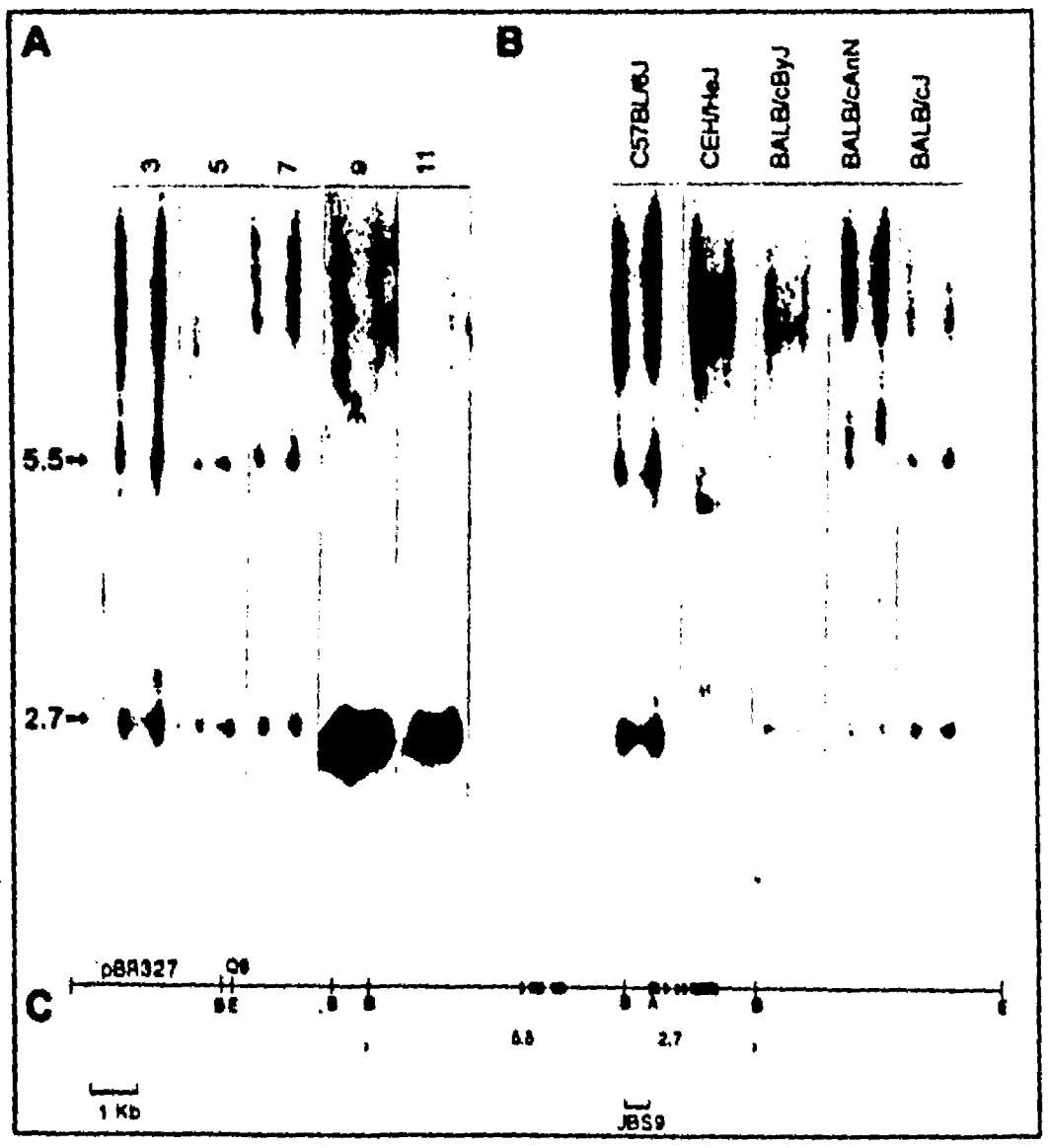

Fig. 1. Sourthern hybridization analysis of Bam H1-digested genomic tail DNA obtained from **(A)** progeny of Q9 microinyected embryos derived from a mating between (C57BL/6J×BALB/cAnN) and **(B)** some non-transgenic syngenic mice. Transgenic mice were identified by high hybridization of DNA to the JBS9 probe for the Q9 transgene in the 2.7 Kb position. **(C)** Restriction map of DNA fragment used for microinjection. (E: EcoRI, B: Bam HI, A: Apa I). Plasmid sequences were removed before injection by EcoRI digestion. The diagram of the bottom shows the position of the JBS9, a 500-pb Bam H1-Apa I fragment from cosmid C5[14] corresponding to a segment of the large intron between exons 3 and 4[13] which hibridize in two regions spanning 2.7 and 5.5-kb.

This Qa-2 antigen is a particularly attractive candidate, considering that Qa-2 antigens are class I-like molecules that can bind a diverse array of structurally similar peptides from intracellular proteins in much the same manner as classical antigen-presenting molecules[11] and could be also involved in skin graft rejection[12]. Thus, we decided to explore the possibility that Qa-2 expression has a direct effect on resistance to *T. crassiceps* cysticercosis using transgenic mice.

Table 1. Qa-2 level expression in transgenic and non-transgenic mice.

Mouse	Level of Qa-2(%)
C57BL/6J	30.91
BALB/cAnN	1.11
(C57BL/6J × BALB/cAnN)F1	13.44
1	27.11
2	26.72
3	7.64
4	3.86
5	9.64
6	4.36
7	13.52
8	15.92
9	57.05*
10	9.14
11	44.45*
12	11.31

Expresion of the transgene product Qa-2 detected by flow cytometric analyses. Qa-2 protein on T cells from peripheral blood samples were stained with an IgG Qa-m2 antibody and goat anti-mouse immunoglobulin conjugated to fluorescein isothiocyanate. The level of expression of Qa-2 protein is represented by the mean fluorescent intensity of positive cells from animals resulting from the injected embryos (referred 1 to 12) and is compared with C57BL/6J ($Qa\text{-}2^+$), BALB/cAnN ($Qa\text{-}2^-$) and (C57BL/6J × BALB/cAnN) F1.

* Selected transgenic mice

Table 2. Effects of Qa-2 overexpression on parasite load measurements in transgenic mice.

	Females	Males
BALB/cAnN	132.9± 6.8♦ (20)•	27.49±1.95 (20)
C57BL/6J	18.6± 5.4 (20)	0.34±0.14 (20)
$B6C_2$	57.8±10.99 (14)	10.33±1.73 (15)
TgB1	25.4± 4.03 (28)	3.88±1.24 (18)
TgB2	16.5± 5.16 (9)	1.33± 0.97 (6)

T. crassiceps cysticerci recovered from the peritoneal cavity of female BALB/cAnN donor mice were washed thoroughly in cold PBS (0.15M NaCl, 0.01 M sodium phosphate buffer, pH 7.2). For infection, ten small non-budding cysticerci were injected intraperitoneally in all mice . Thirty days after infection, mice were sacrificied and the cysts inside the peritoneal cavity were counted. Both female and male mice from the two homozygous transgenic lines generated, TgB1 and TgB2 were infected. Mice from BALB/cAnN (Qa-2-), C57BL/6J (Qa-2+) and an equivalent system cross for the production of the transgenic colony: (C57BL/6J × BALB/cAnN)F1 backcrossed to BALB/cAnN mice and intercrossed between them (referred as $B6C_2$), were used as control of the overexpression effect of the Qa-2 protein in the parasite growth. Trangenic lines (TgB1 and TgB2) were generated by the mating system backcross-intercross. For TgB1 the male founder referred as B6C"9" was backcrossed with BALB/cAnN female mice and for TgB2 line the founder was the mouse referred as B6C"11". Homozygous transgenic mice were produced by setting up heterozygous intercrosses.

♦ Mean number and standar error of the parasite growth

• Number of mice tested

Generation of Q9 transgenic mice

We generated two transgenic mice that carry the Q9 transgene, one of the four genes genes that codify for Qa-2 protein. The Q9 transgene was cloned from C57BL/6[12] and excised from the plasmid vector. Transgenic mice were produced from eggs derived from a mating between (C57BL/6J×BALB/cAnN) and detected by the strong signal in Southern hybridizations analysis using a specific probe for Class I genes of the Q region (JBS9[13]), which hybridize with a 2.7-Kb Bam H-1 fragment (Figure 1). Trangene expression of the two transgenic mice detected was 1.5 to two times that of endogenous Qa-2 (Table 1). Two lines of transgenic mice derived from the two founders referred as TgB1 and TgB2 were generated by backcrossing transgenic founders to inbred BALB/cAnN mice.

Effect of Q9 transgene on the susceptibility to *T. crassiceps* cysticercosis.

Intraperitoneal infection of both female and male TgB1 and TgB2 mice with *T. crassiceps* cysticercy shows a lower parasite growth in contrast with similar backcrossed non-transgenic mice. (Table 2). Thus the high expression of the class 1b Qa-2 protein increase the innate resistance to this parasitosis.

Ensuring resistance to this parasitosis at the genetic level could provide control strategies aimed at decreasing the risk of infection to both commercially important livestock and the human population. In adition, the identification that Qa-2 protein control the host resistance to this parasitosis is a crucial step in elucidating the mechanisms of the host involved in this host-parasite relationship.

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Ratones transgénicos en México

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Microinyección del ADN en los embriones inmovilizados con un capilar de vidrio

Durante la última década, el desarrollo de la biología molecular ha permitido conocer detalladamente las moléculas que contienen la información genética, separarlas del organismo completo, modificarlas y hasta sintetizarlas en el laboratorio. Este conocimiento, aunado a los avances en embriología, ha hecho posible la modificación de varias especies animales, mediante la introducción de genes particulares (en los que se esté interesado) a embriones recién formados, que al integrarse al material genético (ADN) del individuo puedan entonces transmitirse a su descendencia.

ANIMALES TRANSGÉNICOS

Esta tecnología, conocida como transgénesis, se desarrolló en animales de laboratorio tales como ratones y conejos; los animales así modificados se denominan transgénicos y al gene introducido artificialmente se le conoce como transgene. Actualmente, la transgénesis se ha extendido a otros mamíferos como vacas, ovejas y cerdos. En el caso del ratón, este procedimiento se ha aplicado principalmente para investigar la función de genes específicos en un animal completo y para generar modelos experimentales de enfermedades. En cambio, en animales de importancia económica, aunque todavía en etapa experimental, con la transgénesis se pretende mejorar su crecimiento, la

calidad de los productos derivados de ellos y aumentar la resistencia a enfermedades.

TRANSGENES HOMÓLOGOS Y HETERÓLOGOS

Recientemente se ha comenzado a explorar la posibilidad de producir proteínas de interés farmacéutico y de diagnóstico induciendo su secreción en la leche de animales transgénicos. Los transgenes pueden ser propios de la especie (homólogos) o ajenos a ella (heterólogos). De esta forma, por ejemplo, se han integrado genes humanos al material genético del ratón para simular enfermedades humanas y genes de la hormona de crecimiento bovina a cerdos con el fin de mejorar la ganancia de peso y la eficiencia en la alimentación.

¿Cómo se hace un animal transgénico? En los primeros intentos se utilizaron retrovirus como acarreadores de los transgenes; el ADN viral es capaz de integrarse al ADN de la célula infectada, en forma estable y sin destruirla. El límite en el tamaño de los genes a introducir, así como el riesgo que implica el uso de los retrovirus, ha determinado que no sea el procedimiento de elección rutinario. Actualmente, el método más utilizado es el de la microinyección de genes purificados en óvulos recién fecundados.

En México, en el Instituto de Investigaciones Biomédicas de la UNAM, logramos producir a mediados de 1993 nuestros primeros ratones transgénicos por el procedimiento de microinyección. Nuestro objetivo al hacer este tipo de ratones fue utilizarlos para estudiar la resistencia a la cisticercosis. En el instituto, desde hace más de 20 años, varios grupos de investigación se han interesado en el estudio de la cisticercosis humana y porcina causada por el cisticerco de la *Taenia solium* (solitaria), que es una parasitosis frecuente en nuestro país. Este parásito causa graves efectos al establecerse en el cerebro de las personas que infecta. Además, afecta únicamente al hombre y al cerdo, lo cual dificulta el estudio de la enfermedad.

RATONES VS. CISTICERCOSIS

Tratando de superar estos obstáculos, inicialmente el doctor Carlos Larralde propuso utilizar la cisticercosis del ratón (causada por el cisticerco de *Taenia crassiceps)* como una alternativa para estudiarla esperando que el conocimiento derivado pudiera ayudar a entender mejor la enfermedad humana. Desde un principio, en nuestros estudios se hizo evidente que ratones genéticamente distintos diferían en la susceptibilidad al cisticerco de *Taenia crassiceps.* Así, al infectar ratones de diferentes tipos con el mismo número de cisticercos, observamos que en algunos los parásitos casi no se multiplicaban (ratones resistentes) mientras que en otros, los cisticercos se reproducían extensamente (ratones susceptibles). Aunque no es nuevo que hay variación en la susceptibilidad de individuos de la misma especie a una enfermedad, en la cisticercosis del ratón observamos que estas diferencias de susceptibilidad se presentaban entre individuos genéticamente muy similares, y pudimos relacionar estas diferencias con una región cromosómica en la que se encuentra un gran número de genes conocidos como genes del Complejo Principal de Histocompatibilidad (conocido por las siglas del inglés como MHC).


Transferencia de embriones en una solución con colorante azul al oviducto de hembras seudoembarazadas.

Este hallazgo es interesante porque algunos de estos genes contienen la información para producir proteínas que son fundamentales en la respuesta inmunitaria, la cual participa de manera muy importante en el control de la extensión de infecciones. A partir de estas observaciones, decidimos identificar los genes del MHC que se asocian a la resistencia de los ratones a la cisticercosis. Con este objetivo utilizamos varios tipos de ratones con una composición genética definida y encontramos así al menos dos genes del MHC (H-2Db y Q9) que siempre estaban presentes en ratones resistentes. Con el fin de confirmar si efectivamente estos genes confieren resistencia al ratón contra el cisticerco de *Taenia crassiceps*, decidimos introducir uno de ellos (Q9) mediante transgénesis a ratones naturalmente sus-

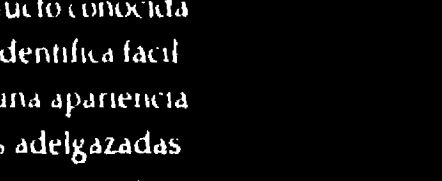
ceptibles y estudiar en ellos el crecimiento del cisticerco. Los resultados fueron muy satisfactorios; la presencia del transgene en ratones inicialmente susceptibles provocó la reducción significativa del número de parásitos, haciéndolos más resistentes. Estos hallazgos abren la posibilidad de utilizar la transgénesis como una nueva alternativa para el control de las enfermedades parasitarias. Dados los intereses de nuestro grupo de investigación, entre los que figuran desarrollar procedimientos tendientes a controlar la cisticercosis humana y porcina, estamos estudiando la susceptibilidad natural del cerdo considerando su posible modificación por transgénesis. Reduciendo la cisticercosis porcina se interrumpiría el ciclo biológico de la *Taenia solium* y por consiguiente la cisticercosis humana.

Nota: Este proyecto se realizó con el apoyo económico proporcionado por DGAPA y CONACyT.

¿Cómo se hace un ratón transgénico?

En la producción de un ratón transgénico se distinguen tres etapas: a) La recuperación de una gran cantidad de óvulos recién fecundados (embriones) b) La microinyección del transgene y c) La implantación de los embriones inyectados en hembras seudoembarazadas. Para recuperar una gran cantidad de embriones y favorecer además la maduración y salida de los óvulos de los ovarios, previo a la cruza de las hembras con los machos, las hembras son estimuladas con hormonas sexuales. Estos embriones se recuperan del oviducto (conducto por el cual los óvulos pasan del ovario a los cuerpos uterinos). Los embriones se suelen agrupar en una zona del oviducto conocida como ámpula, la cual se identifica fácilmente porque presenta una apariencia inflada y con las paredes adelgazadas. La ruptura de esta zona permite entonces recolectar a los óvulos fecundados. Con el fin de aumentar la probabilidad de que los genes inyectados se integren al ADN del embrión, la microinyección se realiza en el pronúcleo, generalmente el masculino. La microinyección se realiza bajo un microscopio al cual se le ha adaptado un equipo para la micromanipulación del embrión. Este equipo consiste esencialmente en un aparato que permite el sostén de los embriones, y otro con el que se realiza la microinyección. Una vez sostenido el embrión y visualizado el pronúcleo masculino, se atraviesa la membrana del pronúcleo con la microaguja cargada con el ADN en solución inyectándose en pequeñísimas cantidades. Para que estos embriones continúen su desarrollo hasta la producción de un ratón transgénico, deben ser introducidos en una hembra que se encuentre en las condiciones fisiológicas del embarazo. A fin de evitar que los embriones inyectados se confundan con los embriones propios de un embarazo normal, la transferencia se realiza a hembras seudoembarazadas, es decir, cruzadas con un macho estéril (vasectomizado). A estas hembras se les transfieren quirúrgicamente al oviducto los embriones microinyectados. Al término de la gestación, se estudian las crías que logran terminar su desarrollo para identificar cuáles presentan el transgene y, además, en cuáles la proteína codificada por el gene se expresa y es funcional.

Los autores, Gladis Fragoso, Edmundo Lamoyi, Otto Tomeh, Andrew Mellor, Marcela Hernández y Edda Sciutto se encuentran adscritos al Instituto de Investigaciones Biomédicas de la UNAM



Ampula del oviducto donde se agrupan los embriones sostenida por una pinza quirúrgica

ARTICULOS EN COLABORACION

IMMUNIZATION AGAINST *TAENIA CRASSICEPS* CYSTICERCOSIS: IDENTIFICATION OF THE MOST PROMISING ANTIGENS IN THE INDUCTION OF PROTECTIVE IMMUNITY

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ABSTRACT: Cross immunity between *Taenia solium* and *Taenia crassiceps* parasites points to *T. crassiceps* cysticercosis as a convenient model to test promising antigens aimed at the development of a vaccine against *T. solium* cysticercosis. Since total antigens from *T. crassiceps* metacestodes induce significant levels of protection in pigs against *T. solium* cysticercosis, we initiated this work to identify the most interesting antigens involved in protection. Twelve different antigen fractions isolated from *T. crassiceps* cysticerci were evaluated with respect to their capacity to induce resistance against a challenge with 10 *T. crassiceps* cysticerci in male BALB/cAnN mice. Mice were intraperitoneally immunized with 2 doses of each antigen, 5 or 15 μg per mouse. The 12 antigen fractions were classified as protecting (200, 123, 74, 66, 56, 40–50, 27, and 8–14 kDa), facilitating (220–205 kDa), or irrelevant (150–160, 93, 108 kDa), according to their effect on the parasite load. The 3 most promising antigen fractions were reevaluated via subcutaneous immunization with Freund's complete adjuvant. A high level of protection was obtained when antigen fractions of 56, 66, and 74 kDa were used together. Interestingly, antigens with similar molecular weights were also detected in early steps of differentiation in *T. solium* cysticercosis. These observations may be helpful in the development of a synthetic or a recombinant vaccine against cysticercosis.

Taenia solium cysticercosis is an important socioeconomic problem in Mexico, Latin America, Asia, and Africa (Mahajan, 1982; Schenone et al., 1982; Gemmell et al., 1985). In Europe it stopped being a health problem during this century, having been eradicated by the application of sanitary engineering, personal hygiene, effective inspection of slaughterhouses, improved hog rearing, and a general improvement in living conditions. In contrast, in Mexico as in other developing countries, conditions that favor transmission persist: more than 40% of pork consumed in Mexico is from areas where sanitation is poor (Mazón-Rubio, 1991), meat inspection is inadequate, and unsound environmental and behavioral habits prevail (Aluja, 1982; Sarti-Gutiérrez et al., 1988; Keilbach et al., 1989). The life cycle of this parasite includes a larval phase that may occur either in the pig or in man, and the cycle is completed when the man ingests infected pork. Porcine cysticercosis is, therefore, indispensable in maintaining the life cycle. This offers the possibility of interfering with this life cycle by modifying the prevalence of pork cysticercosis through vaccination, which is probably a more realistic measure than those mentioned above to interrupt transmission successfully.

We used the experimental *T. crassiceps* murine cysticercosis as a model to evaluate immunogens in the design of a vaccine. The value of this system reflects the antigenic cross-reactivity between both cestodes (Kunz et al., 1989; Larralde et al., 1990; Ramos-Kuri et al., 1992), the fact that the life cycles of the 2 cestodes are remarkable similar and that there is a high growth rate of this parasite within the peritoneal cavity of the mouse (Larralde et al., 1988, 1989).

MATERIALS AND METHODS

BALB/cAnN male mice 4–6 wk of age were employed. They were bred in our animal facilities by the brother–sister inbreeding system over 20 generations, starting with original stock from the Jackson Labs in 1982. *Taenia crassiceps* cysticerci used in this study were of the ORF strain, supplied by Dr. B. Enders (Behringwerke, Marburg, Germany) in 1986 and maintained in our laboratory by intraperitoneal inoculation of 20 small larvae in female BALB/cAnN mice 4–6 wk old (Freeman, 1962). The parasites were harvested after 60–90 days postinfection (PI).

Parasites from infected BALB/cAnN female mice were recovered in cold 0.01 M phosphate-buffered saline (PBS), pH 7.2, containing protease inhibitors (0.006% phenylmethylsulfonyl fluoride [PMSF] and 0.04% sodium *p*-hydroxymercuribenzoate [PCMB]; Sigma Chemical Co., St. Louis, Missouri), and washed in the same buffer with a 1:10 (parasite : PBS) ratio. To recover cysticerci vesicular fluid, parasites were suspended in a minimal amount of buffer and centrifuged at 25,000 *g* for 60 min, at 4 C. The pellet containing the cysticerci membranes was discarded; the protein content of the vesicular fluid was determined using Lowry's method (Lowry et al., 1951).

Sera from *T. crassiceps*-infected mice and from hu-

Received 10 January 1994; revised 26 May 1994; accepted 26 May 1994.

TABLE I. Protection against murine *Taenia crassiceps* cysticercosis by intraperitoneal immunization with *T. crassiceps* antigens separated and included in 7% SDS-PAGE.

Groups	Dose (μg)	*Number of cysticerci (n)	†Protection efficiency, PE (%)	Number of tested mice
Total antigens‡	100	6.3 ± 7.8§	82.3	9
Separated antigens (kDa)				
56	5	4.8 ± 3.7§	86.5	8
	15	18.5 ± 6.1§	48.1	8
66	5	14.7 ± 16.8§	58.8	8
	15	13.3 ± 12.3§	62.7	8
74	5	8.0 ± 6.6§	77.5	8
	15	15.5 ± 9.7§	56.5	8
93	5	34.1 ± 13.7	4.4	10
	15	21.9 ± 8.4	38.6	10
108	5	31.4 ± 21.2	12.0	10
	15	39.7 ± 12.3	−11.2	10
123	5	23.7 ± 12.2	33.6	10
	15	10.1 ± 4.1§	71.7	10
150–160	5	23.8 ± 8.9	33.3	8
	15	35.0 ± 12.7	1.9	8
200	5	13.0 ± 7.9§	63.5	9
	15	11.8 ± 7.7§	66.9	8
220–205	5	27.5 ± 9.4	22.9	9
	15	52.4 ± 24.4‖	−49.7	10
Controls	–	35.7 ± 12.6	–	17

* Mean and standard deviation of individual parasite load recovered after 30 days of infection from nonimmunized (control) and immunized mice.
† PE = (n in control mice − (n in immunized mice)/(n in control mice).
‡ Total antigens include antigens of high and low molecular weight in 7% gels.
§ Significant protective differences with control value at 99% confidence level.
‖ Significant facilitating differences with control value at 99% confidence level.

TABLE II. Protection against murine *Taenia crassiceps* cysticercosis by intraperitoneal immunization with *T. crassiceps* antigens separated and included in 15% SDS-PAGE.

Groups	Dose (μg)	*Number of cysticerci (n)	†Protection efficiency, PE (%)	Number of tested mice
Total antigens‡	100	15.1 ± 11.4§	78.6	9
Separated antigens (kDa)				
8–14	5	33.0 ± 16.5§	53.3	15
	15	16.2 ± 12.3§	77.1	15
27	5	28.5 ± 35.3§	59.7	10
40–50	5	27.9 ± 18.3§	60.5	16
	15	19.7 ± 14.8§	72.1	14
Controls	–	70.5 ± 42.0	–	11

* Mean and standard deviation of individual parasite load recovered after 30 days of infection from nonimmunized (control) and immunized mice.
† PE = (n in control mice − (n in immunized mice)/(n in control mice).
‡ Total antigens include antigens of high and low molecular weight in 7% gels.
§ Significant protective differences with control value at 99% confidence level.

man neurocysticercotic patients were used. Sera from pigs experimentally infected with *T. solium* (250,000 eggs/pig) were used 30 (early) and 60 (late) days after infection.

Antigens from *T. crassiceps* cysticerci were separated by SDS-PAGE following the protocol of Laemmli as described elsewhere (Larralde et al., 1986). High (56–220 kDa) and low (8–50 kDa) molecular weight antigens were separated in 7% and 15% polyacrylamide gels, respectively, to optimize the resolution of the complex antigen mixture, using 3 or 8 mg of total antigen for analytic or preparative gels. After protein separation the edge of the gel was cut, fixed, and stained with 0.1% Coomasie/blue R-250 and used as a reference. Percentage quantitation of each identified antigen fraction relative to the total protein in the mixture was calculated using a Beckman DU-8B spectrophotometer. Bands to be cut were visualized with 4 M sodium acetate for 20–40 min (Higgins and Dahmus, 1979). The cut bands were washed with distilled water to remove the remaining sodium acetate before homogenizing the gel for immunization. Immunoblotting of the vesicular fluid was performed as described elsewhere (Larralde et al., 1986).

Eight to 16 mice per group were intraperitoneally or subcutaneously immunized. Intraperitoneal (i.p.) injections consisted of 5 or 15 μg/mouse of each antigen fraction. Subcutaneous (s.c.) injections consisted of 5 μg of each antigen fraction per mouse. Control animals were immunized with 7% or 15% polyacrylamide gels containing total vesicular fluid antigens or no antigens.

For s.c. immunizations, either Freund's complete adjuvant (FCA) was used at a ratio of 1:1 antigen/adjuvant, or muramyl dipeptide (MDP, 15 μg per mouse) previously homogenized in incomplete Freund's adjuvant at a ratio of 1:1 antigen/adjuvant. Aluminum hydroxide was also employed at 4 μg/mouse for i.p. immunizations.

Mice were i.p. challenged 15 days after immunization with 10 nonbudding cysticerci (2–3 mm). Thirty days after the challenge, mice were killed and the number of cysticerci in the peritoneal cavity was determined by counting the parasites recovered from each mouse. Results were statistically analyzed by multifactorial analysis of variance ANOVA (Anonymous, 1985).

RESULTS

To identify the antigens of *T. crassiceps* involved in protective immunity against murine cysticercosis, mice were immunized with several antigen fractions and challenged with larvae 15 days later. The concentration of each fraction was calculated by gel densitometry scan (Fig. 1). Mice were immunized with 12 different antigenic complexes ranging from 220 to 56 kDa (Table I) and from 50 to 8 kDa (Table II). Both immunization doses, i.e., 5 and 15 μg, of antigens of 200, 74, 66, 56, 40–50, and 8–14 kDa provoked a statistically ($P < 0.01$) significant de-

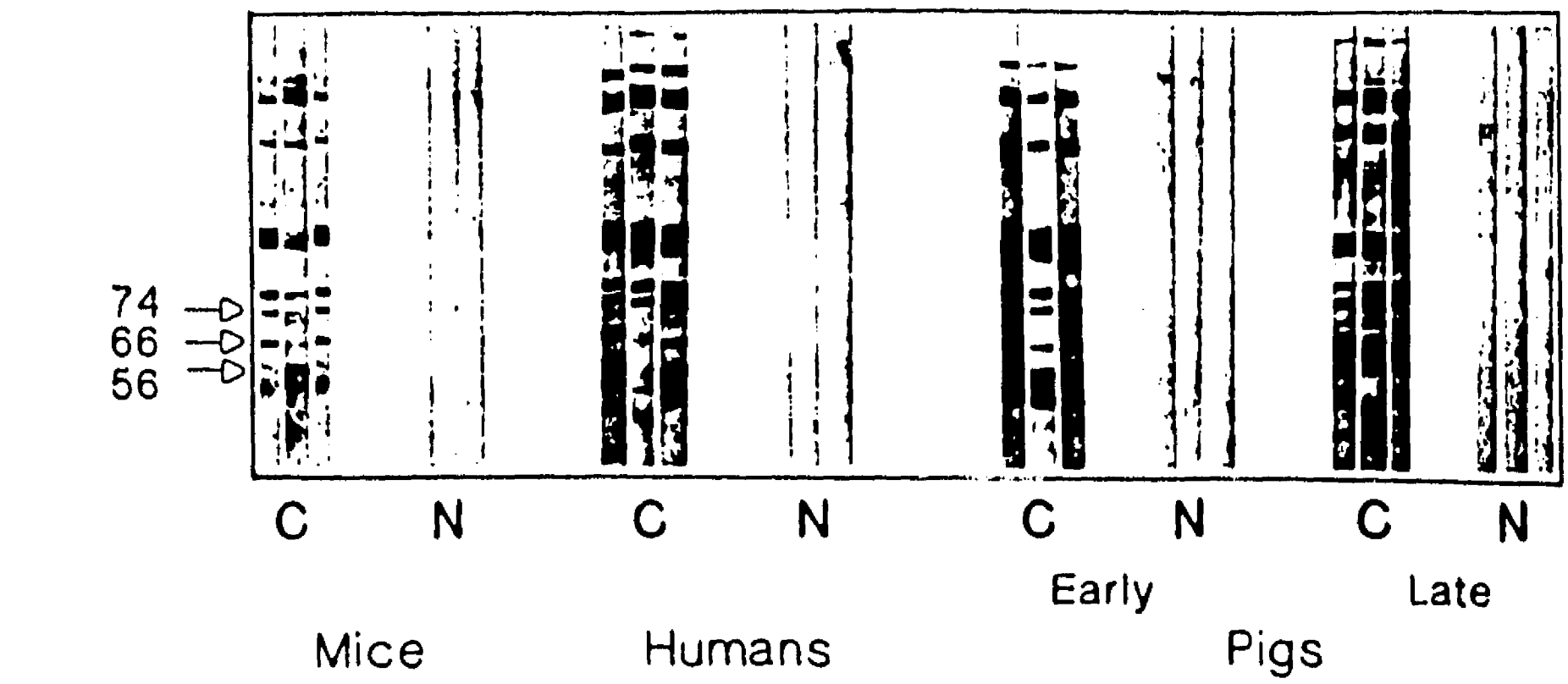

FIGURE 1. Western blots of vesicular fluid of *Taenia crassiceps* cysticerci reacting with sera of cysticercotic and healthy humans, pigs, and mice. C, cysticercotic; N, normal.

crease in the parasite load when compared with nonimmunized animals; antigens of 150–160, 108, and 93 kDa at both doses, i.e., 5 and 15 μg, and 220–205 kDa at the low dose (5 μg) did not significantly affect ($P < 0.01$) the parasite load. Antigens of 123 kDa induced protection only with the higher dose and 27-kDa antigens protected with the low dose. On the other hand, antigens of 220–205 kDa at 15 μg/mouse significantly increased ($P < 0.01$) the parasite load (Tables I, II). All the cysticerci recovered both from immunized and nonimmunized mice were viable by appearance (cysts containing a clear transparent fluid); 7% or 15% gel-injected mice were, respectively, used as nonimmunized controls with immunizations of >50 kDa or <50 kDa.

From the 8 protective antigen fractions detected (56, 66, 74, 123, 200, 40–50, 27, and 8–14 kDa), the 3 most effective (56, 66, and 74 kDa) were selected to reevaluate their protective capacity. These antigen fractions were selected because they induced the highest level of protection independent of the dose used and they could be cut without contamination of other antigen fractions because they appeared in the gels as single bands (Fig. 1), although densitometric scanning revealed some fractions have more than 1 component. Since we observed that s.c. immunizations with total antigens induced protection only when injected in FCA, while no protection was observed when aluminum hydroxide or muramyl dipeptide was used in incomplete Freund's adjuvant (data not shown), we reevaluated these 3 most promising antigen fractions. From the 3, only those of 2, 66, and 74 kDa maintained their protective capacity when injected subcutaneously in FCA and were equally effective whether used individually or in combination. Immunization efficiency was also as high when the 3 antigen fractions were used subcutaneously as with total antigens (74.5 vs. 62%; Table III). Antigens of similar size from *T. crassiceps* were recognized by sera from humans and pigs infected with *T. solium* cysticerci as well as by sera from *T. crassiceps*-infected mice (Fig. 2). Interestingly, the 3 antigens are recognized early in the cysticercus development as shown by western blot performed with sera from pigs with early *T. solium* infections (Fig. 2).

DISCUSSION

The protective capacity of 12 different antigenic fractions of *T. crassiceps* cysticercus vesicular fluid was evaluated in mice. In the present study we identified 6 antigenic groups (8–14, 40–50, 56, 66, 74, and 200 kDa) capable of inducing high levels of protection against experimental murine cysticercosis, regardless of whether they were administered in high or low doses. The protective effect induced independent of the dose used is of special interest considering previous results that emphasize a strict relationship between dose and protection-inducing capacity.

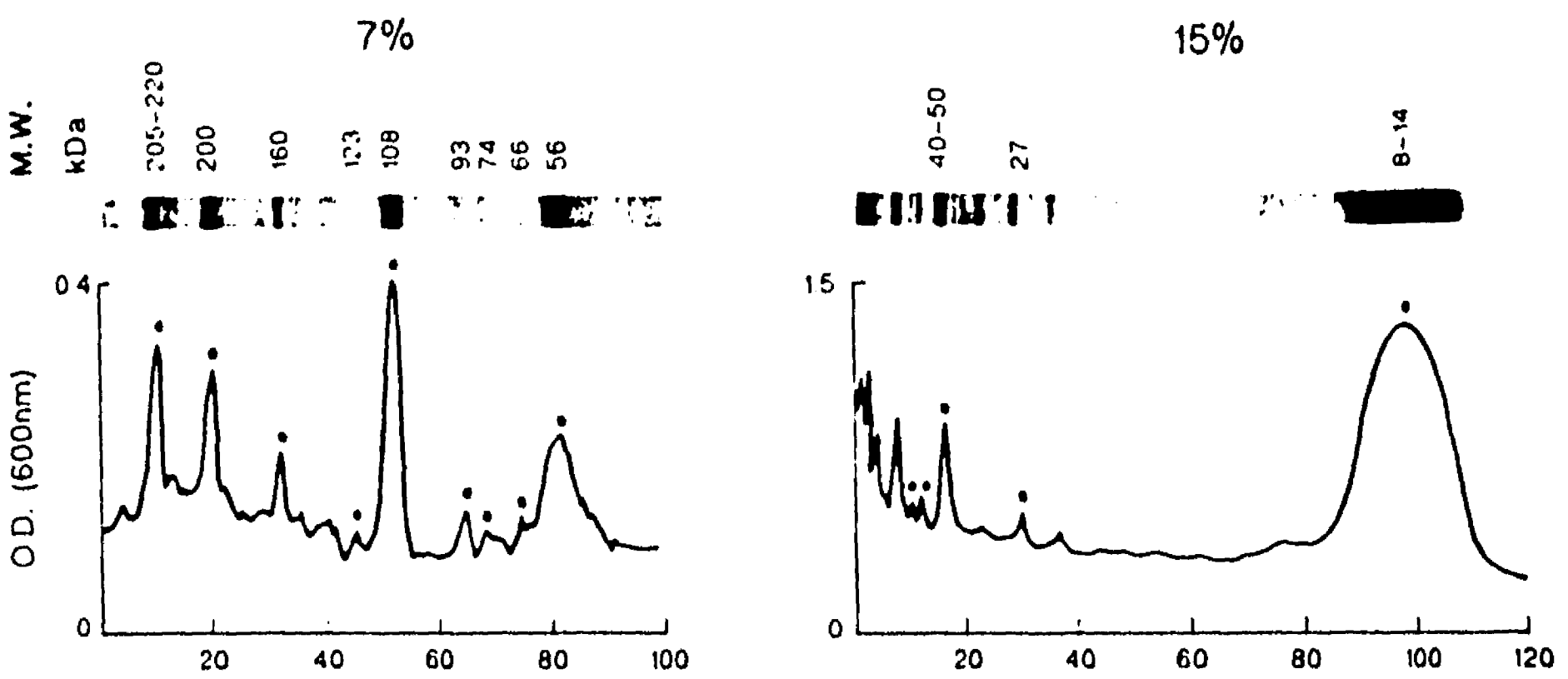

FIGURE 2. SDS-PAGE (7% and 15%) chromatogram and densitometry analysis after staining with 0.1% Coomasie blue R-250 illustrate the protein composition of soluble antigens of the *T. crassiceps* cysticercus. Bands localized at 8–14 kDa represent 60.5% of the total protein content and appear systematically in all different cysticerci antigens tested. *Fraction used for immunization.

This was reported for total cysticercus antigens used as immunogens to prevent murine cysticercosis (Sciutto et al., 1990), or porcine cysticercosis. In pigs, immunization with high antigen doses not only did not reduce parasite numbers, but it clearly facilitated their increase (Rodarte, 1992).

Of the 6 protective antigenic fractions identified in this study, we selected the 3 most promising (56, 66, and 74 kDa) to continue their evaluation as candidates for protective immunization. These 3 antigen fractions were selected because they were prominent bands in the polyacrylamide gels used for their separation as single bands. This suggested that we were dealing with a simple combination and opened possibilities regarding future production by means of recombinant DNA techniques. Since it seemed scarcely possible that we could affect the destiny of a complicated relationship such as that of host and parasite with only 1 antigen, we considered combining the 3 antigen fractions. Protection efficiency obtained is at least as high as levels achieved with total antigen. The use of a combination of antigens, instead of a unique one, increases the probability of inducing a protective immune response capable of controlling the parasite in its different developmental stages. Moreover, it increases the probability of obtaining protection despite parasitic heterogeneity due to various genetic and environmental factors (Correa et al., 1987; Aluja and Vargas, 1988). It is also relevant considering the variable susceptibility of the host population, which may be associated to the heterogeneous recognition of parasitic antigens. Thus, important differences in susceptibility to cysticercosis have been reported not only in porcine (Rodarte, 1992) and ovine cysticercosis caused by *T. ovis* (Gemmel and

TABLE III. Combined use of electophoretically separated antigens in subcutaneous immunization against murine cysticercosis. All immunizations were carried out in Freund's complete adjuvant.

Groups	Dose (μg)	*Number of cysticerci (n)	†Protection efficiency, PE (%)	Number of tested mice
Total antigens‡	100	14.3 ± 7.2§	62.0	16
Separated antigens (kDa)				
56	5	27.5 ± 11.2‖	27.8	14
66	5	17.9 ± 6.9§	53.0	13
74	5	16.6 ± 10.7§	56.4	13
56 + 66	10	12.6 ± 7.0§	66.9	15
56 + 74	10	14.2 ± 6.7§	62.7	15
66 + 74	10	12.6 ± 7.7§	66.9	14
56 + 66 + 74	15	9.7 ± 6.6§	74.5	11
Controls	–	38.1 ± 20.3‖	–	9

* Mean and standard deviation of individual parasite load recovered after 30 days of infection from nonimmunized (control) and immunized mice.

† PE = (n in control mice) − (n in immunized mice)/(n in control mice).

‡ Total antigens include antigens of high and low molecular weight in 7% gels.

§ Significant protective differences with control value at 99% confidence level.

Data labeled with the same symbol are not significantly different at 99% confidence level

Lawson, 1982), but also in experimental murine models where a group of antigens recognized at higher frequencies by antibodies from genetically resistant individuals have been reported (Sciutto et al., 1991).

To evaluate the potential capacity of the identified *T. crassiceps* antigenic fractions to prevent porcine cysticercosis, we investigated if *T. solium* cysticerci shared them and found that sera from both cysticercotic humans and pigs recognize antigens with similar molecular weights. Since these possibly shared antigens are recognized early in the infection, they are likely to be present in oncospheres and young larvae. This lends importance to our 3 antigenic fractions because they could induce an immune response capable of interfering not only with cysticercus proliferation but also with transformation from oncosphere to cysticercus. In fact, some cysticerci antigens of *T. crassiceps* are capable of effectively protecting rats infected with *T. taeniaeformis* oncospheres (Ito et al., 1991).

The identification of antigens of interest for immunization against cysticercosis has not been limited to porcine cysticercosis. Fruitful efforts in the development of immunogens against cysticercosis by *T. ovis* have been made (Johnson et al., 1989). The production of this vaccine was based on the use of rodent experimental models, which allowed the identification of protection-inducing antigens. Johnson et al. (1989) identified and cloned an oncosphere antigen of 45 kDa, which efficiently prevents ovine cysticercosis.

Finally, the strategy of the present study allowed the identification of 3 antigenic fractions (56, 66, and 74 kDa), which seem promising for the prevention of porcine cysticercosis. We are presently evaluating them against the challenge of pigs with *T. solium* eggs, and initial observations are encouraging. Should this protection-inducing capacity turn out to be effective then we will consider antigen production with recombinant DNA techniques.

ACKNOWLEDGMENTS

We thank M. Ramos-Kuri for kindly supplying sera from human neurocysticercotic patients, Gerald W. Esch, Rafael Saavedra, and Enrique Ortega for critical reading of the manuscript, discussion, and helpful comments, Violeta Aguilar for her secretarial work, Isabel Pérez Montfort for revision and correction of the manuscript, and Mercedes Baca for her technical assistance. This research was supported by DGAPA IN209393 and CONACyT, México.

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For the Editors
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Advances in the Development of a Recombinant Vaccine against *Taenia solium* Pig Cysticercosis

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Taenia solium cysticercosis is a frequent cause of neurological illness of humans in México and other countries of Latin America, Asia, and Africa and causes important economic losses in pig rearing because of meat infection. The life cycle of this parasite includes a larval phase affecting either pigs or humans after ingestion of eggs present in foods contaminated with human feces. The cycle is completed when individuals ingest improperly cooked infected pork and develop the adult tapeworm, which produces millions of eggs. The disease is no longer a health problem in developed countries, having been eradicated through strict meat inspection and other sanitary measures. In contrast, in México, as in other developing countries, conditions that favor transmission persist: More than 40% of pork consumed in México is from areas where sanitation is poor, meat inspection is inadequate, and open air fecalism prevails. In the United States, neurocysticercosis is more frequently found in immigrants from endemic areas, but there are some autochthonous cases.

Humans are the only carriers of the adult form of the parasite tapeworm and are solely responsible for the transmission to pigs and other humans through inadequate disposal of feces or the deliberate feeding of pigs with human excrement. Because pigs are indispensable hosts for maintaining the life cycle, the possibility of interfering with the transmission of the parasite by modifying the prevalence of pig cysticercosis through vaccination has been proposed as a more realistic control measure, since vaccination would find better acceptance than meat inspection, which is confiscatory. Besides, immunologically protected pigs may better breed under the rustic conditions forced by poverty and yet aspire to better value in the meat market.

Several antigenic preparations from eggs and larvae have been shown to produce different degrees of protection against the metacestode stage of several cestodes, and one recombinant vaccine effective against *T. ovis* cysticercosis has been developed (Johnson et al. 1989). It thus seemed feasible to develop a vaccine against *T. solium*.

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Experimentation leading to a vaccine against porcine cysticercosis is hampered by the high cost, genetic variability, and slow data retrieval involved in testing pigs. However, another cestode, *T. crassiceps*, whose metacestodes develop in mice, and closely resemble those of *T. solium*, are most suitable for experimentation. They are smaller, have a similar biological cycle, and can be mantained in the laboratory by simply injecting metacestodes into the peritoneal cavity of mice, where they rapidly reproduce by budding. Thus, grams of metacestodes can be recovered within a few weeks after infection. But, most importantly, we found that an extensive cross-reactivity and cross-protecting immunity between both cestodes has been documented (Larralde et al. 1989; Sciutto et al. 1990). Thus, experimental murine cysticercosis caused by *T. crassiceps* is a suitable laboratory model in which to test promising antigens and is a plentiful source of potentially relevant cysticercal antigens. We first identified and isolated antigens from *T. crassiceps* that effectively protected mice against *T. crassiceps* and then showed that they also cross-protected pigs against *T. solium* (Sciutto et al. 1994). We now have finally succeeded in their production by recombinant DNA techniques.

DISCUSSION

Cross-reactivity between *T. solium* and *T. crassiceps* Cysticercal Antigens

We present here the extensive antigen homology between humans and the mouse parasite as detected by Western blot analysis (Larralde et al. 1989). As shown in Figure 1, a high number of *T. crassiceps* antigens are recognized by both mouse and human sera infected with *T. crassiceps* and *T. solium*, respectively. Such extensive antigen sharing is of relevance for the development of vaccine technology based on whole extracts or partially purified parasite antigens.

Cross-immunity in *T. solium* and *T. crassiceps* Murine Cysticercosis

Considering the sharing of antigens, we evaluated the effectiveness of vaccination with *T. crassiceps* and *T. solium* antigens against *T. crassiceps* cysticercosis in mice (Sciutto et al. 1990). BALB/cAnN male mice were immunized with 100 μg of total extract from either of both cestodes, and 15 days later, mice were challenged intraperitoneally with 10 cysticerci per mouse. Thirty days later, they were sacrificed, and the number of parasites in each mouse was determined. Table 1 shows that both antigens effectively protected mice against *T. crassiceps* cysticercosis.

Cross-immunity in *T. solium* and *T. crassiceps* Porcine Cysticercosis

We evaluated whether the inverse cross-protection also worked in pigs (Sciutto et al. 1994). Seven pigs were vaccinated subcutaneously with 400 μg/kg of a total extract from *T. crassiceps* with Freund's complete adjuvant (FCA) and seven pigs with FCA alone. Four months later, pigs were orally challenged with 250,000 *T. solium* eggs each. Two months later, they were sacrificed humanely, and the parasite load in each pig was measured by counting the number of cysticerci in approximately 10% of the total skeletal muscle weight. Table 1 shows that vaccination reduces approximately 58% of the parasite load in challenged pigs.

Identification of the Most Promising *T. crassiceps* Antigens in the Induction of Protective Immunity

To identify the most important antigens involved in protective immunity, mice were immunized with 12 different antigen fractions ranging from 220 kD to 8 kD separated in 7% and 15% SDS-PAGE. Vaccination with eight of these antigens caused a statistically significant decrease in parasite load when compared with nonimmunized mice (Valdez et al. 1994). Of these eight antigens, the three most effective were reevaluated for protective capacity (using them together in FCA). Table 1 demonstrates the effect of the subcutaneous immunization with these three antigen fractions in FCA upon the parasite load and shows that they induced levels of protection as high as those obtained with total vesicular fluid antigens inoculated under equivalent conditions. These antigens are also recognized by sera of pigs experimentally infected with *T. solium* (Valdez et al. 1994). The fact that antibodies against these three antigens are expressed in early phases of cysticercus establishment, where metacestodes appear to be most susceptible to immune attack, indicated that they could be used in vaccination trials against pig cysticercosis. Indeed, using the three antigen fractions for the prevention of pig *T. solium* cysticercosis resulted in six of seven immunized pigs being completely protected. These results, although preliminary, encouraged us to begin their production by recombinant DNA techniques and to explore further their efficacy in preventing pig cysticercosis by *T. solium*.

Construction of a *T. crassiceps* cDNA Library

A cDNA library was constructed in Uni-ZAP XR (Stratagene) vector using mRNA from *T. crassiceps* cysticerci. A total of 180,000 individual recombinant clones were obtained.

To detect clones that codified for antigens of 56 and 74 kD, rabbit polyclonal antibodies against these antigen fractions were produced. The serum was tested for its specificity by Western blot analysis, and pseudoscreening was carried out to remove anti-*E. coli* activity previously used in the immunoscreening of the library. The cDNA library was plated at a concentration of 10^4 pfu/plate on XL1-Blue cells, and 2 x 10^4 clones were screened. Thirteen positive clones with insert sizes from 0.5 to 2.0 kb were identified. To detect those of interest for *T. solium* vaccination, pooled antisera from *T. solium*-infected pigs were used. Five of these clones were recognized by these antisera (Fig. 2). Further experiments have been carried out using these five clones, designated KETc1, 4, 7, 11, and 12 with insert sizes of 1.2, 1.1, 0.5, 0.9, and 1.4 kb, respectively. Sequence analysis demonstrated that all clones were unique in view of the fact that sequence homology was not found in the 400 bp from the 3′ portion of genes already sequenced. However, some degree of immunological cross-reactivity has been found between the proteins encoded by KETc12 and 1, 4, 7, and 11 and between KETc11 and 1, 7, and 12. KETc1 has no reactivity against other clones except KETc4. Interestingly, only 329 bp of the KETc11 antigen clone was found in the GenBank (Zarlenga et al., unpubl.) and is considered an antigen of diagnostic interest for bovine cysticercosis.

By using the proteins produced from these clones, we prepared affinity-purified antibodies from the rabbit hyperimmune sera and then used these antibodies to probe immunoblots of *T. crassiceps* vesicular fluid antigens. These antibodies specifically recognized antigens at the expected molecular weights.

Vaccination Trials with Recombinant Antigens

In an effort to test their potential for cysticercosis prevention, crude lysates of the five clones mixed with FCA were used to vaccinate BALB/cAnN susceptible male mice

(Table 1). As controls, mice injected with FCA and a lysate of a clone without the insert mixed with FCA were included. Very promising results were obtained. Mice vaccinated with KETc1, 7, and 12 lysates showed a high level of resistance against *T. crassiceps* murine cysticercosis, reducing about five times the expected parasite load.

CONCLUSIONS

We reported here the identification of three recombinant antigens isolated from a *T. crassiceps* cDNA library of potential interest for the development of an effective vaccine against *T. solium* cysticercosis in pigs. The evaluation of these recombinant antigens in protection against *T. solium* cysticercosis in pigs is now being carried out, but preliminary results in mice are promising. Meanwhile, we are using different vectors to better express and purify these antigens and to identify the protective antigenic determinants involved. A more extensive vaccination protocol and studies of the immune response involved in protection are currently under way in our laboratories.

ACKNOWLEDGMENTS

This work was supported by Dirección General de Asuntos del Personal Académico, UNAM and CONACyT. We are grateful to the Facultad de Medicina Veterinaria y Zootecnia, UNAM, for maintenance of the pigs. We are also indebted to Dr. Juan Pedro Laclette for computer analysis of sequences, to Dr. Edmundo Lamoyi for his useful comments, and to Violeta Aguilar for her fine secretarial work.

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update ?

Figure 1
Western blots of protein antigens from vesicular fluid of *T. crassiceps* and *T. solium* cysticerci reacting with sera of pooled *T. solium* neurocysticercotic patients and *T. crassiceps*-infected mice.

Figure 2
Detection of recombinant antigens that cross-react with *T. solium* antisera using spot-lysis approach. Positive clones (KETc1, 4, 7, 11, 12) are indicated.

Table 1
Induction of Protective Immunity against Cysticercosis

	Mouse		*Pig*	
Immunized with	*control*	*vaccinated*	*control*	*vaccinated*
T. crassiceps (total extract)	25.8 ± 1.9[a]	0[b]	519 ± 49.2	216.6 ± 53.8[b]
T. solium (total extract)	25.8 ± 1.9	1 ± 0[b]		
T. crassiceps (vesicular fluid)	38.1 ± 6.7	14.3 ± 7.20[b]	5 ± 0.85	2.5 ± 1.19[b]
T. crassiceps (gel cut-out 56, 66, and 74 kD fractions)	38.1 ± 6.7	9.7 ± 6.60[b]	5 ± 0.85	0.16 ± 0.17[b]
KETc1	28.2 ± 18.38	6.6 ± 4.72[b]		
KETc7	28.2 ± 18.38	7.6 ± 7.57[b]		
KETc11	28.2 ± 18.38	40.6 ± 22.2		
KETc12	28.2 ± 18.38	4.4 ± 5.30[b]		

[a]Mean ± standard error of the cysticerci recovered. The number of animals in each group varies from 5 to 15. KETc1, 7, 11, and 12 are *T. crassiceps* recombinant antigens. Mice were challenged with 10 *T. crassiceps* cysticerci each, and pigs were challenged with *T. solium* eggs.

[b]Significant protective differences with control.

CROSS-REACTIVITY BETWEEN *T.solium* and *T.crassiceps*

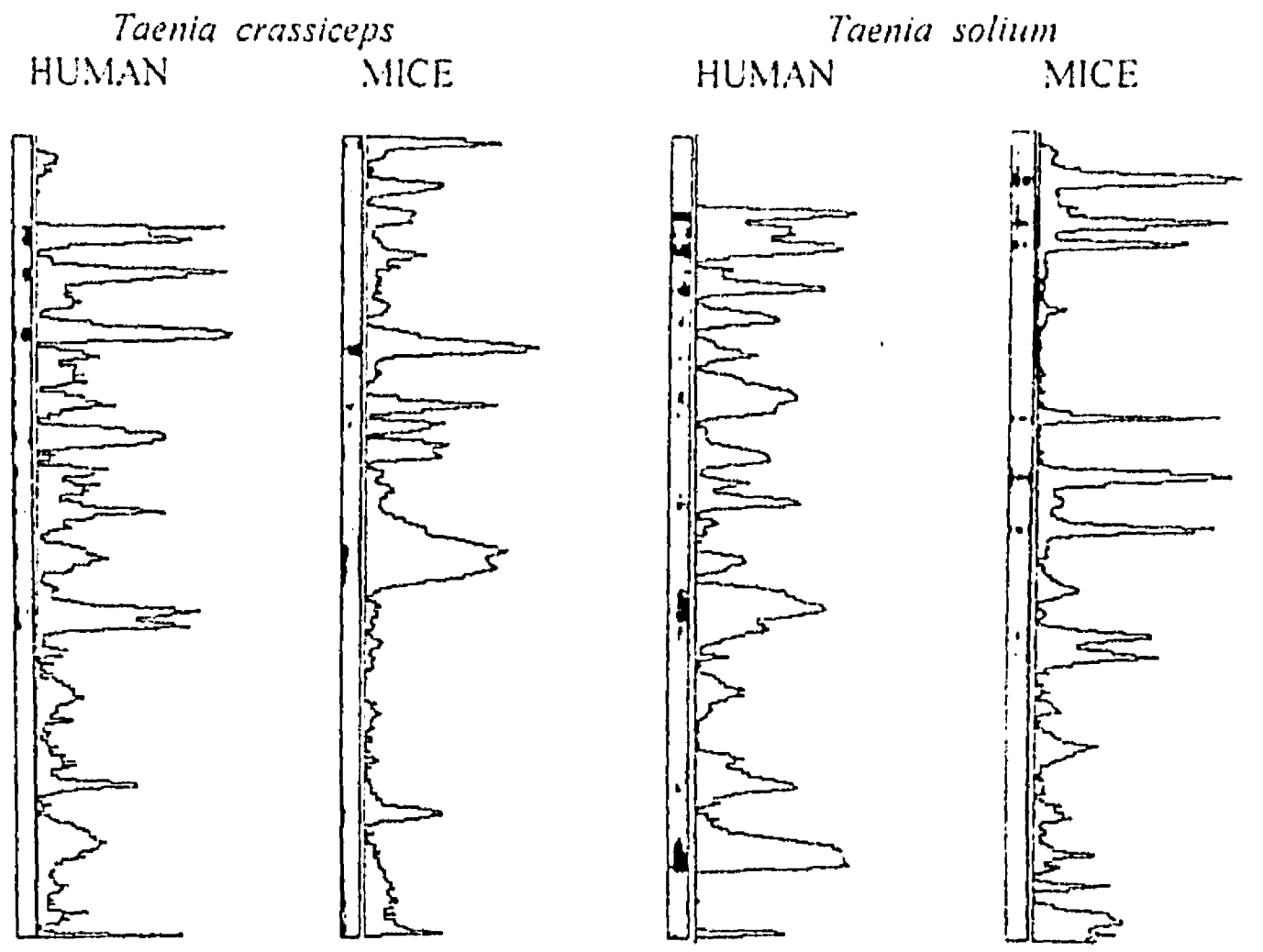

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Fig. 1

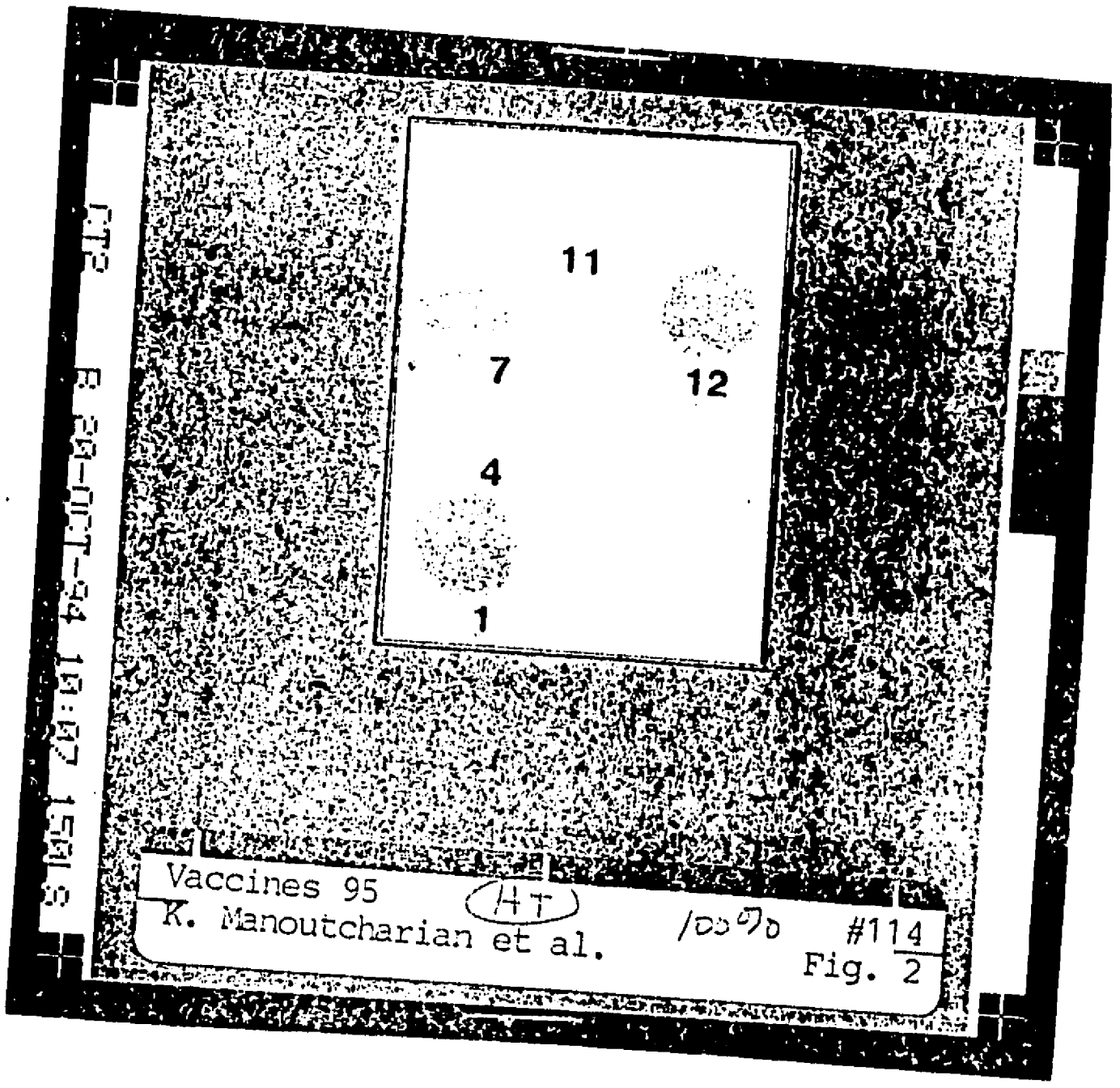
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Fig. 2

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Dr. Tony Holder
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Editor

Dear Dr. Holder

The enclosed manuscript entitled : "Cysticercosis: Identification and Cloning of Protective Recombinant Antigens" by Manoutcharian et al. is hereby submitted for publication in the Molecular and Biochemical Parasitology.

Results reported in the present manuscript were partially reported in the proceedings of the meeting : Molecular Approaches to the Control of Infectious Diseases, in Cold Spring Harbor. Editors of these proceedings inform us that results including in Vaccines.95 can be published in more detail in a journal. We hope this will not be a problem, however we want to inform you and the information list and the galleys of the report is also enclosed for your convenience.

I hope it will be found acceptable.

Sincerely yours

Edda Sciutto, D. Sc.
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Fax: (525) 550-0048.

CYSTICERCOSIS: IDENTIFICATION AND CLONING OF PROTECTIVE RECOMBINANT ANTIGENS.

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Abstract

We herein describe the cloning and characterization of five recombinant antigens expressed in the cysticercus stage of both *T. crassiceps* and *T. solium*. A cDNA library was constructed in bacteriophage λZAP using mRNA isolated from larvae of *T. crassiceps* ORF. The recombinant phage library was screened with polyclonal antibodies against 56- and 74-kD protective antigen fractions. This screening identified 13 recombinant clones, 5 of which were also strongly recognized by pooled sera from *T. solium* experimentally infected pigs. The native antigens are proteins of 56 (clones KETc1, 4, 7) and 74 and 78 kD (clones KETc11, 12) of *T. crassiceps* cysticerci. Vaccination experiments using these five recombinant clones against murine cysticercosis, point to the relevance of KETc1, 4, 7, and 12 in host-protection while the clone KETc11 does not modify the expected parasite load in females and facilitates the parasitosis in males.The complete sequence of the KETc7 antigen clone is presented, with its deduced amino acid sequence. This gene does not reveal high homology with any other reported antigen sequence.

1. Introduction

Cysticercosis caused by *Taenia solium* seriously affects human health and is responsible of important economic losses in developing countries [1, 2, 3]. In developed countries it's frequency is extremely low, but recently, due to immigrants from endemic areas in Mexico and Central America, its incidence has risen in the United States [4, 5].

The essential role of pigs as obligatory intermediate hosts, offers the possibility of interfering with transmission by vaccinating pigs, thus modifying the prevalence of cysticercosis. This has been proposed as a realistic control measure, since it does not pretend to modify the rustic breeding conditions forced by poverty and allows for aspirations to better value in the meat market.

Several antigenic preparations from oncospheres and larval stages of different cestodes have been fruitful in the development of vaccines against cysticercosis, and one recombinant vaccine effective against *T. ovis* has been developed [6]. Among these antigenic preparations, *T. crassiceps* cysticercal antigens are capable of protecting mice against *T. crassiceps* experimental infection [7], as well as rats and pigs infected with *T. taeniaeformis* [8] or *T. solium* [9] oncospheres, respectively. 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* [9]. Here we report the protective capacity of *T. crassiceps* separated antigens against pig cysticercosis and the production and characterization of four of these antigens by recombinant DNA methods which effectively protect mice against *T. crassiceps* murine cysticercosis.

2. Materials and Methods

Parasites, challenge and measurement of the parasite load. Larval *T. crassiceps* ORF utilized in this study were from a stock kindly supplied by Dr. B. Enders (Behringwerke, Marburg, Germany) in 1984. The parasites have been maintained by serial intraperitoneal inoculation in young BALB/cAnN female mice ever since. The parasites used to challenge control and vaccinated mice were obtained from donor mice -also BALB/cAnN- infected with ten small larvae and allowed to reproduce for 1 to 4 months inside the donor host before harvesting.

Vaccinated and non-vaccinated mice were each infected intraperitoneally with 10 small cysticerci 15 days after vaccination as described elsewhere [10]. Parasite load was measured in each mouse by counting the number of larvae recovered from the peritoneal cavity.

T. solium eggs used for challenging pigs were isolated from a tapeworm recovered from one man in the state of Chiapas, México, after treatment with a single oral dose (2g) of Niclosamide (Yomesan, kindly supplied by Bayer, México). The tapeworm was classified and eggs were recovered as previously described [9]. For infection, pigs were administered 10,000 eggs each, orally, in a single meal of Purina Chow , 60 days after vaccination. Sixty days after infection pigs were euthanized 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.

Parasite antigens and anti-parasite antibodies. Soluble antigens of *T. crassiceps* cysticerci were obtained from cysticerci recovered from the peritoneal cavity of BALB/cAnN female mice after 30 to 60 days of infection by a procedure published elsewhere [10]. Pigs were experimentally infected with *T. solium* oncosphere and 30 days after sera from these pigs were pooled.

Affinity purification of anti-parasite antibodies reactive with recombinant clones and immunoblot analysis. New Zealand white rabbits were immunized by subcutaneous injection with gel cut-out acrilamide including 56- and 74-kD *T. crassiceps* cystercal antigen fractions in Freund's complete adjuvant (FCA). After three biweekly boosts with Freund's incomplete adjuvant, blood

samples were taken and the presence and specificity of the antibodies produced were tested by Western Blot analysis according to a previously reported protocol [11]. Antibodies to antigens expressed by recombinant clones were affinity-purified from these immune sera according to the method described by Ozaki et al [12]. Affinity-purified antibodies from the KETc1, 4, 7, 11,and 12 were used to probe immunoblots of *T. crassiceps* cysticercal antigens to identify the native antigens that correspond to recombinant proteins.

Construction of cDNA library. mRNA from 15 ml of *T. crassiceps* cysticerci was isolated using a comercial kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. The mRNA (5μg) was transcribed into cDNA using the ZAP-cDNA synthesis kit (Stratagene). The cDNA was 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 four packagings was $1.8x10^5$ plaque forming units (pfu).

Screening of the expression library and selection of recombinant clones. To detect clones that codify for antigens of 56- and 74-kD, the specific rabbit immune sera, produced as described above, were used following conventional techniques [13]. The cDNA library was plated at a concentration of 10^4 pfu/plate on XL1-Blue cells and $2x10^4$ clones were screened using specific polyclonal rabbit antisera after removal of anti-*E. coli* antibodies by *pseudoscreening* with conventional procedures [14]. To detect positive clones ^{125}I-PA (Amersham, UK) was used as a second antibody. To detect those of interest in *T. solium* vaccination, a screening with sera from *T. solium* infected pigs was performed.

Vaccination. Groups of 6 and 7 York-Landrace pigs, 40 days old, were immunized with *T. crassiceps* total antigens at the base of the ear in a single dose of 0.4 mg per kg (2.5ml) homogenized in the same volume of FCA. Other group of pigs was immunized under equivalent conditions with gel cut-out bands of 56-, 66- and 74-kD together. Antigens were prepared as previously reported [10]. Control pigs were inoculated with FCA in saline at a dose of adjuvant similar to that received by the immunized animals.

Crude lysates of recombinant clones were prepared following the procedure described by Snyder et al [13]. Soluble antigens from crude lysates were used for vaccination. Groups of 5 to 8 male and female BALB/cAnN mice were vaccinated by injecting subcutaneously 400 μl of a crude lysates from each recombinant clone homogenized in an equal volume of FCA. Control mice were immunized with crude lysate from λ clone without insert in FCA.

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 [14]. Sequencing was performed by the dideoxynucleotide chain termination method [15] using "-40" and Reverse primers to sequence the two chains and [α-^{35}S] dATP (Amersham, UK). For sequencing double stranded plasmid DNA, was employed using Sequenase as recommended by the manufacturer (U.S. Biochemicals, Cleveland, OH). The obtained DNA sequences and deduced amino acid sequences were compared with the GeneBank DNA Databases [16] and other available databases.

3. Results

Effect of vaccination with T. crassiceps antigens against T. solium pig cysticercosis. Table I shows the parasite load in non-vaccinated control pigs after 60 days of oral infection with *T. solium* oncospheres and those obtained in pigs vaccinated either with vesicular fluid or a mixture of gel cut-out *T. crassiceps* antigens (74+66+56kD). Vaccination with vesicular fluid antigens resulted in a significant decrease of the parasite load. Higher protection was observed in pigs vaccinated with gel cut-out antigens of 56-, 66- and 74-kD, where 6 of 7 showed no parasites.

Construction of a T. crassiceps cDNA library and immunoscreening. A cDNA library was constructed using mRNA isolated from *T. crassiceps* cysticerci. In order to perform an antibody screenning of the cDNA library, specific polyclonal antibodies were prepared in rabbits against 56, 66 and 74 kD *T. crassiceps* cysticerci antigens. Screening of approximately $2x10^4$ recombinant phage yielded 13 clones, recognized by antibodies against 56 and 74 kDa. After secondary screening, to detect antigens of interest for *T. solium* vaccination, positive clones were screened with pooled *T. solium* sera from infected pigs using the spot-lysis approach. By this procedure five positive clones were detected. These clones were designated as KETc1, 4, 7, 11 and 12. DNA fragments 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).

Nucleotide Sequencing. λDNA and rescued phagemid (pSK) DNA were obtained from the 13 clones, and restriction mapping revealed insert sizes from 0.5 to 2.0 Kb. Sequence analysis demonstrated that all clones were unique in view of the fact that no sequence homology was found in the aproximately 400 bp of the 3′ portion of the sequenced genes except for the insert of KETc11 which was previously reported a *T. crassiceps* HYG sequence. Figure 2 shows

the complete sequence of KETc7 which is the only one entirely sequenced; while the sequencing of others are now in process. This nucleotide sequencing demonstrates that the cloned cDNA (KETc7) sequence is 445 bp in length (Fig. 2). The coding strand was identified by the presence of a polyA tail. The presence of EcoRI and XhoI sequences in flanking regions of the gene shows that the cloning procedure was carried out correctly. From three possible reading frames of these sequences only one appeared to be appropriate (300bp), which was also in frame with the lacZ gene of λZAP and coded for a 100 amino acid peptide.

Identification of the native parasite proteins corresponding to the recombinant antigens. *T. crassiceps* vesicular fluid antigens were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose filters. Each lane was probed with anti-KETc 1, 4, 7, 11, and 12 affinity-purified antibodies from rabbit anti-56 and 74kD specific antisera. A single band of antigens of approximately 56kD was detected with antibodies purified with KETc1,4,and 7 and two bands of 74- and 78-kD with antibodies purified with KETc11 and 12.

Effect of vaccination with λ lysates from recombinant clones against murine cysticercosis. To test the potential of recombinant antigens for cysticercosis prevention, crude lysates of the 5 clones in FCA were used to vaccinate BALB/cAnN female and male mice (Table 2). As controls, mice injected with lysates from clone without insert in FCA were included. As shown in Table 2, significant protective effects were obtained with 4 (KETc1,4,7,12) from the 5 clones employed. 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 30.5 to 64.1% in females.In contrast, immunization with KETc11 clone lysate significantly increased the expected parasite load in males and did not modify that obtained in females.

4. Discussion

In this report we confirm, in pigs, the host-protective nature of 56-, 66- and 74- kD *T. crassiceps* antigens previously observed in mice against murine cysticercosis. Pigs immunized with SDS-PAGE cut-out regions from total antigens were significantly (96.8%) protected against infection. To produce these antigens in plentiful supply we prepared a cDNA library from *T. crassiceps* cysticerci. Polyclonal specific antisera were produced in rabbits to isolate genes encoding for 56, 66 and 74 kD protective antigens, and used to screen the expression library. Specific antibodies against the 56 and 74 kD antigen region enabled us to isolate two types of clones that represent parts of the serologically related molecules of these two regions. Using this screening we identified 13 recombinant clones. To reduce the number

of antigen-expressing cDNA clones of interest in the prevention of *T. solium* pig cysticercosis, a pooled serum from *T. solium* early infected pigs was used for screening. This screening let us identify 5 clones. The Western blots using affinity purified antibodies against each recombinant clone confirmed that KETc1, 4, and 7 correspond to a native antigen of 56-kD whereas KETc11 and 12 correspond to 74-kD (Figure 1).

We determined the efficacy of our recombinant antigens as a vaccine, in the first instance, 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 [17], males and females of this strain were employed in the study. For controls, mice were immunized with lysates from control clones. As Table 2 shows, 4 of these 5 recombinant antigens induce high levels of protection in mice (but not complete). This result points for the first time towards the existence of recombinant antigens of potential interest in *T. solium pig* cysticercosis prevention. On the other hand, mice immunized with lysates from KETc11 showed a higher parasite load than control male mice, while no effect was observed in susceptible females in which the parasitosis facilitating effect could be overriden by the naturally high susceptibility of females. A systematic study of the immune response underlying these phenomena is in process with the aim of understanding the immunological mechanisms involved in this host-parasite relation.

The KETc11 facilitating antigen could be an interest tool to modify the natural susceptibility to this parasitosis by the induction of tolerance to the mechanisms which facilitate parasite establishment and/or its growth by transgenesis. Our clone KETc11 was isolated from the ORF strain and its estimated size is 0.9 kb. Interestingly, only 329 bp of this antigen was previously isolated from the HYG strain by Zarlenga D. S. et al. This author reported in the GenBank this antigen as an antigen of diagnostic interest for bovine cysticercosis. In this regard, their use in *T. solium* diagnosis should be explored.

Comparisons of the nucleic acid sequences and predicted amino acid sequences of our clones with electronic databases did not reveal any significant homology with known genes or proteins except KETc11. Nevertheless, although we only have the 3´ portion of the KETc7 native gene, this short segment provides the expression of at least one protective epitope of the native protein as demonstrated in the vaccination experiments shown on Table 2. Interestingly, herein we found an open reading frame encoding a polypeptide of 100 amino acids which is highly hydrophobic and proline rich (29%) aminoacids(Figure 2).

Varying degrees of protective immunity against some cestodes have been shown to be effectively induced in their mammalian hosts [9, 17, 18, 19, 20] with a variety of antigens, nevertheless, the epidemiological impact of a widespread vaccination program upon transmission dynamic is not trivial [1]. Oncospheral antigens - natural or recombinant - are held to be the most effective in protecting rats and sheeps against challenge with eggs of *T. taeniaeformis* [21] or *T. ovis* [6], respectively, allegedly because the onchosphere's greater sensitivity to antibodies compared with the metacestode stage [6]. 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 a *T. crassiceps* cysticercus, and this species can divide by budding apart from going through a sexual stage, and therefore probably presents a much more heterogeneous group of antigens, some of which could share oncospheral determinants. Although their presence in oncospheres remains to be evaluated, the vaccine designed using these early cysticercal antigens would cover against oncospheres that managed to reach the metacestode stage. Although 100% protection has not been achieved yet, increasing levels of protection could be obtained through an optimal combination of the protective antigens detected and using more effective expression systems to purified recombinant antigens. The use of more appropriate adjuvants to increase levels of protection remains to be studied. Our results allow us to begin the identification of protective epitopes and their chemical synthesis for use as an alternative approach in designing a vaccine.

Studies on the role of vaccination in pig cysticercosis are comparatively scarce. It has been reported that various antigen preparations can effectively induce protection in pigs [9, 21, 22, 23]. At present we are evaluating our recombinant antigens against the challenge of pigs with *T. solium* eggs.

In summary, we presently report the identification of *T. crassiceps* recombinant antigens which demonstrate significant protective capacity against murine cysticercosis and of potential interest in the prevention of pig cysticercosis, together with the first reported molecular characterization of a *T. crassiceps* antigen gene.

Acknowledgments

We thank Dr. Roberto Coria and Dr. Juan Pedro Laclette for help with database comparision. Biol. Mercedes Baca for their expert assistance, and Dr. Carlos Larralde and Dr. Edmundo Lamoyi for their comments. 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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Table 1. Effect of vaccination with *Taenia crassiceps* antigens on *Taenia solium* pig cysticercosis.

Controls	[a]6,6,7,3,3,5	[b]5 ±2.06
Immunized with:		
Total Antigens	1,3,3,2,0,0,9	2.5 ±3.1 ([c]48.5%)+
Gel cut-out antigen fractions (56+66+74 kD)	0,0,0,0,0,1	0.16±0.4 (96.8%)+

Pigs were immunized 60 days before challenge infection in FCA. Sixty days after challenge pigs were sacrificed and parasites were counted in aprox. 10 kg of meat each.

aNumber of cysticerci recovered per pig. bMean(N)±standard deviation of the parasite load recovered per group. c Protection efficiency=(N in control pigs- N in immunized pigs/ N in control pigs). + $P<0.05$ (ANOVA test)

Table 2. Effects of immunization against *Taenia crassiceps* cysticercosis in BALB/cAnN mice.

	Control	KETc1	KETc4	KETc7	KETc11	KETc12
M	[a]28.2±18.4	6.6±4.7*	N.D.	7.6±7.5*	40.6±22.2*	4.4±5.3*
		[b]76%		73%	-44%	84.4%
F	61.3±29.7	28.5±17.5*	22±15.9*	42.6±6.4*	46.0±17.4	26.3±7.2*
		53.5%	64.1%	30.5%	24.9%	57.1%

aMean(N)±standard error of the parasite load recovered in groups of five to eight male (M) and female (F) mice. bProtection efficiency: (N in control mice - N in immunized mice)/ N in control mice. *Significant difference with control values at 95% confidence level (ANOVA test).

Legends

Figure 1. Identification of native proteins of *T. crassiceps* cysticerci encoded by the recombinant clones. Lanes probed with: (1)rabbit anti-56 kD antibodies affinity-purified from clone KETc1, (2) from clone KETc4; (3) from clone KETc 7 and rabbit anti-74 kD antibodies affinity purified (4)from clones KETc11 and (5) from clone KETc12. Lane (6) probed with pooled sera from three infected pigs; (7) from normal pig sera, (8) from infected mice and (9) from normal mice.

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 EcoRI 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.

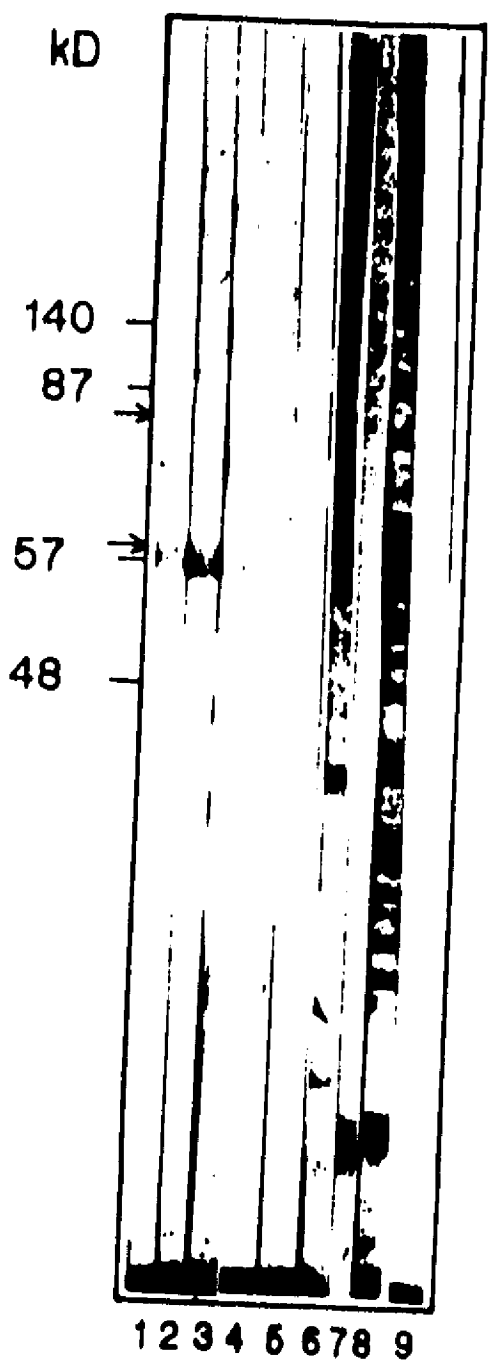

Figure 1

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 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 ATTTCGTCTCCATACGCAAAAAAAAAAAAAAAAAAAAACTCGAG
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VETERINARY PARASITOLOGY

An International Scientific Journal
EDITORIAL OFFICE, P.O. BOX 181, 1000 AD AMSTERDAM, THE NETHERLANDS

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Re.: VETPAR 862 Amsterdam, December 7, 1994

Dear Dra. Sciutto,

I am pleased to be able to inform you that the manuscript *Immunization of pigs against Taenia solium cysticercosis: factors related to effective protection* by Sciutto, E., Aluja, A., Fragoso, F., Rodarte, L.F., Hernández, M., Villabos, M.N., Padilla, A., Keilbach, N., Baca, M., Govezensky, T., Diaz, S. and Larralde, C. has now been found acceptable for publication in our journal. The manuscript has been transferred to the desk editor for preparation for press. Proofs will be sent to you in due course.

Yours sincerely,
AGRICULTURAL SCIENCES SECTION

pp

Mrs. N. de Jong

IMMUNIZATION OF PIGS AGAINST Taenia solium CYSTICERCOSIS: FACTORS RELATED TO EFFECTIVE PROTECTION

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ABSTRACT

Fifty-six (56) pigs were immunized against Taenia solium cysticercosis with antigens from Taenia crassiceps metacestodes, in a variety of protocols, and then challenged orally with Taenia solium proglottids or eggs. Results of immunization -expressed as individual parasite loads- ranged from significant reduction of parasite loads (host protection) to clear increase (parasite facilitation) in apparent relation with immunogen dose, adjuvant employed and genetic background of pigs. In all trials, however, immunized pigs harbored more damaged cysticerci than controls, indicating that immunization does induce some restrictions to parasite survival even if these are eventually overwhelmed by other parasite-promoting factors. Western Blots in immunized-protected pigs indicated to antigens of MW 242, 234, 118, 77, 55 and 45 Kd's as possibly involved in immunological protection.

INTRODUCTION

Human and pig cysticercosis caused by Taenia solium seriously affects health and economy of developing countries (Gemmel et al., 1985; Larralde et al, 1992): neurocysticercosis is it's most dreaded complication in human disease (Del Bruto et al., 1988) and skeletal muscle cysticercosis in pigs lowers meat value and is cause of meat confiscation in abbatoirs (Aluja, 1982). Transmission factors are deeply rooted in social, educational and economic circumstances: meat inspection is avoided for a substantial fraction of the rustically bred pigs to evade confiscation and destruction by health authorities (Aluja, 1982); ignorance, distrust and reluctance to change ancient forms of pig-rearing lessen the impact of educational efforts (Keilbach et al., 1989) and the poverty of the rural populations in endemic areas impose risky pig-breeding methods based on free-roaming, random scavenging and deliberate feeding with human excrements (Aluja, 1982). Transmission is furthered by the increasing intensity of pig rearing and the turpitude of social change in these conditions. Meat inspection has failed to completely curb transmission because there are not sufficient nor adequately located abbatoires and also because many rustically bred pigs are deliberately slaughtered domestically to avoid the possibility of confiscation. Intensive and extensive compaigns search and treatment of tapeworm carriers are presently being launched in hope of diminishing transmission (Pawlowski, 1989). The essential role of pigs as an obligatory intermediate host, offers the possibility of interfering with transmission through their

vaccination. Vaccination is thought to be better accepted than meat inspection because it is not confiscatory, protected pigs would be better fit to resist the rustic breeding methods forced by poverty, and effective vaccination could increase the economic return of the meat should it meet with inspection standards in abbatoirs.

Varying degrees of protective immunity against some cestodes have been shown to be effectively induced in their mammalian hosts (Johnson et al., 1989; Bogh et al., 1990; Ito, Bogh et al., 1991; Ito, Onitake et al., 1991; Rickard and Williams, 1982) with a variety of antigens. Nevertheless, the epidemiological impact of a thorough vaccination program upon transmission dynamics is not trivial (Gemmell et al., 1985). Oncospheral antigens -natural or recombinant- are held to be the most effective in protecting rats and sheep against challenge with eggs of Taenia taeniaeformis (Ito, Bogh et al., 1991) or Taenia ovis (Johnson et al., 1989), respectively, allegedly because of the oncosphere's greater sensitivity to antibodies compared to the more impregnable metacestode stage (Johnson et al., 1989). Fortunately, however, in experimental murine Taenia crassiceps cysticercosis, an effective protective role for cellular immunity against the metacestode stage is emerging (Bojalil et al., 1993). Thus, a vaccine designed towards the induction of cellular immunity together with protecting antibodies would cover against all the parasite's stages, including the few oncospheres that manage to survive antibody attack and reach the metacestode stage.

Studies on the role of vaccination in pig cysticercosis are comparatively scarce; they inform of various antigen preparations as inducing some degree of protection in pigs (Molinari et al., 1993; Nascimiento et al., 1987). However, we are convinced that vaccination of pigs against Taenia solium cysticercosis should be more thoroughly explored before massively applied. Genetically controlled experiments in murine Taenia crassiceps cysticercosis stress the relevance of the major histocompatibility complex (MHC) (Sciutto et al., 1991) and sex (Bojalil et al., 1993; Huerta et al., 1992) in innate and acquired immunological resistance to metacestodes, cautioning against simple expectations in genetically heterogeneous populations, as are humans and pigs. Hence, the purpose of this study was to test the effects of immunization of pigs against subsequent experimental challenge with Taenia solium eggs in a variety of conditions. Taenia crassiceps was chosen as source of antigens because of it's extensive antigenic similarity with Taenia solium (Larralde et al., 1990) and of previously reported crossprotection between Taenia solium and Taenia crassiceps in experimental murine cysticercosis (Sciutto et al., 1990; Valdez et al., 1994). Should the inverse crossprotection hold in pigs, the murine model of cysticercosis would provide unlimited amounts of vaccine for more extensive application in the field.

MATERIALS AND METHODS

Experimental Design

We report results obtained in four different immunization trials, varying in the number and breed of pigs, as well as in their age at immunization, dose of immunogen and adjuvant employed. Each immunization trial included a corresponding control group. All pigs were challenged orally with Taenia solium eggs, some in suspension and others still inside proglottids, at differing times after immunization because of the impossibility of securing sufficient eggs at fixed times. So variable an experimental design was accepted as the only way of obtaining first guiding clues toward firmer designs, and was even welcomed as a more realistic modeling of future vaccination field trials in endemic areas, where genetic control of pigs and homogeneity in risk is presently utopic.

Pigs

One trial included 19 hybrid pigs Yorkshire and Landrace, 19 days old; a second trial included 6 hybrid hogs Pelon Mexicano and York-Landrace, 17 days old; the third included 16 hybrid hogs Yorkshire, York-Hamshire, York-Duroc, Duroc-Hamshire, 60 days old; and the fourth included two litters, one of 7 hybrid York-Landrace and another of 8 hybrids from a Spotted dam and a Duroc sow, Hamshire Yorkshire and Landrace all 60 days old.

All pigs were immunized with anticholerae vaccine 20 to 45 days after birth, housed in the Facultad de Medicina Veterinaria y Zootecnia, UNAM, where no transmission of cysticercosis occurs, and fed ad libitum until used for experimentation at various times according to the availability of Taenia solium eggs.

Immunogen preparation

The immunogen consists of total antigens recovered from Taenia crassiceps metacestodes grown in the peritoneal cavity of young Balb/c female mice infected for 1-4 months, as previously described (Sciutto et al., 1990).

Cysticerci were collected in cold phosphate buffered saline (PBS, 0.15 M NaCl, 0.01 M phosphate) in sterile conditions and homogenized (Polytron, Brinkman Instruments) after extensively washing in PBS. This antigen preparation contains aproximately 50 different molecular weight bands in Western Blot, some corresponding to glycoproteins and others to proteins and peptides (Larralde et al., 1989). The final protein concentration was adjusted to 9 mg/ml, as estimated by the method of Lowry (Lowry et al., 1951), and the immunogen preparation was stored sterile at -70^{o}C until used.

Immunization schedules

Four different immunization protocols were followed as shown in Figure 1. In the first and second trials (High Dose Protocols) pigs were vaccinated by subcutaneous inoculation at the base of one ear with of antigens suspended in aluminium hydroxide (alum) (30 mg protein per 1 mg of alum); the

immunogen was administered twice, two weeks apart for the first trial (HD2dose) and only once in the second (HD1dose). Control non-immunized pigs were injected with alum alone at a dose approaching the average dose of adjuvant received by the immunized animals. For the third trial (Low Dose Protocol, LD.4) a ten times lower dose (0.4 mg/kg in 2.5ml) of antigens in Freund's complete adjuvant (FCA, Sigma St. Louis MO, v:v), was applied only once at the base of the ear, while control pigs were injected with the aquivalent amount of FCA alone. For the fourth trial (Low Dose Protocol, LD.1) we employed one dose of 0.1 mg/kg in 2.5 ml of vaccine in FCA (v:v) while control pigs were injected with the equivalent amount of FCA alone.

Pigs were housed, fed and observed until sacrificed; they were also bled before and, after immunization and immediately after to sacrifice to keep track of their immune response.

Parasites

Taenia solium eggs used for the challenge came from two tapeworms: one recovered from a man in the state of Guerrero, Mexico, and the other from a woman in Sinaloa, Mexico. The tapeworms were recovered after treatment with a single oral dose (2g) of Niclosamide (Yomesan, kindly supplied by Bayer, Mexico). The tapeworms were washed in saline, plus antibiotics (100 U per ml penicillin + 100 μg per ml streptomycin) and classified as Taenia solium by counting the number of uterine ramifications under light microscopy (Molinari et al., 1985). When eggs were used for challenge, gravid proglottids were separated from one tapeworm with the help of two dissection needles and eggs were uniformly suspended and counted in a hemocytometer. The suspension was adjusted to 10,000 eggs per ml of saline solution. Pigs were challenged within 30 days after recovering the tapeworms from the human donors.

Eggs challenge

All pigs used in these experiments were challenged with Taenia solium eggs. Pigs of the first trial (here referred as HD2dose), were infected with 3 complete gravid proglotids by oral administration through a cannula, while only 1 proglotid was administered to each pig of the second trial (here referred as HD1dose). Pigs of the third (LD.4) and of the fourth trial (LD.1) were challenged with 250000 eggs per pig. The egg suspension was placed in a single controlled meal of Purina Chow, after 18 hours of food deprivation to increase their eagerness to eat. Pigs ingested the contaminated meal quite thoroughly in a few minutes and licked the plate clean which assured us of the complete ingestion of the challenging eggs. We strongly recommend this method as a safe and controlled oral experimental challenge in pigs, contrary to physical restrain and forceful swellowing of the eggs, which might leak out.

Necropsies

After 73 to 90 days of infection all pigs were killed with a captive bolt stunner (Schermer) and then bled. Sera obtained from pigs before, after and during the experimentation were prepared as usual and kept at -70°C until assayed. Parasite load was measured in each pig after necropsy by counting the number of parasites in complete masseters, tongue and diaphragm, in 200g of right and left abdominal muscles, in intercostal muscles and in 400g of the right and left shoulder and hind legs. Parasites counted were classified according to their appearance as live (cysts containing a clear transparent fluid and a scolex) or damaged (containing colloid or caseous material) cysticerci. Parasite load was expressed as the number of cysticerci per gram of meat explored.

Serology

Antibody titers in sera were assessed by ELISA as previously described (Larralde et al., 1990; Ramos-Kuri et al., 1992). Also, Western Blots were performed in all the sera following procedures described elsewhere (Larralde et al., 1989).

Immunoplots

To identify the most relevant antigens in a recognizedly complex antigen mixture, we plotted the frequency of reaction in Western Blot of each individual antigen band from the set of sera collected in one experimental group (i.e. protected) against the frequency of the same antigen band when reacted with sera from pigs of another experimental group (i.e. not-protected), as detailed elsewhere (Larralde et al., 1989). When present, antigens related to protection clearly stand out in the plot.

Statistical analysis

The statistical significance of the effect of experimental variables (immunization, dose, number of doses) on the parasite load was studied by multifactorial analysis of variance (ANOVA, SAS Institute, 1985).

RESULTS

Parasite load

Table I shows the parasite load found in all immunization trials: in immunized pigs and in non-immunized control pigs. The HD2dose (where alum was employed as adjuvant) resulted in facilitation of parasite establishment, i.e., statistically significantly ($p<0.01$) higher numbers of live parasites were found in immunized than in non-immunized pigs. No significant effect of immunization upon parasite load was observed with the HD1dose. The LD.4 (where FCA was employed as adjuvant) induced protection, i.e., a statistically significant lower (average 58% decrement) live parasite load was found in immunized pigs compared with controls ($p<0.01$), with exception of one non-immunized pig which had a low parasite load (parasites=282) and one immunized pig which had a parasite load similar (parasites=535) to controls. With LD.I (where FCA was employed as adjuvant) parasite load was similar in immunized and control pigs. However, in this LD.1 trial, one of the litters (litter 2) was significantly less affected

than the other (litter 1) ($p<.05$): litter 2 included three pigs without live parasites, one pig with only two cysticerci, two pigs with less than two hundred live parasites, and two more in the lower range of the live parasites found in litter 1, which were all affected by several hundreds of parasites. In all trials the number of calcified parasites was higher in the immunized than in control pigs, and also in litter 2 of the LD.1 trial. Variation among controls within experiments are probably due to differences in challenge size and to intrinsic differences among the groups of pigs (breed; age; sex).

Antibody response in immunized and non-immunized control pigs

Serum levels of anticysticercus antibody registered before and after challenge are shown in Table II (corresponding to the HD2dose and the LD.4 trials). Very low levels of antibodies were present after immunization -slightly higher in immunized than in control pigs- but levels increased significantly in both groups 10 weeks after infection. As shown in Figure 2 there is a positive correlation between the total number of parasites found in each pig and antibody level in pigs from the HD2dose trial (where immunization induced facilitation) but not in those from the LD.4 trial (where immunization induced protection).

Figure 3 shows the Western Blots of Taenia crassiceps antigens with sera from pigs in which protection was documented (LD.4 trial). Clearly, there are many background bands prior to immunization (specially 90, 98, 105, 154 and 165 Kd), but immunization brings out a stronger and more plentiful antigen profile, albeit somewhat variable from pig to pig. However, 38 days after challenge a dramatic increase in antigen bands intensity is observed in all sera, very notably so at molecular weights of 118 Kd and lower than 80 Kd: bands of 77, 55 and 45 Kds being the most apparent (frequencies >70% in the immunized group and <10% in the non-immunized group, Figure 4A). Later along infection (73 days), the antigen profile is strong but main reactivity moves to higher molecular weight antigens, excepting 77 Kd and 45 Kd bands, which remain clearly visible in the blots. Figure 4B shows that in later infections antigens cluster better about the unit slope (i.e. antigen profiles of immunized and non-immunized pigs tend to greater similarity) and the most prominent antigens shift to the upper right quadrant of equally high reactivity for both groups, with the exception of bands 242 Kd and 234 Kd, which remain as indicative of previous immunization (frequencies > 70% in the immunized group and < 30% in the non-immunized). Similar patterns of antigen recognition are observed between non-immunized pigs and those immunized with the HD2dose trial in which parasite facilitation is induced by immunization (data not shown).

DISCUSSION

Our attempts to immunize pigs against Taenia solium cysticercosis with Taenia crassiceps cysticercal antigens lead

to three different sorts of results with respect to parasite loads: they protected the pigs against experimental infection with eggs in one trial, favored parasite establishment in another, and were irrelevant in two trials. These diverse results bring out many important points that we believe are worth considering in advancing towards an effective and controllable vaccine, some dealing with immunization protocols, others with the right choice of antigens, and still others with the genetic background of porcine hosts.

The great range of effects caused by the Taenia crassiceps antigens upon parasite loads of live cysticerci in the porcine host is a most prominent result: high doses of immunogen seem to be either non-effective (HD1dose trial) or to promote parasite survival (HD2dose trial), while low doses, specially the LD.4 trial, resulted in a clear (58%) decrement of parasite load in immunized pigs; however, no protocol resulted in total protection (parasite load = 0). Such dose-dependent results of vaccination had been noticed before in murine experimental cysticercosis (Sciutto et al., 1990) as well as in a number of other parasitic diseases (Nascimiento et al., 1987), when using complex antigen mixtures, and argues in favor of the identification and purification of protective antigens from those irrelevant or parasite-promoter. The role of the dose, however, is obscured by the possibility that differences may be attributed to the type of adjuvant employed: FCA in the LD.4 trial and alum in the HD2dose trial. It is well recognized that FCA is generally more effective. The protective results found in one trial (LD.4 trial) are encouraging but the range of effects warns against simple expectations from non-optimized immunization protocols. Parasite promotion resulting from high dose immunization discloses the complexities of the immunological network activated in parasitic disease, where immune interventions upon the host may simultaneously activate both protective and parasite-promoting events (Parkhouse and Harrison 1989). That all immunized pigs harbored more damaged (probably-dead) cysticerci than the controls furthers the notion that the immunogen managed to induce restrictive conditions for parasite growth in all animals, whatever the overall net effect proved to be in each trial and at that particular time after infection. An additional source of variation in parasite load between trials would be innate differences in T. solium infectivity or vulnerability to immunological attack -as there is in antigenic content (Yakoleff-Greenhouse et al., 1982; Prokopic et al., 1988) - so much so, in fact, that one specimen seemed to thrive in immunized hosts (HD2dose trial), while the others ignored or resented immunization (LD.4 and HD_1 dose trials). Heterogeneity in pathogenicity in Taenia solium would greatly weigh on the design and evaluation of a generally effective vaccine.

About the antigens employed in the immunogen, we can see that those from Taenia crassiceps metacestodes induce important and relevant immunological

changes towards Taenia solium antigens in the immunized pigs, as has been shown with Taenia taeniaeformis (Ito, Takami and Itoh., 1991) and in experimental murine Taenia crassiceps cysticercosis itself (Sciutto et al., 1990). The Western blot profiles we show here, contrasting those of immunized with non-immunized pigs, are unique in the literature and provide some important clues for immunodiagnosis and immunoprevention. Firstly, the sera of non-immunized pigs strongly react with a number of T. crassiceps antigens, many of which cross-react with those of T. solium, most notably with those of 126 to 259 Kds. The stimulus originating these antibodies is unknown, but one would expect they may interfere with quantitative serological tests, using non-purified antigens. Secondly, the Western blot profiles point to antigens associated to immunization and to protection. The immunization induced changes in Western blot profiles were most apparent after infection with Taenia solium eggs: sixteen different antigen bands in the Western Blots increased in the immunized pigs (LD.4 trial) early after infection - most notably 118 Kd, 77 Kd, 55 Kd and 45 Kd - many of which have been shown to be crossreactive with Taenia solium (Larralde et al., 1989). Even later in infection, when serological differences between immunized and non-immunized pigs tended to disappear, two antigen bands - 242 Kd and 234 Kd - were more frequently recognized in previously immunized pigs. When immunization induced facilitation of infection no specific antigens were found in Western Blots to be associated to either protection or facilitation (data not shown). Serum antibody levels were slightly higher in immunized than in control pigs prior to infection but not so afterwards: it would seem that antigen stimulation from challenge overides quantitative differences in whole antibody response due to immunization. However, in the trial where facilitation resulted from immunization (HD_2 trial), a statistically significant positive correlation was found between parasite load and total antibody level, hinting that some antibodies may actually help rather than hinder infection, as shown for Taenia crasssiceps in mice (Sciutto, 1990). Thus, in ongoing efforts to develop recombinant antigens in the design of a pig-cysticercosis vaccine, one should avoid selecting those very immunogenic but irrelevant or even parasite-promoting, a vast collection in crude parasite extracts. The Western blot profiles shown here point to some epitopes carried by antigens 242, 234, 118, 77, 55 and 45 Kd as those most strongly associated with immunization and protection.

One final point on the possible participation of genetic background in the response to challenge with Taenia solium eggs comes from the LD.1 trial where 4 of 7 pigs from the same litter were practically not infected (0, 0, 0, 2 cysticerci/pig), whether vaccinated or not, while the rest of the pigs were infected by the hundreds. This suggestion of strain-associated resistance in Spotted-Duroc pigs is encouraging, and in no way outlandish, since genetic background associated to the major histocompatibility complex (H_2) is a well established factor in

murine susceptibility to Taenia crassiceps (Sciutto et al., 1991), as well as in other parasitic diseases (Wakelin 1985). Breeding a pig strain resistant to Taenia solium cysticercosis, be it naturally resistant or transgenically induced with resistant genes, is no extravagance nowadays, and potentially useful in curbing transmission.

Our experiences immunizing pigs against Taenia solium cysticercosis with a complex antigen mixture from Taenia crassiceps metacestodes demonstrate significant corss reaction between these two cestodes and effective immunological changes in the immunized porcine hosts, some related to immune protection and some related to immune facilitation. Further, results hint towards the importance of vaccine dose, type of adjuvant employed, antigen selection and innate differences in susceptibility among pigs. Difficulties in securing T. solium eggs to use in challenge must resolved in order to control variation in this key factor of vaccination protocols. Other sources of variation, such as differences in pathogenicity in the eggs of T. solium tapeworms must be investigated, although its control would seem hopeless and unrealistic, as would also be that of a uniform susceptibility in the porcine host.

ACKOWLEDGEMENTS

This work was supported, in part, by the Dirección General de Asuntos del Personal Académico, UNAM, Consejo Nacional de Ciencia y Tecnología and Secretaría de Agricultura y Recursos Hidráulicos de México. We would like to thank Violeta Aguilar for her secretarial help. The authors also thank the Departamento de Cerdos de la Facultad de Medicina Veterinaria y Zootecnia, UNAM, for support in keeping the pigs during experimentation, and Bayer Co., for the Yomesan (Niclosamide). Isabel Pérez Montfort corrected the English version of the manuscript.

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LEGENDS

Table I. Individual parasite load recovered from control (non-immunized) and immunized pigs in all immunization trials.

Table II. Levels of serum anticysticercus antibodies measured by ELISA in immunized and control pigs before and after various challenge instances with Taenia solium eggs. Immunized pigs showed slightly higher antibody level than control pigs ($P<0.01$) before they were infected, but differences dissapeared after infection.

Fig. 1. Scheme of events in the immunization protocols of each immunization trial to illustrate differences in sequence, in time relation and in the age of pigs.

Fig. 2. The scattergrams show a statistically significant ($m=1.34$, $r^2=0.382$, $P=.0062$) positive correlation between serum antibody levels and parasite load when the immunogen facilitated the establishment of cysticerci (HD2dose trial) than when it hindered it (LD.4 trial).

Fig. 3. Challenge with Taenia solium eggs of control (non-vaccinated) and immunized pigs from the LD.4 trial, where protection was induced, caused a major qualitative change in the serologic profile of Taenia crassiceps antigen band recognition in Western Blots, specially in the region of molecular weights lower than 90 Kd, and in the intensity of band recognition in the Kd 260-105 interval. These changes are more noticeable in the sera of immunized pigs (Fig 4B) than in controls (Fig 4A).

Fig. 4. Immunoplots of Western Blots (Figure 4) contrasting serology of immunized versus non-immunized pigs (LD.4 trial; where immunogen induced protection) early and late after experimental infection with Taenia solium eggs. Differences were more marked in early infection, as evidenced by the many antigen bands plotted in high-left quadrant of the plane (corresponding to bands recognized by 50% or more of the immunized pigs, and by 50% or less of the non-immunized) and the tighter scatter of points in late infection. Antigen bands 118, 77, 55 and 45 Kd, in early infection, and 242, 234, in late infection, most significantly differ between immunized (protected) and non-immunized (unprotected) pigs.

TABLE I

Immunization Trials	State of Cysticerci	Control	Immunized
High dose (2 dose) & Alum◊	Live	1*, 9, 5, 0, 12	31, 137, 14, 12, 559, 450, 20, 108, 86, 10, 4, 6, 7, 21
		5.4• ± 4.1	**104.6 ± 175.8**
	Damaged	3, 3, 1, 0, 9	9, 123, 6, 23, 46, 25, 0, 16, 2, 1, 2, 5, 10, 9,
		3.2 ± 3.5	**19.8 ±2.2**
High dose (1 dose) & Alum	Live	18, 11, 8	34, 8, 1
		12.3 ± 5.1	**14.3 ± 17.4**
	Damaged	12, 9, 4	25, 6, 5
		8.3 ± 4	**12.0 ± 11.3**
Low dose (.4 mg/kg) & FCA	Live	586, 607, 282, 632, 611, 478, 376, 583	147, 112, 178, 193, 130, 292, 147, 535
		519 ± 128.7	**216.8 ± 140.0**
	Damaged	0, 0, 0, 8, 0, 3, 0, 7	41, 82, 18, 10, 0, 52, 14, 4
		2.3 ± 3.4	**27.6 ± 28.4**
Low dose (.1 mg/kg) & FCA	Live Litter 1	1219	933, 1129, 81, 1190, 405, 861
			766.5 ± 435.4
	Live Litter 2	0, 152, 0, 111	0, 517, 2, 786
		65.8 ± 77.7	**326.3 ± 391.3**
	Damaged Litter 1	0	26, 0, 51, 0, 5, 0,
			13.66 ± 20.9
	Damaged Litter 2	3, 20, 5, 1240	0, 76, 185, 0
		317 ± 55.9	**65.3 ± 87.5**

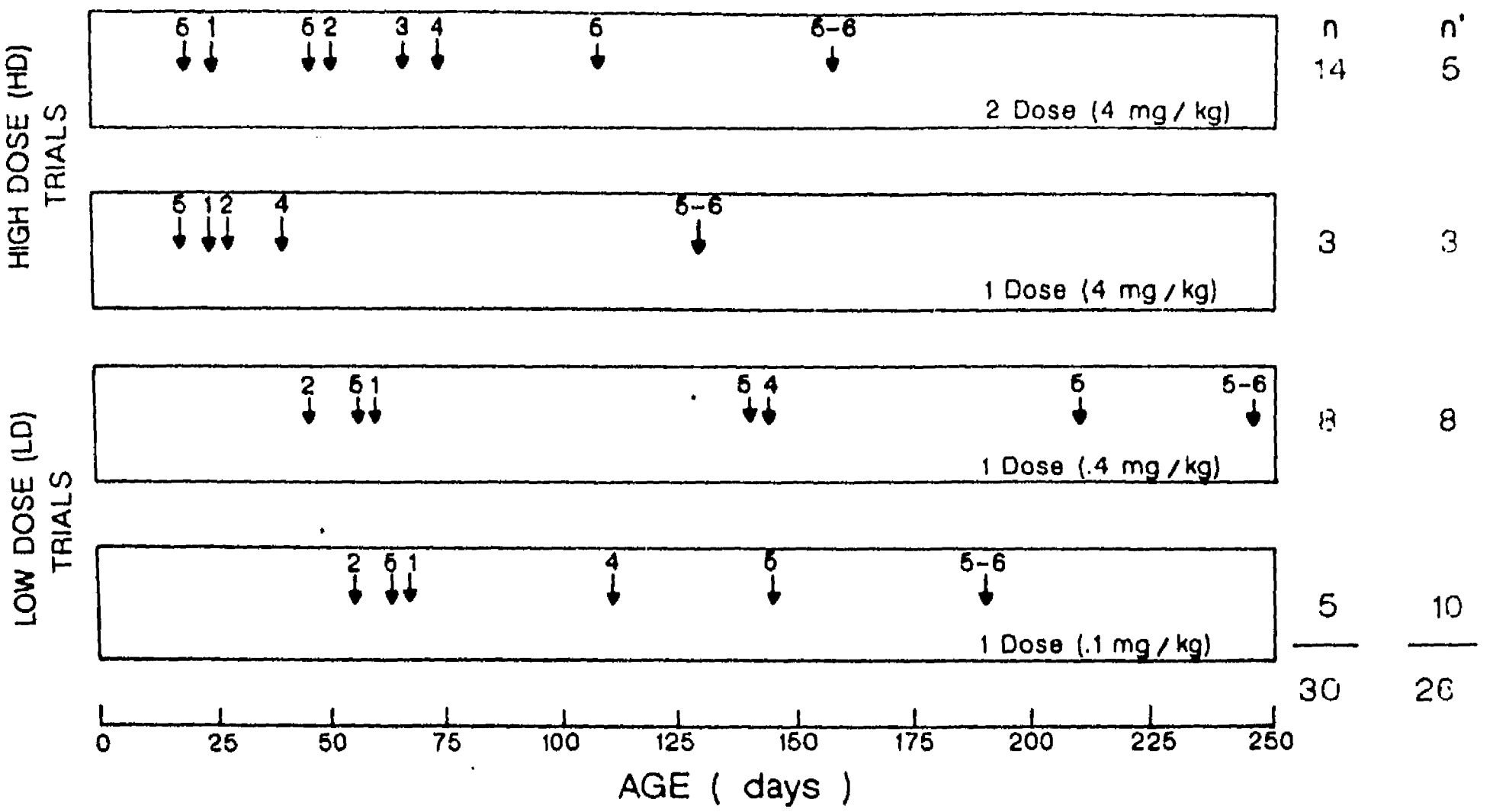
* *Total number of cysticerci found in each pig (individual parasite load)*
• *Mean and standard deviation of individual parasite load*
◊ *Type of adjuvant: Alum or Freund's complete adjuvant (FCA)*

TABLE II

SERUM ANTIBODY LEVELS IN IMMUNIZED AND NON-IMMUNIZED PIGS

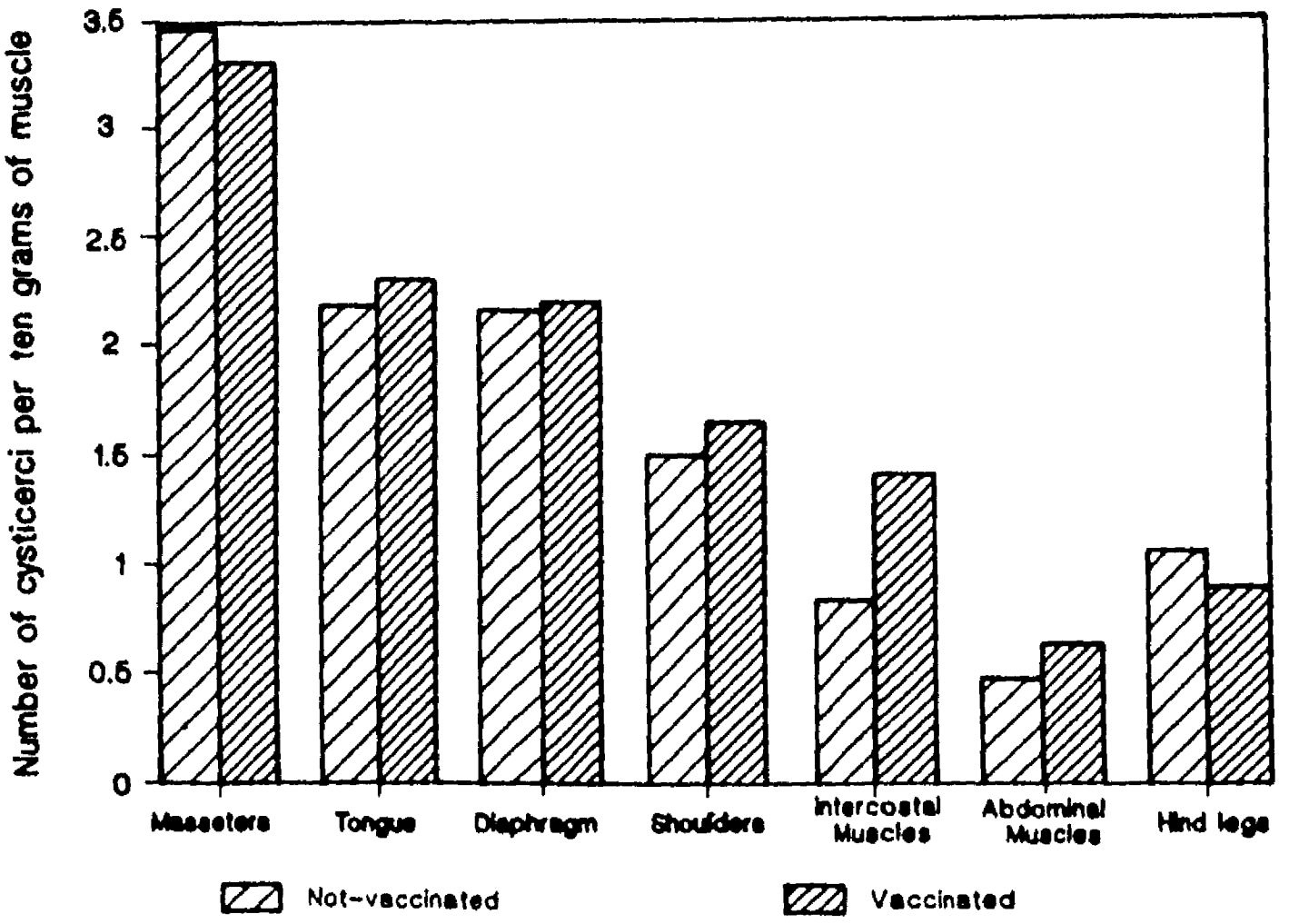
		BEFORE CHALLENGE	AFTER CHALLENGE
NON-IMMUNIZED		0.027 ± 0.014	0.699 ± 0.525
IMMUNIZED	HD2dose trial	0.077 ± 0.069*	0.413 ± 0.300
	LD.4 trial	0.073 ± 0.043*	0.713 ± 0.326

IMMUNIZATION PROTOCOLS AGAINST EXPERIMENTAL PORCINE *Taenia solium* CYSTICERCOSIS

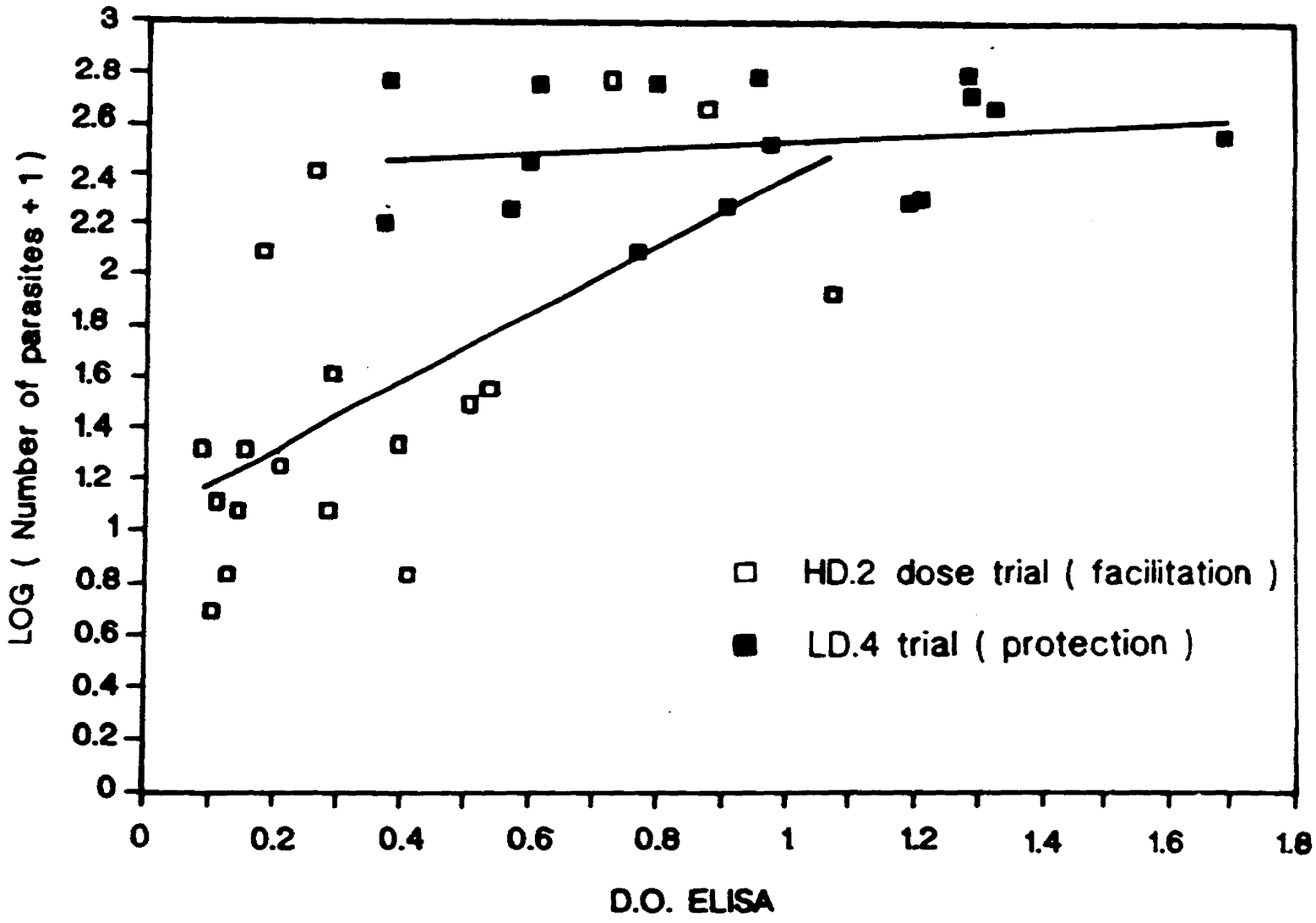


1 – 1ST Immunization with T. crassiceps antigens. 2 – Cholera vaccination. 3 – 2nd Immunization with T. crassiceps antigens. 4 – Challenge with T. solium eggs. 5 – Bleeding. 6 – Sacrifice . n= is number of vaccinated pigs in the trial and n'= is that of controls in each trial

PARASITE DISTRIBUTION IN DIFFERENT MUSCLES OF INFECTED VACCINATED AND NOT-VACCINATED PIGS POOLED FROM ALL TRIALS



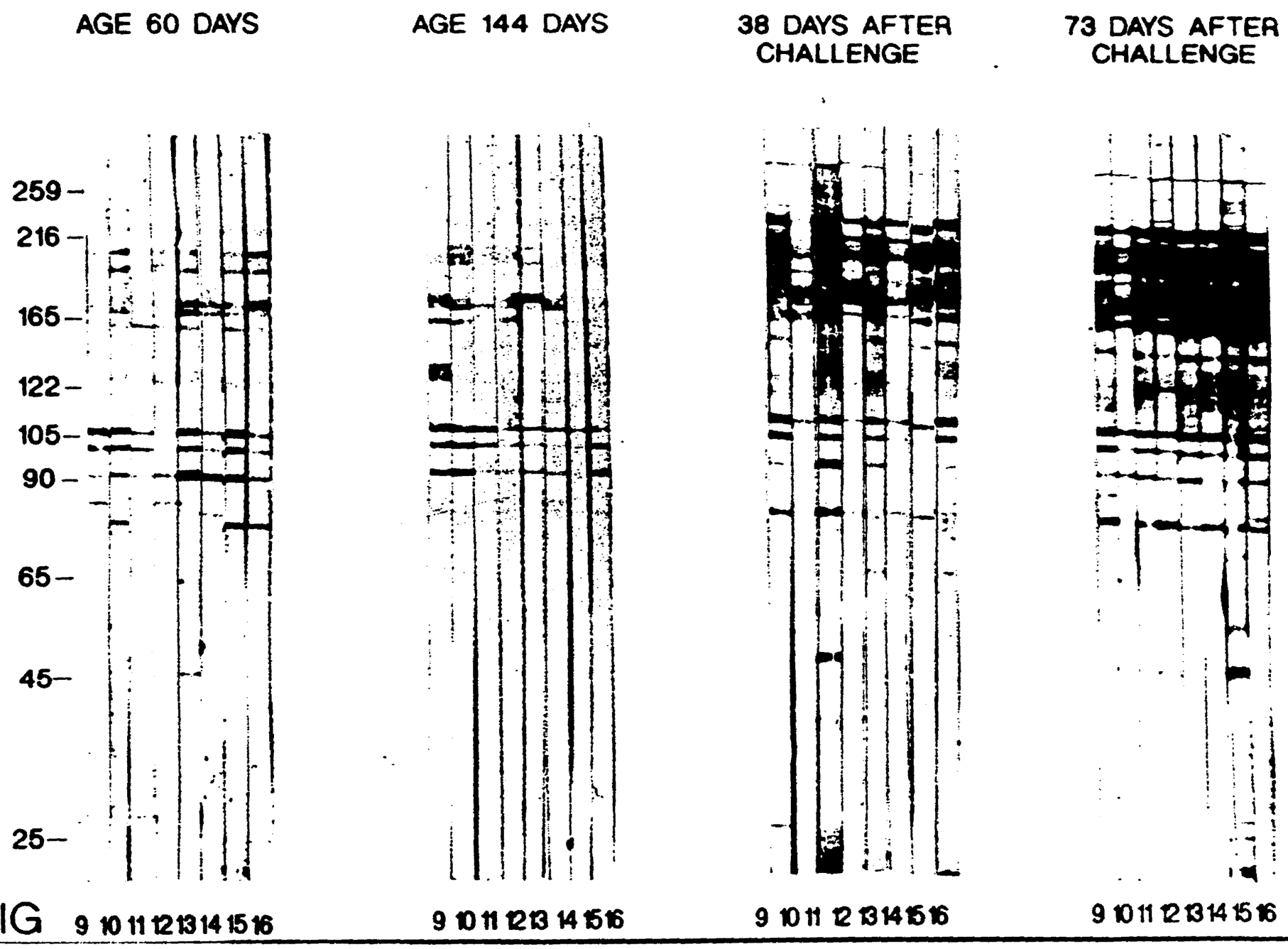
CORRELATION BETWEEN THE TOTAL NUMBER OF CYSTICERCI PER PIG AND THE ANTIBODY LEVEL IN VACCINE INDUCED PROTECTION AND FACILITATION



SEROLOGIC PROFILE IN WB OF NOT-VACCINATED PIGS BEFORE AND AFTER CHALLENGE WITH T. SOLIUM EGGS

A

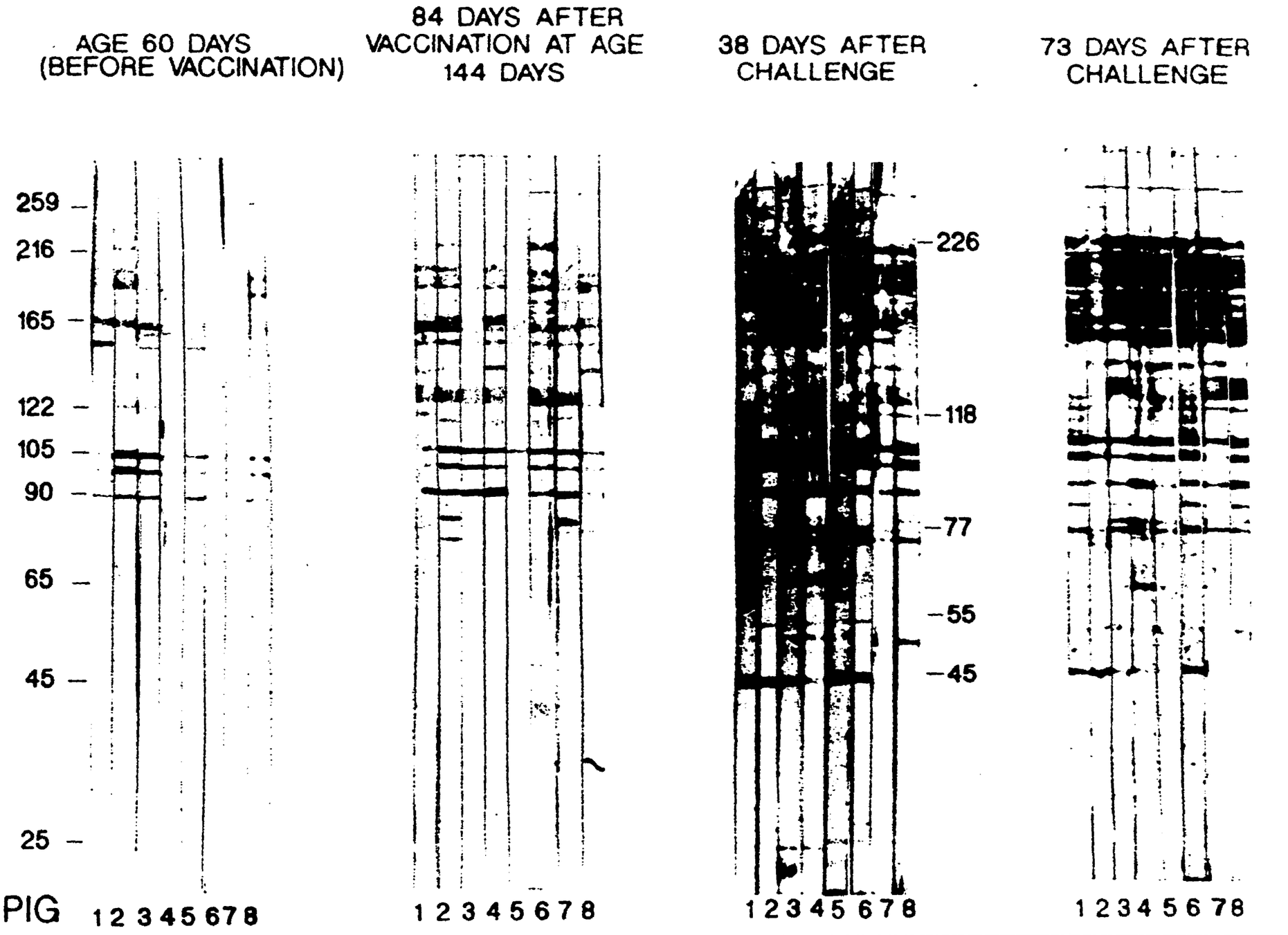
NOT-VACCINATED



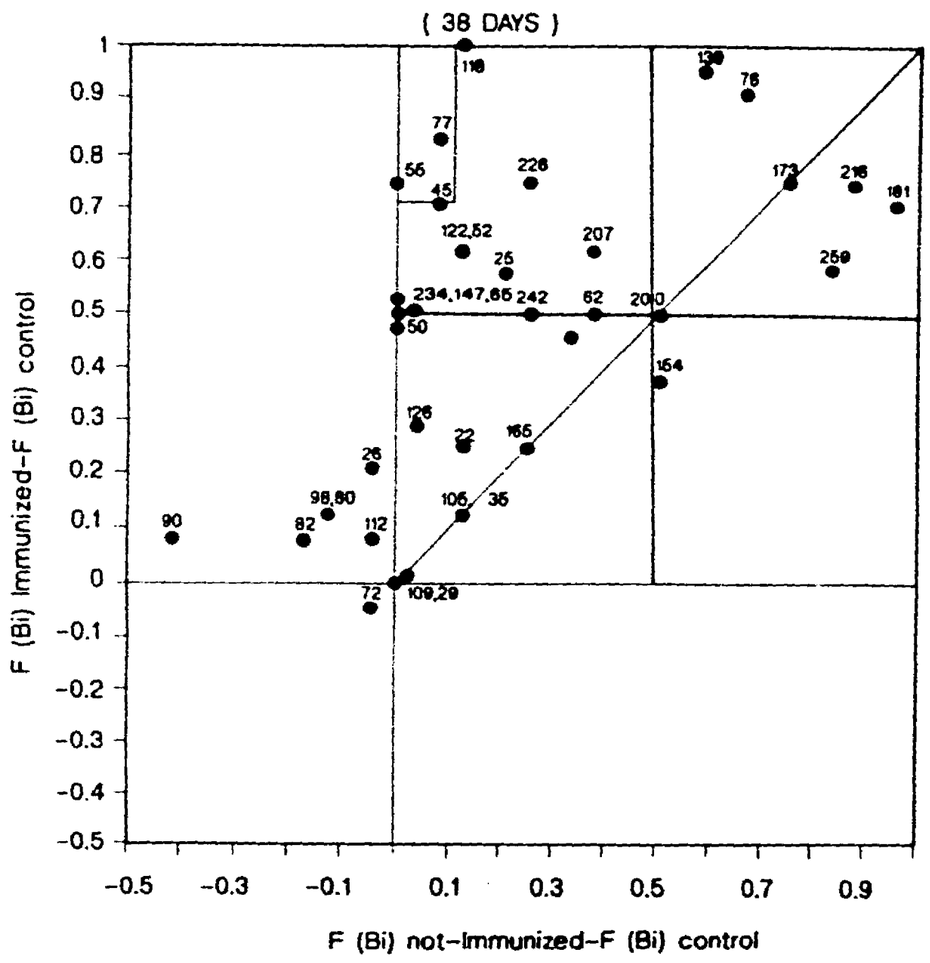
SEROLOGIC PROFILE IN WB OF VACCINATED PIGS BEFORE AND AFTER CHALLENGE WITH T. SOLIUM EGGS

B

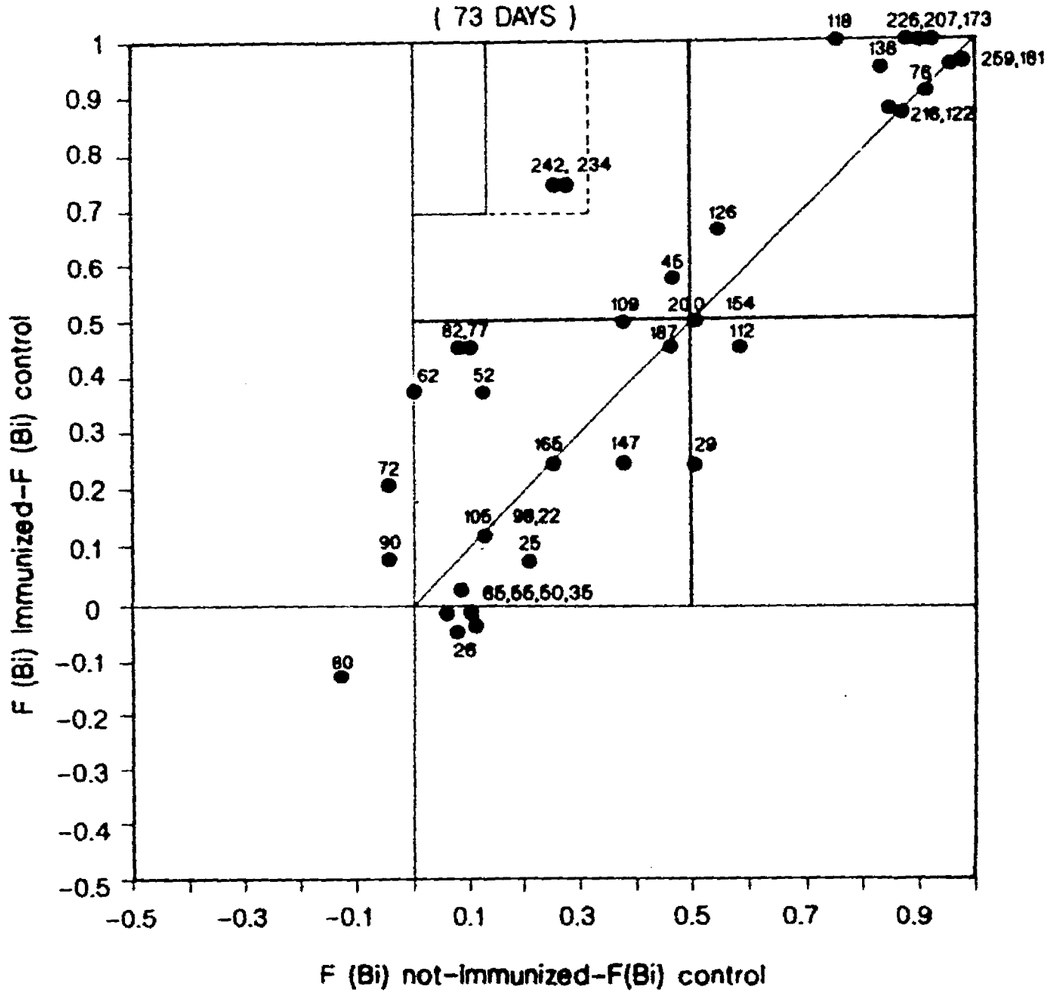
VACCINATED



A : EARLY INFECTION



B : LATE INFECTION



INFECTION AND IMMUNITY

A publication of the American Society for Microbiology

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March 20, 1995

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IAI 1262-94R

Dear Dr. Sciutto,

Your manuscript, IAI 1262-94R entitled "Depressed T-cell proliferation associated with susceptibility to experimental Taenia crassiceps infection," has been accepted for publication by Infection and Immunity. It will appear in the section on Fungal and Parasitic Infections. The ASM Journals Office will be in contact with you regarding publication date, proofs, and other details.

Thank you for selecting Infection and Immunity as the journal in which to publish the results of your work.

Sincerely,

Dexter H. Howard

Dexter H. Howard, Ph.D.
Professor

DHH/lfh

DEPRESSED T-CELL PROLIFERATION ASSOCIATED WITH SUSCEPTIBILITY TO EXPERIMENTAL *Taenia crassiceps* INFECTION

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Running Title : Immunodepression in experimental cysticercosis

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ABSTRACT

Peritoneal infection with *Taenia crassiceps* cysticerci of naturally resistant (C57B1/10J, C57Bl/6J) and susceptible (BALB/cAnN) mice induces a cellular immune depression. T-cell proliferation in response to Concanavalin A (ConA) or anti-CD3 was significantly depressed in infected mice of all strains tested. However, in resistant mice, the diminished response to ConA was transient and animals recovered normal responsiveness at day 40, whereas susceptible mice remained suppressed throughout the 40 days of the experiment. In contrast, the proliferative response to anti-CD3 was lower in infected mice than in non-infected controls regardless of differences in natural susceptibility of the strains. Intraperitoneal injection of mice with a parasite extract also induced a depression of the response to ConA although not as strong as that produced by the parasite itself. This depression is not due to direct effects by parasite antigens over host lymphocytes as proliferation is not affected by the presence of cysticercal antigens added *in vitro*. Diminished IL-2 production during the parasitosis accounts at least in part for the diminished responses to ConA. A primary infection favors parasite establishment after a second challenge, pointing to the relevance of the immunodepression in generating a host-environment favorable to the parasite.

INTRODUCTION

Taenia crassiceps cysticerci cause a chronic infection in laboratory animals such as mice (10, 27) and rats (5). Cysticerci grow in the peritoneal cavity where they reproduce by budding, and from where they can be harvested and individually counted to estimate how susceptible different strains are. Host-susceptibility and the intensity of this murine cysticercosis depend on a combination of genetic (25); sexual (6, 13, 25) and anatomical factors (19). In addition, survival and growth of the cysticerci within the host probably involve mechanisms of immune response evasion as it occurs in other parasitosis (29). Thus, *T. crassiceps* peritoneal infection induces a concomitant decline in antibody response against sheep erythrocytes (12), and inhibits mast-cell degranulation (26). However, the importance of these immune response modifications in cysticerci establishment and persistence within the host remains uncertain. In an attempt to improve our understanding of the role of immunity in experimental cysticercosis, we undertook a study of immune functions of infected animals from strains differing in natural resistance to this parasite. We describe now, the appearance of cellular immune depression in *T. crassiceps*-infected mice as evidenced by a diminished lymphocyte proliferation, attributable at least in part to insufficient supply of endogenously produced IL-2. Furthermore, our results point to the relevance of immune depression in parasite establishment and/or in its growth within the host.

MATERIALS AND METHODS

Mice. Three strains of mice differing in susceptibility to *T. crassiceps* were used: C57Bl/10J and C57B1/6J (resistant) and BALB/cAnN (susceptible). Original stocks were obtained from the Jackson Laboratory, Bar Harbor, Maine and from M. Bevan (Seattle University) respectively, and then bred in our animal facilities by brother-sister mating. All mice used were 5-7 weeks 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, USA.

Parasites. The ORF strain of *T. crassiceps* (Zeder 1800) Rudolphi 1810, isolated by Freeman (10), and supplied by Dr. B. Enders (Behringwerke, Marburg, Germany) has been mantained by serial intraperitoneal passage in BALB/cAnN female mice for 8 years in our Institute. Parasites for infection were harvested from the peritoneal cavity of mice, 1 to 3 months after inoculation of 10 cysticerci per animal.

Infections and Immunizations. Mice were routinely injected intraperitoneally (ip) with ten

small (2 mm in diameter), non-budding *T. crassiceps* larvae, suspended in phosphate-buffered saline (PBS). Either 5, 12, 30 or 40 days after infection mice were sacrificed, their spleens were removed for proliferation assays and *T. crassiceps* cysts inside the peritoneal cavity were counted. For some experiments, mice were inoculated ip with 100 mg of a mixture of soluble *T. crassiceps* antigens in PBS, prepared as previously reported (18).

Proliferation inducers. Concanavalin A obtained from Sigma Chemical Co.(St. Louis, Mo.) was prepared as previously described (8). IL-2-rich supernatant from the 929 cell line (IL-2-transfected fibroblasts) was kindly provided by Dr. José Moreno (Hospital de Especialidades , Centro Médico Nacional Siglo XXI, Instituto Mexicano del Seguro Social, México). Anti-CD3 antibodies from hybridoma 145.2C11 were prepared as described (21).

Proliferation assay. Spleen cells from normal, immunized or parasitized mice were cultured in RPMI 1640 medium, supplemented with L-glutamine (0.2 mM), sodium pyruvate (1 mM), non-essential aminoacids (0.01 mM), penicillin (100 U/ml), streptomycin (100 µg/ml) and fetal calf serum (10%). Cells were incubated with the appropriate concentrations of ConA or anti-CD3 at 37°C in a 5% CO_2 humidified atmosphere, in flat-bottom microtiter plates, at a concentration of $5x10^5$ cells per 200 µl final volume. After 48h, cells were pulsed (1 µCi/well) for a further 18h with [^{3}H] thymidine (TdR) (New Products, Du Pont, Boston, MA). They were then harvested and the amount of incorporated label was measured by counting in a liquid scintillation spectrometer. All assays were performed in triplicate in at least four individual mice. To test for inhibitory effects of *T. crassiceps* antigens on cell proliferation, cultures from normal mice prepared as above were treated with 50-5000 ng/ml of soluble vesicular fluid antigens added either simultaneously with ConA , 3 or 17 hours later.

Effect of IL-2 on proliferation of cells from *T.crassiceps* infected mice. IL-2 activity in the supernatant of the 929 cell line was determined by measuring [^{3}H]TdR uptake by the CTLL-2 cell line according to Gillis et al (11); IL-2 units were calculated relative to a recombinant IL-2 standard (Sigma Chemical Co., St. Louis Mo). The effects of IL-2 on ConA-stimulated cultures from normal and 5-day infected mice was evaluated by adding 10 to 100 U/ml of rIL-2 concomitantly with ConA.

Effect of previous immunization and a first challenge on susceptibility. The effect of a primary infection with *T. crassiceps* on the establishment of a secondary infection with the same parasite was determined as follows: C57Bl/6J male mice were infected with *T. crassiceps* as described above and five days later they were inoculated again with 10 cysticerci each. As controls, normal mice were infected once either with ten or twenty parasites each. Cysticerci were counted after 30 days of infection.

Statistical analysis. The statistical significance of the effects of the experimental variables was determined by multifactorial analysis of variance (ANOVA) and by the non-parametric test Wilcoxon Scores (rank-sums).

RESULTS

Proliferative responses to ConA by spleen cells from resistant and susceptible mice during the course of *T. crassiceps* infection. We have reported previously that inbred strains of mice differ in susceptibility to T. *crassiceps;* BALB/cAnN strain was among the most susceptible, whereas C57Bl/6J was comparatively resistant (25). Results in Table 1 show that

C57Bl/10J mice are also resistant to the parasite and confirm our previous finding that female mice are more susceptible than males to intraperitoneal cysticercosis (13, 25).

To evaluate T-lymphocyte function during experimental infection with *T. crassiceps*, proliferative responses to ConA were determined in spleen cells from BALB/cAnN and C57Bl/10J mice at different times after infection. As shown in Figure 1, diminished proliferative responses to ConA stimulation were observed both in resistant and in susceptible mice, as early as five days after infection. This effect was more marked in the resistant C57Bl/10J where a reduction of 79% with respect to control mice was observed. However, at later stages of infection (40 days), spleen cells from resistant mice recovered the ability to respond to ConA, whereas the response of cells from susceptible mice remained at a lower level (55%) than that of cells from uninfected mice. Similar diminished cell-proliferation to ConA and kinetics was observed in *T. crassiceps*-infected C57Bl/6J (e.g. Table 4) and in another resistant strain, namely B10.D2 (data not-shown).

Next, we determined if the depressed responses to ConA observed in cells from infected mice could also be observed in cells from mice injected with soluble cysticercal *T. crassiceps* antigens. As shown in Figure 2, the response of cells from infected and from antigen-treated mice was significantly lower than that of normal mice when stimulated with 0.5 and 2.5 µg/ml of ConA. However, at these ConA doses a statistically significant lower response was observed in cells from infected mice than in those from antigen-treated mice.

In order to ascertain if immunodepression was also induced by *T. crassiceps* antigens in vitro, we investigated their effect on normal mouse lymphocyte proliferation. Different amounts of cysticercal antigens were added to the cultures either at the time of ConA addition, at 3 or 17 hours later. As Table 2 shows, proliferation of cells from normal mice cultured in the presence of *T. crassiceps* antigens was not affected at any of the concentrations tested.

T-cell activation by cross-linking of CD3 antigens. T-cell proliferation induced by anti-CD3 antibodies was also evaluated. For these experiments, spleen cells from BALB/cAnN and C57Bl/6J mice that had been infected 40 days previously, were incubated with different amounts of supernatant containing anti-CD3 Mab. The results are shown in Table 3. At this stage of infection, the resistant strain C57BL/6J had recovered the normal responsiveness to Con A (see also Figure 1). In contrast, the anti-CD3 induced proliferation was diminished in both strains regardless of differences in susceptibility.

Effect of exogenous rIL-2 on mitogen-induced proliferation of spleen cells from infected mice. In order to determine if the depressed ConA response observed in spleen cells from infected mice was due to an inadequate amount of IL-2 in the cultures, and to examine if these cells were capable of responding to IL-2, we evaluated the effect of exogenously added rIL-2 on the ConA-induced proliferative response of non-infected and infected mice. As Table 4 shows, addition of IL-2 (10 to 100 U/ml) did not modify the maximal level of the ConA response in spleen cells from non-infected mice. In contrast, rIL-2 significantly increased the response to ConA in spleen cells from infected mice. However, the level of response in the presence of rIL-2 did not reach that of normal mice even when large doses of rIL-2 were employed. These results indicate that the diminished responsiveness to ConA observed in spleen cells from infected mice is

partially due to insufficient supply of endogenously produced IL-2.

Effect of a first infection with *T. crassiceps* cysticerci on a second challenge. To investigate if the depressed cellular response of infected mice may have a bearing on the establishment of the parasite, we analyzed the effect of a first infection as well as immunization on a secondary challenge with the parasite, using C57Bl/6J male mice. As Table 1 and 5 show, this is a highly resistant strain, from which no parasites could be recovered, even when mice were intraperitoneally inoculated with 20 cysticerci. However, as shown in Table 5, 33 to 46% of these mice displayed the parasitosis when they were challenged with 10 parasites 5 days after a previous infection or antigen injection, a time when the maximal cellular depression is observed (see Figure 1). These results indicate that parasites as well as parasite antigens may facilitate the establishment of cysticerci upon a second challenge even in highly resistant hosts .

DISCUSSION

Our results clearly show that infection with *T. crassiceps* cysticerci induces a depression of the T-cell proliferative response to ConA and to anti-CD3 mAb. The reduction of this response was very pronounced in both naturally susceptible and resistant mice. In susceptible mice, the depressed response to ConA appears as early as 5 days after infection and sustained for 40 days while in resistant mice, the depression decreases after 30 days, and is no longer present at day 40 postinfection. Restoration of normal levels of ConA-induced proliferation observed 40 days after infection in resistant mice may be related to the presence of only a few parasites at this time of infection whereas in susceptible BALB/c mice, the long term depression may reflect that *T. crassiceps* continues to reproduce in the peritoneal cavity. Diminished anti-CD3 induced T-cell proliferation was also found, in both resistant and susceptible mice, even at late stages (40 days) of infection (Table 3). At this time, the low anti-CD3 response in resistant mice contrasts with the recovery of Con A responsiveness; and, while we have no explanation for this finding, it may simply reflect differences in the mechanisms of T-cell activation by these two agents. Thus, in well established chronic parasitosis the T-cell response is defective, seemingly because of this low proliferation to anti-CD3 stimulation. These observations suggest that *T. crassiceps* cysticerci can develop ways to evade the host defense allowing the establishment of a balance between the host and the parasite, to the point that they coexist, and the parasite multiplies albeit with some restraint.

Interestingly, not only living cysticerci can induce this T-cell depression, *T. crassiceps* cysticercal antigens can also induce it, though to a lesser degree, as early as five days after their injection into mice (Figure 2). This antigen-induced depression prompted the speculation that larval products may bind to the cell surface and interfere with mitogens and antigens. However, this explanation was ruled out since no effect was observed when parasite antigens were incubated in vitro with cells and ConA (Table 2). This in turn, suggests that cysticercal antigens neither damage host's lymphocytes, nor occupy or block lymphocytes receptors, at least in vitro and strongly support the notion that the observed depression is not a consequence of parasite components that directly affect T-cell proliferation.

Immunodepression has been observed in several parasitic infections. There are many instances of immunosuppression induced by phylogenetically-unrelated parasites, with very

different life cycles and hosts range and pathological consequences (1, 2, 4, 20). In addition, a variety of mechanisms leading to this low responsiveness have been uncovered. Thus, some infections inhibit phagocytic cell activity, decrease lymphokine production (3, 22, 30), or of their cell receptors (3, 16) as well as the expression of molecules necessary for T-cell activation (28). Also, the presence of T suppressor cells has been reported (14, 15, 29). Here, we have shown that suppressed ConA response observed in cysticercosis could be attributed at least in part to defects in IL-2 production since the addition of rIL-2 to cell cultures partially restore responsiveness. It remains to be explored if defects related to IL-2 receptor also play a role in these processes. Since unpurified splenocytes were used in these studies we cannot rule out a possible contribution from macrophages or their products to the diminished T cell proliferation or IL-2 secretion as has been shown in other parasitic infections (17, 20).

Recent data indicate that immunosuppression may be associated with persistence or exacerbation of some parasitic infections (29). Our evaluation of a possible role of immunodepression on the establishment of cysticerci in the host suggests that an initial infection or parasite contact (which effectively depressed T-cell proliferation) can modify the natural high resistance of a host (Table 5). Thus, after a moderate challenge, no parasite growth was observed in untreated resistant C57Bl/6J mice; in contrast, a previous infection or antigen-treatment allowed parasite establishment. However, since parasites were not found in all inoculated mice, other factors, such as parasite heterogeneity might also be involved. This parasite growth facilitation observed early after antigen inoculation, contrasts with the late effects of immunization. Mice challenged 15 or 30 days after immunization with total cysticercal antigens, showed significantly lower parasite load than those not-immunized (24, 32). This finding is not surprising in view of the multiplicity of mechanisms that appear to be involved in the control of larval cestodes, and it also suggests that the timing between vaccination and challenge should be considered in the evaluation of vaccine efficacy. In this context, it should be mentioned that immunization with some cysticercal antigen fractions (220-205, 108kD) can facilitate parasitosis, causing an increase in cysticerci load even if the challenge was given late after immunization (32). We are currently investigating if this phenomenon is the result of specific immunosuppression.

Previous studies on murine *T. crassiceps* cysticercosis revealed that cellular immune mechanisms are involved in resistance. Thus, an increase of DTH response to parasite antigens was found to correlate with enhanced resistance. Moreover, neonatal thymectomy significantly decreased resistance and it could be restored by T-cell transfer (6). More recently, preliminary evidence of a Th1-Th2 disbalance favoring Th2 has been obtained (31). As it stands, resistance to *T. crassiceps* is more clearly associated with cellular immunity than with bulk antibodies (6), although the latter are quite conspicuous components of the host immune response and some could be involved in subtle ways in resistance. In addition, it has been recently demonstrated that NK cells, through the production of IFN-gamma which induces Th1 lymphocyte differentiation, play a major role in natural resistance to intracellular parasites (7, 23). If NK or other cell types (9) are important for the control of cysticercosis is not known and should be explored.

Thus, the data in this report suggest that cysticerci are more effective than cysticercal antigens in inducing immune depression by affecting T-cells, modifying their IL-2 secretion and favoring the installation and/or the development of the parasite. Exploration of the mechanism by which *T. crassiceps* induces depression of lymphocyte functions could help not only to further the knowledge of immune mechanisms that participate in this host-parasite relationship, but also to advance our understanding of the regulatory events governing lymphocyte activation.

ACKNOWLEDGMENTS

We would like to thank Dr. Victor Gold for the revision of this manuscript, Dr. Albert Zlotnik for his comments, Maricela Hernández for her technical support, Tzipe Govezensky for the statistical analysis, Dr. Ciro Lomeli for providing mice and Violeta Aguilar for her secretarial work. This work was supported by grants from the Consejo Nacional de Ciencia y Tecnología, México and from the Dirección de Asuntos del Personal Académico, Universidad Nacional Autónoma de México.

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TABLE 1. Growth of *T. crassiceps* in the peritoneal cavity of susceptible and resistant mouse strains.

Mouse Strain	Sex	Average parasite load[a]
BALB/cAnN	Female	122.1±8.07
	Male	30.3±2.1
C57Bl/6J	Female	3.07 ±2.7
	Male	0
C57Bl/10J	Female	15.8 ±5.6
	Male	0

[a] Mean ± standard error. Cysticerci recovered 30 days after infection with 10 cysticerci per mouse.

TABLE 2. Lack of *in vitro* effect of *T. crassiceps* antigens on the capacity of normal C57Bl/10J splenocytes to incorporate [^{3}H]TdR in response to stimulation with ConA

Amount of *Taenia* *crassiceps* antigens added (ng/ml)	Time of antigen addition after ConA (hrs)		
	0	3	17
	[^{3}H]TdR incorporation (kcpm± sd)[a]		
0	131.1 ± 27.8	124.2 ± 19.4	135.1 ± 34.5
50	148.5 ± 7.3	116.3 ± 62.2	131.6 ± 9.4
500	156.6 ± 7.9	124.4 ± 66.8	134.6 ± 2.1
5000	124.4 ± 41.2	152.4 ± 12.9	189.8 ± 45.5

[a] Mean ± standard deviation of three mice per group. The differences between T-cell proliferation levels in the absence or in the presence of *T. crassiceps* antigens were not statistically significant. This set of data is representative of three repeat experiments.

TABLE 3. Effect of chronic *T. crassiceps* infection on ConA- or anti-CD3-induced T-cell proliferation from susceptible (BALB/cAnN) or resistant (C57Bl/6J) mice.

Strain of mice	[^{3}H] TdR incorporation[a]			
	ConA (0.5 µg/ml)	Anti-CD3 (µl)		
		1	10	50
BALB/cAnN				
Noninfected	324.1±28.8	122.0±30.7	152.8±14.7	106.7±17.6
Infected	179.0±53.4	25.7±12.4	48.9±4.2	26.4±2.20
C57Bl/6J				
Noninfected	187.1±21.2	34.0±10.2	39.6±3.3	34.7±6.2
Infected	205.0±11.2	9.0±7.2	12.1±3.3	16.8±0.4

[a] Mean ± standard deviation of T-cell proliferation from three individual mice per group. Mice were infected 40 days previously to the assay. All values of [^{3}H]TdR incorporation are expressed as kcpm. All diferences between values obtained from infected and noninfected mice stimulated with anti-CD3 were statistically significant ($P \leq 0.05$, t-student test). The sets of data are representative of three repeat experiments.

TABLE 4. Partial restoration by exogenous rIL-2 of the depressed ConA T-cell responses in *T. crassiceps* infection.

rIL-2 added (U/ml)	[^{3}H] TdR incorporation[a]	
	Non-infected	Infected
0	187.5 ±38.3[b]	58.86 ± 4.9[c]
10	186.9 ±33.4[b]	54.34 ± 2.5[c]
50	212.4 ± 7.5[b]	129.63 ± 32.5[d]
100	173.8 ±12.5[b]	105.79 ± 5.7[d]

[a]Mean ± standard deviation (kcpm). Proliferation was measured in pooled cells from three normal and three 5-day infected C57Bl/6J mice .

[b,c,d]Data labeled with the same letter are not significantly different from each other, whereas those with different superscript letters are significantly different, ANOVA test ($P<0.01$). These data are representative of three separate experiments.

TABLE 5. Effect of a primary infection or antigen treatment on susceptibility of male C57B1/6J mice to a second *T. crassiceps* challenge.

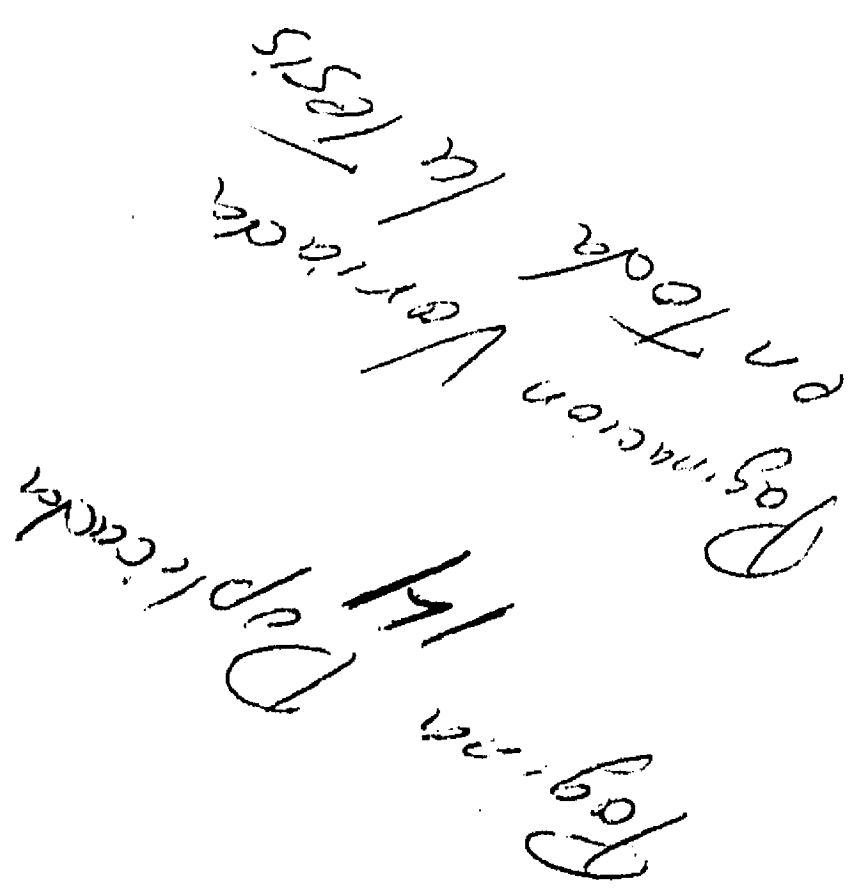
First Treatment day 0	Number of larvae in a challenge day 5	Total number of larvae recovered day 35
nil	10	0 (0/15)[a]
nil	20	0 (0/15)
100 μg antigen per mouse	10	7 (6/13)
10 larvae per mouse	10	27 (4/12)

[a]Number of mice bearing larvae/total number of mice challenged. For statistical analysis the non-parametric test wilcoxon scores (rank-sums) was used.

FIGURE LEGENDS

Figure 1. Effect of *T. crassiceps* infection on ConA-induced proliferation of splenocytes from susceptible (BALB/cAnN) and resistant (C57Bl/10J) mice. Data were calculated as percentage of the response of control noninfected mice. Mean ± standard deviation of the kcpm incorporation was 265.7±83.1 for control BALB/cAnN and 163±60.7 for C57Bl/10J. Each point represents the average response ± standard error of three to eight individual mice. The response of infected mice of both strains was significantly lower ($P<0.01$) than that of control mice on days 5, 12, and 30. On day 40, only T cell proliferation of susceptible BALB/cAnN mice was significantly lower.

Figure 2. ConA dose-responses of spleen cells from noninfected , 5-day infected or immunized C57Bl/10J male mice. Points and vertical bars represent the mean of triplicate values and their standard deviations. Immunization and infection significantly reduced the Con A responses ($P<0.05$) with a higher significantly decrease due to the infection.



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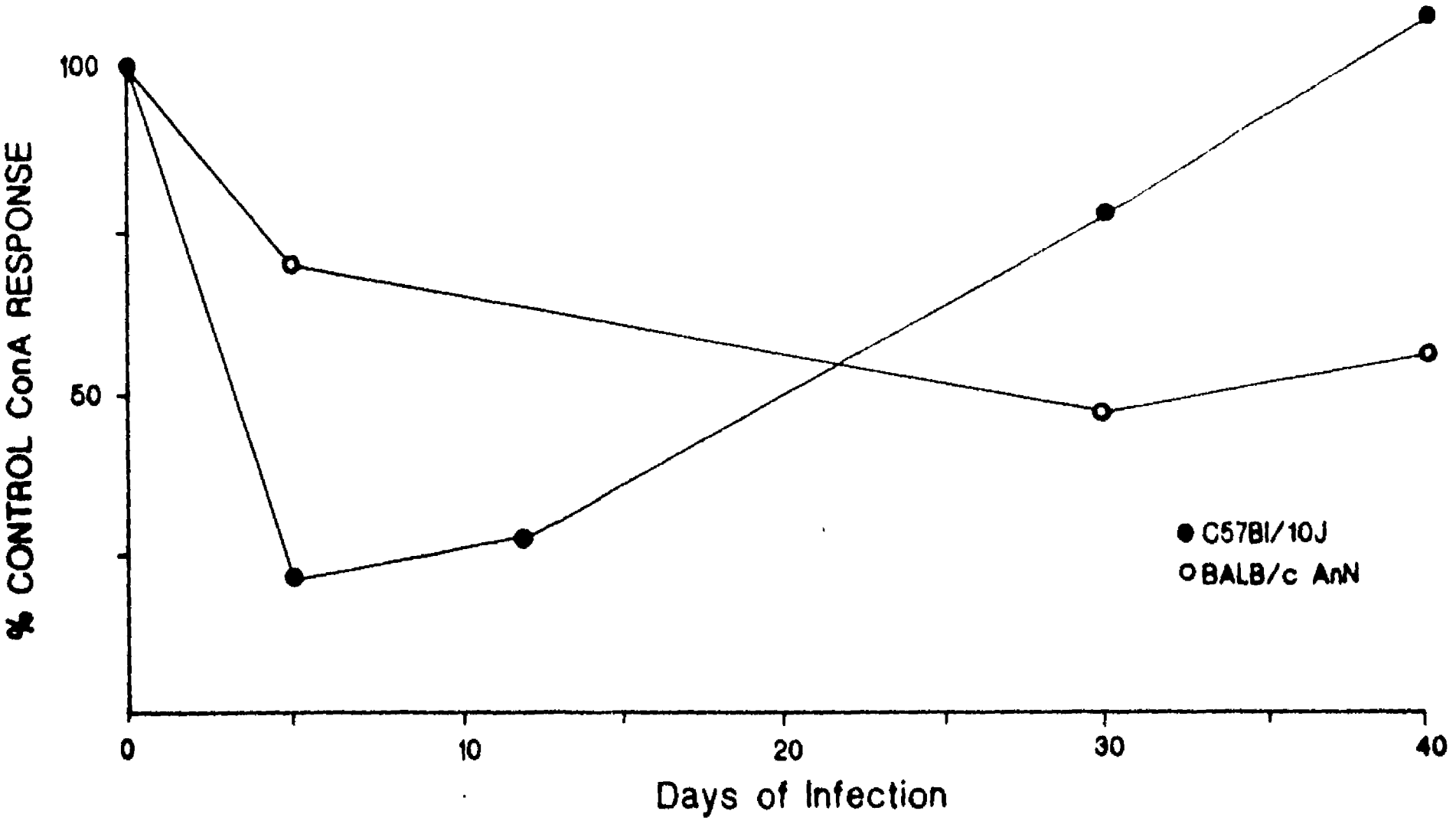

Figure 1

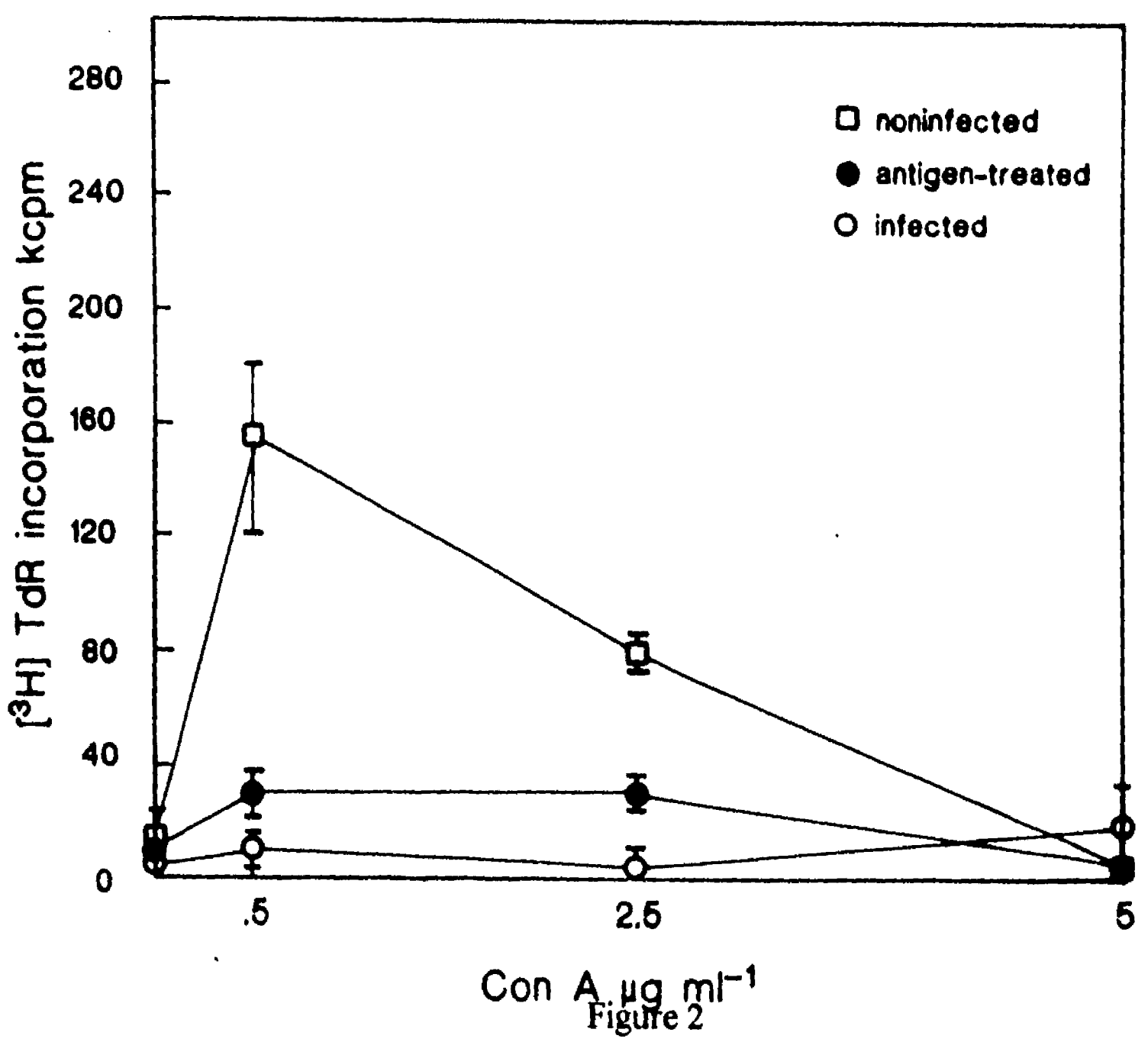

Figure 2

DISCUSION Y PERSPECTIVAS

DISCUSION

En la cisticercosis humana, los factores de riesgo para adquirir la enfermedad parecen estar relacionados principalmente con la magnitud y la frecuencia con la que el hospedero se expone a los huevecillos liberados por la *Taenia solium*. La elevada frecuencia de esta parasitosis así como los daños tanto en salud pública como socioeconómicos justifican los intentos de prevenir la enfermedad. Considerando que la cisticercosis es una enfermedad que depende de las medidas sanitarias y el nivel cultural de la población, existen grupos de investigación que han sugerido que mediante un programa de educación y mejoramiento de las condiciones higiénicas de la población expuesta a un mayor riesgo, se podría interrumpir el ciclo biológico de éste parásito. Esta medida se propone esencialmente considerando el hecho de que la cisticercosis no se presenta en países desarrollados y sí en países en vías de desarrollo (Mahajan 1982). Otras alternativas son las evaluadas en este trabajo de investigación cuyo fundamento inicial se basa en la modificación de la relación hospedero-parásito tanto por inducción de una respuesta inmunológica eficaz contra el parásito así como por la modificación genómica del hospedero susceptible introduciendo en el mismo genes de resistencia. Considerando que para que el ciclo biológico de este parásito se complete es indispensable la infección del cerdo, consideramos a este hospedero como el blanco más accesible en el cual incidir en la parasitosis. Utilizando como modelo experimental la cisticercosis murina causada por la larva *de T. crassiceps*, se determinó que por vacunación es posible inducir una inmunidad protectora con eficiencias mayores del 50% tanto en el ratón como en el cerdo, y que por inserción de un gene identificado como "gene asociado a la resistencia" también se puede disminuir el número de parásitos que se desarrollan en la cavidad peritoneal del ratón.

Respecto a la vacunación, cabe recordar que las vacunas que han tenido mayor eficacia, son aquellas que estan dirigidas hacia el control de enfermedades virales y bacterianas. Esto se debe en gran medida a la relativa sencillez de ambos agentes infecciosos, comparada con la de los parásitos, así como por el limitado repertorio de antígenos que le presentan al hospedero, aunque cabe señalar relaciones complejas establecidas entre agentes virales y el hospedero como lo es el caso del virus del SIDA. En el caso de vacunas parasitarias, parece probable que la gran complejidad estructural y de interacción con el hospedero, así como la falta de conocimiento de los mecanismos involucrados en la relación hospedero-parásito, sean factores que han obstaculizado el desarrollo de vacunas anti-parasitarias. A la fecha, son muy pocas las vacunas contra enfermedades parasitarias, humanas y veterinarias, que han demostrado tener cierta eficacia. Entre ellas podemos mencionar la vacuna de uso humano anti-malaria (Patarroyo *et al.* 1987) y la producida

contra la cisticercosis ovina (Johnson *et al.* 1989).

En el campo de la cisticercosis, la inmunoprotección cruzada entre los antígenos del cisticerco de la *Taenia solium* y de la *Taenia crassiceps* hace razonable utilizar a estos últimos como una fuente de antígenos interesantes para la prevención. Además, la cisticercosis por *T. crassiceps* resulta un modelo experimental útil como herramienta con la cual profundizar en la búsqueda de antígenos de interés en contra esta parasitosis (Sciutto *et al.* 1990). En los esquemas de vacunación contra la cisticercosis murina y porcina se demostró que al vacunar a los animales con antígenos totales del cisticerco de *T. crassiceps* se podían conseguir eficiencias de protección (en las mejores condiciones) hasta del 100% en el ratón y del 50% en el cerdo (Sciutto *et al.* 1990; Sciutto *et al.* 1995). Estos resultados nos permitieron definir mejores estrategias para la optimización de la vacuna. Así, la vacunación con los antígenos de un extracto completo obtenido del cisticerco de *T.crassiceps* era prometedora y los antígenos fáciles de adquirir en cantidades adecuadas para comenzar las pruebas de campo, sin embargo, nuestra experiencia respecto a su evaluación experimental en la prevención de la cisticercosis porcina indicó ciertos riesgos. La capacidad protectora resultó dependiente de la dosis empleada pudiendo inducir facilitación cuando se utilizan dosis altas (4 mg/Kg de peso) e incrementar la carga parasitaria en más del 50% de lo esperado. Parece entonces probable que el conjunto de antígenos del parásito incluya algunos que ayudan a su crecimiento en el hospedero y otros que lo hacen especialmente vulnerable. Comenzamos entonces a identificar fracciones antigénicas aisladas y evaluar su capacidad protectora con el fin de identificar antígenos vulnerables del parásito. Mediante la separación de los antígenos del cisticerco de *T. crassiceps* se pudieron evaluar doce distintas fracciones antigénicas en su capacidad de inducir resistencia en la cepa más susceptible (BALB/cAnN). De estas doce fracciones, se observaron tres distintos patrones de comportamiento: protectores del hospedero (200, 123, 74, 56, 66, 40-50, 27 y 8-14 Kd), facilitadores del crecimiento parasitario (200-205) e irrelevantes (150-160, 93 y 108). Considerando que con las fracciones de 56, 66 y 74 Kd utilizadas conjuntamente como inmunógeno se obtuvo la mejor protección de los ratones (Valdez *et al.* 1994), se evaluaron en su capacidad de proteger al cerdo contra la cisticercosis por *Taenia solium*. La notable disminución en la carga parasitaria de los cerdos vacunados (0.16 cisticercos en promedio en los animales vacunados a diferencia de 5 en los no vacunados) confirmaron la capacidad de inducir una respuesta inmune protectora contra la cisticercosis con estas tres fracciones aisladas (Manoutcharian et al. 1995). El hallazgo de que existen en el cisticerco fracciones antigénicas que facilitan el crecimiento parasitario en la cavidad peritoneal del ratón, constituye una

justificación muy importante de la necesidad de optimizar la vacuna a fin de estimular la inmunidad del ratón hacia efectos protectores. Aunque con la separación de las fracciones antigénicas se pudieron identificar antígenos protectores, el tipo de aislamiento utilizado no es el adecuado para la provisión de estos antígenos con el fin de utilizarlos masivamente, por lo que recurrimos a producirlos por métodos de DNA recombinante y acceder a cantidades adecuadas de estos antígenos. Para ello se realizó una biblioteca de cDNA a partir de mRNA del cisticerco de *T. crassiceps*, se seleccionaron por inmunodetección (con anticuerpos policlonales específicos producidos en conejo: anti-56, anti 74 y con un suero anti-*T. solium*) cuatro clonas recombinantes que codifican para los antígenos 56 y 74 kDa y compartidos por *T. solium* lo que los hace de interés en la prevención contra la cisticercosis por *T. solium* en cerdos. De las clonas identificadas cuatro (KETc1, KETc 4, KETc7 y KETc 12) tuvieron capacidad de actuar como antígenos inductores de protección contra la infección en ratón, (6.6, 7.6 y 4.4 promedio de cisticercos, respectivamente contra 28.2 en los no vacunados) mientras que una clona, KETc11, mostró capacidad de facilitación de la parasitosis (40.6 en los inmunizados y 28 en los no vacunados) (Manoutcharian *et al.* 1995). La evaluación de estas tres clonas en su capacidad de prevenir la cisticercosis porcina se encuentra actualmente en curso y los resultados serán muy importantes para proponer a estos antígenos como candidatos para la obtención de una vacuna de uso sistemático en el cerdo así como para continuar con los estudios de los mecanismos inmunológicos asociados a la enfermedad y a la protección. Respecto a la presencia de moléculas en el parásito facilitadoras de la infección, resulta interesante resaltar que la infección por si misma genera inmunodepresión (Sciutto *et al.* 1995b) y este fenómeno podría estar asociado a la presencia de moléculas que depriman las capacidades inmunológicas del individuo para rechazar al parásito y la participación de antígenos facilitadores podría intervenir en este tipo de mecanismos.

Un punto importante a discutir lo constituye el hecho de que todas las evaluaciones de vacunación se han realizado en animales con un *status* de vida controlado: alimentación, genética, higiene, etc. que no representan a la población de animales naturalmente expuestos a la cisticercosis. Todos estos factores son muy importantes en el desarrollo de una inmunidad protectora, por lo que es necesario evaluar la eficacia de la vacuna en animales de campo. No sería sorprendente que en este tipo de estudios, se detecte variabilidad en la capacidad protectora de estos antígenos, considerando que la respuesta inmune inducida por vacunación puede verse modificada con la heterogeneidad genética de los individuos.

Tomando en consideración las dificultades para lograr proteger totalmente a la población por vacunación, decidimos explorar un método complementario para el control de la cisticercosis cuyas bases son la generación de individuos genéticamente resistentes mediante transgenesis de genes asociados a la resistencia. Este método complementario implica identificar genes que se encuentren asociados a resistencia. Estudios previos realizados en el modelo murino mostraron que la susceptibilidad y/o resistencia de hospedero es un fenómeno multifactorial en el que participan, al menos, factores inmunológicos, endocrinológicos y genéticos (Larralde *et al.* 1989; Sciutto *et al.* 1991; Huerta *et al.* 1992). Para identificar los factores genéticos, utilizamos un conjunto de cepas singénicas y congénicas en H-2 y las estudiamos respecto a su susceptibilidad a la cisticercosis por *T. crassiceps*. De estos resultados observamos que las cepas de haplotipo $H\text{-}2^d$ (BALB/cAnN, DBA/2J) fueron mucho más susceptibles que las cepas de haplotipo $H\text{-}2^k$ (BALB.K, C3H/HeJ) y $H\text{-}2^b$ (BALB.B, B6, B10). La elevada carga parasitaria recuperada de la recombinante natural A/J (K^b, IA^b, IE^b, S^d, D^d), sugirió que los genes *D* y/o *S* podrían estar asociados al fenotipo de parasitosis observado (Sciutto et al. 1991). Para confirmar la influencia de estos dos genes en la susceptibilidad, recurrimos a evaluar el patrón de susceptibilidad en una serie de cepas con fondo genético B10 y diferencias específicas en el H-2: congénicas (B10 y B10.D2) y congénicas recombinantes (B10.D2(R103), B10.D2(R107) y B10.A(2R) que fueron seleccionadas en función de la presencia de los alelos *d* y *k* y/o *b* en los genes sospechosos. Los resultados mostraron que todas las cepas estudiadas fueron resistentes, sin importar los alelos diferentes. Estos resultados sugieren que el fondo B10 existen gene(s) de resistencia que pueden enmascarar el efecto de H-2 observado inicialmente en un fondo BALB. Decidimos utilizar entonces un conjunto de sulíneas BALB/c en las cuales encontramos importantes diferencias de susceptibilidad: BALB/cJ fue muy resistente, BALB/cAnN muy susceptible y BALB/cByJ de resistencia intermedia. Este resultado es especialmente interesante considerando que entre estas sublíneas existen muy pocas diferencias fenotípicas reportadas de las cuales dos son candidatos que podrían estar asociados a las diferencias de susceptibilidad existentes entre ellas: el alto nivel de testosterona sérica y la expresión de la proteína Qa-2. Se ha detectado que los machos de la cepa BALB/cJ expresan niveles muy elevados de testosterona sérica (Roderick et al. 1985; Potter, 1985) lo cual podría ser un factor de resistencia en especial considerando que el ambiente hormonal en el que el parásito se desarrolla es importante para su crecimiento (Terrazas et al. 1994) y que las interacciones inmunoendocrino-lógicas del hospedero intervienen en el control de la parasitosis (Huerta et al. 1992). La proteína Qa-2 es un antígeno no clásico de clase I del MHC del ratón cuyas funciones aún no se han identificado. Esta proteína se expresa en

cantidades muy bajas en células T (Sharrow et al. 1989), a diferencia de los antígenos clásicos de clase I, cuyas funciones reconocidas son las de mediar el rechazo de antígenos extraños (Lechler *et al.* 1991). Mellor y colaboradores han mostrado que la baja expresión de la proteína Qa-2 es la que limita sus funciones como posible seleccionador de lo propio y lo extraño (Mellor *et al.* 1991), y muy posiblemente también sea la razón por lo cual no se ha identificado su papel como molécula importante en la presentación de antígenos sobre todo considerando que sí es capaz de unir péptidos pequeños derivados de proteínas intracelulares (Joyce et al., 1994). Esta proteína parece asociarse a la mayor resistencia de BALB/cJ a cisticercosis murina, ya que es la única de las tres sublíneas que puede expresarla (Mellor *et al.* 1985;) y que además fue la más resistente. Para evaluar si esta proteína se asocia a resistencia, decidimos transferir al gene Q9 (uno de los cuatro genes que codifican a esta proteína) (Mellor *et al.* 1991) a ratones muy susceptibles a la cisticercosis (BALB/cAnN). La transgénesis es un procedimiento cuyo éxito depende de muchos factores: cepa a modificar por transgénesis, tamaño del gene a inyectar, dosis del gene, pureza y lote de los reactivos empleados para el cultivo de embriones. Uno de los obstáculos para la producción de este ratón transgénico fue la selección de la cepa a inyectar. De acuerdo con los resultados de control genético, la cepa BALB/cAnN es la más susceptible y no expresa la proteína Qa-2, por lo que resultaba el candidato idóneo a modificar genéticamente. Sin embargo, no es una cepa recomendable para realizar transgénesis, tanto por la baja producción de embriones como por la labilidad de los mismos (Hogan et al. 1986), características que verificamos en nuestra colonia de BALB/cAnN. Utilizamos entonces como cepa receptora del gene Q9 un híbrido entre BALB/cAnN x C57BL/6J, que se sabe es efectivo para la producción de ratones transgénicos (Hogan *et al.* 1986). Debido a que estos híbridos expresan la proteína Qa-2 y son además tan resistentes como C57BL/6J, decidimos realizar las evaluaciones de susceptibilidad/resistencia en la descendencia de animales transgénicos retrocruzados hacia BALB/cAnN [(BALB/cAnN x C57BL/6J)F1 x BALB/cAnN], cuyos controles son de susceptibiidad intermedia. De los embriones inyectados se pudieron identificar dos ratones transgénicos (el 9 y el 11) con los cuales se realizó la retrocruza a BALB/cAnN para establecer las líneas (TgB1 y TgB2, respectivamente). De los resultados obtenidos observamos que en las dos sublíneas de ratones transgénicos la carga parasitaria se logró disminuir en más del 50% respecto a los controles. Con la evaluación de estas dos sublíneas (TgB1 y TgB2) se reduce la posibilidad de que el transgene se haya integrado en un sitio genético importante para el establecimiento de parásito, ya que la probabilidad de que el sitio de inserción sea el mismo en las dos líneas TgB es muy baja. Si bien no se conoce el mecanismo a través del cual se

medie este efecto de disminución de la carga parasitaria por la proteína Qa-2, un mecanismo que cabe considerar es que la elevada expresión de estas proteínas en ciertos linfocitos T favorezca el desarrollo de una población linfocitaria más efectiva para el control de la parasitosis.

Considerando que en estudios recientes se observó que con la vacunación (tanto con extracto total como con los antígenos recombinantes) es posible proteger por completo a cepas de ratones naturalmente menos susceptibles (C57BL/6J, C57Bl/10J y BALB/cJ), contemplamos la posibilidad de utilizar como estrategias complementarias de protección, a la vacunación y a la transgénesis. Así, generando una población por transgénesis naturalmente menos susceptible (en la cual sabemos que se puede reducir la carga parasitaria hasta en un 50%) y sometiéndola a vacunación con los antígenos recombinantes protectores y bajo las mejores condiciones de protección (dosis, mezcla de fracciones recombinantes, etc.) es posible que se pueda controlar con mayor eficiencia la parasitosis. Con la generación de una población menos susceptible a través de la introducción de genes asociados a resistencia, se produciría además una población más homogénea en genes relacionados con la parasitosis. Esto parece importante considerando que como consecuencia de la heterogeneidad genética es esperable una amplia distribución de los eventos inmunológicos que se generan ante la presencia de un agente infeccioso y en respuesta a la vacunación. Así, las dificultades aún presentes para lograr mejorar las eficiencias de protección por vacunación se verían reducidas, no sólo por la generación de una población hospedera con menor variabilidad genética, sino además con menor susceptibilidad innata.

Finalmente, los hallazgos reportados en este trabajo incrementan las esperanzas de poder acceder al control de la cisticercosis porcina mediante la obtención de razas menos susceptibles por transgénesis y sometidas a programas de vacunación. Si bien se desconoce el equivalente de la proteína Qa-2 en el cerdo, el hecho de que se hayan generado cerdos transgénicos con xenogenes (incluyendo genes del MHC) (Pursel *et al.* 1990) y ratones transgénicos con genes del MHC del cerdo (Bluestone *et al.* 1987) que se expresen y sean capaces de conservar sus funciones en un contexto diferente, apoya las espectativas de que el transgene Q9 pueda disminuir la susceptibilidad a la cisticercosis porcina.

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APENDICES

EL COMPLEJO PRINCIPAL DE HISTOCOMPATIBILIDAD DEL RATON:
Antígenos de clase I

EL COMPLEJO PRINCIPAL DE HSTOCOMPATIBILIDAD DEL RATÓN: ANTIGENOS CLASE I

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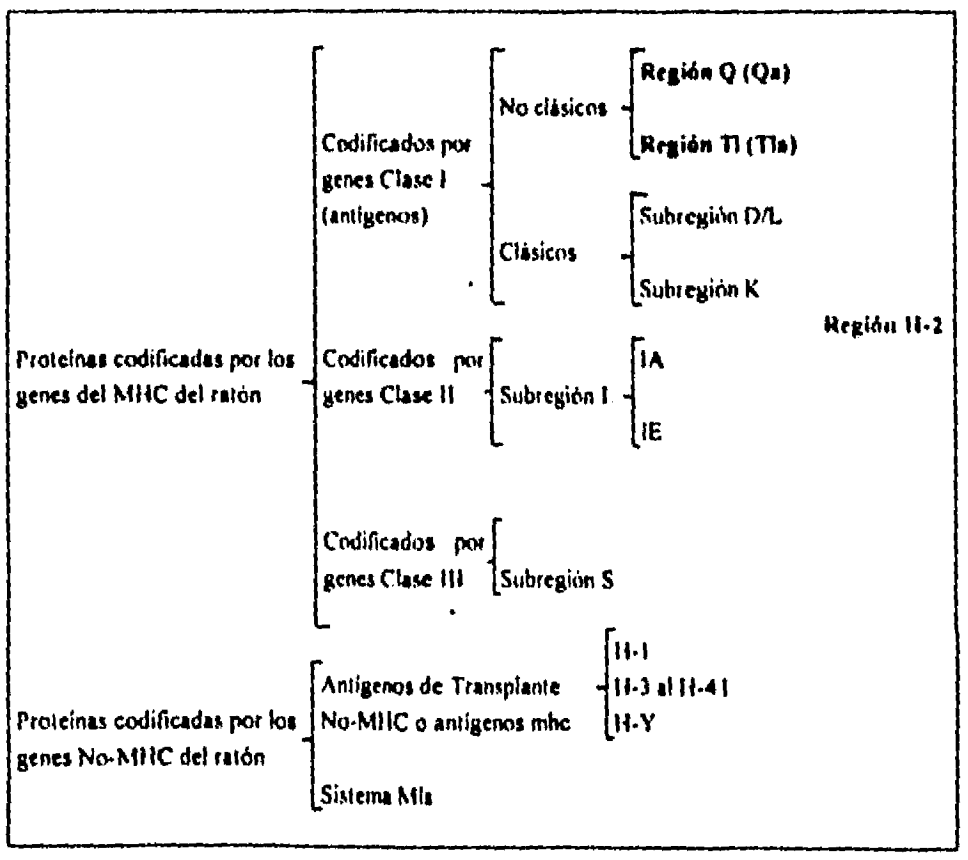
Resumen:El Complejo Principal de Histocompatibilidad (MHC) es un conjunto de genes localizados en serie en una misma región cromosómica. Su estudio ha cobrado gran importancia dado el papel relevante de las proteínas que ahí se codifican, en los fenómenos de citotoxicidad contra moléculas extrañas, así como en la regulación de la respuesta inmune. En el ratón se ha identificado una región en este complejo que contiene la mayor parte de los genes de histocompatibilidad y que se considera como región de Clase I no clásica de histocompatibilidad. Su estudio resulta de gran interés pues sus productos son homólogos estructuralmente a los antígenos clásicos de clase I y sin embargo no parecen estar involucrados en las mismas funciones según lo sugiere su distribución celular y su anclaje a la membrana. De esta región de histocompatibilidad falta aún mucho por conocer, así se desconocen los productos fenotípicos de la mayor parte de sus genes y la función que desempeñan. En esta revisión se presenta la información actualizada acerca de los antígenos de clase I del ratón incluyendo una descripción estructural y funcional comparativa de los dos grupos de antígenos presentes (clásicos y no clásicos) a fin de señalar la importancia del estudio de esta región del MHC del ratón no sólo como modelo para el estudio del MHC de otras especies, sino por la posibilidad de que se identifiquen algunos eslabones aún no identificados que participan en los procesos que ocurren durante la respuesta inmunológica.

Introducción. Los antígenos de histocompatibilidad fueron inicialmente definidos como un conjunto de proteínas criticamente involucradas en los fenómenos de rechazo de transplantes de tejidos (Gorer, 1937). Dentro de este conjunto de proteínas, se encuentra un grupo con características muy inmunogénicas que se codifican principalmente en el Complejo Mayor o Principal de Histocompatibilidad (MHC) y otro con características menos inmunogénicas, al que se le ha designado como proteínas del complejo menor de histocompatibilidad (mhc).

En el ratón, el MHC se localiza en el cromosoma 17 y desde 1937 fue designado por Peter Gorer como *H-2* (Gorer, 1937). Por mucho tiempo el término H-2 se consideró como sinónimo de MHC del ratón, sin embargo en la actualidad, existe la tendencia a dividir al MHC del ratón en dos regiones: *H-2 y Q/TL/M.* De todos los genes que se ubican en el MHC del ratón, sólo algunos codifican para los antígenos propios de transplante (moléculas clásicas de histocompatibilidad), mientras que el resto de los genes codifican para diversas proteínas, algunas de las cuales se sabe que desempeñan funciones en el sistema inmune (Benacerraf, 1981). A pesar de que éste conjunto génico ha sido estudiado muy extensamente, aún no se conoce la función de algunos de sus productos fenotípicos.

La terminología para designar a todas estas moléculas y sus genes, ha cambiado en los últimos años y a pesar de los intentos para simplificarla, se ha ido haciendo más compleja y confusa. Una agrupación que facilita el estudio de las moléculas y sus genes, comprendidas bajo el término de histocompatibilidad, es la que se presenta en la Figura I.

FIGURA1
CLASIFICACION DE LOS ANTIGENOS DE HISTOCOMPATIBILIDAD DEL RATON



Los genes del MHC del ratón se han agrupado en tres clases en función de la homología estructural que presentan sus productos fenotípicos: genes clase I, II y III. En esta clasificación, las moléculas codificadas por los genes H-2 de Clase I y II se han reconocido estar involucradas en los fenómenos de rechazo rápido de transplantes (Lechler y cols, 1991). Los genes de clase II y III se caracterizan porque sus funciones más relevantes se encuentran asociadas con la respuesta inmune humoral que se desencadena contra antígenos extraños (Guillemot y cols, 1984). Así, los genes Clase II codifican para proteínas que son reconocidas junto con el antígeno extraño, por receptores presentes en los linfocitos T cooperadores permitiendo fenómenos com-plejos de activación celular que influyen en la activación de las células B (Benacerraf, 1981). Los genes Clase III codifican para algunos de los componentes del sistema del complemento como C2, C4A, C4B (responsables de la destrucción celular mediada por anticuerpos) y el factor B (Campbell y cols, 1988), así como para la enzima 21 hidroxilasa (White y cols, 1984), el factor de necrosis tumoral y la proteína Slp (Muller y cols, 1987).

Dentro de los antígenos histocompatibles codificados por genes que no pertenecen al MHC (no-MHC) se encuentran aquellos que desencadenan o median respuestas inmunológicas débiles (mhc) y aquellos que son capaces de producir señales estimulatorias fuertes sobre células T citotóxicas (como los antígenos clásicos de histocompatibilidad) pero que no se codifican en el MHC, sino que se caracterizan por ser los productos de muchos loci genéticos no ubicados en serie, en el ratón (Janeway, 1990). Estos antígenos se denominan determinantes del sistema Mls (productos estimulatorios linfocitarios menores) (Festenstein, 1973; Janeway y cols, 1980). Recientemente se les ha encontrado que son codificados por virus de tumores mamarios del ratón (MMTV), los cuales se integraron dentro de la línea germinal como provirus de DNA (Acha-Orbea y Palmer, 1991).

Esta revisión se concentra principalmente en la descripción estructural y funcional de algunos de los genes de clase I del MHC y sus productos. Estos genes codifican para dos grupos de moléculas muy relacionadas estructural y genéticamente: los antígenos clásicos *(H-2)* y no clásicos de histocompatibilidad (éstos últimos también denominados antígenos de diferenciación linfoide, antígenos no polimórficos o moléculas clase Ib) (Hood y cols, 1983; Lew y cols, 1986a; Strominger, 1989). De los 36 genes clase I identificados en la cepa BALB/c (Steinmetz y cols, 1982) y de los 25 en C57BL/6J, (Weiss y cols, 1984) sólo 8 codifican proteínas clásicas de histo-compatibilidad, y el resto de los genes codifican a los antígenos no clásicos de histocompatibilidad (Winoto y cols, 1983).

Antígenos clase I clásicos de Histocompatibilidad.

1.- <u>Nomenclatura genética y molecular.</u>

Los loci que se encuentran integrados en este grupo, las proteínas que codifican, así como la nomenclatura más reciente acordada para designar a éstas moléculas, se ilustran en la Tabla I.

TABLA I

DESIGNACION DE LOS LOCI *H-2* CLASE I CLASICOS Y SUS PRODUCTOS FENOTIPICOS.

SUBREGION	LOCI COMPRENDIDOS		PROTEINA
	BALB/c	C57BL/10	
K	K^d	K^b	H-2K
	$K2^d$	$K2^b$	
D	D^d	D^b	H-2D
	$D2^d$		
	$D3^d$		
	$D4^d$		
L	L^d		H-2L

Basado en las reglas de nomenclatura establecidas en el International Committe for Mouse Genetic Nomenclature (Klein *et al.*, 1981)

Se han establecido pocos cambios en la nomenclatura de los loci *H-2* que codifican para los antígenos de clase I clásicos. En términos generales, se ha acordado designar a los loci omitiendo el *H-2*, mientras que para referirse a la proteína, se comienza con el término *H-2* seguido de la letra que designe la región de que se trate (Klein y cols, 1990). Una nomenclatura más rigurosa al designar a los genes y sus proteínas entre cepas de distintos haplotipos *H-2*, requiere que además se especifique el haplotipo al que pertenece cada uno de los genes; así, en la cepa BALB/c (haplotipo $H\text{-}2^d$) D^d designa al gene y $H\text{-}2D^d$ a la proteína, mientras que en C57Bl/10 (haplotipo $H\text{-}2^b$), D^b designa al gene y $H\text{-}2D^b$ a la proteína.

2.- <u>Organización de los genes que los codifican.</u>

Los genes del MHC que codifican a los antígenos clásicos de histocompatibilidad, pertenecen a la región *H-2* y se han agrupado en tres subregiones: *H-2D*, *H-2K* y *H-2L* localizadas en la región más centromérica del MHC. Estas tres subregiones no se encuentran en serie, puesto que *H-2K* se encuentra separado de *H-2D* y *H-2L* por genes de Clase II y III (Figura 2).

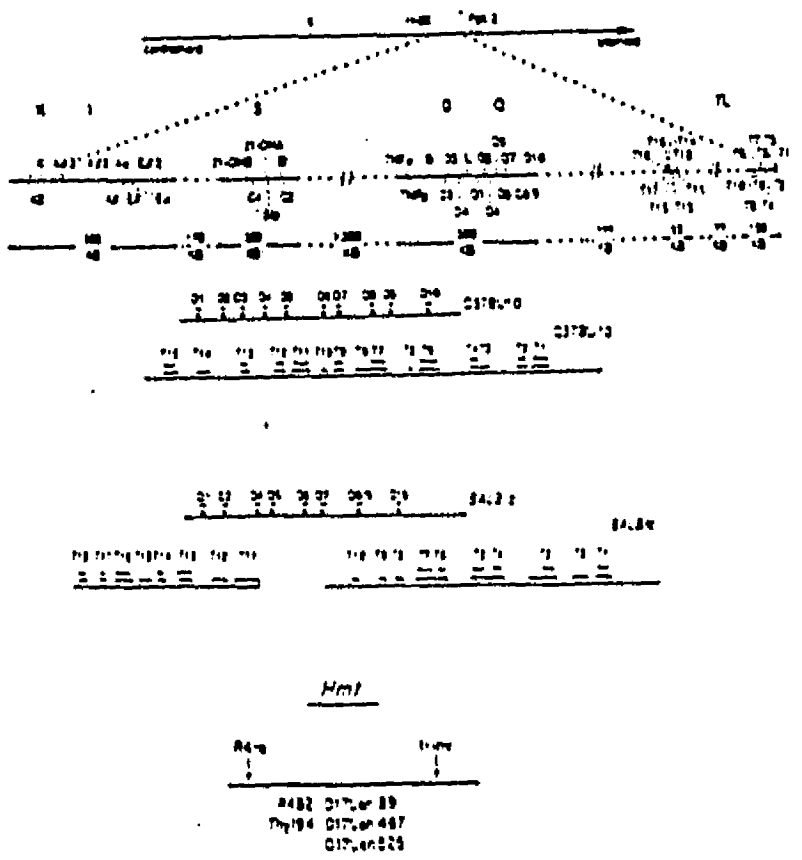


Figura 2. Mapa del MHC del ratón: región H-2, Q, TL y Hmt. (organización de los genes comprendidos en esta región)

Cabe mencionar, que entre cepas congénicas de distinto haplotipo de histocompatibilidad, existen diferencias notables en cuanto al número de genes que están incluidos en cada una de las subregiones *D*, *K* y *L*. Así, en la cepa BALB/c, ($H\text{-}2^d$) se han identificado 4 genes para la subregión *H-2D*: *D*, *D2*, *D3* y *D4* (de los cuales sólo D es funcional mientras que en *D2*, *D3* y *D4* se han detectado deleciones como dm1 y dm2 que los hacen no funcionales (Stephan y cols, 1986); en C57BL/10 ($H\text{-}2^b$), solo un gene funcional *D* se ha identificado el cual codifica para la proteína. En el caso del gene *L* (subregión *H-2L*) y de su producto fenotípico, sólo se encuentra presente en la cepa BALB/c ($H\text{-}2^d$). Los genes comprendidos en la subregión *K* (*K* y *K2*) se encuentran presentes en ambas cepas (Klein y cols, 1990). Un aspecto interesante lo constituye el hecho de que el gene *D2* (de BALB/c) parece tener más homología con los genes no clásicos de histocompatibilidad (Stephan y cols, 1986) que con los genes clásicos clase I .

3.- <u>Estructura de los genes y las proteínas.</u>

Los antígenos de Clase I clásicos son dímeros formados por una cadena pesada de naturaleza glicoprotéica de 45,000 daltones unida no covalentemente a una cadena ligera de aproximadamente 12,000 daltones (la β2 microglublina) que no se codifica en el *H-2* (Lew y cols, 1986a). El polipéptido de 45 Kd está constituido por tres dominios, denominados α1, α2 y α3 cada uno con un resto hidrocarbonado asociado covalentemente. Los dominios α1 y α2 son los más polimórficos y en ellos residen las funciones antigénicas y biológicas de las molécula; el dominio α3 es más conservado y presenta una homología genética y molecular con el dominio constante de la IgM. Esta cadena se ancla a la membrana por un pequeño segmento transmembranal y presenta además un dominio citoplasmático que comprende el extremo carboxilo terminal (Mallisen, 1986; Figura 3).

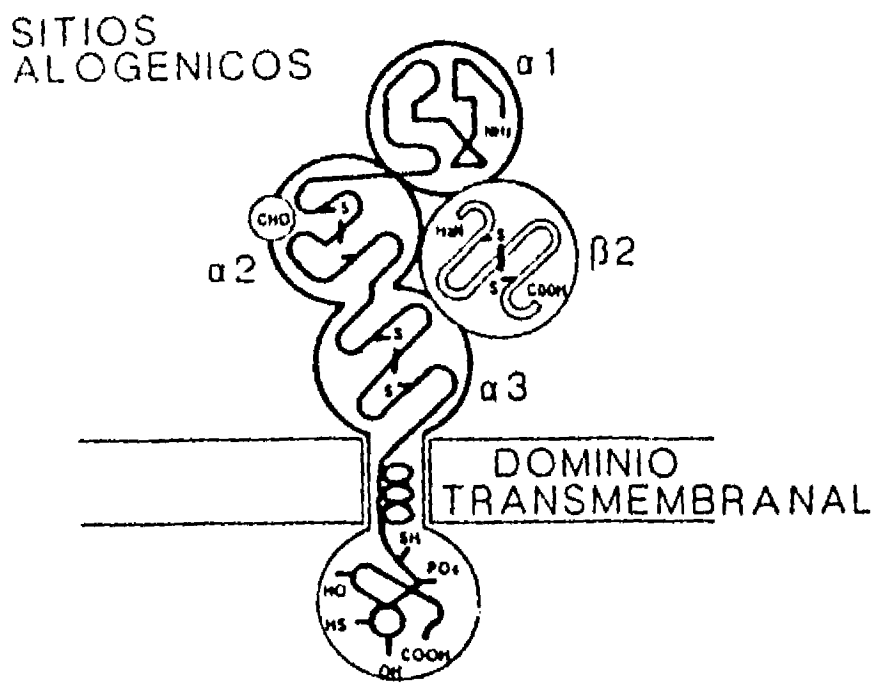


Figura 3. Estructura de los antígenos de clase I del MHC del ratón,

Cada dominio de la molécula es codificado por exones separados por secuencias no codificantes (intrones). El exón 1 codifica para el péptido señal, el cual es removido después de la transcripción por enzimas proteolíticas. Los tres dominios extracelulares (α1, α2 y α3) con aproximadamente 90 aminoácidos cada uno, son codificados por los exones 2, 3 y 4, respectivamente. El exón 5 codifica para la región transmembranal, mientras que la región citoplasmática está codificada por los exones 6-8 (Figura 4).

4.- Distribución y Función

Los antígenos clásicos se expresan ubicuamente como glicoproteínas de superficie en casi todos los tipos celulares, aunque más abundantemente en macrófagos y células linfoides, principalmente linfocitos B. Son moléculas cuya función se encuentra íntimamente relacionada con su alto grado de polimorfismo y se piensa que están involucradas en la presentación de antígenos extraños, sintetizados endógenamente, a elementos del sistema inmune. Así, estos antígenos son elementos que permiten el reconocimiento por parte de los linfocitos T citotóxicos (CTL), de marcadores celulares de lo no propio (rechazo rápido de transplantes) (Zinkernagel y Doherty, 1989), así como células alteradas ya sea por mecanismos naturales (envejecimiento y degeneración) o por infecciones virales (Klein, 1979). De esta manera, los receptores αβ específicos de las CTL reconocen sólo glicoproteínas extrañas, propias (alteradas o virales) cuando éstas se asocian a las moléculas de clase I clásicas de histocompatibilidad (Song y cols, 1988), desencadenando una serie de eventos que culminan con la destrucción celular (Figura 5).

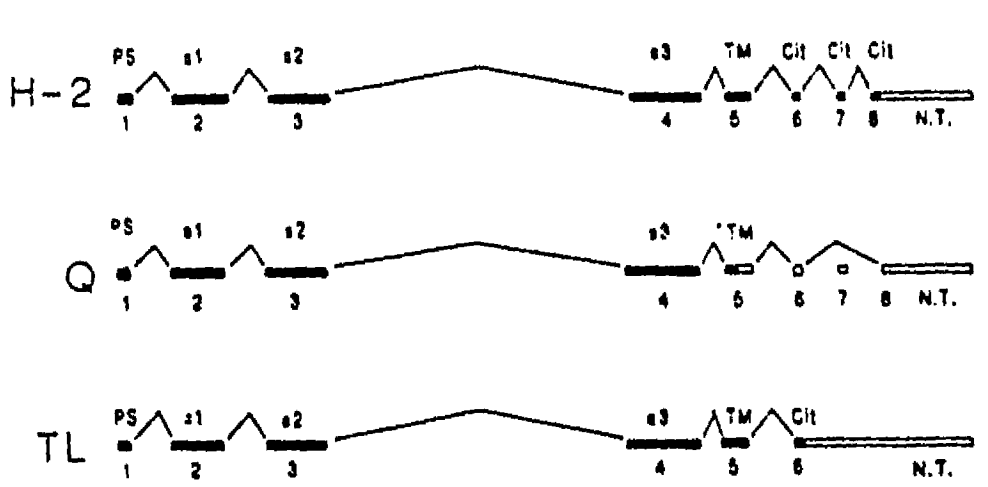


Figura 4. Relación entre la estructura de los intrones-exones de genes de la región H-2, Q y Tl y las regiones de las molécula prototipo que codifican. PS: secuencia del péptido señal. TM: región transmembranal; Cit: dominio citoplasmático y NT: región no traducible

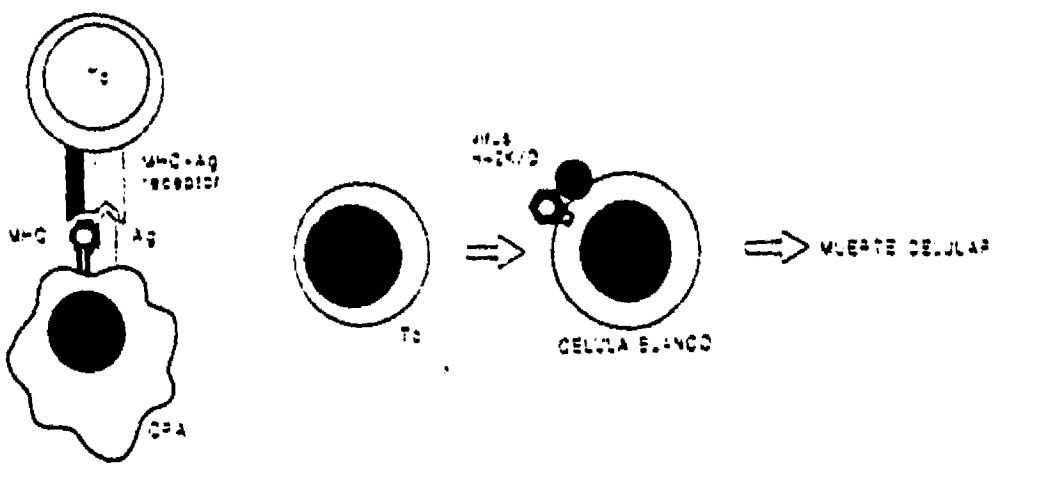


Figura 5. Destrucción celular mediada por antígenos clase I del H-2

Antígenos clase I no clásicos de Histocompatibilidad.

El estudio formal de los antígenos de Clase I no clásicos de histocompatibilidad se inició a partir de 1980 aunque se desconoce las funciones biológicas de la mayor parte de ellos; se trata de un conjunto de proteínas que también se codifican en el MHC del ratón.

Los loci que codifican para los antígenos no clásicos de histocompatibilidad, se ubican en las regiones *Q*, *TL* y *M* (antes designadas como *Qa*, *Tla* y *Hmt*, respectivamente), localizadas en la región más distal del MHC, adyacentes a la región *D* del *H-2*. Estos antígenos no clásicos, constituyen un grupo de proteínas que se han incluido dentro del grupo de Clase I, dado la gran homología estructural molecular y genómica que presentan con los antígenos clásicos (Lew y cols, 1986b; Robinson, 1985). Sin embargo, la mayor parte de estas proteínas no parecen desempeñar las mismas funciones y presentan además características que hacen muy atractivo su estudio, como su bajo grado de polimorfismo, su expresión tejido específica y la manera como se encuentran ancladas a la membrana de las células que las expresan. El conocimiento que se tiene sobre éstos loci se ha obtenido de dos cepas congénicas de ratones, principalmente: BALB/c y C57BL/10, por lo que la información que se proporcione en ésta revisión, se concentrará en ellas.

1.- <u>Nomenclatura genética y molecular</u>

La nomenclatura más reciente con la que se ha acordado nombrar a este conjunto génico se presenta en la Tabla II. La designación de estos genes y sus productos fenotípicos ha cambiado considerablemente en los últimos años debido a la identificación de nuevos genes y a la inconsistencia respecto a la presencia de estos genes en las distintas cepas de ratones.

Cabe mencionar que de todos los genes que se han identificado en cada una de las regiones, sólo se conocen de algunos sus productos fenotípicos. Así, para la región *Q*, de los 10 genes que la constituyen, sólo de seis se conocen las proteínas que codifican: *Q4*, *Q6*, *Q7*, *Q8*, *Q9* y Q10 en donde Q4, codifica para la proteína *Qb-1* (Robinson, 1985), *Q10* codifica la proteína *Q10* (Maloy y cols, 1984) y *Q6*, *Q7*, *Q8* y *Q9* codifican a la proteína Qa-2 (Mellor y cols, 1985; Soloski y cols, 1988). Esta colección de genes *Q6-Q9* se ha sugerido que provienen de la duplicación de un gene ancestral y posterior duplicación del par de genes, de ahí que todos codifiquen a la misma proteína. (Mellor y cols, 1985).

TABLA II

DESIGNACION DE LOS LOCI Q Y TL QUE CODIFICAN A ANTIGENOS CLASE I NO CLASICOS DE HISTOCOMPATIBILIDAD

Región Q y Hmt				Región TL	
GENE	PROTEINA	GENE			PROTEINA
		C57BL/10	BALB/c		
Q1 (Mh1)*		*T1* ^{b}	*T1* ^{d} *(T1c)*		
Q2 (Hx 19.4)		*T2* ^{b}	*T2 d (T2c)*		
Q3 (Hmt)	Mla	*T3* ^{b}	*T3* ^{d} *(T3c)*		TL (TL 1)
Q4 (C3H1)		*T4* ^{b}	*T4 d (R5')*		
Q5 (C3H2)		*T5* ^{b}	*T5 d (T4c)*		
Q6 (C3H3)		*T6* ^{b}	*T6 d (T5c)*		
Q7		*T7* ^{b}	*T7 d (T6c)*		
		T8 ^{b}	*T8 d (T7c)*		
		T9 ^{b}	*T9 d (T8c)*		
Q1		*T10* ^{b}	*T10 d (T9c)*		
Q2		*T11* ^{b}	*T11 d (T10c)*		
Q3		*T12* ^{b}	-		
Q4	Qb-1	*T13* ^{b}	-		
Q5		*T14* ^{b}	-		
Q6	Qa-2	*T15* ^{b}			
Q7	Qa-2	-	*T16 d (T11c)*		
Q8	Qa-2	-	*T17 d (T12c)*		
Q9	Qa-2	-	*T18 d (T13c)*		TL (TL 1,TL 2)
Q10	Q10	-	*T19 d (T14c)*		
		-	*T20 d (T15c)*		
		-	*T21 d (T16c)*		
		T22 ^{b}	*T22 d (T17c)*		
		T23 ^{b}	*T23 d (37 c)*		Qa-1
		-	*T24 d (T18c)*		

Basado en las reglas de nomenclatura establecidas en el International Committe for Mouse Genetic Nomenclature (Klein *et al*, 1990)

* Nombre anterior con el que se conocían

Para la región *TL*, de los 18 genes identificados en BALB/c, sólo se conocen tres productos fenotípicos: *T3* y *T13* que codifican a la proteína TL (Chen y cols, 1985; Matsuura y cols, 1987) y *T23* que codifca a la proteína Qa-1 (Wolf y Cook, 1990). También en ésta región se han detectado genes duplicados, principalmente en la cepa *BALB/c*: $T1^c \rightarrow T11^c$, $T2^c \rightarrow T12^c$, $T3^c \rightarrow T13^c$, $T16^c \rightarrow T14^c$, $T7^c \rightarrow T15^c$, $T18^c \rightarrow T16^c$, $T9^c \rightarrow T17^c$ y $T10^c \rightarrow$ *"37"* (Stroynowski, 1990).

En los antígenos Qa-2 (Flaherty, 1976), se han identificado distintas formas serológicas utilizando anticuerpos monoclonales. Este conjunto de formas serológicas se designan como familia de antígenos Qa-2 (Flaherty, 1990), e incluyen: Qa-3, Qa-4, Qa-5, Qa-6, Qa-7, Qa-8, Qa-9, Qa-11 y Qc-1 (Harris y cols, 1984; Flaherty, 1990). La proteína Qa-1, se designó Q pues en un principio se consideró que estaba codificada por alguno de los genes de está región Q, y aunque en la actualidad se sabe que está codificada por el gene T23 de la región TL (Wolf y Cook, 1990), el nombre se ha mantenido.

Los genes de la región M fueron designados en un principio como genes Hmt (Fisher Lindahl y cols, 1983), ya que se desconocía su naturaleza y el número de loci que integraban ésta región. Dentro de esta región se ha mantenido el nombre de gene Hmt para designar al responsable de la expresión del antígeno Mta (antígeno transmitido maternalmente) (Fisher-Lindahl y cols, 1983) que posee propiedades intermedias de distribución y función entre los antígenos H-2D/H-2L/H-2K y los antígenos de las regiones *Q* y *TL*. Actualmente se sabe que son ocho genes los que conforman ésta región y su nomenclatura se resume en la Tabla II.

En general, se designan como genes *M* (por su asociación con la proteína Mta) seguida de un número según el orden como se han ido identificando (Klein y cols, 1990). De los 8 genes sólo se ha logrado identificar la proteína codificada por el gene *Hmt* (M3), mientras que para *M4* y *M7* se ha identificado por análisis de secuencias de nucleótidos que se trata de pseudogenes.

Es importante destacar que a diferencia de los antígenos clásicos, en los que dado el gran polimorfismo que existe entre cepas de distinto haplotipo *H-2* se necesita designar el haplotipo al que corresponden, en los antígenos Qa-2 basta con aclarar si la proteína se expresa o no, es decir sólo se consideran dos alelos: ***a*** que denota que el gene es activo y ***b*** que no lo es.

2.- <u>Organización de los genes que los codifican.</u>

La propuesta de la organización de los genes que conforman este fragmento génico *Q/TL/M*, ha cambiado en los últimos años y difiere entre cepas de ratones con distintos haplotipos de histocompatibilidad. En la figura 4 se ilustra la organización de estos genes en las cepas BALB/c y C57BL/10. Muchas de las diferencias que se observan entre estas dos cepas se debe a que no se han identificado algunos de los genes faltantes, a duplicaciones de genes ancestrales así como a varias deleciones que se han originado, sobre todo en los genes que conforman la región *Q*. De esta manera, en los diferentes subtipos de BALB/c se reflejan las diferencias en ésta región por la expresión de distintos niveles de la proteína Qa-2. En BALB/cJ la fusión de la región 5' del gene *Q8* con el extremo 3' del gene *Q9* en el exón 4 provocó la generación de un gene híbrido con varios cambios en la secuencia de nucleótidos que tuvieron como consecuencia 6 cambios en los aminoácidos de la proteína original, en especial una cisteína necesaria para la estabilidad estructural de la molécula. Estos cambios provocaron una diminución en la expresión de la proteína Qa-2 (Mellor y cols, 1985; Nakayama y cols, 1991) dada por los genes *Q8* y *Q9*, quedando sólo la proteína codificada por los genes *Q6* y *Q7*. En las cepas BALB/cAnN y BALB/cByJ una doble mutación en *Q6-Q7* y *Q8-Q9* tuvo como consecuencia la pérdida total de su expresión (Mellor y cols, 1985; Flaherty y cols, 1985).

En el caso de la región M, de los ocho genes identificados (*M1-M8*) aún se desconoce la organización de la mayor parte de ellos e incluso la proteína que codifican. Sólo para *M2* se ha encontrado que se transcribe en muy bajos niveles en células del timo; y para *M4* y *M7*, la presencia de codones de terminación en los exones 3 y 4, respectivamente, sugieren que se trata de pseudogenes (Fisher-Lindahl y cols, 1991).. A diferencia de los genes de las regiones *Q* y *TL*, los genes de ésta región no se encuentran en serie, ya que el gene *M1* se localiza entre los genes de la reglón *Q* y *TL* mientras que el resto de los genes (*M2-M8*)

se localizan en el extremo distal de la región TL. La expresión de la proteína Mta, requiere no sólo de la participación de los genes de la región *M (M3)*, sino que también de dos genes más, no codificados en el MHC: el gene extracromosómico mitocondrial *Mtf* (que codifica para el factor de transmisión materna (Fisher-Lindahl y Burki 1982) y el gene que codifica para la b2 microglobulina (Fisher-Lindahl y cols, 1988).

En resumen, en la región Q se han detectado de 8-10 genes (*Q1-Q10*), en la región TL, dependiendo de la cepa estudiada, de 10 a 20 genes y en la región *M*, 8 genes. Al igual que para los genes que codifican a los antígenos clásicos, muchos de los genes de estas regiones se han secuenciado, pudiéndose establecer una correlación entre secuencias del gene y la región protéica que codifican (Figura 4).

3.- Distribución

Una de las características más sobresalientes de los antígenos de Clase I no clásicos es que la mayor parte de ellos presentan una distribución tejido especifica, a diferencia de los antígenos clásicos que se localizan en casi todos los tipos celulares del organismo. Esta distribución característica, en células principalmente de orígen linfoide, es uno de los factores que contribuyen a considerar que sus funciones puedan estar asociadas con procesos de diferenciación celular y suponer que no desempeñen las mismas funciones que los antígenos clásicos de histocompatibilidad.

3.1 *Distribución de los antígenos codificados por la región Q*

En general todas las proteínas codificadas en la región Q (Qa-2, Qb-1 y Q-10) tienen una distribución limitada principalmente a células de orígen hematopoyético (Vernachio y cols, 1989; Robinson, 1985; Sharrow y cols, 1989). La proteína más estudiada en este grupo es Qa-2. Esta molécula ha sido detectada en nódulos linfáticos, bazo, timo, médula osea e hígado (Flaherty, 1981; Lalanne y cols, 1985), siendo mayor su expresión en células T activadas principalmente de fenotipo CD5/CD3 (con cualquiera de las siguientes combinaciones: $CD4^+/CD8^-$, $CD4^-/CD8^+$ ó $CD4^+$ /$CD8^+$ y menor en células T inmaduras, linfocitos B y macrófagos (Vernachio y cols, 1989). Una característica importante en la molécula Qa-2 es que no sólo se expresa en la superficie de las células T activadas, sino que puede ser secretada por la célula, a diferencia de la proteína Qb-1 que sólo existe en su forma secretada o intracelular pero no se expresa en la superficie de las células (Robinson, 1985). Esta molécula Qb-1 se ha detectado principalmente en linfocitos T y B de médula ósea y nódulos linfáticos. La proteína Q10, a diferencia de las otras dos proteínas de la región Q, sólo se ha encontrado en hepatocitos y debido a una deleción en el exón 5, sólo se expresa en su forma soluble (Mellor y cols, 1984).

3.2 *Distribución de los Antígenos codificados en la región TL.*

Las dos proteínas reconocidas en ésta región (TL y Qa-1) presentan una diferencia en cuanto a su distribución. En general los antígenos TL tiene una distribución limitada, característica de las proteínas de clase I no clásicas de histocompatibilidad, y se expresan en células del timo (Old y cols, 1983). Una característica, poco común, que presentan éstas moléculas es que aparecen anormalmente en células leucémicas, de ahí la designación que tuvieron en un principio como antígenos Tla (antígenos de leucemias tímicas). En relación con los aloantígenos Qa-1, estos presentan una distribución extensa y ubicua muy similar a los antígenos clásicos de histocompatibilidad (Aldrich y cols, 1988). Esta característica, aunada con el hecho de que la molécula es altamente polimórfica (Widacki and Cook 1986) y de que puede presentar antígenos a células T (Vidovic y cols, 1989) la coloca en una posición estructural y funcional intermedia entre los antígenos clásicos y no clásicos de histocompatibilidad. Una característica interesante de ésta molécula Qa-1 es que su expresión parece ser modificada por un gene unido a la región *H-2D*, denominado *Qdm* (Aldrich y cols, 1988). Este hallazgo permitiría pensar que la expresión de otras moléculas de clase I no clásicas de histocompatibilidad pudiese también estar regulada por otros genes.

3.3 *Distribución de los antígenos codificados por la región M: antígeno Mta.*

El antígeno transmitido maternalmente (Mta), es una proteína determinada por el fenotipo materno que se expresa en una gran variedad de células (Chan y Fisher-Lindahl, 1985). Esta proteína al igual que Qa-1, puede considerarse como un antígeno con propiedades intermedias entre los antígenos clásicos y los no clásicos de histocompatibilidad, ya que no sólo se expresa en una gran variedad de células, sino que también sirve de blanco a linfocitos T citotóxicos de una manera no restringida por *H-2* (Fisher-Lindahl y cols, 1980).

4.- <u>Estructura y posibles funciones asociadas.</u>

Los antígenos no clásicos de histocompatibilidad presentan una estructura general muy similar a los antígenos de clase I clásicos. Ambos tipos de moléculas están constituidas por una cadena pesada, a, de 43-45 Kd, asociada no covalentemente con la β2 microglobulina (Michaelson y cols, 1981). La cadena a presenta en ambas moléculas tres dominios α1, α2 y α3, aunque a diferencia de los antígenos clásicos que tienen glicosilaciones en las porciones 86, 176 y 256, la mayor parte de los antígenos de las regiones *Q* y *TL* carecen de la glicosilación en la porción 176 (Landolfi y cols, 1985). Cabe mencionar que la mayor parte de las proteínas no clásicas de histocompatibilidad son muy poco polimórficas (con excepción de Qa-1, Widacki y Cook, 1986), y Mta (Fisher-Lindahl y cols, 1991).

4.1 *Características estructurales y de expresión de los antígenos codificados por genes Q y funciones asociadas a ellas.*

Una característica única de los productos de la región *Q* (Qa-2, Qa-1 y Q10), es su capacidad para poder ser secretados, proceso que está íntimamente relacionado al hecho de que el segmento hidrofóbico transmembranal, característico de los antígenos clásicos, se encuentra reemplazado por una secuencia hidrofílica (Mellor, 1984). Sin embargo, la vía de secreción parece ser diferente entre estos antígenos; por ejemplo Qb-1 se secreta directamente, mientras que Qa-2 primero se expresa en la superficie de las células y luego es procesada a su forma soluble (Robinson, 1987a). Qa-2, ha sido la proteína más estudiada de todos los antígenos de clase I no clásicos y se sabe que posee una asociación a la membrana celular poco usual respecto a los antígenos de histocompatibilidad. A diferencia de los antígenos H-2D, H-2L y H-2K que se encuentran unidos a la membrana celular mediante una estructura glicosídica, la proteína Qa-2 lo hace a través de una unión de fosfatidilinositol (PI) (Stienberg y cols, 1987). Aún cuando todavía se desconocen los eventos biológicos que puedan depender de ésta forma de unión, se han sugerido dos fenómenos que puedan estar asociados con ella: i) se refiere a su presentación en forma soluble que pudiese estar mediada por la presencia de fosfolipasas endógenas (Stienberg y cols, 1987; Robinson, 1987b) y ii) se asocia esta forma de unión con propiedades mitogénicas, considerando el hecho de que en procesos de proliferación celular se encuentran involucrados mecanismos mediados por PI (Gunter y cols, 1984).

La producción de ratones transgénicos empleando diferentes construcciones genómicas quiméricas, han mostrado que las diferencias en cuanto a las funciones de los antígenos clásicos y la proteína Qa-2 no se debe a sus características estructurales, sino más bien a su baja expresión tejido específica (Mellor y cols, 1991). En estos estudios tres diferentes genes clase I quiméricos *Q9/D*^{b} (con diferencias en el exón 1, que codifica para los elementos promotores transcripcionales, y en los exones 5, 6, 7 y 8, que codifican para los dominios transmembranales), así como genes clase I no quiméricos *Q9* y *D*^{b} (controles) fueron inyectados a embriones de ratones de haplotipo *H-2*^{k}, y *Qa-2*^{b}. Los autores no encontraron diferencias en cuanto a la capacidad de estos animales de rechazar tejidos de piel ni en ensayos de citotoxicidad celular empleando células blanco haplotipo-específicas. Además, la línea transgénica que integró el gene *Q9* en

varias copias y por lo tanto manifestó una mayor expresión de la proteína Qa-2, presentó funciones similares a los antígenos de clase I clásicos de ratón. Así pues, las diferencias funcionales que existen entre los antígenos clásicos y no clásicos (Qa-2) pueden estar asociada a una expresión débil tejido específica de éstos últimos.

4.2 *Características estructurales de los antígenos codificados por la región TL y funciones asociadas a ellas.*

De las dos proteínas reconocidas en esta región, es la proteína Qa-1 en la que más se ha avanzado en el conocimiento de sus propiedades estructurales y funcionales. Esta proteína además de tener la estructura general de los antígenos clase I, también se encuentra unida a membrana a través de uniones glicosídicas (como los antígenos clásicos de histocompatibilidad). Esta característica, aunada con los hallazgos de que puede presentar antígenos a través del receptor γδ en hibridomas T cooperadores (Boneville y cols, 1989; Vidovic y cols, 1989) constituye una primer avance en el conocimiento de las posibles funciones de algunos de los antígenos no clásicos de histocompatibilidad.

4.3 *Características estructurales y funcionales de las moléculas M (región Hmt).*

El antígeno transmitido maternalente (Mta), posee una estructura tipo clase I (Fisher- Lindahl, 1983), y se asocia la membrana de las células a través de enlaces glicosídicos. Una característica estructural de esta molécula que la relaciona más con los antígenos de clase I clásicos, es que su asociación con la β2m le permite unir pequeños péptidos (codificados por el gene *Mtf*) y presentarlos en su superficie a células T citotóxicas (Fisher-Lindahl y cols, 1983). Esta proteína también parece desempeñar funciones que la relacionan más con los antígenos de clase I de histocompatibilidad: se comporta como un antígeno de rechazo rápido (aloantígeno) y parece ser reconocida por el receptor ab de las células T (Fisher-Lindahl y cols, 1991). Recientemente se ha encontrado que la proteína Mta es capaz de presentar péptidos mitocondriales (Loveland y cols, 1990) y procarióticos (Kurlander y cols, 1992) al sistema inmune. Este último observó que una proteína del MHC del ratón, identificado como el H-2M3 es capaz de presentar antígenos derivados de ***Listeria monocytogenes*** a células CD8+ específicos para ***Listeria***. Estos hallazgos resultan de gran interés dado que abre la posibilidad de que otras moléculas de clase I no clásicas de histocompatibilidad cuya función aún se desconoce puedan también participar en los mecanismos de defensa contra otros agentes infecciosos.

Perspectivas. De todos los antígenos clase I estudiados, se ha avanzado notoriamente en definir las funciones que desempeñan los antígenos clásicos de histocompatibilidad. Sin embargo, en relación con los antígenos no clásicos, aunque en los últimos años varios grupos de investigación se han dedicado a ahondar en el estudio de éstas moléculas, los avances logrados se han obtenido principalmente a nivel de descripción de la estructura de estas proteínas así como la organización de los genes que las codifican. Falta aún por identificar muchos de los productos fenotípicos que se codifican en la regiones *Q, TL* y *M* así como el papel biológico que desempeñan. Es posible que el avance que se obtenga en ésta área pueda aclarar muchos de los eventos del sistema inmune que aún faltan por decifrar.

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PRODUCCION DE RATONES TRANSGÉNICOS

Producción de ratones transgénicos

M. en C. Gladis Fragoso González

Uno de los avances importantes que se ha desarrollado en el campo de la genética ha sido la introducción de genes extraños en la línea germinal de células de mamíferos. Los pioneros de este logro fueron el grupo de Gordon y cols. quienes evaluaron la posibilidad de insertar DNA de cualquier orígen directamente en el ratón a través de una técnica conocida como microinyección al pronúcleo (Gordon y cols. 1981). A partir de entonces los animales modificados por este tipo de estrategias se han denominado "Animales transgénicos". Esta metodología iniciada en ratones se ha extendido a una gran número de especies de animales.

Este apéndice tiene como objetivo el describir detalladamente la metodología que utilizamos para la producción de ratones transgénicos en el Instituto de Investigaciones Biomédicas de la UNAM.

En la Figura 1 se ilustra un esquema que resume los pasos esenciales para la producción de ratones transgénicos, que esencialmente consisten en:

1) Obtención y purificación del gene a introducir (transgene)
2) Obtención e inyección de los embriones a transformar (embriones de 12 hrs de gestación)
3) Producción de machos vasectomizados y de hembras pseudoembarazadas.
4) Transferencia de los embriones inyectados a hembras pseudoembarazadas.

Uno de los aspectos más importantes a controlar para tener éxito en la producción de ratones transgénicos es el horario de los ciclos de luz-oscuridad que deben tener los animales con el fin de no modificar su ciclo estral. De acuerdo con las facilidades proporcionadas por el bioterio del Instituto, el horario que se estableció para la producción de ratones transgénicos fue el siguiente:

Acción	Horario
Ciclo de luz-obscuridad de las instalaciones del Bioterio	Luz: de las 6:00 am a las 18:00 pm Obscuridad: de las 18:00 pm a las 6:00 am
Inyectar Gonadotrofina sérica (obtenida de hembra preñada) a hembras prepúberes.	Día -3: 17:00 hrs.
Inyectar Gonadotrofina coriónica humana a las hembras y aparearlas con machos completos	Día -1: 9:00-10:00 hrs.
Sacrificar a las hembras: Obtención y manipulación de los embriones	Día 0: 9:00-11:00 hrs
Microinyección del DNA	Día 0: 14:00-15:00 hrs
Aparear hembras maduras con machos vasectomizados: Hembras Pseudoembarazadas	Día 0: 13:00-16:00 hrs
Transferir los embriones a hembras pseudoembarazadas	Día +1: 9:00-11:00 hrs.
Revisar embarazo y parto	Días: 18-21

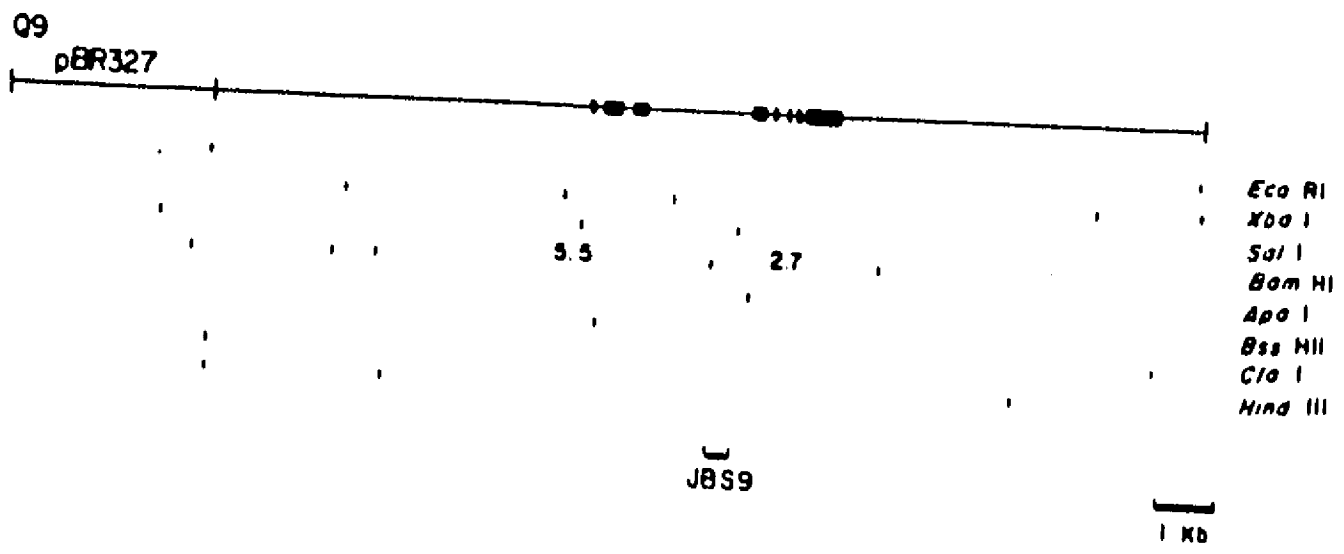


Figura 2. Mapa de restricción del gene *Q9*

A) Preparación del DNA.

El gene que se microinyectó (Q9 del H-2 de ratón) fue clonado de la cepa C57BL/6J y se uso al plásmido PBR327 como vector de expresión. Para amplificar el plásmido, se transfectó a la cepa HB101 de *Escherichia coli* y las colonias que integraron el plásmido se seleccionaron utilizando Ampicilina como marcador de resistencia. Posteriormente se sometió a una separación en gradiente de cloruro de cesio y por extracción con fenol y cloroformo se obtuvo el plásmido en forma pura.

El mapa de restricción del gene Q9 se ilustra en la Figura 2. Para la liberación del gene, el plásmido se cortó con la enzima de restricción EcoRI de acuerdo al siguiente protocolo:

Gene Q9 (2μg/μl)	10μl
Buffer 10X (EcoRI)	2μl
H_20	5μl
EcoRI (10U/μl)	3μl
	20μl

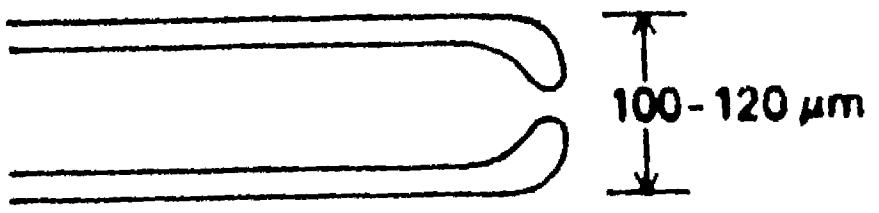
Incubar a 37°C durante 4 horas

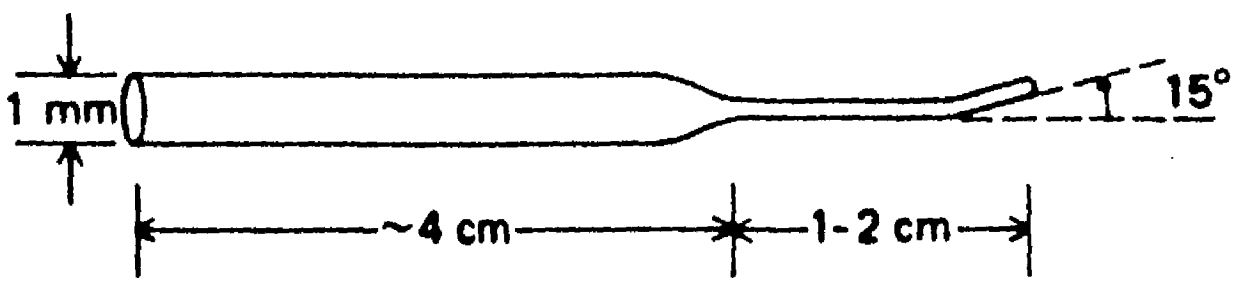
La mezcla resultante se separó electroforéticamente en geles de agarosa (de bajo punto de fusión) al 0.7% en un amortiguador de 0.04M Tris-Acetato-0.001M EDTA (TAE) . Una vez terminada la corrida, el gel se tiñó con una solución en agua de Bromuro de Etidio (0.5μg/ml) durante 15 minutos y la banda de 9 Kb correspondiente al gene Q9 se cortó y se colocó en un tubo eppendorf de 1.5 ml. La tira de agarosa se fundió calentándola a 65°C durante 30 minutos y una vez estimado el volumen correspondiente se separó el DNA de la agarosa de acuerdo al siguiente esquema:

1. Se adicionó un volumen igual de fenol equilibrado con NaOAc 0.2M, se mezcló con el vortex y se centrifugó en una microfuga durante 5 minutos. La fase acuosa (superior) se removió y se guardó en un tubo eppendor.
2. La fase fenólica se reextrajo con 10mM Tris-Cl-0.2mM EDTA, pH 7.5 (TE) y al final la fase acuosa obtenida se mezcló con la obtenida en el punto A.
3. Ambas fases acuosas (1y2) se extrajeron con fenol y por último con una mezcla de fenol:$CHCl_3$: Alcohol Isoamílico [1:1 (25:1)]
4. Para la precipitación del DNA se adicionó NaCl a una concentración final de 0.1M y 2.5 volumenes de alcohol absoluto. La mezcla se congeló a -70°C durante 2 horas.
5. La mezcla se centrifugó a 10,000 rpm por 30 minutos a 4°C, se descartó el sobrenadante y se dejó que se secara el botón formado.
6. Por último el botón se resuspendió con 20 μl de TE (10:0.2) y se preparó para someterlo a un segundo nivel de purificación utilizando el protocolo del Kit de GENCLEAN.
7. Purificación por GENECLEAN:

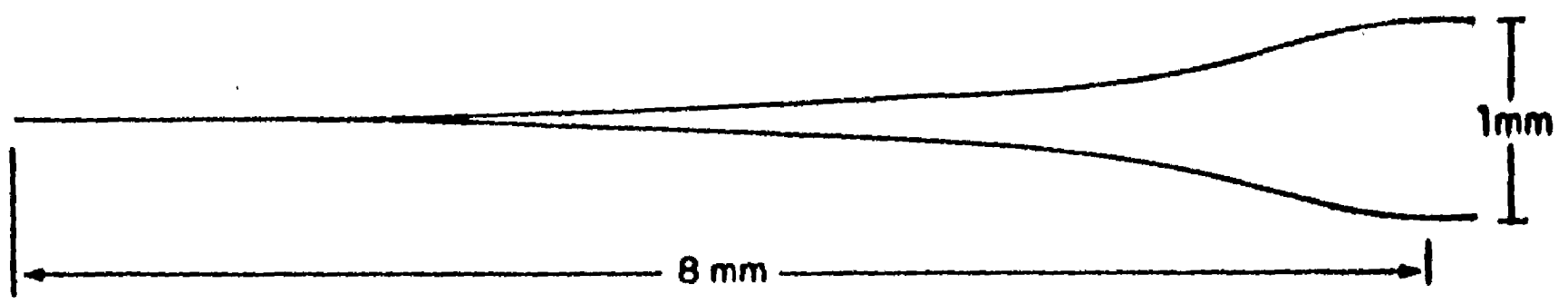
- El DNA se corrió electroforéticamente en un gel de agarosa al 0.6% en Tris-Acetato-EDTA (TAE). Una vez terminada la corrida, se cortó la banda donde se localizó el gene y se colocó en tubo eppenderof de 1.5 ml
- Posteriormente se adicionó de 2.5 a 3 volumenes de una solución de NaI por volumen de la solución de DNA y se incubó durante 5 minutos a 55°C con agitación cada 2 minutos
- Una vez fundido el gel se adicionó una suspensión de "Glass Milk" (5μl de solución de Glass Milk por 5 μg o menos de DNA). Se mezcló y se incubó 5 minutos a 4°C en hielo
- Se centrifugó por 5 minutos, se eliminó el sobrenadante y el paquete celular se lavó tres veces con una solución de New Wash (700 μl/lavado).
- En el último lavado, el sobrendante se eliminó totalmente y el paquete celular se resuspendió en 20 μl de TE (10:0.1mM). Posteriormente se calentó

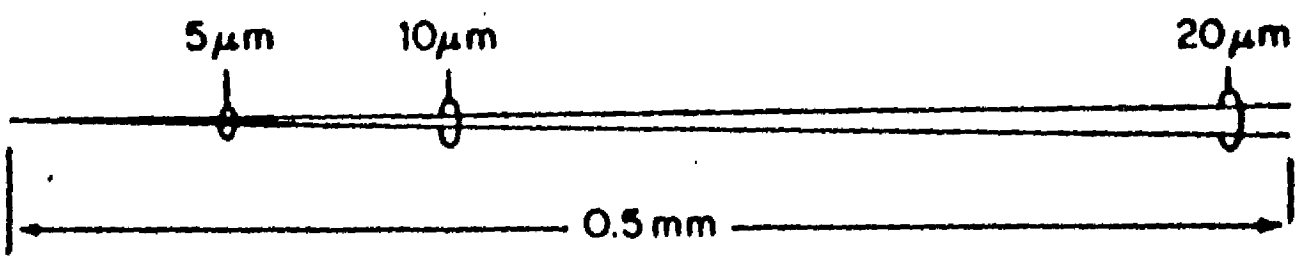
Figura 3.



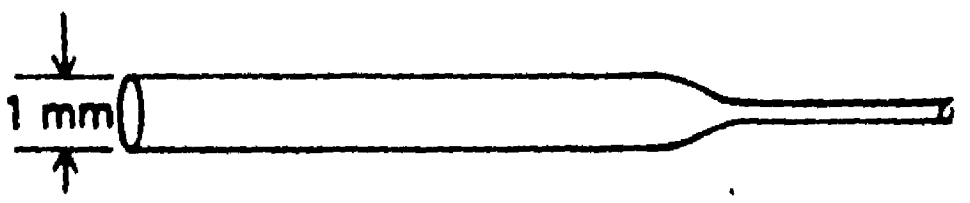


a) Pipeta de sostén de los embriones: dimensiones y forma del extremo que sostiene al embrión (arriba); dimensiones y forma de la pipeta completa.





b) Pipeta para la microinyección: dimensiones y tamaño del extremo para la microinyección de los embriones.



c) Pipeta para el manejo y transferencia de los embriones

a 55°C durante 3 minutos se centrifugó y se evaluó la cantidad de DNA recuperada en el sobrenadante tanto por densidad óptica a 260 nm como por visualización directa en un gel de agarosa.

- El DNA se diluyó en TE (10-0.1) a una concentración final de microinyección de 1-2 μg/ml y se guardó en alícuotas de 2μl a 4°C para la microinyección.

B) Elaboración de pipetas de sostenimiento, transferencia y microinyección de embriones.

En la tabla 1 se describe el equipo y material necesarios para la elaboración de los diferentes tipos de pipetas empleadas.

TABLA 1

	Pipetas de sostén de embriones	Pipetas de Microinyección	Pipetas para manipulación y transferencia de embriones
Material	Tubo capilar de 10 cm de largo y 1mm de diámetro interno Leitz (No. 521009)	Tubo capilar de pa-red delgada de 1 mm de diámetro interno. World Precision Instruments TW100f-6	Tubo capilar de paredes gruesas de 1 mm de diámetro interno. Clark Electromedical Instruments (No. GC150T - 10
Equipo	Lápiz de Diamante Microforge MF-9 (Narishige, Nikon)	Micropippet Puller PB-7 (Narishige, Nikon)	Lápiz de Diamante Micromechero de Bunsen

Pipetas de sostén: Las pipetas de sostén se realizan calentando el capilar en la parte más azúl de la flama y con movimientos rotatorios. Una vez que el vidrio se ha reblandecido, se le da un tirón fuera de la flama hasta que el vidrio se ha estirado a tener un diámetro interior de aproximadamente de 100-120 μm. El capilar se corta con un lápiz de diamante de manera que la distancia del vidrio estirado comprenda unos 4 cm. La formación de la estructura propia del capilar para el sostenimiento del embrión se logra utilizando el microforge. En la Figura 3a se ilustra la forma que debe tener la pipeta de sostén.

Pipetas de microinyección. La pipeta de microinyección se realiza colocando el capilar en el micropippet puller de manera que la resistencia quede aproximadamente a la mitad del capilar. Se ajusta las condiciones de calor-tiempo de la resistencia y del contrapeso de manera que se puedan obtener pipetas con un estiramiento de 1.5 cm y con una punta muy fina que en la mayor parte de las veces quedará cerrada. La Figura 3b muestra la forma de las pipetas de microinyección.

Pipetas para el manejo y transferencia de los embriones al oviducto. Los requisitos importantes que deben reunir estas pipetas son: 1) La dureza del capilar de manera que no se doble fácilmente durante el proceso de transferencia de los embriones, y 2) La textura suave que debe tener el borde del capilar de manera que los embriones no se dañen. El primer punto

se logra utilizando capilares de pared gruesa, como los especificados en la Tabla 1. Para la elaboración de las pipetas se sigue el mismo procedimiento de flameado y estirado que el descrito para las pipetas de sostén. El diámetro interno es más amplio de unos 150-200 μm. El capilar también se corta con un lápiz de diamante permitiendo una distancia del capilar estirado de unos 4 cm y calentando el borde muy suavemente con la parte menos caliente de la flama, de manera que el extremo del capilar quede con los bordes lisos y redondeados (Figura 3c).

C) Medios empleados para el aislamiento y cultivo de los embriones.

Soluciones concentradas uilizadas para la preparación del medio M2 (aislamiento y manipulación de los embriones) y del medio M16 (cultivo de embriones)

STOCK A	COMPONENTE	g / 50 ml
Concentración 10X	NaCl	2.767
	KCl	0.178
	KH2PO4	0.081
	MgSO4.7H20	0.148
	Lactato de Sodio	1.7 ml
	glucosa	0.500
	Penicilina	0.030
	Streptomicina	0.025
STOCK B	**COMPONENTE**	**g / 50 ml**
Concentración 10X	$NaHCO_3$ Rojo de Fenol	1.0505 0.005
STOCK C	**COMPONENTE**	**g / 50 ml**
Concentración 10X	Piruvato de sodio	0.018
STOCK D	**COMPONENTE**	**g / 50 ml**
Concentración 10X	CaCl2.2H20	0.126
STOCK E	**COMPONENTE**	**g / 50 ml**
Concentración 10X	HEPES Rojo de fenol Ajustar el pH a 7.4 con NaOH 0.2N	2.979 0.005

Las soluciones se llevan a 50 ml con agua bidestilada muy pura y se filtran a través de filtros estériles Acrodisc de 0.2μm (Gelman Sciences), se alicuotean y se guardan a -20°C.

Preparación del Medio M2 de los Soluciones Concentradas

SOLUCION	M2		M16	
	25 ml	50 ml	10 ml	25 ml
A (X10)	2.5 ml	5.0 ml	1.0 ml	2.5 ml
B (X10)	0.4 ml	0.8 ml	1.0 ml	2.5 ml
C (X10)	0.25 ml	0.5 ml	0.1 ml	0.25 ml
D (X10)	0.25 ml	0.5 ml	0.1 ml	0.25 ml
E (X10)	2.10 ml	4.2 ml	--	--
H_2O	19.5 ml	39.0 ml	7.8 ml	19.5 ml
BSA (4mg/ml)	125.0 mg	250.0 mg	50.0 mg	125 .0 mg

Para el medio M2 el pH debe ser ajustado a 7.2-7.4 con NaOH 0.2N. Una vez aforado al volumen final deseado, se filtra a μm y se guarda a 4°C por no mas de 1 semana. El M16 usualmente queda con el pH ajustado, en caso de no tener el pH de 7.2-7.4 se debe ajustar con gas CO_2 al 5% por 30 segundos o bien con el gas producido por hielo seco. El medio también se filtra y se guarda a 4°C por no más de 1 semana.

D) Superovulación y recolección de oviductos y embriones.

Un aspecto importante que hay que cuidar es el tipo de cepa que se va a emplear como donadora de los embriones, ya que la cantidad de embriones que se puedan obtener y la resistencia de los mismos ante la microinyección serán factores decisivos para el éxito en la producción de ratones transgénicos. En la Tabla 2 se muestran los resultados de un estudio de algunas de las cepas del bioterio del Instituto de Investigaciones Biomédicas respecto al rendimiento en el número de embriones recuperados frente a una inducción de superovulación, así como la resistencia de los mismos al proceso de microinyección

Tabla 2

CEPA	Facilidad de obtención de un gran número de embriones.	Resistencia de los embriones a la microinyección
BALB/cAnN	****	*
BALB/cByJ	***	*
C57BL/6J	****	***
A/J	**	*
C3H	**	*
DBA/2	**	*
C57BL/10J	****	***
BALB/cAnNxC57BL/6J /F1	****	****
BALB/B	***	*

El número de asteriscos indican el nivel de respuesta positiva obtenida.

* Mala
** Regular
*** Buena
**** Muy Buena

Para la inducción de una buena superovulación se inyectaron hembras prepúberes BALB/cAnNxC57BL/6J/F1 de 3 semanas de edad y de 11.5-12 g de peso con una mezcla de gonadotropinas provenientes de suero de hembra preñada (SHP). La dosis de SHP fue de 5 UI inyectadas intraperitonealmente y de acuerdo al ciclo luz-obscuridad establecido la hormona se administró a las 17:00 del día -3. El SHP puede ser obtenido como un producto denominado Folligon (Intervet, Ltd, Inglaterra). La segunda hormona necesaria para que se de la ovulación, la hormona gonadotropina coriónica (Intervet, Ltd, Inglaterra), fue administrada intraperitonealmente a las 10:00 am del día -1, en una dosis similar de 5 UI / ratón. Existe una amplia bibliografía en la que se puede consultar la fisiología de la

Figura 4. Ampula del oviducto donde se agrupan los embriones sostenida por una pinza quirúrgica (a la izquierda) .

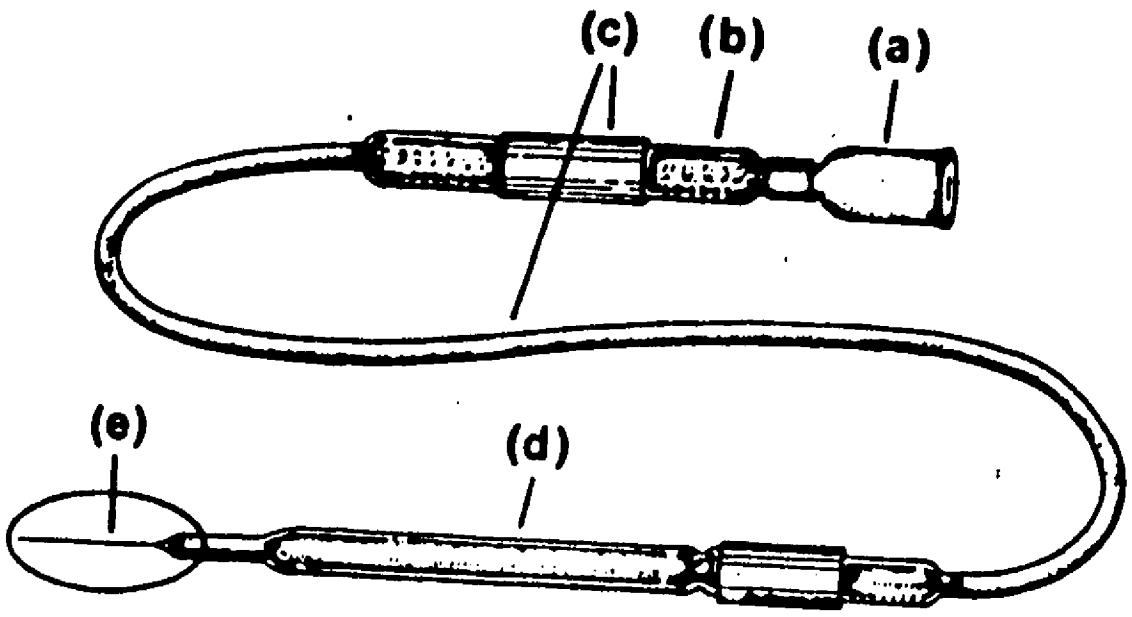


Figura 5. Sistema para manejar a los embriones: a) boquilla, b) tubo de plástico, c) trampa de algódón d) pipeta de manejo y transferencia de los embriones, e) puntade la pipeta

reproducción del ratón en la que se explica el fundamento del uso de las dos hormonas, así como la importancia en el tiempo de la administración de las mismas. El apareamiento de las hembras con los machos se realizó 2-3 horas después de la administración de la segunda hormona.

El día siguiente de la administración del Chorullon, se checaron las hembras apareadas por la presencia del tapón vaginal, se sacrificaron y se separaron los oviductos, en los cuales se pudo identificar claramente la zona de acumulación de los embriones, por la apariencia de una zona de ámpula, de paredes delgadas y estriadas en el segundo tercio del oviducto (Figura 4). Para no lesionar a los embriones, se extrajeron los oviductos cortando primero la unión entre oviducto-ovario y después entre oviducto-cuerno uterino. Los oviductos se recolectaron en 3 ml de el Medio M2 (en cajas para cultivo de tejidos de 35x10mm, Lux Scientific Corporation). Para la recuperación de los embriones, los oviductos fueron trasladados de uno en uno, a otras cajas adicionadas con M2 y Hialuronidasa (Sigma) (300ug/ml).
Empleando unas pinzas Watchmakers de punta fina del No. 4 perfectamente limpias, se rompió el tejido en la región de la ámpula y con un movimiento ligero se liberaron los embriones, los cuales salieron rodeados de un cúmulo de proteínas. Para la limpieza de los embriones, se añadió al medio M2 la hialuronidasa, la cual disgrega fácilmente el cúmulo protéico. Esta fase de la liberación y limpiado de los embriones se realizó bajo un microscopio estereoscópico (Nikon) utilizando un iluminador de fibra óptica como fuente de luz indirecta (Schott Glaswerke KL 1500). Para la limpieza de los embriones, se pasaron varias veces a través de la pipeta de manejo de embriones, la cual estaba conectada a un tubo de silicón cuyo otro extremo terminaba en una boquilla a través de la cual se hizo la succión de los embriones (Figura 5). Una vez libres de partículas visibles, los embriones se pasaron a otra caja de cultivo con M2 y hialuronidasa donde recibieron un último lavado. Para retirarles la hialuronidasa, los embriones se lavaron en medio M2 solamente y después se colocaron en una gota de medio M16 (previamente calentado a 36°C en una incubadora a 37°C y 5% de CO_2) de manera quedaran impreganados de el medio de incubación. Por ultimo los embriones se trasladaron a gotas de medio M16, colocando no más de 30 embriones/ gota. Las gotas de M16 se protegen de los cambios de temperatura y del medio en general cubriéndolas con aceite mineral (Boots, Ltd). Los embriones se incubaron a 37°C, 5% de CO_2, hasta que el cuerpo polar y los pronúcleos fueran perfectamente distinguibles al microscopio (aproximadamente 2-4 hrs).

E) Microinyección de los embriones

En la fase de microinyección, se pueden distinguir tres etapas:

La primera consiste en el llenado de la micropipeta de inyección con el DNA. Para esto es necesario tomar la micropipeta por la mitad de manera que ninguno de los dos extremos (el borde recto, y el que ha sido alargado) tengan

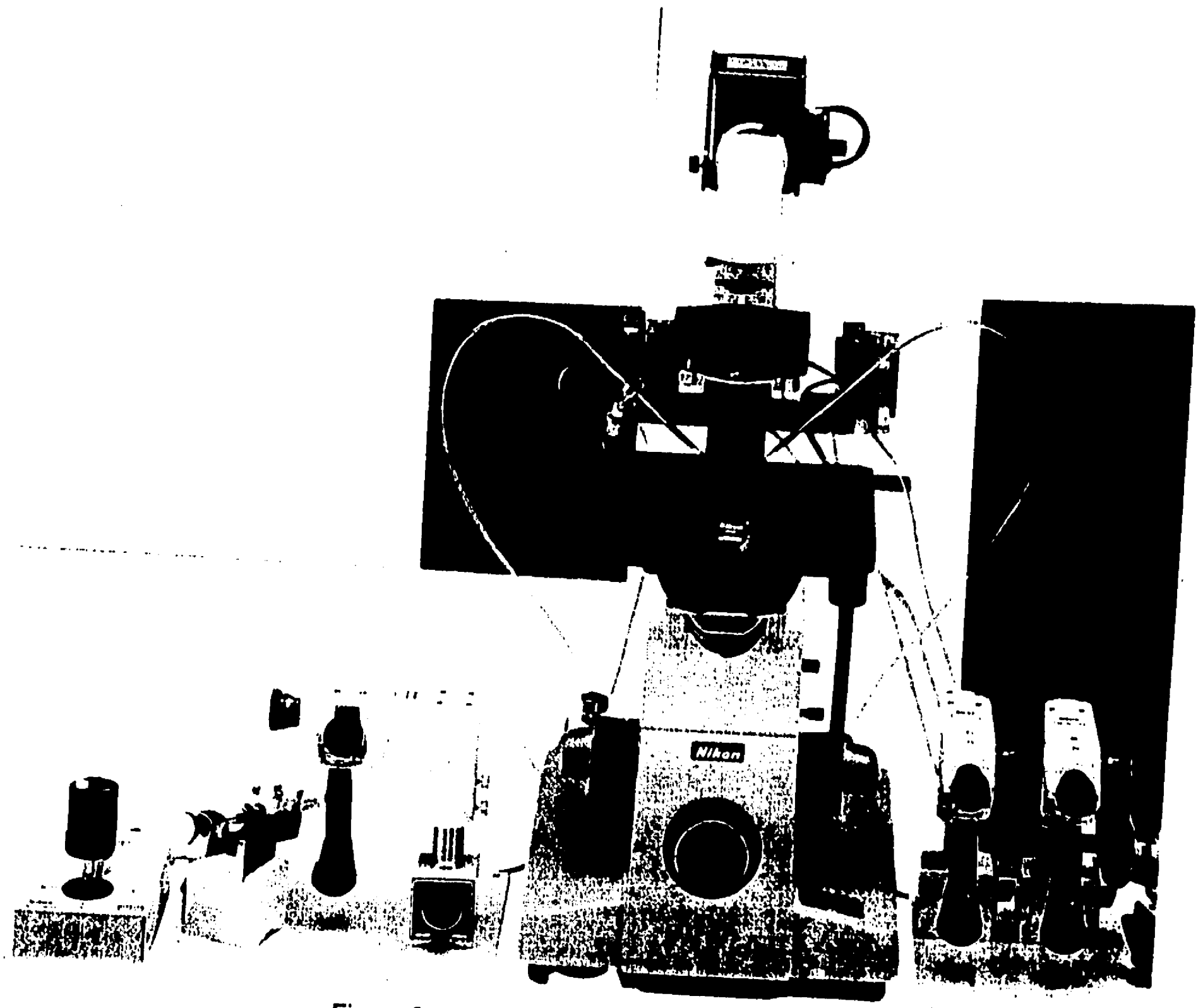


Figura 6. Equipo de microinyección.

contacto con la piel. La pipeta se llena sumergiendo el borde recto en la solución de DNA el cual sube hasta el otro extremo por capilaridad. La micropipeta se adapta al sostenedor de un sistema de micromanipulación hidraúlico (Narishige M0-188) por uno de los extremos (el que tiene el tornillo de sostén). Por el otro extremo del sostenedor se ajusta un tubo de plástico el cual se conecta a una jeringa de vidrio con capacidad como para 50 ml.

La segunda fase consiste en la preparación del sistema que permitirá el sostén de los embriones. La micropipeta de sostén se rellena con una sustancia inerte (Fluorinert , Sigma). La micropipeta se adapta al extremo con tornillo de otro sistema de micromanipulación hidráulico (Narishige M0-188). El otro extremo del sostenedor tiene adaptado un tubo de hule que termina en el sistema de microinyección (Narishigue, IM-6). Todo el sistema de microinyección hasta la entrada de la pipeta de sostén se rellena con aceite mineral, de manera que los movimientos de el tornillo de control de succión representen pequeños ajustes. En la Figura 6 se ilustra el microscopio con todo el sistema empleado para la micro-inyección.

La tercera etapa consiste en la microinyección de los embriones. Los embriones se colocan en la concavidad de un portaobjetos de bordes esmerilados (Assistent No. 2410), en medio M2 y cubiertos con aceite mineral. Para distinguir los embriones inyectados de los no inyectados se divide la zona de visión en 3 partes, colocando en cada vértice los embriones deseados: inyectables, muertos y inyectados. El portaobjetos se coloca en un microscopio invertido (Diaphot TMD, Nikon). La pipeta de sostén se coloca al mismo nivel vertical y horizontal de la pipeta de microinyección. Posteriormente se manipulan los micromanipuladores de manera que ambas pipetas penetren hasta la zona donde se encuentran los embriones. Para la microinyección primero se seleccionan los embriones aptos a ser inyectados (aquellos a los que se les distinguen los pronúcleos) y se atrapan de uno en uno con la pipeta de sostén de manera que el pronúcleo masculino quede lo más expuesto posible. Con el micromanipulador de microinyección se penetra el embrión, hasta que la punta de la aguja penetre la membrana del pronúcleo y una vez adentro se presiona la jeringa con lo cual el DNA penetra libremente. La aguja debe ser retirada en un movimiento rápido y suave en el momento en que se observe que la membrana del pronúcleo ha crecido al doble de su tamaño original, evitando de esta manera dañar al embrión. Los embriones no deben estar por más de 15 minutos fuera del sistema de incubación. Una vez inyectados los embriones se lavan con medio M16 fresco y se introducen en gotas de medio M16 nuevo para ser incubados a 37°C, 5% de CO_2 durante toda la noche.

F) Transferencia de los embriones a hembras pseudoembarazadas.

La tranferencia de los embriones requiere dos tipos de cirugías en el ratón,

una en los machos (vasectomía) y la otra la transferencia de los embriones a hembras. El instrumental quirúrgico requerido para ambos se presenta en la Tabla 3.

TABLA 3

[illegible]	[illegible]
Tijeras para cirugía rectas y de punto fino (Holborn B727)	Tijeras para cirugía rectas y de punto fino (Holborn B727)
Pinzas para sostenimiento y ligadura. Curvas de 10 cm (Holborn BN541)	Pinzas para sostenimiento y ligadura. Curvas de 10 cm (Holborn BN541)
	Pinzas rectas y de punto muy fino (No. 4 y No. 5 y de 11 cm de largo) (Holborn, MIC/JF-5)
Grapas quirúrgicas: Autoclips 9mm (Clay Adams)	Grapas quirúrgicas: Autoclips 9mm (Clay Adams
	Clips de acción cruzada y rectos (Buildog Clips, Holborn B748)
Hilo de Sutura (Catgut) (Holborn B2082E)	Hilo de Sutura (Catgut) (Holborn B2082E)
Agujas de Sutura curvas No. 20 (Holborn B1120/S)	Agujas de Sutura curvas No. 20 (Holborn B1120/S)

a) <u>Obtención de hembras pseudoembarazadas.</u>

Para la obtención de hembras pseudoembarazadas, un día antes de la transferencia de los embriones, se aparean hembras maduras en ciclo (de preferencia se selecciona una cepa con un alto índice de eficiencia reproductiva, por ejemplo una línea híbrida o una cepa singénica B6, de unos 25 gr. de peso) con machos vasectomizados (1 hembras con un macho por jaula). Al día siguiente se seleccionan las hembras que presenten tapón vaginal para ser utilizadas como hembras receptoras de los embriones micro-inyectados.

b) <u>Obtención de machos vasectomizados</u>.

La vasectomía es un procedimiento quirúrgico en el cual se cortan los conductos deferentes para que los espermatozoides no lleguen hasta la uretra. Por lo general se utilizan machos híbridos de unas 6 semanas de edad. Los animales se anestecian con pentobarbital (Anestesal, Smith Kline) en una dosis de 1 ml por cada 2.5 Kg de peso por vía peritoneal. Una vez dormido el ratón, se coloca en un plano frontal y se desinfecta la región abdominal. Posteriormente, con unas tijeras de disección de punta muy fina se realiza una incisión transversal de aproximadamente 1 cm largo en la región abdominal que queda en el mismo plano del inicio de las piernas. Se detectan los testículos y con una pinzas curvas se

Figura 7. Vasectomía de machos. Identificación y corte de las conductos deferentes.

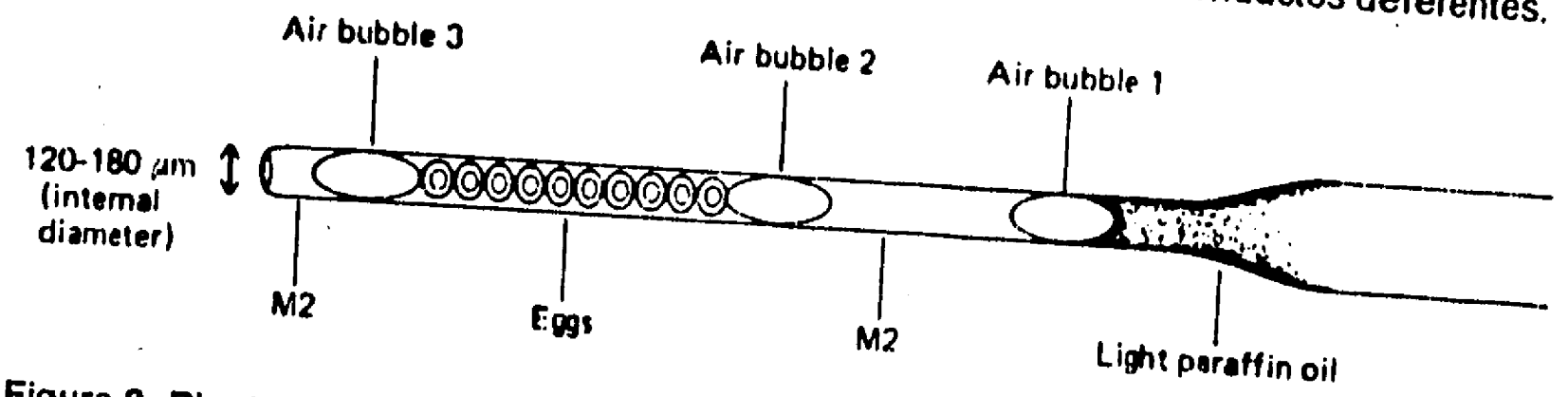


Figura 8. Pipeta de transferencia. Los embriones se colocan entre burbujas de aire y medio M2.

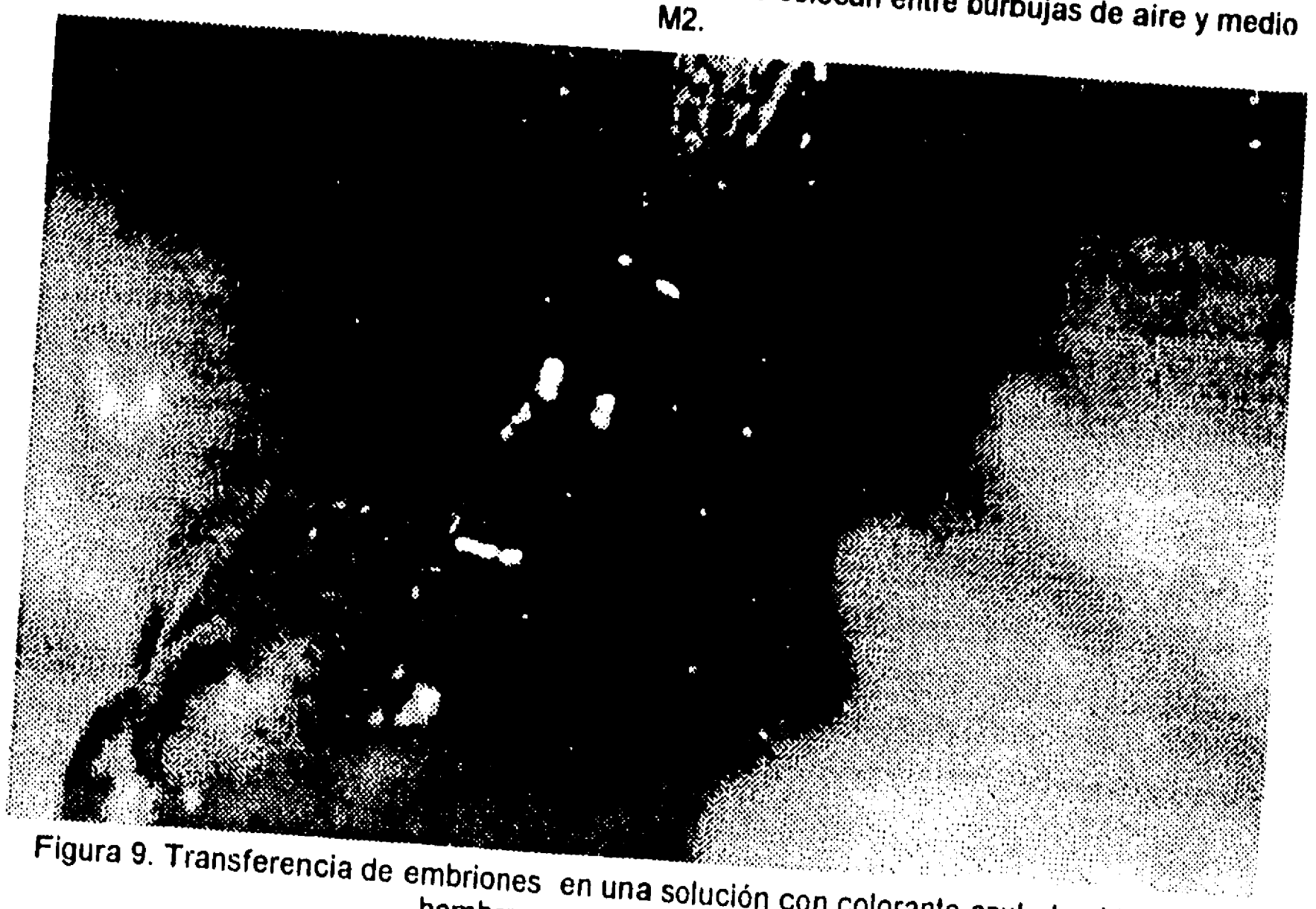


Figura 9. Transferencia de embriones en una solución con colorante azul al oviducto de hembras pseudoembarazadas .

extrae el tejido tomándolos de la región grasa que los rodea. Se identifican los conductos deferentes y se cortan con las tijeras de disección (Figura 7). El testículo se coloca de nuevo en su lugar y se realiza el mismo procedimiento del otro lado. Por último se realiza una sutura en la pared del peritoneo aplicando de 2 a 3 puntos y por último se sutura la piel uniendo los bordes cortados con grapas quirúrgicas (autoclips 9mm, Clay Adams). Dos semanas después de la cirugía, se checa la incapacidad de los machos operados de preñar a las hembras, a fin de confirmar una adecuada vasectomía.

c) Transferencia de los embriones.

Para la transferencia de los embriones, las hembras pseudoembarazadas se anestecian con pentobarbital, siguiendo el mismo esquema realizado para los machos vasectomizados. Mientras las hembras quedan anesteciadas, se procede a preparar la pipeta de transferencia. Para ello se sacan los embriones de la incubadora, se revisan bajo microscopio estereoscópico y se seleccionan aquellos que lograron dividirse en dos células (embriones viables). Los embriones se lavan con medio M2 y se colocan en la pipeta de transferencia la cual previamente se ha llenado con aceite mineral. El modo como debe de prepararse la pipeta para la transferencia de los embriones se ilustra en la Figura 8. Para asegurar que la hembra quede preñanda se prefiere transferir varios embriones por oviducto (20 a 25), ya que aún cuando el aspecto de los embriones pueda ser sano, puede ser que no puedan continuar con la división.

Una vez preparada la pipeta se toma a la hembra, se desinfecta la región posterior del lado derecho o izquierdo, dependiendo de con cual lado se va a comenzar la transferencia. Como la transferencia se realiza en el oviducto se hace una pequeña incisión en la piel, siguiendo el plano vertical de aproximadamente 1 cm y antes de cortar tejido subcutáneo, se localiza en dónde se encuentra el cuerpo reproductor. Una vez distinguido se corta el tejido y con una pinzas curvas se extrae el ovario, oviducto y parte del cuerno uretral, sujetándolo por el tejido graso que rodea al ovario. Bajo el microcopio se detecta la zona en la que se encuentre el inicio del oviducto y con mucho cuidado debe de comenzar a romperse membranas dejando un orificio muy pequeño. La pipeta de transferencia se acopla a un sistema de expulsión como el utilizado para el manejo de los embriones y con mucho cuidado se introduce la pipeta en el oviducto. Los embriones se depositan suavemente. Para saber que la transferencia fue adecuada se debe observar la presencia de las burbujas de aire dejadas entre los embriones y las gotas de M2 (Figura 9). Como se ha mencionado en diversas revisiones de transgénesis, para que se de una transferencia exitosa el muy importante no lesionar mucho el tejido. Para ello se debe cuidar, a) tratar de que se libere el mínimo fluido, b) exponer el tejido al aire lo menos posible. La transferencia debe realizarse en 5 minutos como máximo y utilizando lámparas de luz

fría. Una vez terminada la transferencia debe de regresarse con mucho cuidado el tejido a su lugar. Para la sutura se utiliza el mismo procedimiento que para la cirugía de los machos vasectomizados.